SEROLOGICAL DIAGNOSIS OF *BRUCELLA* INFECTION

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ABSTRACT

The automated complement fixation test (CFT) and the brucellosis card test (BCT) have been widely used as official tests in the New Zealand Bovine Brucellosis Eradication Scheme. During the course of the eradication programme it was observed that a significant proportion of cattle reacted to the BCT yet remained negative to the CFT and this often occurred on more than one occasion for any particular animal.

Twenty cows, from reactor herds, that had been BCT+/CFT- on at least three successive occasions were slaughtered. Despite extensive sampling, attempts at isolating Brucella abortus organisms from tissues of these animals were unsuccessful. Serum from one cow was found to be positive to a wide range of serological tests and it also caused a strong prozone reaction in the CFT, which could easily have been overlooked. The possibility that the automated CFT, which is essentially a one dilution test, was unable to detect such prozoning sera was investigated. It was shown that providing a suitable choice of antigen concentration was made, such sera would be detected by the automated test.

Brucella - specific IgG_1, IgG_2, and IgM levels in prozoning and non prozoning sera were measured using the single radial immunodiffusion test. It was shown that serum containing a high proportion of specific IgG_2 was likely to exhibit prozoning and that various degrees of prozoning could be induced by varying the ratio of specific IgG_1 to specific IgG_2.

Cattle, previously sensitized by calfhood Br. abortus strain 19 vaccination, were experimentally inoculated with killed Br. abortus. It was shown that although serum agglutination test (SAT) and CFT titres appeared for a short period, titres to the BCT in some cattle tended to remain longer thus allowing an animal to be BCT+/CFT-.

An analysis of herd testing data indicated that BCT+/CFT- animals were more likely to exist in infected herds than in non-infected herds. In heavily infected herds up to 16% of CFT- animals were BCT+ whereas in non-infected or very lightly infected herds less than 4% were CFT-/BCT+. It was concluded that in sensitized cattle at least exposure to the organism without true infection is capable of stimulating antibody which is detected by the BCT, but not necessarily able to provoke positive CFT titres.

The performance of the Auto-Analyzer adaptation of the CFT as used in the New Zealand eradication scheme was assessed. Various prozoning sera from known infected animals were tested and the effect of varying antigen concentrations on these and other sera was noted. Significant differences in antigen concentration required for optimal complement fixation were detected. Prozoning sera required more antigen than non-prozoning sera and even sera that did not exhibit prozoning had varying optimal antigen requirements.

By using I^{125} labelled bovine gama-globulin the dilution gradient of serum within the Auto-Analyzer system was estimated. Knowledge of the serum dilution gradient being obtained was essential for proper understanding of unusual traces given by prozoning sera.

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INTRODUCTION

Bovine brucellosis is a contagious disease of cattle resulting from infection with the bacterium *Brucella abortus*.

The disease is primarily manifest as a placentitis in the pregnant female with a resultant abortion. Humans may also be infected by the causative organism in which case the disease is known as undulant fever.

Because of the contagious nature of the disease, the prevalence of infection within an unprotected herd may be very high and persons in close contact with such an infected herd run a very real risk of contracting undulant fever.

Apart from the human involvement the importance of the disease lies in the loss of calves and in the resultant loss of milk production from aborted cows.

The organism responsible for contagious abortion of cattle was first described by Bang (1897), and termed *Bacillus abortus*.

Some years earlier, Bruce who had been investigating the cause of Malta fever in troops succeeded in culturing a micrococcus from the spleens of four fatal cases (Bruce,1887). He termed this organism *Micrococcus melitensis*.

The close relationship between *Bacillus abortus* and *Micrococcus melitensis* was not recognised until 1918 when Evans (1918) correctly identified *M. melitensis* as a bacillus and suggested the designation *Bacillus melitensis*. She also noted that the agglutination test performed on human serum could not distinguish infection due to *B. melitensis* from that caused by *B. abortus*.

Meyer and Shaw (1920) confirmed the work of Evans and proposed the term *Brucella* be adopted as the generic name in honour of the pioneering work of Bruce.

In the 1920's the role of *Brucella abortus* in human infection was established and *Brucella suis* was recognised as a distinct species.

Three further species of *Brucella* were later isolated.

Buddle and Boyes (1953) described *Brucella ovis* for the first time and in 1957 Stoenner and Lachman (1957) described *Brucella neotomae* which they had isolated from the Desert Wood Rat. The

latest member of the genus, Brucella canis was first described by Carmichael and Bruner (1968). A recent review of the disease in dogs has been prepared by Carmichael (1976).

By the late 1920's it had been established that Brucella abortus could infect man and that raw milk was an important source of infection. By this time most of the basic epidemiology of the disease had been established and the serum agglutination test had been shown to be a useful diagnostic tool.

Formal attempts at eradication or control of the disease in cattle began in the United States in 1934 as a drought relief programme. In Denmark serological testing began in 1936. These schemes along with initial attempts in other Scandanavian countries did not, however, make great progress. Difficulties in test standardisation and interpretation and the advent of World War II hindered eradication attempts.

Renewed attempts at eradication gathered momentum in the late 1940's and these were aided by the development and use of the live attenuated vaccine Br. abortus strain 19. (Cotton and Buck, 1934). Another important aid to eradication was the introduction of the Milk Ring test (Fleischhauer, 1937), which eliminated the need for bleeding of cattle.

Success in eradicating bovine brucellosis has largely been a function of the intensity of effort applied along with the type of cattle husbandry practised. Scandanavian countries have applied considerable legislative and testing pressure since the 1920's (Thomsen, 1957) and have generally been free of the disease since 1970.

In the United States the Co-operative State-Federal Brucellosis Eradication Programme commenced in 1934, but was given emphasis only in the early 1950's. It aimed for completion by 1975 but due to various reinfection problems a more realistic date of 1984 has now been set (Schilf, 1972; Becton, 1976).

The European system of farming with small intensely managed herds has favoured eradication attempts. Control of the disease in countries which practise grassland cattle grazing with large herds and outdoor calving patterns is more difficult. Such differences in cattle management and in public acceptance of the resources required, mean that the technology used successfully in Northern Europe may not be as effective in the more extensive cattle

raising nations.

The New Zealand brucellosis eradication scheme began in September 1971 with the stated objective of bringing every eligible animal under compulsory test by August 1977. By this time 69% of the beef herds and 81% of the dairy herds in the country were accredited free of brucellosis.

Because of the rapidity with which the New Zealand scheme has moved and the limited resources available, there has been little continuing assessment of the effectiveness of technical procedures used.

The object of this thesis is to describe laboratory aspects of the New Zealand scheme, to assess the performance of automated serological techniques and to investigate some aspects of the serology of certain sera which have given anomalous test results.

CHAPTER 1. LITERATURE REVIEW

Pathogenesis of Brucella abortus infection in Cattle

Brucella abortus may be described as a facultative intracellular micro-organism. It is a Gram-negative bacillus about 400 nm in diameter and although aerobic it requires 5 to 10 percent CO_2 to sustain growth during initial isolation.

The characteristic clinical feature of brucellosis in the bovine is the localisation and multiplication of Br. abortus in the epithelial cells of the chorion (Smith, 1919). Later, by spread of infection into the placental fluid a foetal pneumonia may be induced (Smith, 1925).

According to Huddleson (1943), the cause of foetal death and abortion may be due to interruption of the vital functions of the placenta, or to the toxic effects of an endo-antigen.

Payne (1959) attempted to follow the course of Br. abortus infection in eleven pregnant cows experimentally infected via the conjunctival sac. After one week the parotid nodes were found to contain organisms suggesting that Br. abortus penetrates the conjunctival epithelium and proceeds directly via the lymphatics to the local lymph gland. After two weeks organisms could be isolated from the spleen and supramammary glands and after four weeks Br. abortus was consistently isolated from the uterine lumen. Payne's conclusion was that following colonisation of the local lymph node the organism is distributed by the blood and localises in the interstitial connective tissue. Inflammation results and the bacteria find their way into the uterine glands and then into the uterine lumen. Multiplication of Br. abortus occurs in macrophages and the exudate contains these cells packed with bacteria.

In the pregnant uterus the interstitial inflammation leads to a severe ulcerative endometritis and by the time of abortion nearly all of the endometrial mucosa is eroded and replaced by sub-acute inflammatory granulation tissue.

Infection of the placental cotyledons follows the involvement of the uterine lumen although this part of the process is slow in onset. Around the periphery of the cotyledons Br. abortus induces ulceration of maternal tissue and multiplies extensively within chorionic trophoblast cells. Bacteria then migrate through the

connective tissues of the allantochorion and enter the foetal blood vessels eventually invading the placental fluids and the foetus.

After calving or abortion Br. abortus leaves the uterus to localise in the udder and supramammary lymph nodes (Manthei and Carter,1950; Lambert et al., 1961). Philippon et al. (1970) recovered Br. abortus from the supramammary lymph nodes of 44 out of 46 pregnant animals infected at five to six and a half months of gestation and slaughtered six weeks after parturition. The recovery rate from the udder was only 60% and from the uterus only 51%. Other tissues where the organism frequently proliferates are the iliac lymph nodes, pharangeal lymph nodes and spleen. Brucellae are also found in a high proportion of knee hygromas (Doyle,1935).

In an attempt to determine why the reproductive organs should be so receptive to Br. abortus, Payne (1960) experimentally infected non-pregnant cows. He divided these into two groups and infected all animals with one group receiving daily treatments of progesterone. The invasiveness of Br. abortus was much reduced in the non-pregnant animals and although progesterone treatment induced profound changes in the genitalia it had no effect on the pathogenesis of brucellosis.

In an investigation of the chemical factors associated with the growth of Br. abortus Pearce et al.(1962) found that erythritol was present in high levels in bovine foetal fluids and that its presence was required to enable these fluids to stimulate growth of Br. abortus in bovine phagocytes. A search of various maternal and foetal tissues of pregnant cows showed that erythritol was concentrated in those tissues (chorion, cotyledons and foetal fluids) which in brucellosis are prone to heaviest invasion. Erythritol stimulated the growth of Br. abortus in vitro and in vivo in one to five day old calves, and hence was incriminated as the cause for the predilection of Br. abortus for foetal tissue in bovine contagious abortion (Williams et al., 1962; Smith et al., 1962). Similarly in the bull, brucellae tend to localise in the genitalia and on investigation erythritol was found in the seminal vesicles and testes (Keppie et al., 1965).

In later studies Keppie et al. (1967) and Meyer (1967) found that erythritol inhibited the growth of Br. abortus strain 19 and

this difference in comparison with virulent strains was suggested as a chemical basis for the relative "avirulence" of strain 19.

Braude (1951a) detailed the expected effects of challenge with different species of Brucella and highlighted the fact that the granuloma is the basic histological lesion in the tissues of animals and man after infection. In a further study Braude (1951b) detailed the evolution of the hepatic granuloma in experimental infection of guinea pigs and mice.

After challenge Brucellae are rapidly cleared from the blood as they are ingested by polymorphonuclear phagocytes and carried to the liver, spleen and cells of the reticulo-endothelial system. The primary host response appears to be a massive proliferation and/or migration of polymorphonuclear and later mononuclear cells at or towards the focus of infection where they form a granuloma walling off the infected cells (Braude, 1951b). In the case of tuberculosis infection, the granuloma is termed a tubercle - the analagous name in brucellosis, a brucercle, does not seem to have been seriously proposed.

Another characteristic feature of Brucella infection is the ability of the organism to survive within the phagocytes of the host (Holland and Pickett, 1958; Stinebring and Kessel, 1959; Elberg, 1960). Smith (1919) described the intracellular localisation of Br. abortus within the epithelial cells of bovine foetal membranes. The bacteria were not only contained in these cells, but were protected so that multiplication of the organism could proceed without interruption. Dickey and Forbus (1945) noted the immediate phagocytosis of Br. suis by polymorphonuclear cells and the resulting multiplication of the organism. They postulated that such phagocytosis might actually be detrimental to the host since the bacteria may not be killed but protected by this localisation.

The intracellular localisation and growth of Brucella has been confirmed by many investigators. Even in the blood, unless there is overwhelming infection, the bacteria are present within leukocytes (Braude, 1951b). Study of the ability of Brucella to multiply within mononuclear phagocytes maintained in tissue culture has shed much light on the nature of virulence. Virulent strains have this ability, while avirulent strains do not (McCullough, 1970).

In the infected bull two clinical syndromes have been reported and these may or may not occur in the same animal. The first involving the testicle and epididymis is often characterised clinically by orchitis (Buck et al., 1919; King, 1932; Bendixen and Blom,1947; Rankin,1965). The second,and apparently more common syndrome involves the seminal vesicles and ampullae (Buck et al., 1919; Bendixen and Blom,1947; Rankin.1965). Few reports relating to experimental infection and pathogenesis of the disease in bulls are available (Seddon,1919; Lubbehusen and Fitch,1926; Christensen. 1948). The bull, however, is not recognised as playing a significant role in the transmission of the disease (Thomsen,1943; Rankin,1965).

Thus, in summary, a Brucella challenge can lead to a chronic long term infection, characterised by a low level,intermittent or fluctuating antibody titre. The infected cow tends to abort once, and then settles down into a chronic carrier condition. The Brucellae become localised, particularly in the supramammary lymph nodes and udder, and may be excreted in the milk, intermittently, for many years (Morgan,1969).

The Epidemiology of Bovine Brucellosis

A sound knowledge of the sources of infection, host susceptibilities and organism survival are required before any disease control programme can be attempted. A great deal has been written about the epidemiology of bovine brucellosis and a certain amount of confusion has arisen because of earlier conflicting studies.

Sources of Infection

(a) The infected cow

The importance of the aborted cow as a source of infection was recognised by Bang (1897). Large numbers of brucellae are shed in genital discharges following abortion or after an apparently normal calving from an infected cow. Numbers rapidly decrease one to two weeks after calving. Chronically infected cows may excrete organisms following subsequent calvings. Thus the primary hazard within a herd is the aborting cow during the first 10 days after the abortion (Fitch et al., 1938; Manthei and Carter, 1950).

In the chronic disease organisms localise in the udder and related lymph nodes. Up to 2×10^5 organisms per ml may be found in the colostrum or milk within the first few days following calving or abortion. Numbers decrease rapidly after the first week but intermittent excretion will often continue for the remainder of this and subsequent lactations. Strain 19 vaccination will reduce but not necessarily eliminate this excretion (Morgan and McDiarmid, 1960).

(b) The infected bull

Infected bulls may shed brucellae via the semen, seminal fluid, faeces, urine and hygroma fluid. Semen may contain large numbers of organisms during the initial stages of infection but as the disease progresses excretion is reduced and may cease entirely (Lambert et al., 1963; McCaughey and Purcell, 1973).

(c) Secondary or transient hosts

The FAO/WHO Expert Committee on Brucellosis (Anon., 1971) notes

that the wide host range is one of the important characteristics of the genus Brucella, even though particular biotypes may have a limited host range.

As mentioned previously Br. abortus infection of man is well known although there are no reports of the agent causing abortion in women. Transmission from man to cattle is not known to occur.

Of the domestic animals, infection of the horse is recognised and is usually associated with 'fistulous withers' and arthritis (Stableforth, 1959). Hutchins and Lepherd (1968) noted that horses in contact with infected dairy herds had a higher incidence of positive Brucella titres than horses without such contact. McCaughey and Kerr (1967), Shortridge (1967), Crossman and Bonson (1968) and Robertson et al. (1973) have all reported abortions in mares apparently due to Br. abortus infection.

Br. abortus appears to have low pathogenicity for sheep but sporadic natural infections have been reported (Luchsinger and Anderson, 1967; Allsup, 1969). Shaw (1976a) recovered Br. abortus, biotype I from the foetuses of five out of eight aborting ewes. These ewes were from a group of 20 known to have had contact with cattle having the same biotype. In a subsequent study Shaw (1976b) experimentally infected four ewes by administering a suspension of infected bovine cotyledon orally. Thus although sheep can sustain infection and may abort, the low prevalence and incidence of the disease on a flock basis points to sheep as being of minor importance as reservoirs of infection (Allsup, 1974).

A case in New Zealand reported by Wallace (1959) suggests that pigs should be considered as a possible reservoir or carrier of Br. abortus. A sow which had access to infective material from infected cows aborted and although organisms could not be cultured from foetal membranes, which showed a necrotic placentitis, they were recovered from foetal stomach contents.

In the USA, McCullough et al. (1951) obtained 35 Brucella isolates, 10 of which were Br. abortus, from cultures of sub-maxillary lymph nodes of 5 000 pigs at slaughter. Surveillance of human brucellosis indicates that Br. abortus infections of swine may be more common than previously thought. Four out of eight isolates of Br. abortus in humans in the United States in

1971 were from patients infected as a result of contact with pigs only (Anon., 1971).

Abortion in the bitch due to Br. abortus has been reported by Morse et al.(1953) in the USA, Philippon et al.(1969) in France, Ehrlein et al.(1963) in Germany and by Taylor et al.(1975) and Bicknell et al.(1976) in Great Britain. It has been suggested that the prevalence of brucellosis in dogs is higher than can be inferred from the infrequent reports in the literature (Clegg and Rorrison,1969).

Of the wild animals known to be infected with Br. abortus, most reports have come from Europe and North America. Hares, reindeer and rodents have been shown to be infected. Camels, yaks, buffalo, mink, foxes and various other ungulates have also been shown to be reservoirs of infection (Anon., 1971). Meyer (1966) in a comprehensive review of the role of other animals concludes that Brucella organisms are not readily transmissible from their preferential host to dissimilar hosts and that no serious or threatening reservoir of infection exists in wild animals in the United States.

Daniel (1966, 1967) and McAllum (1976) have tested New Zealand deer sera for Br. abortus but only one positive and three suspicious titres were obtained from some 700 sera.

In the absence of any evidence to the contrary it appears that no animal other than the bovine has any significant role to play in the spread of brucellosis in New Zealand.

(d) Mechanical hosts

Huddleson (1943) has given the following data for the survival time of Brucella abortus

Direct sunlight		4½ hours
Dry soil	(Room temperature)	1-2 months
Wet soil	" "	2-3 months
Sterile tap water	" "	2-3 months
Milk	" "	2-4 days
Urine	" "	2-4 days
Faeces	" "	3-4 months
Genital discharge	(Ice box)	7 months
Foetus	(Shade)	6-8 months

Ice cream	(0 °C)	1 month
Butter	(8 °C)	1-2 months
Cheese	(Room temperature)	2 months

(e) Milking machines

There is no doubt that Brucellae are excreted in the milk. However, opinions vary on the role played by the milking machine in the transmission of brucellosis. Lapraik et al (1964) found no evidence of spread by excreting cows at milking. Leech et al. (1964) also concluded that brucellosis was not readily spread by the milking machine. In Northern Ireland spread via infected milk at milking was thought a real possibility in infected herds (Kerr and Rankin, 1959) and mastitis workers readily accept that Staphylococci and Streptococci can be transferred from udder to udder by the milking machine (Davidson and Slavin, 1958; Whittlestone et al., (1968).

Methods of Infection

Brucellae can be transmitted to a susceptible cow by:

(a) Ingestion

Bang (1906) originally showed that this was a route of infection. It is thought that this is the most usual natural route although the dose of bacteria needs to be large (Cotton and Buck, 1931; Wilson and Miles, 1967).

(b) Inhalation

Infection by inhalation occurs in guinea pigs (Elberg and Henderson, 1948) and man (Anon., 1971). Challenge via the respiratory tract must be considered a real hazard in cattle.

(c) Direct contact

Transmission may occur through unbroken skin and more readily through broken skin. Challenge via the conjunctiva has been commonly used in experiments and low doses induce infection (Cotton, and Buck, 1931). Infection is also said to be acquired through the udder at milking (Kerr and Rankin, 1959).

(d) Coitus

Cows may be infected via the genital tract when served by an infected bull (Thomsen,1936) or by artificial insemination with infected semen (Seit,1944). It appears that natural service by the bull is of negligible importance in the transmission of brucellosis (King,1940; Thomsen,1943; Rankin,1965; Barclay,1977). However, artificial insemination has been held responsible for causing widespread infection in cows (Bendixen and Blom,1947; Manthei et al.,1950). In natural insemination semen is deposited in the vagina whereas when artificial insemination is used semen is introduced into the uterus. It appears that a much higher dose of organisms is required to infect via the vagina than via the uterus (Manthei et al., 1950).

(e) Congenital passive transfer

Fitch et al.(1941) reared 56 heifer calves in isolation from naturally infected mothers. No evidence of brucellosis was found in these animals during the seven years of the experiment. This work led to the general assumption that congenital transfer of infection did not occur. Plommet et al. (1973) proved otherwise. They showed that calves born of infected dams could harbour the organism without any clinical or serological manifestation until during pregnancy. At this stage the disease became apparent and usually resulted in abortion.

The Susceptible Animal

Br. abortus has a special affinity for genital tissues in the pregnant cow. The non-pregnant cow and the mature unserved heifer are said to show some innate resistance to challenge (Edington and Donham,1939) but the size of the challenge dose is important. In a series of initial herd tests in Great Britain 28% of cows, 3.4% of heifers and 13.2% of bulls reacted (Stableforth, 1959).

Calves are relatively resistant to infection and many experiments have been conducted to determine their susceptibility by feeding them infected milk and letting them suckle infected cows. In most cases they fail to produce any demonstrable serum antibodies (Huddleson,1942). Carpenter (1924) claimed that

Brucellae can be recovered from the organs of calves fed infected milk but disappear shortly after feeding is discontinued.

However, the susceptibility of young animals to Br. abortus remains a controversial subject as Plommet et al.(1973) and Lapraik et al.(1975) have shown that calves born of infected mothers and reared in isolation can retain a latent infection. There is also much circumstantial evidence linking breakdowns of Brucella-free herds to reinfection from carrier calves (Tacken,1964; Rankin, 1965; Cunningham and O'Connor,1971; Tarala,1975). Breakdowns of Brucella-free herds, apparently due to carry-over of latent infection in calves, have also been seen in New Zealand (J.Wallace Pers. Com.). Thus it appears that although congenital and calf-hood infection with Br. abortus can in fact occur, the calf remains relatively insusceptible and such infection is uncommon. It is, nevertheless,an extremely important consideration in the context of eradication programmes because of the long interval between infection and the development of serological titre and/or clinical symptoms.

Incubation Period

Many of the problems associated with the control of brucellosis in cattle are due to the long and variable incubation period of the disease.

The length of the incubation period varies according to the state of pregnancy of the cow. Thomsen (1937, 1949) found that for heifers infected at the time of mating the average period between infection and abortion was 225 days, whereas in cows infected at six and seven months of pregnancy, the incubation period was 67 and 53 days respectively. Animals infected as calves may incubate the disease until late pregnancy which can be up to two and a half years later (Nagy and Hignett, 1967).

There seems to be a relationship between the size of the challenge dose and the development of serological titre and abortion. McEwan et al.(1939) infected heifers in their fifth month of gestation and found that whereas a dose of 1.46×10^9 organisms gave an incubation period of 32-59 days, 1.46×10^5 organisms gave 83-151 days.

Animals vaccinated with strain 19 Brucella abortus are said

to take much longer to develop serological titres to infection than do unvaccinated animals (Schilf, 1968). Presumably the same applies for the development of infection.

Course of the Disease Within the Herd

In an acute outbreak in a fully susceptible herd the abortion rate may exceed 50%. More usually the rate of spread is slow extending over several seasons with a peak abortion rate of about 30%. Heifers do not usually acquire infection until brought into the herd at first calving. Once infection is established it tends to persist indefinitely (Stableforth 1959).

Epidemiological Studies Associated With Eradication Programmes

The object of any epidemiological study in brucellosis is to further knowledge of the way in which the disease spreads so that appropriate targets may be identified for particular attention in the event of an attempt at eradication or control of the disease. The real test of the value of studies previously made comes when eradication is tried. In the final stages of an eradication scheme problems arise and the investigation of these can lead to a greater understanding of the epidemiological factors associated with test effectiveness and disease spread.

The development of testing methods is detailed in a later section.

Bacteriological studies of brucellosis "problem" herds have been made by Nelson *et al.* (1966) and Luchsinger *et al.* (1973). These workers conclude that the biotype of *Br. abortus* responsible for a particular infection has no bearing on the type of eradication problem that may exist. Luchsinger *et al.* (1973) also point out the value of routine biotyping in determining sources of infection in the final stages of an eradication programme.

Kellar *et al.* (1976) in a comprehensive study of a series of reinfected herds in Canada using "case control techniques", were able to weigh the importance of the various factors involved in causing herd breakdowns. A most interesting finding in this study was that strain 19 vaccination *per se* did not appear to adversely influence the interpretation of the serological tests used nor did it appear to protect the individual animal.

The Immune Response of the Bovine to Brucella abortus Infection

(a) Cellular response

Studies on the role of cell mediated immunity in the bovine response to brucellosis have only recently been undertaken with any vigour. There has been some interest in delayed type hypersensitivity (DTH) skin tests for diagnosing Brucella melitensis infection in sheep and goats in Eastern Europe and China (Didovets,1965) and widespread testing has been carried out. Current studies are mainly concerned with the development of DTH skin tests (Jones,1976) and lymphocyte transformation and inhibition tests (Swiderska et al., 1971; Renoux et al., 1976; Kaneene et al., 1978).

Experiments by Jones and Berman (1975) have shown that circulating antibody is made in response to the polysaccharide determinants of the cell wall lipopolysaccharide (LPS) in animals infected with smooth Brucella, and not to the protein components.

Apparently, the fact that Brucella is an intracellular parasite makes it unnecessary to possess a complete or typical (compared with the enteric bacilli) LPS to protect against phagocytosis and complement destruction. Brucellae are resistant to intracellular destruction by inhibiting or blocking the degranulation process normally observed following ingestion. It has been shown that "lipid A" is the mitogenic principle of LPS; thus it appears that Brucella has a unique "lipid A" component (Hase and Th. Rietschel,1976).

According to the National Research Council Subcommittee on brucellosis research (Anon., 1977b): "Studies are non-existent on the role of the complement components in serological and immunological phenomena in brucellosis; membrane receptor studies and surface fixation of the antibody substructures onto cells; studies on the components in, and mode of action of, colostral substances; reinvestigation of the bacteriocidal action of blood and its components in the newer era of immunoglobulin and complement chemistry; genetics of the histocompatability system and susceptibility to Brucella infection. These topics have the capacity to change radically viewpoints and technology in applied immunisation to brucellosis".

(b) Humoral response

There are five bovine immunoglobulins presently recognised: IgG_1, IgG_2, IgM, IgA and IgE (Hammer et al.,1971; Duncan et al., 1972). IgG_1 and IgG_2 are quantatively and functionally important in serum and certain secretions and are not restricted to the blood vascular space (Duncan et al., 1972). IgG_1 is selectively concentrated in bovine colostrum (Mach and Pahud,1971).

IgM is a large molecule and because of its size tends to be more restricted to intravascular spaces than IgG (Husband et al., 1972).

In most animals, the antibody response to an antigenic stimulus consists of two phases - an initial IgM followed by IgG. The mechanism of the conversion of IgM to IgG is not known but it can be blocked,e.g.by 6-mercaptopurine (Sahiar and Schwatz,1965).

There is also evidence that IgG inhibits 19S (IgM) synthesis by neutralising the antigen, thus leading to an IgG steady state (Fink and LoSpalluto,1967; Halliday,1968).

The pattern of immunglobulin production in Brucella vaccinated or infected cattle has received much attention since the early 1960's when the nature and structure of immunoglobulins was elucidated.

The first antibodies detected in sera of cattle exposed to strain 19 or a virulent strain of Br. abortus are heat labile macroglobulins (IgM) followed shortly after by the heat stable IgG. (Amerault et al., 1962; Rose and Amerault, 1964; Rose and Roepke,1964; Rice et al., 1966, 1967; Wilkinson, 1966). In the case of vaccinated calves both types of antibody usually disappear within a few months although agglutinins thought to be due to persistent IgM may often be demonstrated in a proportion of such animals. In field cases of bovine brucellosis the antibodies have been reported to be predominantly or entirely of the 7S (IgG) class (Rice et al.,1966; Rice and Boyes,1971; Beh,1974).

Following strain 19 vaccination of calves, IgM antibody appears at about five days whilst IgG appears simultaneously or a few days later. Whereas peak levels of IgM are reached at about 13 days, IgG levels peak at 28-42 days (Rice et al., 1966; Beh,1974). Because of the often long and variable incubation period of the disease, IgM and IgG antibodies may not appear for some time after infection. IgM antibody usually appears first

and this is followed a few days later by IgG. IgM levels then decline whilst IgG remains to be the predominant and often the only immunoglobulin present (Morgan,1967).

Vaccination Against Brucella abortus in Cattle

Nearly every nation that has attempted to control brucellosis in cattle has employed the use of vaccination at some stage of the programme. These efforts to provide immunity have utilised a wide range of organisms; living and dead; virulent and attenuated; related and unrelated.

Strain 19

(a) Development of strain 19

As early as 1919 the U.S. Bureau of Animal Industry permitted commercial manufacture and distribution of live Br. abortus vaccines. These virulent vaccines were used in the belief that if non-pregnant cows were inoculated they would not abort in subsequent pregnancies.

Following the work of Cotton (1932), who demonstrated that organisms could be established in the udder of cows vaccinated whilst lactating, the production of vaccines from virulent organisms was prohibited (Mohler et al., 1941).

Buck (1930) administered the live attenuated strain 19 Br. abortus to three calves five to eight months of age and challenged them with virulent organisms at the fourth month of pregnancy. All three heifers calved normally whereas controls became infected and aborted. The conclusion drawn from this experiment was that immunity could be induced by early vaccination and a resultant reduction in the level of agglutinins produced added further benefit. In follow-up experiments Cotton et al. (1933) and Buck et al.(1934) concluded that a strain of low virulence (strain 19) conferred as much or more immunity as did more virulent strains. In subsequent experiments Buck et al. (1938) Haring and Traum (1937, 1943) and Haring (1938) verified earlier results and concluded that the immunity persisted until the animals matured.

In 1936 the U.S. Bureau of Animal Industry carried out large scale field trials and the results were so encouraging that calf-hood vaccination was adopted as official policy (Mohler et al., 1941). Since then studies by Gilman and Wagner (1959), King and Frank (1961) and Lambert et al.(1961) have added further evidence

that calfhood vaccination is indeed as effective as adult vaccination. Redman et al.(1967) concluded that the protection afforded by vaccinating at two to three months was comparable to that found in cattle vaccinated at four to eight months of age.

There has recently been renewed interest in strain 19 vaccination of adults. In some "problem" herds, where the eradication of the disease is proving difficult, previously unvaccinated adults have been vaccinated with the object of containing the spread of infection. It is claimed that by applying a series of serological tests vaccination titres can be differentiated from those due to infection (Worthington et al., 1973; Nicoletti,1977).

(b) Pathogenicity

Strain 19 differs from more virulent strains of Br. abortus in that even in large doses it produces in guinea pigs no more than a slight enlargement of the spleen and a blood serum titre seldom over 1:50. Organisms are rarely found in the spleen six weeks after infection (Alton et al., 1975a).

Meyer and Nelson (1969) reported that residual localization of strain 19 occurred following immunisation of female cattle but was infrequent. The residual infection involved the mammary glands but did not spread to or involve the reproductive tract. Nicoletti and Muraschi (1966) and Nicoletti (1969) have reported on the isolation of strain 19 organisms from animals in problem herds. Because no more than one strain 19 infected animal was ever found in any one herd it was concluded that there was no evidence to suggest that this biotype is contagious.

Lambert et al.(1964) reported the development of orchitis and the isolation of strain 19 from the testicles of two bulls vaccinated at five to six months. More recently Lambert et al. (1965) administered strain 19 to 15 bulls four to ten months of age. Mild transient post-vaccinal orchitis developed in eight of these animals. Post-vaccinal serum agglutination titres persisted at diagnostic levels longer than in heifers. When slaughtered at 18 months of age strain 19 was unable to be isolated.

(c) Effectiveness

The resistance conferred by strain 19 vaccination has been

the subject of a number of studies, the most extensive being a review of U.S. National Animal Disease Laboratory data by Manthei (1959). This data indicated that 65-75% of vaccinated animals were completely protected whilst of the remaining 25-35%, although infected, most did not abort.

Safford (1959) reviewed the effectiveness of strain 19 vaccination in Montana and Stuart et al.(1959) have reviewed the Californian experience. Jones and Berman (1976) have reviewed the overall U.S. experience with strain 19 particularly emphasising the use of the vaccine in Wisconsin.

McDiarmid (1957) made an extensive study of the duration of resistance conferred by calfhood vaccination with strain 19. From a seven year experiment involving 500 cattle he concluded that one dose of strain 19 at six months of age conferred adequate immunity for five pregnancies and probably for the complete normal milking life of the animal. Other studies on the duration of immunity conferred have been reported by Goode et al.(1956) and Manthei et al.(1951).

Although there is little doubt about the effectiveness of strain 19 vaccination in protecting individual animals and reducing the overall prevalence of disease, strain 19 vaccination will not keep brucellosis out of a clean herd or prevent it from spreading after it has entered a herd (Nadler, 1978). This view has also been stated by King (1971) who found that herd vaccination status did not significantly reduce herd infection rate. Studies in Ontario have also failed to detect significant differences in vaccination levels between infected and non-infected herds (Kellar et al., 1976).

(d) Route of administration

The use of intracutaneous or intradermal administration of Br. abortus vaccines was first reported by Cotton (1932) and by Cotton et al.(1933). They found that strain 19 organisms became localised in the udder when vaccine was given intradermally but not when administered subcutaneously.

Several investigators have found that good agglutination titres have been produced by intracutaneous administration of smaller doses than are required with subcutaneous inoculation (Campbell and Rodwell, 1945; McDiarmid, 1948, 1950; Cotton, 1953).

Cotton (1953) found that agglutination titres stimulated by intracutaneous administration of strain 19 reached peaks as high as those obtained by subcutaneous inoculation but fell to low levels significantly sooner.

In a recent report Plommet and Fensterbank (1976), indicated that intraconjunctival administration of strain 19 vaccine was at least as effective as subcutaneous vaccination and that the vaccine could be administered at any age without risk of producing a serological response that would interfere with diagnosis.

(e) Effect on diagnosis

The diagnostic problems created by strain 19 vaccination are well known. In both the calf and adult agglutinins appear within a few days and reach a peak at two to three weeks, when titres may be similar to those caused by natural infection, i.e. about 2000 i.u./ml. In animals vaccinated subcutaneously with the usual dose, titres soon decline and most are negative six months after vaccination. In calves vaccinated at six to nine months about 80% can be expected to be negative 12 months later and 90% two years later (Stableforth,1959).

The various modifications to the serum agglutination test (SAT) as detailed in a later section were largely developed with the object of finding a test that would not be sensitive to agglutinins produced by vaccination, but would detect those promoted by infection. Although the complement fixation test (CFT) had been used in the early 1900's and was known to be a particularly efficient test, it was not until its relative insensitivity to vaccination and other non specific titres was realised that it found ready acceptance (Wisniowski,1957; Thomsen,1957). Even though the CFT and various other supplemental tests may help in distinguishing vaccination titres from infection titres, it is claimed that as yet there is no valid data indicating that any single test is capable of making this differentiation (Anon., 1977b).

Schilf (1968) reported on the existence of a "masking" effect of strain 19 vaccination. Vaccinated cattle were claimed to take longer to develop reactor titres after exposure to infection than did non-vaccinated animals. Data from US epidemiologists presented by Schilf (1968) showed that Br. abortus was cultured from 462 cows

vaccinated as calves; of these 194 (42%) were not classed as reactors in the standard test. In a group of vaccinated and non-vaccinated cattle exposed to equal numbers of virulent organisms, 75% of non-vaccinated animals reacted to the agglutination test 30 days post-exposure while in the vaccinated group it took 90 days for 75% of the animals to react.

Renoux et al.(1971) experimentally infected a group of strain 19 vaccinated and unvaccinated heifers at between five and six and a half months of gestation. Antibody responses as measured by the SAT and CFT appeared later in the vaccinated group than in the unvaccinated controls. This delayed development of titre in vaccinated animals was evident in groups of animals that eventually aborted as well as in those whose pregnancy did not terminate in abortion. Fensterbank (1973) found that in an experimentally infected group of vaccinated and unvaccinated heifers, the vaccinated animals developed card and complement fixation test titres well before the unvaccinated group. Unfortunately the relative appearance of agglutination test titres was not detailed.

Anderson et al.(1962) noted that vaccination with strain 19 vaccine appears to increase the difficulty of detecting infected animals with the agglutination test. They suggest that detection of exposure to Brucella would be simplified if vaccination were not practised in areas of very low prevalence where the risk of exposure is negligible.

Strain 45/20 Killed Adjuvant Vaccine

McEwan and Samuel (1955) used an avirulent strain (45), and a strain derived from it by guinea pig passage (20), heat killed in an oily base. The "rough" phase induced a level of immunity comparable to that of the "smooth" phase and raised the possibility of developing from the "rough" phase a vaccine which would not produce agglutinins to standard antigen strains and so interfere with serological tests. This strain (45/20) could not be used as a live vaccine as earlier work (Edwards et al.,1945; Taylor and McDiarmid,1949) had shown that it could revert to a fully virulent smooth form.

The major theoretical advantage of strain 45/20 vaccine is

that agglutination titres following vaccination are transient. However, Cunningham (1968) and Cunningham and O'Reilly (1968) found low level persistent agglutinin and complement fixation test responses. Corbel and Bracewell (1976) and Hall et al.(1976) have also noted such responses particularly following a second dose of 45/20. Beh (1975) and Corbel (1976) have characterised the antibodies produced by such a response.

According to Corbel (1976) animals which have not been previously exposed to smooth Brucella antigens initially produce antibodies of the IgM class against rough Br. abortus when inoculated with strain 45/20 vaccine. As the immune response proceeds, IgG_1 and IgG_2 antibodies appear and become predominant. In animals previously exposed to smooth Br. abortus through virulent infection or strain 19 vaccination, antibodies to both smooth and rough antigens are rapidly produced and are of both IgM and IgG classes.

A significant disadvantage of strain 45/20 vaccine is the development of abcesses at the site of injection. Such abcesses caused by the oily adjuvant used in the vaccine may last for more than 12 months and cause serious carcase damage (Cunningham,1966; Hall et al., 1976).

Whereas with strain 19 vaccination one dose has proven to give adequate protection, it has been shown that two doses 10-12 weeks apart are required for optimal protection with 45/20. Even with two doses the protection afforded is no better than and may be inferior to that given by strain 19 (McDiarmid,1972; Worthington and Horwell,1974; Ray and Hendricks,1974).

Ray (1976) has reviewed investigations conducted in the USA into the effectiveness of 45/20 and concludes that 45/20 does not confer adequate resistance on cattle in infected herds subjected to multiple exposures of virulent organisms.

Strain 53H38

This vaccine is made from a strain of Br. melitensis, heat killed and used with an adjuvant. Renoux and Valette (1967a, 1967b, 1967c) studied the effectiveness of 53H38 vaccine and found it to be at least as efficient as live strain 19 vaccine. Agglutination titres produced in response to vaccination were

found to disappear more rapidly than strain 19 induced titres. Possibly because of problems with local reactions at the point of injection 53H38 vaccine has not found widespread acceptance (Dhennin,1973).

Vaccine "P.B."

Pilet-Bonneau vaccine consists of formalin inactivated strain 19 organisms which have been saturated with specific immune sera to reduce agglutinin formation. It was developed by Pilet and Bonneau (Bonneau et al., 1970) but has not been widely used (Dhennin, 1973)

Rev. I.

Rev. I vaccine is a live attenuated strain of Br. melitensis and is widely used for the control of Br. melitensis infection in sheep and goats. It will protect against bovine brucellosis but is not recommended for use in countries where Br. melitensis does not exist (Anon., 1971).

Soluble Antigens as Potential Vaccines

Although very little work has been done in cattle, several laboratories have examined soluble preparations as potential immunogens against challenge with virulent strains of Brucella in laboratory animals. Foster and Ribi (1962) showed that a substance extracted from cell walls by aqueous ether had immunising potency superior to killed whole cells or cell walls. Markenson et al. (1962) examined the immunising potential in mice of sediment from a sonic extract. Rasooly et al.(1967, 1968) have shown that Brucella cell walls can protect laboratory animals against infection with Br. melitensis and Br. abortus. Guinea pigs injected with ribosomes derived from strain 19 organisms, and mixed with an adjuvant, were shown by Corbel (1975b) to induce immunity to challenge by virulent organisms.

Future developments

There is strong evidence that protection against Brucella

infection is dependent on cell-mediated immunity and not on the presence of humoral antibody (Alton, 1977a). Parish (1972) has suggested that the presence of antibody could interfere with the development of cell-mediated immunity and that chemical modification of the specific antigen could leave intact the part that stimulated cell-mediated immunity while inactivating the portion responsible for stimulating antibody producing lymphocytes.

According to the National Research Council Subcommittee on Brucellosis (Anon., 1977b); "It is unlikely that anything will replace strain 19 although data on laboratory animals indicates cell fractions (e.g. aqueous ether or sodium dodecyl sulphate extracts of cell walls) plus proper adjuvants may replace heat killed whole cell vaccines which cause extensive tissue necrosis and abscess formation at the site of injection". The subcommittee suggest that improvements in diagnostic tests are more urgently required than the development of other immunising agents.

Development of Diagnostic Tests for Bovine Brucellosis

Tests used to diagnose brucellosis may be conveniently divided into three groups.

(i) Tests to demonstrate the presence of specific immunoglobulins in the blood, milk, vaginal mucous or semen.
(ii) Tests to demonstrate the presence of Brucella abortus
(iii) Tests to demonstrate a specific allergic reaction to brucellosis.

As mentioned in a previous section the major specific immunoglobulins active in these tests are believed to be IgG_1, IgG_2, and IgM. There is a complication in that the antigenic stimulus may have come from:

(i) Field infection with Br. abortus.
(ii) Vaccination with live strain 19 vaccine.
(iii) Vaccination with killed 45/20 vaccine.
(iv) Other antigens with common determinates, e.g. Yersinia enterocolitica.

Incontrovertible evidence of Brucella infection is obtained by isolation and identification of the organism. Since it is not always possible to isolate the causal organism from infected patients, serological tests play a major role in the routine diagnosis of brucellosis (Alton et al., 1975a).

The first recorded diagnostic test for brucellosis in animals was an agglutination test. A Maltese physician tested the sera of six goats prior to his attempt at experimentally infecting them with the recently isolated Micrococcus melitensis. The blood of five of the six goats showed a strong reaction and thus the first link between Malta fever and goat milk was established (Spink,1956). An agglutination test had previously been applied to the differentiation of typoid and Malta fever in man by Wright (1897).

Full details of the more commonly accepted test methods currently used are given by Alton et al.(1975a).

Agglutination Tests

Traditionally the serum agglutination test has been the main

test used for the diagnosis of brucellosis in man and animals. It is relatively easy to perform and has been well standardised. It was recognised by many of the early workers that the titre obtained with a given serum is influenced by the test methods used. A dried reference serum was prepared in Britain in 1933 (Stableforth, 1936) and this was adopted by the Office International des Epizooties in 1937 following recognition of the divergence of methods used in different countries (Stableforth, 1959). In 1952 a new batch of dried serum equivalent in titre to the original standard was established by the WHO Expert Committee on Biological Standardisation as the International Standard for Anti-Brucella abortus Serum (ISAbS). It was decided that the International unit should be one thousandth of the dried material from one ml of the original serum (Anon., 1954).

Because stocks of the first ISAbS became depleted a replacement standard was prepared and the second ISAbS was established in 1968. The International Unit was then redefined as the activity contained in 0.09552 mg of the second ISAbS i.e. one thousandth of the material from one ml. The antibodies contained in this serum are almost exclusively IgG (Anon., 1971).

Traditionally the standard agglutination test is carried out in test tubes with the antigen suspended in phenol-saline (0.5% phenol 0.85% saline). Because of the occurrence of the prozone phenomena some workers prefer to minimise this effect by using 5% saline as the diluent (Diaz and Levieux, 1972; Fenske, 1977).

Because it is a comparatively easy test to perform the agglutination test has been readily accepted. Despite its widespread use in Europe and America it has in recent years been subjected to more critical study and has been found to be less efficient than other tests. Nicoletti and Muraschi (1966) reported that it failed to classify 39% of 135 culture-positive cattle as reactors. In a later report Nicoletti (1969) claimed that it failed to correctly identify 48% of a group of 165 culture positive cattle. Alton et al.(1975b) found that 11% of culture-positive cattle had less than 100 i.u. of antibody per ml of serum and 4% had less than 30 i.u. Fenske (1977) in a large study claimed that in acutely infected cattle the agglutination test detected only 50% of the infected animals.

It is also acknowledged that the agglutination test becomes

positive later than does the complement fixation test and that it stays falsely positive longer in strain 19 vaccinated animals than other diagnostic tests (Morgan and Richards,1974).

Corbel (1972), Beh (1973), Allan et al.(1976) and Patterson et al.(1976) have investigated the relative importance of the specific immunoglobulin classes in the agglutination, complement fixation and card (Rose Bengal) tests. It is generally agreed that all the major immunoglobulin classes play a role in the agglutination test. IgG_1, however, is said to agglutinate more efficiently with buffered antigen or antigen in 5% sodium chloride (Diaz and Levieux,1972) although Patterson et al.(1976) found this to be so only with sera from strain 19 vaccinated cattle.

Variations of the Agglutination Test

Because the standard agglutination test requires serum dilutions to be made in tubes, and incubated overnight, a number of variations in methods used have emerged.

The Rapid or Plate agglutination test has been used in the USA for a number of years as one of the official tests (Huddleson, 1943). It can be standardised with the international standard serum and its interpretation can be directly related to the tube test (Anon., 1965b).

In an attempt to differentiate between the specific Brucella agglutinins and non-specific agglutinins Rose and Roepke (1957) introduced a modification to the plate test whereby the antigen was buffered at pH 4.0 immediately before use. This test is termed the acidified plate test. Miller (1971) has described an automated version of this test which provides a semi-quantative result.

Numerous other modifications including varying incubation times and temperatures, prior serum inactivation, centrifugation to hasten deposition of agglutinins, and the addition of various salts have been described. Olitzki (1970) has extensively reviewed the literature regarding these procedures.

The Brucellosis Card Test (BCT) or Rose Bengal Plate Test (RBPT)

Following the observations of Rose and Roepke (1957) that an

acidified antigen would destroy the activity of non-specific agglutinins a card test using a stained antigen buffered at pH 3.6 was developed (Nicoletti,1967). The card has been used as an official test in the US brucellosis eradication programme since 1966.

In the UK a similar development took place at Weybridge which resulted in the Rose Bengal Plate test, this was introduced as an official test in 1970 (Morgan and Richards,1974).

The two tests are essentially the same, both utilising the mixing of equal volumes (usually 0.03 ml) of serum and antigen on a white card or tile and then rocking gently for four minutes. Any degree of agglutination is regarded as positive, there is no doubtful classification.

Whereas the British RBPT antigen is made from Br. abortus strain 99, the American BCT antigen is derived from strain 1119-3 cells. Otherwise preparation of the antigens are similar. The RBPT is conducted on a white tile or in haemagglutination tray wells whilst the BCT is carried out on a white card (Brewer Card Test Kit, Hynson, Westcott and Dunning, Inc., Baltimore, Maryland). Antigen for the kit is prepared by the National Animal Diseases Laboratory, Ames.

The card test is usually used as a screening test with animals showing a positive reaction being subject to further testing.

The Coombs (anti-bovine globulin) Test

Another test based on an extension of the agglutination test is the Coombs test. This is one of the most sensitive serological tests for the detection of antibody, particularly for incomplete or blocking antibodies (Morgan,1967).

An anti-bovine globulin preparation is added to a washed non-agglutinating antigen-antibody complex so that the anti-globulin will combine with and agglutinate the previously formed complex. Details of the test procedure are given by Morgan (1967). The major disadvantage of the test is the need for careful repeated washing of the cells after the first reaction.

There has been some confusion over the use of the terms

"blocking" and "incomplete" antibodies. Jones et al.(1957) advised limiting the term "blocking" antibodies to those revealed by the "blocking" test and the term "incomplete" to those revealed by the Coombs test. A serum shown to have blocking antibodies by the blocking test will usually have higher titres of incomplete antibodies as revealed by the Coombs test (Jones and Wilson,1951; Hall and Manion,1953), the Coombs test is therefore preferred.

Cunningham (1967) demonstrated the presence of anti-bovine globulin titres in several sera that were negative to the agglutination and complement fixation tests and concluded that the Coombs test detects a specific incomplete antibody for Brucella. Hadju (1963) reported on the usefulness of the test in detecting chronic infections in Czechoslovakia and Fenske (1977) has reported on its value but warns that it is often over-sensitive. The particular problem of its over-sensitivity in strain 19 vaccinated animals has been investigated by Beh and Lascelles (1973) who found that it was the IgG_1 incomplete antibody that persisted. They suggested that the use of an anti-IgG_2 immunoglobulin in the Coombs test would be helpful in differentiating vaccination from infection titres.

Disulphide Bond Reduction Tests

IgM, which is a pentametre, is broken down by reduction of the disulphide bonds by certain compounds such as mercaptoethanol, cysteine and dithiothreitol. After reduction the molecule loses its antibody activity. These tests are used as presumptive evidence for the presence of IgG by comparing agglutination titres before and after treatment. The mercaptoethanol version originally described by Anderson et al.(1964) is used as one of the so called "supplemental" tests in the US. Its effectiveness has been reported on by Nicoletti and Muraschi (1966) and Nicoletti (1969). The dithiothreitol test is used in the UK (Morgan et al. 1971).

Rivanol Test

Rivanol (2-ethoxy-6,9-diaminoacridine lactate) precipitates all serum proteins except gamma globulins (Frommhagen and Martins, 1963; Huddleson,1965) and has been used as the basis of a

diagnostic test for brucellosis (Nicoletti and Muraschi,1966). The principle of interpretation is the same as that applied for the disulphide bond reduction tests.

The Complement Fixation Test (CFT)

According to Olitzki (1970) the complement fixation test (CFT) was used along with the agglutination test in the earliest investigations on the serological diagnosis of bovine brucellosis in Europe in the early 1900's. There have been many reports in the literature on the superiority of the CFT over the agglutination test, but despite these the test found little use,except as a confirmatory test in some European countries,until relatively recently (Olitzki 1970).

A number of studies in recent years have indicated that the CFT is more specific than the agglutination test and is especially useful in cattle that have been vaccinated with strain 19 vaccine (Burki,1963; MacKinnon,1963; Nicoletti and Muraschi,1966; Nicoletti,1969; Morgan and Richards,1974; Alton et al., 1975b).

A major problem in the development of the CFT has been the lack of a standardised method between laboratories. This has led to a wide variety of titres being given for tests on standard sera (Morgan et al., 1973).

Vaginal Mucus Agglutination Test

This test was introduced by Kerr (1955) and has been further studied by Christie et al.(1968). It is said to be particularly useful in testing cows following suspect calvings when serological titres may not have developed. The presence of antibodies is not significantly influenced by strain 19 vaccination.

The Milk Ring Test

The milk ring test (MRT) was developed by Fleischhaeur (1937) for detecting the presence of Brucella antibodies in milk using a stained antigen. Its most useful application is in the testing

of bulked milk samples from farm vats to indicate the presence or absence of infection in the herd (Roepke and Stiles, 1970). Patterson and Deyoe (1977) provide an up to date discussion on the mechanisms of the test while Olitzki (1970) has extensively reviewed its development and the manufacture of the stained antigen.

Microscopic Examination

Staining methods usually used for the direct examination of foetal material, placenta, discharges and fluids from joints etc., are the Kosters and the modified Ziehl Neelson methods (Stamp et al.,1950). Leech et al.(1964) recorded the results of the examination of 569 membranes. Positive smears were found in only 1.2% of placentae which were declared non-infected using culture or animal inoculation test results as the criterion of infection.

Lapraik et al.(1967) drew attention to the difficulty in differentiating Br. abortus from Coxiella burnetti by this method. In New Zealand where Coxiella burnetti is not known to exist this difficulty does not arise.

Corbel (1973a) has assessed the direct fluorescent antibody test and obtained consistent results. This method was able to differentiate C. burnetti from Br. abortus.

Cultural Tests

Various workers have compared different media for the culture of Br. abortus, (Morgan,1960; Painter et al.,1966; Farrell and Robertson, 1972). Alton et al.(1975a) detail the suggested tissue and types of culture media recognised for optimal isolation of Br. abortus. Serum dextrose agar plates are commonly used with a variety of selective inhibitors - e.g. bacitracin, polymixin B and cyclohexamide. Plates are incubated in 5 to 10% CO_2 in air as most biotypes will grow only in this atmosphere on initial isolation. Robertson et al.(1977) have discussed the development of methods of isolation of Brucella from contaminated sources and have reviewed the development of modern selective media.

Corbel and Morgan (1975) and Morgan and Corbel (1975) have

proposed minimal standards for the descriptions of species and biotypes of Brucella.

The need for biotyping of cultures as an aid to the epidemiologic knowledge of the disease has been expounded by Nelson et al.(1966) and Luchsinger et al.(1973).

Nine biotypes of Br. abortus are recognised and these are identified on the basis of dye sensitivity tests, CO_2 requirement and H_2S production. Alton et al.(1975a) give full details of biotyping procedures. In New Zealand only three biotypes have been recognised from 1140 cultures typed at the Ruakura Animal Health Laboratory. These are:

biotype 1	93.2%
biotype 2	6.4%
biotype 4	0.18%
strain 19	0.18% (Anon., 1977a)

Guinea pig inoculation is often used in conjunction with plate culture when maximum sensitivity is required. Homogenised tissue is normally injected into two guinea pigs which are killed at three and six weeks post inoculation. Spleens and any other inflammed organs or lymph nodes are cultured and a serum agglutination test is carried out at the time of necropsy. Full details are given by Alton et al.(1975a).

Delayed Type Hypersensitivity (DTH) Tests

Although DTH tests for brucellosis have been widely used in sheep, goats and man in Eastern Europe (Didovets, 1965), they have not been recommended for use in cattle.

Olitzki (1970) has reviewed all earlier attempts at DTH testing. Problems which have arisen include differentiation of infected from strain 19 vaccinated animals, false positive reactions at repeat tests and false positive reactions in cattle infected with tuberculosis.

It is unlikely that a DTH test will replace serological testing of cattle in the near future. However, renewed interest in this test may be stimulated now that the allergen has been characterised (Jones and Berman, 1975, 1976).

Other Tests

Numerous other serological and milk tests have been used for the diagnosis of brucellosis in cattle. Among these are the conglutination complement-absorption (complement fixation) test (Rice, 1952), passive haemagglutination test (Freeman et al., 1955), indirect haemagglutination test (Carrere and Roux, 1952), precipitin test (Reiter, 1936), flocculation test (Hunter and Colbert, 1956) indirect fluorescent antibody test (Biegeleisen et al., 1962), surface fixation test (Castaneda, 1950), gel diffusion test (Bruce and Jones, 1958), 45/20 anamnestic test (Cunningham and O'Connor, 1971), indirect haemolysis test (Plackett et al., 1976), radioimmunoassay (Chappel et al., 1976), indirect enzyme-labelled antibody test (Saunders and Clinard, 1976), milk plate test (Blake et al., 1952), whey agglutination test (Cameron and Kendrick, 1955), whey complement fixation test (Robertson and Farrell, 1968), and lymphocyte transformation tests (Swiderska et al., 1971). Of these the indirect haemolysis, indirect enzyme-labelled antibody test and lymphocyte transformation tests appear to show most promise for future use.

The Evaluation of Diagnostic Tests

When a new test for a disease is being evaluated it is customary to perform the test in two selected groups of animals; one group known not to have the disease and the other group all known to be infected. Test results are usually given as positive or negative or by some numerical value which can later be evaluated in terms of a particular degree of positivity or negativity.

An alternative method, particularly useful in preliminary observations of the effectiveness of a test, is to compare the new test results with those of another accepted reliable test.

A third method which has received little attention but which can be applied in certain areas of veterinary medicine, is the trial use of a particular test in an eradication programme. This method is at present being used in a large scale field evaluation of the indirect haemolysis test in Victoria, Australia, and will be the eventual means by which the automated complement fixation test is judged in the New Zealand eradication scheme.

Publications describing the mathematical concepts of test evaluation include those of Thorner and Remein (1961) and Schwabe et al. (1977).

Sensitivity and specificity are two important attributes of a test. Sensitivity is the ability of a test to correctly detect a diseased animal. Specificity is the ability of a test to correctly detect animals which are not diseased.

$$\text{Sensitivity} = \frac{\text{diseased animals with positive test}}{\text{diseased animals tested}}$$

$$\text{Specificity} = \frac{\text{non-diseased animals negative to test}}{\text{non-diseased animals tested}}$$

Martin (1977) has discussed the evaluation of tests with respect to specificity and sensitivity and graphically illustrated errors inherent in using inadequate tests. Vecchio (1966) showed how the "predictive value" of tests varies according to the infection status of the population under test.

$$\text{Predictive value (PV+) (of positive test)} = \frac{\text{diseased animals with positive test}}{\text{total animals with positive test}}$$

$$\text{Predictive value (PV-) (of negative test)} = \frac{\text{non-diseased animals with negative test}}{\text{total animals with negative test}}$$

Predictive values of positive and negative tests at varying disease prevalences when sensitivity and specificity each equal 95% are given in the following table taken from Vecchio (1966).

Actual disease prevalence %	PV+ %	PV- %
1	16.1	99.9
2	27.9	99.9
5	50.0	99.7
10	67.9	99.4
20	82.6	98.7
50	95.0	95.0
75	98.3	83.7
100	100.0	-

The important concept illustrated by this example is that in terms of an eradication scheme where the disease prevalence is diminishing, a test that was adequate early in the programme may not be suitable later when diseased animals are uncommon.

Another aspect of the evaluation of tests is that of the practicability and precision of a given test. Even though a test may be well suited according to its sensitivity and specificity it may be difficult to perform, subject to errors in its performance and interpretation, or require expensive reagents or equipment. The United States experience with the complement fixation test for brucellosis has been that it is too difficult to standardise and that a combination of simpler tests (Rivanol, mercaptoethanol, rapid plate) will give an equivalent result for less effort (Nicoletti, 1969; Pietz, 1977). Other workers have obviously felt that the extra time and care required for complement fixation testing is justified by its superior sensitivity and specificity. Tests such as the radioimmunoassay procedure (Chappel et al., 1976) require expensive and complex equipment and for this reason are probably limited to use as a research tool.

An important consideration when evaluating tests against results of culture is that whereas the sensitivity of a given test is readily derived, the specificity is not. Since a negative culture result cannot be taken as proof of the absence of infection it is usual for estimations of specificity to be made in known non-infected populations (e.g. McKinnon, 1963). However, such populations may not be representative and development of titres by animals exposed to, but not infected by, the disease may cause the test specificity to be quite different in a partially infected population from that in a disease free population.

Evaluation of the Brucellosis Card Test (BCT) or Rose Bengal Plate Test (RBPT)

The brucellosis card test was developed following the observations of Rose and Roepke (1957) that the rapid plate agglutination test would, with an acidified antigen, destroy the activity of non-specific agglutinins.

Both the card and Rose Bengal plate tests utilise a Rose Bengal stained antigen buffered at pH 3.65 and adjusted to contain 8% of cells by volume. The two tests are sometimes known as Buffered Brucella Antigen tests (BBA). Details of antigen preparation and test methods are given by Alton et al.(1975a).

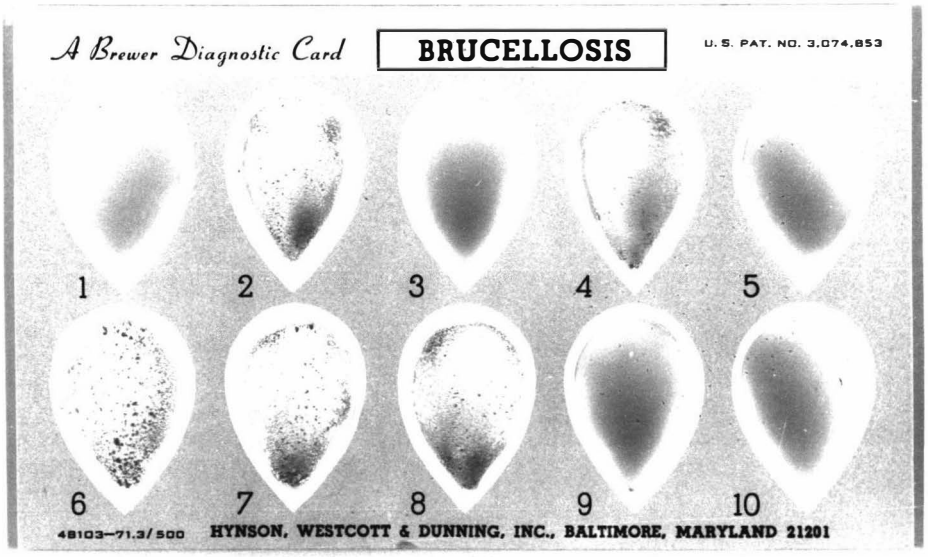
Nicoletti (1967) initially evaluated the use of the card test and found that it was positive in all of 184 culturally positive cattle whereas the tube agglutination test detected only 48% of these as being reactors. Morgan et al.(1969) using the same antigen as that prepared for the card test but using serum instead of plasma for the test, found good agreement with the complement fixation and serum agglutination tests.

Following this early work the card test gained acceptance as a rapid screening test whereby any sera positive to it were retested using traditional techniques. The early acceptance of the card test in the US was also prompted by increasing farmer agitation for a more rapid testing method, particularly from beef cattle ranchers in the southern states (Becton,1976).

Studies relating bacteriological culture results to the test have been few. Nicoletti (1967) showed that all 184 culture positive cattle that he examined gave positive card test reactions. Fensterbank (1973) in a study of experimentally infected heifers found that 1.6% of the serum samples from known infected animals were negative to the Rose Bengal test. Mylrea (1972) in an investigation of serological tests in naturally infected cows found that two out of 42 culture positive animals gave a negative RBPT (i.e. 4.8% false negatives). In a further study (Mylrea and Fraser,1976) all of 40 culture positive cows gave a positive RBPT. Alton et al.(1975b) in a study of naturally infected animals, found that only one of 79 animals gave a negative RBPT. The worst correlations appear to be those obtained in trials

Figure 1 Brucellosis card test

Nos.	1	3	5	9	10	negative
No.	4				++	positive
Nos.	2	6	7	8	+++	positive


A Brewer Diagnostic Card
BRUCELLOSIS
U. S. PAT. NO. 3,074,853
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48103—71.3/500
HYNSON, WESTCOTT & DUNNING, INC., BALTIMORE, MARYLAND 21201

preceding the New Zealand eradication scheme when 27 out of 213 culture positive animals (12.7%) were negative to the card test (Adlam,1978).

Studies in Great Britain and Ireland have attempted to relate the RBPT to the complement fixation (CFT) and serum agglutination (SAT) tests. Morgan et al.(1969) tested 6424 unselected cattle sera and found the majority of sera with positive CF tests were also positive to the RBPT.

There seems to have been some difficulty in the early stages in deciding whether to compare the RBPT with the CFT or the SAT or both. When compared to the CFT the RBPT has shown good sensitivity (Morgan et al.,1969; Davies,1971; Morgan and Richards,1974), whereas when compared to the SAT, especially in the suspicious (marginally positive) range, it has been less enthusiastically received (O'Reilly and Cunningham,1971; Prior et al., 1975). Canadian investigators examining sera from a largely Brucella-free population found that 99.8% of the negative card test results were confirmed by the serum agglutination test, which was the official test. On the other hand 56 out of 152 (37%) agglutination test reactors were missed by the card test whereas only 32 out of 482 (6.6%) sera suspicious or positive to the complement fixation test were card test negative (Prior et al.,1975). The question of whether to accept the CFT or SAT test as the definitive test has been coloured somewhat by the traditional acceptance of the SAT as the official test and the practical difficulties of using the CFT on a wide range of unselected sera or sera from known non-infected herds.

In terms of test sensitivity the card test is generally recognised as being too sensitive to use as a definitive test but because of its ease of application and good specificity it has been used extensively as a screening test. The bacteriological studies reported by Nicoletti (1967), Mylrea (1972), Alton et al. (1975b), Mylrea and Fraser (1976) and Adlam (1978) all include observations on culture negative animals which indicate that the card test is oversensitive, especially in strain 19 vaccinated animals.

In New Zealand the relationship between the BCT and the CFT has been investigated by Timbs et al.(1978a) who emphasised that

the dynamics of the population under test must be considered when assessing the sensitivity of a test. It was found that in heavily infected herds 78.6% of BCT positive sera were also CFT positive whereas in lightly infected herds only 12.6% of the BCT positives were CFT positive. Thus the sensitivity of the BCT with respect to the CFT had changed from .786 to .126 simply by considering a different sample. No doubt the sensitivity of the CFT changes in a like manner with respect to true infection. Nicoletti (1967) found a similar change in sensitivity when evaluating the BCT with respect to culture in two groups of herds with different infection rates.

Reasons for the apparent oversensitivity of the card test are not clear and have apparently not been specifically investigated. Allan et al.(1976) have drawn attention to at least two types of non-specificity in agglutination reactions.

(i) That due to immunologically non-specific agglutination. This is due to non-specific agglutinins which can agglutinate a variety of unrelated bacterial antigens.

(ii) That due to immunologically specific but non-diagnostically specific agglutins, e.g. prior strain 19 vaccination.

Innumologically non-specific agglutination has been investigated by Hess (1953a, 1953b) and Rose and Roepke (1957). Organisms that have been reported as cross-reacting with Brucella include Pasturella (Mallman,1930; Berman,1956; King,1961; Morse et al.,1953). Vibrio (Morse et al.,1953; Kiggens et al., 1955), Salmonella (Morse et al.,1953; Corbel,1975a) and Yersinia (Ahvonen et al.,1969; Corbel and Cullen,1970).

Another aspect of the evaluation of the card test which requires consideration is its effectiveness in detecting early infections. Because of the long incubation period of the disease it would be advantageous to employ a test that is efficient at detecting early or latent infections. Early investigators observed that with infected animals the card test became positive before the CFT or the SAT (Nicoletti,1967; Morgan et al.,1969; Davies,1971). As thorough investigation of this aspect requires experimental infection of cattle, there have been few comprehensive studies of it. Fensterbank (1973), in an experiment

involving the inoculation of 50 two year old heifers, found that the RBPT became positive earlier than did the CFT or SAT. On average the RBPT became positive 40.5 days after infection for unvaccinated cattle and 29.5 days after infection for animals vaccinated as calves with strain 19. These figures compare with 49.5 and 34.3 days respectively for the CFT. The serum agglutination test took an average of 63.2 days to become positive in the group as a whole. Pietz (1977) found that in experimentally infected animals it took an average of 62 days after exposure for the agglutination test to detect infected animals whilst the card test took only 43 days.

Immunoglobulins active in the Card Test

Results of studies to identify the class or classes of immunoglobulin active in the card or RBP tests have been conflicting. Corbel (1972, 1973b, 1973c) Diaz and Levieux (1972) and Wood and Corbel (1973) considered that the only immunoglobulin active in the test was IgG_1. Levieux (1974) found that IgG_1 and IgM were active but that IgG_2 activity was inhibited by the low pH. Other studies have indicated that IgG_1, IgG_2, and IgM are all active (Beh,1973; Allan et al.,1976; Patterson et al.,1976). A further attempt at identifying immunoglobulin classes active in the card test is made in this thesis.

It has been suggested that the discrepancies in results may be due to different methods chosen for analysis and because sera vary so much in their immunoglobulin content (Jones,1977). In the knowledge that it is IgM agglutinins that tend to persist after strain 19 vaccination, and that the card test is more "over sensitive" in strain 19 vaccinated populations, especially in younger animals (Timbs et al.,1978a),then the theory that IgM is active in the card test reaction tends to carry most weight. Further circumstantial evidence for the role of IgM in the card test is provided by the fact that IgM is the first immunoglobulin produced following infection and the card test detects infection earlier than other tests (Rice et al.,1966,1967; Rice and Boyes, 1971; Fensterbank,1973).

Evaluation of the Complement Fixation Test for Brucellosis

The complement fixation test was used along with the agglutination test in the earliest investigations into bovine abortion in Denmark and England. Although most of the early workers agreed that the test was more efficient than the agglutination test, it was apparently considered too complex for routine use. Jones et al.(1963) and Olitzki (1970) have extensively reviewed the early literature and Jones et al.(1963) have highlighted the observation that comprehensive reviews of brucellosis by Spink (1956), Stableforth(1959) and Dalrymple-Champneys (1960) have dismissed the test with a brief statement.

Of the many variables in the test method, two which have great influence on its effectiveness are the time and temperature of the primary incubation and the type of antigen used.

Some workers have expressed a preference for the cold fixation (4 ^{o}C for 18-24 hours) method (Zeissig and Mansfield,1930; Trilenko,1957; Isayama,1961) and it has been the experience of workers at Wallaceville that the cold test is able to detect a small number of infected animals which are otherwise missed by the warm method (Te Punga,unpublished). Many laboratories prefer to use the warm fixation (37 ^{o}C for 30-60 minutes) technique and of 25 laboratories in 18 countries participating in a recent survey 10 used warm fixation and 13 used cold fixation with two laboratories using both (Morgan et al.,1973). Preference for the warm method is generally due to its convenience and the rapidity with which results are obtained.

Various different antigen preparations have been used including a trichloracetic acid extract (Renoux and Alton,1957) and soluble antigens (Renoux,1957). The heat killed whole cell antigen as used for the agglutination test has, however, proved to be quite satisfactory and is almost universally used as the standard antigen.

There are relatively few reports of studies relating complement fixation test results to bacteriological findings. McKinnon (1963), in a comprehensive work, laid the framework for the International Standard Anti-Brucella abortus Serum. He examined sera from 1134 vaccinated, unvaccinated, Brucella-free and infected cattle and proposed determinant titres for

Figure 2 Auto-Analyzer adaptation of the complement fixation test for brucellosis


0 2 4 6 8
Auto Analyzer
SAMPLER
3

classification of negative, suspect and reactor animals.

Lambert and Amerault (1962) demonstrated positive CFT titres in 16 experimentally infected cattle, while among 20 which resisted challenge only one showed a transient titre. In more recent studies Nicoletti and Muraschi (1966) found that 114 out of 117 cattle (97.5%) which gave positive cultures also gave positive CF tests; two of the three cattle improperly identified gave suspicious reactions. Nicoletti (1969) in a further study obtained 116 CFT positive titres from 119 culture positive cattle (97.5%). Renoux et al.(1971) found that 10 out of 47 experimentally infected heifers gave negative or doubtful agglutination tests at abortion or calving whereas only three gave negative complement fixation tests. Fensterbank (1973) experimentally infected 43 heifers in mid-gestation and they all gave a positive CFT response, the average time between infection and development of titre was 34.3 days for strain 19 vaccinated heifers and 49.5 days for those unvaccinated. Mylrea (1972) isolated Br. abortus from 42 cows and sera from 41 of these gave a positive CF titre and in a later study (Mylrea and Fraser,1976) 47 positive CF titres were obtained from the sera of 47 culture positive cows. Alton et al.(1975b) showed that the CFT detected all of 79 cattle giving positive lymph node cultures. New Zealand trials undertaken to assess the potential of various tests also showed the cold CFT to be the most sensitive test although only 110 out of 131 infected cattle (84%) were detected by it. This compared with 84% detected by the card test and 80.1% detected by the SAT (Adlam,1978; Te Punga,unpublished). An interesting aspect of the New Zealand trials was that 19 of the 131 culture positive animals were negative to all serological tests applied.

All of the above investigators have concluded that the complement fixation test is the best single serological test available for brucellosis in cattle.

Immunoglobulins Active in the Complement Fixation Test

Investigations into the various classes of immunoglobulins active in the CFT have generally been conducted in parallel with investigations of the card and serum agglutination tests. Rice

et al.(1966) have claimed that IgM does not fix complement, however, Beh (1973), Levieux (1974), Allan et al.(1976) and Patterson et al. (1976) all agree that both IgG_1 and IgM immunoglobulins fix complement whilst IgG_2 does not. Allan et al.(1976) maintain that IgM probably fixes complement twice as efficiently as IgG_1 on a weight basis, however, because it is heat labile in serum, it may be destroyed during the inactivation process commonly performed prior to conducting the CFT.

The Prozone Phenomenon

The use of the complement fixation test for brucellosis is complicated by the occasional occurrence of "prozones". Sera exhibiting this effect appear to give negative reactions in low test dilutions while giving positive reactions in higher dilutions. Although the occurrence of prozones in the agglutination test is well known and has been investigated (Glenchur et al., 1961; Cho and Ingram,1972) there has been little comment until recently on the significance of prozones in the CFT although they were recognised by Burki (1957). Alton et al.(1975b) noted the existence of prozones in both the warm and cold fixation tests but they went to much higher dilutions in the warm test. Plackett and Alton (1975) and McNaught et al.(1977) investigated the immunogenic mechanism of prozones and found that specific IgG_2 antibodies to Br. abortus could block complement fixation by IgG_1 and IgM antibodies. McNaught et al.(1977) also noted that the relative antigen concentration has a marked influence on the extent of prozoning, a high antigen concentration inhibits prozoning while a low concentration enhances it.

The importance of this IgG_1/IgG_2 interaction is highlighted by the fact that the ratio of the two classes can vary widely in cattle and in particular, serum IgG_1 is depleted shortly before parturition by transfer to the mammary gland while serum IgG_2 levels remain high (Brandon et al., 1972; Williams and Green, 1976). Thus prozone reactions may be more likely to occur at about the time of parturition.

Development of Automated Serological Testing Methods for Bovine Brucellosis

Automated serological testing methods have been a recent development compared with the much earlier introduction of automated biochemistry. An excellent review of the methods and machinery in current use for automated serology is given by Kwantes (1976). A bibliography on rapid methods and automation in microbiology and immunology has recently been published by Palmer and LeQuesne (1976).

Automated testing methods are generally divided into continuous flow and discrete sampling systems (Stevens 1973). In continuous flow systems samples separated by air bubbles are fed through a tube and reagents are added to the stream so that reactions take place and are measured within the tubes. In the discrete sampling systems individual samples are processed separately.

The Technicon Auto-Analyzer* was developed during the 1950's to provide a rapid method of performing clinical biochemical analyses. The principle used was that of continuous flow analysis and in 1957 the Auto-Analyzer became the first commercially available fully automated testing system.

Joubert et al.(1967) provided the first description of a complement fixation test for brucellosis adapted to the Auto-Analyzer, and later, Miller et al.(1973), described a similar system developed in Britain. Auto-Analyzer adaptations of the agglutination test have been described by Joubert et al.(1967), Vargues et al.(1968) and Miller (1971a; 1971b). An automated Rose Bengal Plate test has been developed at the Central Veterinary Laboratory, Weybridge, and has been documented by Gower et al. (1974). The Autotape machine used in this application has the capacity to perform 1200 tests per hour.

The automated complement fixation test used in the New Zealand brucellosis eradication scheme was developed at the Wallaceville Animal Research Centre and was originally described by Te Punga (1971). It has received further mention by Elliot and Pullan (1973) and Timbs et al.(1978b). The initial evaluation of the

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* Technicon Equipment Corp. N.Y.

test with respect to its correlation with animals known to be infected and free of disease, and with other manual tests has not been published. This thesis further examines the efficiency of the test with particular respect to its capacity to detect prozoning sera.

Another semi-automated testing method which is becoming widely used in serology is the micro adaption of standard tube tests. A machine may be used to perform dispensing and serial dilution functions thus automating an otherwise manual micro-test system. Micro complement fixation tests are used in the Australian and New Zealand brucellosis eradication schemes, Alton (1977b) and Timbs et al.(1978b) have described the methods used.

Brucellosis Eradication Schemes

Bovine brucellosis control or eradication schemes have been attempted in many countries. Such programmes have been justified on the grounds of economic loss by infected cattle and because of public health considerations. In New Zealand the major stimulus for eradication was the threat of discontinued market access for meat products, particularly to the European Economic Community.

The initial approach to control has generally been to declare brucellosis a notifiable disease. An Act of 1903 in Norway required cattle owners to notify cases of contagious abortion and in 1920 brucellosis was made a notifiable disease in Denmark (Thomsen,1957). Following the development of the serum agglutination test control programmes based on a test and slaughter policy were begun in 1934 in the USA, Denmark 1936, Norway 1935, Finland 1938 and Sweden 1938, (Thomsen,1957; Schilf,1972).

Countries having completed or virtually completed bovine brucellosis control or eradication schemes include Denmark (declared free 1970), Norway (1952), Sweden (1957), West Germany, United States, Canada, Finland, Northern Ireland, Italy, Luxembourg, Switzerland, Czechoslovalia and Tasmania.

By 1976 extensive programmes were operating in Japan, Austria, Australia, New Zealand, Hungary, East Germany, Yugoslavia, USSR, and Great Britain.

CHAPTER 2. CULTURAL AND SEROLOGICAL STUDIES

Introduction

Isolation of *Brucella abortus* from the tissues of an infected animal is the only indisputable evidence available that the animal is infected. Because of the absence of any clinical signs or gross pathology, and the need to culture a variety of tissues, slaughter of the suspect animal is generally required before an assessment of its true infection status can be made.

In the New Zealand Brucellosis Eradication Scheme the brucellosis card test (BCT) is often used as a screening test and positively reacting sera are then submitted to the complement fixation test (CFT) for definitive diagnosis. In many instances all sera from herds are card tested and submitted for CF testing despite the card test result.

Herds are retested regularly at 2-4 month intervals until declared free of brucellosis (Adlam *et al.*, 1978a).

It has been noticed that many animals may be repeatedly BCT positive and CFT negative for some months (Timbs *et al.*, 1978a). Such BCT+/CFT- reactions could be the result of some related or non-specific antibody which is detected by the card test but not by the CF test.

This section describes cultural and serological studies carried out on a group of BCT+/CFT- animals.

Materials and Methods

Selection of Cattle for Slaughter

Twenty cows which had been BCT+/CFT- for at least three previous tests were selected for slaughter from 12 herds in South Taranaki. The age of these animals ranged from three to 10 years. As far as could be determined, all had been vaccinated as calves with live *Brucella abortus* strain 19 vaccine.

Selection was solely on the basis of each having had at least three previous tests BCT+/CFT-, no account was taken of the previous herd testing history. The herds from which these animals came had histories of continuing reactors despite repeated testing. These reactors did, however, tend to occur sporadically so that the total number removed was not unduly large and the herds could not be

said to be heavily infected.

Collection of Specimens

Because of financial constraints it was necessary for the purchased cows to go directly for slaughter. It was not possible to collect serum or milk specimens or to observe them for a period prior to slaughter. The animals were routinely slaughtered at an export freezing works and approximately 3 ℓ blood and various tissues were collected from each animal during carcase proceessing.

Sections of uterus, spleen, udder, and mesenteric, supra-mammary, iliac, retropharyngeal, sub-maxillary, and parotid lymph nodes were collected in individual plastic bags and refrigerated overnight before culturing. Sera were stored at -20 ^{o}C.

Culture Technique

Each tissue was defatted, dipped in 95% ethanol, flamed and placed in a sterile plastic bag with an approximately equal volume of 0.85% saline. The bag was placed in a Colworth Stomacher* and the tissue pounded for approximately 30 seconds. Two drops of the resultant homogenate were spread onto culture media and 0.5 ml was retained for guinea pig inoculation.

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* A.J. Seward Ltd., Lond.

Brucella selective medium was prepared according to the method of Alton and Jones (1975a) using a serum dextrose agar base and cycloheximide, bacitracin and polymixin B as antibacterials. Blood agar was prepared by adding 5% bovine blood to a nutrient agar base. Homogenate from each tissue was inoculated onto the medium. All plates were incubated at 37 °C in an atmosphere of 5% CO_2-in-air and examined at three and five days for evidence of growth. Each batch of medium was tested for its ability to sustain growth by inoculating with Br. abortus biotype 2.

Suspect Brucella colonies were examined microscopically and if necessary replated and later tested using a slide agglutination test against control anti-serum. Any other uniform growth on blood agar plates was also examined carefully and subjected to routine biochemical tests as required to establish identity. Use was made of API 20* multi-test strips to identify entero-bacteriaceae.

Guinea Pig Inoculation

Following homogenisation 0.5 ml of each sample was placed in a single sterile bottle so that a composite sample of homogenates from each animal was available for guinea pig inoculation.

One ml of each of the 20 composite homogenates was injected intramuscularly into the left hind leg of each of 20 guinea pigs. Six weeks later the guinea pigs were bled and euthanased. At autopsy all organs were examined carefully for any sign of abnormality and all spleens were taken aseptically for direct culture onto blood agar and Brucella selective medium. Serum agglutination and complement fixation tests were performed on all sera.

Preparation of Whole Cell Antigen

For most of the standard serology a standardised concentrate of Br. abortus strain 99 preserved with 0.5% phenol was used. This was obtained from a commercial source+.

When required, fresh unpreserved antigen was prepared by

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* Analytab Products Inc. N.Y.

+ Brucella abortus standardised concentrate. I.C.I. Tasman Vaccine Ltd., Upper Hutt, New Zealand.

culture of strain 99 or strain 19 Br. abortus on potato infusion agar in Roux flasks as described by Alton et al.(1975a). After harvesting, the organisms were heat killed at 60 °C for 1 hr, centrifuged and resuspended in phosphate buffered saline (PBS) pH 6.4. Suspensions were kept at 4 °C until required, but being unpreserved were used within 14 days.

Preparation of Ultrasound Treated Antigen

Wellcome opacity tubes+ were used to prepare a suspension of 1 x 10^{11} cells/ml from freshly harvested, heat killed, Br. abortus strain 99 or strain 19. The suspension was made in distilled water. Cells were sonicated for 20 minutes in an M.S.E. ultrasound disintegrator+. An ice jacket was used to prevent excessive heating. Following ultrasound treatment the suspension was centrifuged at 4000 g for 30 minutes to remove cell debris. The supernatant was then used as a soluble antigen.

Preparation of Ether Treated Antigen

The method used was essentially that of Ribi et al.(1959). Both commercially prepared and preserved Br. abortus strain 99 and freshly prepared strain 19 organisms were used.

Washed cells were suspended in 0.85% sodium chloride to a concentration of 1 x 10" cells/ml, and shaken with excess ether in a separatory funnel for 1 min. The suspension was left to stand overnight. The aqueous phase which had settled to the bottom was then removed and air was bubbled through it to remove any excess ether. Insoluble residue was removed by centrifuging at 4000 g for 30 minutes.

Brucellosis Card Test (BCT)

In its commercially available form this test is known as the Brewer Card Test*. Full instructions accompany the kit.

+ Wellcome Research Laboratories, Beckenham, Kent.

+ Measuring and Scientific Equipment Ltd. Lond.

* Brewer Diagnostic Kits, Hynson Westcott and Dunning Inc., Baltimore, Maryland, U.S.A.

0.03 ml volumes of serum and antigen were measured on to white card, each card held 10 samples. After mixing with a stirrer the card was placed on a rocking machine and rocked for four minutes, results were then read immediately.

Reactions were scored as follows:-

- \- no visible agglutination
- \+ very fine particles present
- ++ fine particles observed with some clumping
- +++ complete clumping of antigen

Serum Agglutination Test (SAT)

This test was performed according to the method of Alton et al.(1975a). A series of doubling dilutions of each test serum was made in phenol saline and an equal volume of diluted "standardised concentrate" antigen added. Incubation took place overnight at 37 ^{o}C. Titres were read according to the degree of agglutination present after incubation. The degree of agglutination in each tube was graded from 0, no agglutination, to 4, 100% agglutination.

Coombs Test

Following reading of the agglutination test, tubes with 2+ or more agglutination were discarded. All remaining tubes were centrifuged at 2000 g for 20 minutes and the supernatant discarded. One ml of phenol-saline was added to each tube and the deposit resuspended with the aid of a pasteur pipette or vortex mixer. Centrifugation and resuspension was repeated and following the third centrifugation 0.45 ml of phenol-saline was added and the deposit resuspended. At this stage 0.05 ml of diluted rabbit anti-bovine globulin serum was added and the tubes incubated overnight. Results were read as for the agglutination test.

Titration of Rabbit Anti-bovine Globulin (Coombs reagent)

A known high titre Coombs positive serum was taken and diluted in doubling dilutions to 15 tubes. This was repeated for five sets of tubes. A serum agglutination test was performed by

adding antigen and incubating. The following day tubes showing less than 2+ agglutinations were centrifuged and the antigen washed as described above. After the final centrifugation 0.45 ml phenol-saline was added to each tube. Suitable dilutions of rabbit anti-bovine globulin (Coombs reagent) were made, 1:5, 1:10, 1:20, 1:40 and 1:80. 0.05 ml of each dilution of Coombs reagent was added to each tube in its corresponding series, thus each test series had a different dilution of Coombs reagent. The tubes were incubated overnight and results read the following day as for an agglutination test.

The Coombs reagent dilution in the series of tubes having the highest titre was taken as the optimal dilution to use in the test proper.

Rivanol Agglutination Test

A 1% solution of Rivanol (2 ethoxy-9 diamino acridine lactate) in distilled water was prepared. Equal volumes of 1% Rivanol and serum (0.5 ml each) were prepared, shaken and allowed to stand at room temperature for 15-30 minutes. The suspension was centrifuged at 1000 g for 10 minutes and the supernatant used in an agglutination test, replacing normal serum. Account was taken of the differing equivalent serum dilution obtained after Rivanol treatment i.e. the supernatant contained the equivalent of 50% serum.

2-Mercapto-ethanol Test (2-ME)

Two methods were used for this test.

(a) Serum was treated by preparing a 0.1 M solution of 2-ME containing serum in a 1:5 concentration (i.e. 0.7 ml saline, 0.2 ml serum, 0.1 ml of 1M 2-ME). This solution was then used as the first tube in an agglutination test series which used normal saline as the diluent in the remaining tubes.

(b) The agglutination test was performed in the standard fashion except that a 0.05M solution of 2-ME in normal saline was used in place of phenol-saline as the diluent.

The antigen concentrate used in the 2-ME test was centrifuged to remove the phenol-saline diluent and resuspended in normal saline to a dilution equivalent to that obtained by diluting the standardised concentrate 10 times. This phenol-free antigen was then used in each 2-ME test and in the agglutination tests performed in parallel to them.

Complement Fixation Test (CFT)

Both automated and manual complement fixation tests were used. The automated CFT is described in Chapter 6.

The method used for manual CF tests resembled that described in the U.S. Public Health Service Monograph No. 74 (Anon., 1965a). Both macro and micro test variations were used. Barbital buffered saline was used as the diluent for all reagents (see Appendix I).

Reagents were titrated in macro volumes. Haemolysin* was titrated by the plateau method designed to give optimally sensitised erythrocytes. Five 50% haemolytic units of guinea pig complement+ were used. A commercially available standardised concentrate of Br. abortus+ as used in the agglutination tests was used as the antigen. Sonicated and ether-water extracts of Br. abortus prepared as described previously were also used as antigens. Antigens were titrated in a block titration against a positive serum. Sheep blood was collected in Alsever's solution (see Appendix I) and left to stand at 4 °C for at least one week before being centrifuged and the cells washed three times with barbital buffer. The erythrocytes were standardised to 2% by centrifuging an approximately 50% suspension at 1000 g for 10 minutes, in a graduated tube, recording the concentration, and diluting accordingly.

Both warm fixation (37 °C for 30 mins) and cold fixation (4 °C for 18-24 hrs) methods were used. For the warm fixation test doubling serum dilutions commencing at 1:4 were used while for cold fixations the test series started at 1:10. Reactions were described according to the degree of haemolysis in each tube.

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* Commonwealth Serum Laboratories, Parkville, Vic.

\+ Becton, Dickinson and Co., Cockeysville, Maryland.

\+ I.C.I. Tasman Vaccine Ltd. Upper Hutt, N.Z.

0%	haemolysis	4
25%	"	3
50%	"	2
75%	"	1
100%	"	0

Serum and complement controls and a test serum of known titre were included with each batch of tests.

Sera were inactivated at 58 ^{o}C for 50 minutes. For the macro test 0.25 ml volumes of diluted serum, complement, antigen and haemolytic system were used. For the micro system 0.025 ml unit volumes were used.

Rivanol Complement Fixation Test

After inactivation at 58 ^{o}C for 50 minutes serum was treated with an equal volume of 1% Rivanol. In some tests any surplus Rivanol was removed from the supernatant by treating it with 5% w/v NaCl and leaving to stand overnight. A precipitate containing the Rivanol formed and was centrifuged off. The treated serum was then dialysed against barbital buffer (pH 7.4) to reduce the salt content. In other tests the supernatant recovered directly after Rivanol treatment was used despite the presence of some colour due to surplus Rivanol.

The Rivanol treated serum was used in the complement fixation test as a direct substitute for normal serum except that it was only half normal serum strength due to dilution with the Rivanol solution.

Gel Diffusion Test

This test was performed according to the method of Ouchterlony (1953). The diffusion medium was 1.2% Agarose in 0.85% saline containing 0.1% sodium azide. Sonicated and ether-water treated antigens were used, plates were incubated at room temperature and read daily for three days.

Indirect Haemolysis Test

The method of Plackett et al.(1976) was used. After making

doubling dilutions of serum in barbital buffer (pH 7.4) in micro-titre plates, an equal volume of washed antigen-sensitised bovine red blood cells was added and after 15 minutes incubation at room temperature one drop of a 1:10 dilution of guinea pig serum (complement) was added. Plates were incubated for 60 min at 37 ^{o}C with constant shaking and stored overnight at 4 ^{o}C to allow unlysed cells to settle. The endpoint was taken as the highest dilution showing complete haemolysis.

Recording and Interpretation of Titres

To facilitate result notation while still providing an indication of the degree of reaction at each dilution of the test series the following system of result recording was adopted.

The degree of reaction within each tube of a series was graded between 0 and 4. In the case of agglutination tests

0	=	no agglutination
1	=	25% "
2	=	50% "
3	=	75% "
4	=	100% "

For complement fixation tests

0	=	100% haemolysis
1	=	75% "
2	=	50% "
3	=	25% "
4	=	no "

This degree of reaction in each tube of the test series was recorded. Thus for a series in which the first two tubes exhibited a reaction of 4 and the third tube showed a reaction of 2 the notation used was 442.

To simplify the expression of higher titres an index figure was used to indicate that a particular reaction occurred in a series of consecutive tubes. e.g. The series, 444443 may be expressed as $4^{5}3$ where the first five tubes had a reaction of 4 and the sixth tube had a 3 reaction. Using this system it is understood that the reader is familiar with which dilutions were made

in a particular test. For all agglutination tests serum-dilutions were 1:10, 1:20, 1:40, 1:80 etc. For the warm fixation CFT dilutions were 1:4, 1:8, 1:16 etc. For the cold fixation CFT dilutions were 1:10, 1:20, 1:40 etc.

Using the result notation described above some examples of equivalent titre values are:-

SAT	$4^{2}3$	(443)	= 3 at 1:40
Warm CFT	$4^{4}2$		= 2 at 1:64
Cold CFT	4^{6}		= 4 at 1:320

It should be noted that when referring to agglutination tests the dilution figure refers to the proportion of serum in the final test reaction volume. In the case of the complement fixation test the dilution figure refers to the serum dilution before other reagents such as antigen, complement and haemolytic system are added.

Other Sera

A selection of other sera was available from known infected animals and this was used on occasions in comparisons of various tests. A laboratory standard serum, of known titre with respect to the 2nd International Standard Anti-*Brucella abortus* Serum (2nd ISAbS), was included in each series of tests.

Results

Microbiological Examination

No growth appeared on any of the Brucella selective media plates. Various light mixed growths were observed on blood-agar plates and a range of organisms were identified including *E. coli*, *Klebsiella*, *Streptococcus*, *Pseudomonas* and *Corynebacterium pyrogenes*. Again no *Brucella* were identified.

Guinea Pig Inoculation

At necropsy no abnormalities were seen in any of the guinea pigs. Spleen culture revealed no growth on either blood-agar or *Brucella* selective media. Serum agglutination and complement fixation tests on the serum taken immediately prior to death were negative.

Serological Tests

(i) Titration of antigens

Because commercially prepared antigen is preserved in phenol-saline, cell walls of the organisms tend to be relatively resistant to disruption, thus freshly prepared organisms were used for ultra-sound and ether-water treatments. Freshly prepared antigen (Br. abortus strain 19) was first titrated against a laboratory standard serum and a "standardised concentrate" prepared. A portion of this concentrate was then treated and titrations of this treated antigen were compared with untreated antigen for use in complement fixation tests.

Table I indicates the titration of antigen against a standard serum to find the dilution at which 50% agglutination was given against a 1:500 dilution of the standard serum.

1:16 was the dilution of antigen required for use in the serum agglutination test and for preparation of standardised concentrate the antigen was diluted 1:1.6 with PBS.

(ii) Complement fixation test titrations

Tables II, III and IV show complement fixation test antigen titrations for untreated, ultra-sound treated and ether-water treated antigens.

Table I Strain 19 antigen titration for agglutination test, against laboratory standard serum (equivalent to 2nd ISAbs).

Serum dilution	1:300	1:400	1:500	1:600	1:700
Antigen dilution					
1:10	-	2	-	-	-
1:12	3	4	-	-	-
1:14	3	4	1	-	-
1:16	4	4	2	1	-
1:18	4	4	4	3	-

Table II. Whole cell strain 19 antigen, titration for warm fixation CFT.

Serum dilution	1:4	1:8	1:16	1:32	1:64	1:128
Antigen dilution						
1:100	4	4	4	-	-	-
1:200	2	4	4	4	3	-
1:400	-	-	-	1	4	3
1:800	-	-	-	-	-	-
1:1600	-	-	-	-	-	-

Table III. Sonicated strain 19 antigen, titration for warm fixation CFT.

Serum dilution	1:4	1:8	1:16	1:32	1:64	1:128
Antigen dilution						
1:50	4	4	4			
1:100	4	4	4	2	-	-
1:200	1	3	4	3	-	-
1:400	-	-	1	3	4	-
1:800	-	-	-	-	-	-
1:1600	-	-	-	-	-	-

Table IV. Ether-water treated strain 19 antigen, titration for warm fixation CFT.

Serum dilution	1:4	1:8	1:16	1:32	1:64	1:128
Antigen dilution						
1:50	4	4	2			
1:100	4	4	4	-	-	-
1:200	-	4	4	-	-	-
1:400	-	2	3	2	-	-
1:800	-	-	-	-	-	-
1:1600	-	-	-	-	-	-

(iii) Serological results

Table V displays results obtained for the agglutination type tests for sera from each of the repeat BCT+/CFT- animals slaughtered.

Complement fixation tests results are given in Tables VI, VII and VIII for tests using standard commercial strain 99 antigen, sonicated strain 19 and ether-water treated strain 19 antigens. A laboratory standard serum with equivalent complement fixing activity to that of a 1:30 dilution of the 2nd International Standard Anti-Brucella abortus Serum (2nd ISAbs) was included in each series of tests.

The Rivanol complement fixation test was negative for all samples in the series 1-20 except for numbers 1 and 20, see Table IX.

Gel-diffusion tests were conducted using both sonicated and ether-treated antigens. Of the twenty test sera (Numbered 1-20) only serum 1 showed a precipitation line. Both sonicated and ether treated antigens could produce this single line which appeared to be common to them both. The line was also produced with high titred sera from known infected animals. Upon diluting serum 1 to 1:10 lines disappeared.

The indirect haemolysis test was negative for sera 2-19.

Serum 1 gave a titre of 4^6

Serum 20 gave a titre of 4

and the laboratory standard gave 4^2.

Table V. Titres to agglutination type tests of repeat BCT+/CFT- animals slaughtered.

Animal No.	BCT	SAT	Riv	ME(1)	ME(2)	Coombs
1	+++	$4^{7}2$	4^{7}	$4^{7}2$	$4^{7}1$	$4^{13}2$
2	++	41	2	42	41	41
3	++	4	-	3	3	4^{2}
4	-	42	-	21	1	42
5	+	$4^{2}2$	4	$4^{2}1$	42	$4^{2}2$
6	-	42	-	42	4	4^{2}
7	+	4^{2}	3	43	4	$4^{2}2$
8	++	$4^{3}2$	-	4^{3}	43	$4^{3}2$
9	-	43	-	43	3	4^{2}
10	+	4^{2}	4	4^{2}	43	$4^{2}3$
11	-	42	3	31	3	42
12	-	43	-	4^{2}	2	4^{2}
13	-	$4^{3}1$	-	4^{3}	4^{2}	$4^{3}1$
14	+	2	-	2	1	42
15	-	42	-	42	4	4^{2}
16	+++	$4^{2}3$	-	$4^{2}2$	43	$4^{2}3$
17	++	43	2	43	4	43
18	+	$4^{2}2$	3	43	43	$4^{2}2$
19	++	$4^{2}31$	-	$4^{2}3$	43	$4^{2}31$
20	+++	$4^{3}2$	4	4^{4}	4^{3}	4^{5}

(1) method (a) see text

(2) method (b) see text

Table VI. Complement fixation test results for repeat BCT+/CFT- animals.

(a) Cold fixation: commercial strain 99 antigen

Animal No.	Antigen concentration			
	1:100	1:200	1:400	1:600
1	$4^{7}2$	$4^{7}3$	$2^{2}4^{7}$	$-^{7}4^{2}3$
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	2	1	-
7	2	41	2	1
8	2	2	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	1	3	3	1
17	-	-	-	-
18	-	-	-	-
19	2	3	4	1
20	43	432	$4^{2}1$	1^{3}
Std(1/30 ISAbS)	4	42	43	42

(b) Warm fixation: commerical strain 99 antigen

Animal No.	1:100	1:200	1:400	1:600
*				
1	$-^{4}13^{2}2^{2}$	$-^{4}123^{3}2$	$-^{5}2^{3}3^{2}$	$-^{5}13^{3}1$
20	32	42	42	43
Std(1/30 ISAbS)	43	4^{2}	4^{2}	42

* Sera 2-19 all negative.

Table VII. Complement fixation test results for repeat BCT+/CFT- animals.

(a) Cold fixation: sonicated strain 19 antigen

Animal No.	Antigen concentration			
*	1:20	1:50	1:100	1:200
1	$4^{6}2$	$4^{6}3$	$2^{2}4^{5}3$	$-23^{2}4^{3}3$
7	3^{2}	43	43	32^{2}
8	2	1	2	-
19	3	1	4	4
20	3	431	$4^{2}1$	$4^{2}2$
Std (1/30 ISAbS)	41	42	42	4

(b) Warm fixation: sonicated strain 19 antigen

Animal No.	Antigen concentration			
*	1:20	1:50	1:100	1:200
1	$-^{5}34$	$-^{5}21$	$-^{5}1^{2}2$	-
20	4^{2}	$3^{2}1$	4^{3}	$23^{2}2$
Std (1/30 ISAbS)	4	41	42	42

* Results for sera not listed - all negative.

Table VIII. Complement fixation test results:

Use of ether treated, sonicated and whole cell antigens in testing of prozoning sera from known infected animals.

Sample No.	Ether Ag 1:100		Sonicated Ag 1:100		Whole cell Ag 1:400	
	WCFT*	CCFT**	WCFT	CCFT	WCFT	CCFT
S4982	$-^{20}$	$-^{20}$	$-^{20}$	$-^{2}{}_{14}{}^{6}{}_{2}$	$-^{20}$	$-^{6}{}_{24}{}^{3}$
L3846	$-^{20}$	$-^{7}{}_{3}{}^{3}$	$-^{20}$	$-^{3}{}_{34}{}^{6}$	$-^{20}$	$-^{5}{}_{34}{}^{3}$
A5639 684	$4^{6}{}_{1}$	$4^{5}{}_{3}$	4^{5}	$4^{5}{}_{3}$	$34^{6}{}_{1}$	$4^{6}{}_{2}$
598	$-^{4}{}_{4}{}^{2}{}_{1}$	$-^{4}{}_{3}{}^{2}$	$-^{2}{}_{4}{}^{3}$	$-234^{4}{}_{3}$	$-^{3}{}_{234}{}^{2}{}_{2}$	$34^{5}{}_{3}$
165	$-^{5}{}_{24}{}^{3}$	$-^{5}{}_{34}{}^{2}{}_{3}$	$-^{3}{}_{24}{}^{4}$	$-^{3}{}_{4}{}^{7}$	$-^{4}{}_{234}{}^{3}$	$234^{7}{}_{3}$
199	134^{6}	4^{8}	4^{7}	4^{8}	4^{9}	4^{10}
L3991	-	-	-	-24^{3}	-	$4^{3}{}_{3}$
Std(1/30 ISAbS)	$4^{2}{}_{1}$	42	41	43	42	42

* WCFT = Warm fixation, complement fixation test
** CCFT = Cold fixation, complement fixation test

Table IX. Rivanol complement fixation test commercial strain 99 antigen 1:400

Serum No.	WCFT*	CCFT**
1	$-^{5}{}_{2}{}^{2}{}_{3}{}^{2}$	$2^{2}{}_{4}{}^{6}{}_{2}$
20	4	3

* WCFF = Warm fixation, complement fixation test
** CCFT = Cold fixation, complement fixation test

Discussion

The antigenic relationships of *Brucella* to other organisms have been reported on by Harris (1950), Spink (1956), Berman (1956), Nicoletti and Holmes (1968), Ahvonen *et al* (1969) and others. Although *Pasturella*, *Salmonella*, *Shigella* and *Vibrio* sp. have all been implicated, only *Yersinia enterocolitica* type IX has consistently shown strong cross-reactivity (Ahvonen *et al*., 1969; Corbel and Cullen, 1970). It was considered that in the absence of *Br. abortus* infection it may have been some other agent stimulating a cross-reacting antibody that caused the card test to react but did not fix complement.

In the event it transpired that neither *Brucella abortus* nor any other likely non-specific agent could be isolated from any of the twenty cattle examined. A series of serological tests was conducted in an attempt to clarify the situation.

Animals purchased for this experiment were selected on the basis of having reacted to previous card tests but not to previous complement fixation tests. Herds from which they came generally had a long testing history although in some cases the actual number of reactors taken over the series of tests was very low. The prevalence of such repeat BCT+/CFT- animals in infected herds and in non-infected herds is not known. This is because herds are not necessarily subjected to card testing at each test and herds which become accredited are not subjected to any further testing, thus there is no opportunity to apply the card test on a repetitive basis in non-infected herds. Timbs *et al*.(1978a) investigated the subsequent testing history of a group of these animals and concluded that the probability of any animal eventually becoming CFT positive was not related to its previous card testing history. The problem of repeat BCT+/CFT- animals is probably not unique to New Zealand although it has not been recorded elsewhere as being of any significance. A further consideration of this matter is made in chapter 7.

Following initial preparation, the strain 19 antigen was titrated in a checkerboard fashion (Table I) to enable it to be standardised prior to aliquots being taken for ether treatment

and sonication. In this way the antigenic activity was equated to the commercial strain 99 antigen used for the standard agglutination and complement fixation tests. Table II compares complement fixation test antigen titrations of commercial strain 99 antigen and the freshly prepared strain 19 antigen. (Both antigens having the same agglutination activity). Thus, although strain 19 antigen is a little less sensitive than strain 99 it remains quite suitable. Tables III and IV compare the effectiveness of sonicated and ether treated strain 19 antigens; there appears to be little difference in the sensitivities of these two antigens and in each case a dilution of 1:100 was taken as the optimal dilution to use in the CF test.

Sonicated and ether-water treated antigens were used in the CFT specifically to determine if prozoning could be avoided by altering the type of antigen employed. As indicated by results illustrated in Tables VI, VII and VIII neither type of soluble antigen appears to offer any advantage over the standard whole cell antigen. Ether-water treatment of phenol-preserved commercial whole cell strain 99 Br. abortus antigen proved to be quite successful. On titration the complement fixing activity of this extract proved to be equivalent to that obtained from treatment of freshly prepared cells.

The outstanding features of the results obtained in this section are the high titres given by serum from animal No. 1. When the initial complement fixation tests were performed on this animal's serum, prior to it being selected for this experiment, it was thought to be negative. This may have been because of true absence of titre or because there was such a high degree of prozoning that no complement was fixed. Although Br. abortus was not isolated, the serological results indicate that this animal may well have been infected. The nature of the CFT reaction was such that it required further investigation and this formed the basis of studies outlined in chapter 5.

Although serum 20 had a low CF titre, the marked reduction, following Rivanol treatment, in both the CF and agglutination tests, indicated that the titre may have been due to residual vaccination (IgM) antibodies (Morgan,1967).

All other sera in this series were CFT negative and were

regarded as having insignificant agglutination titres.

Two mercapto-ethanol methods were compared with the Rivanol test. If it is accepted that animals 2-20 were not infected and that the agglutination titre present was due to residual strain 19 stimulated IgM or a cross reacting antibody, then it appears that the Rivanol test is best at clarifying the situation as it markedly reduced the SAT titres. The mercapto-ethanol test performed using buffer containing 0.05M 2-ME appeared to be more effective than the method of using 0.1M 2-ME in the first tube prior to making dilutions. Despite the extra time required to perform the Rivanol test it proved to be superior and also avoided the use of mercapto-ethanol which is dangerous to use and has a most unpleasant pungent odour.

The high Coombs titre of serum 1 was noteworthy (1:81920), titres of this order are often seen in other strongly prozoning sera.

Gel diffusion tests were conducted in an effort to detect any abnormal pattern of antibody diffusion that serum 1 may have had. The single line that was detected was identical to a line produced by another serum from a known infected cow. No abnormal pattern was seen. No other sera in the series produced any detectable lines. Only serum 1 reacted to the indirect haemolysis test and no evidence of prozoning was seen. This test was specifically developed to overcome the problem of prozoning in the complement fixation test (Plackett et al., 1976).

CHAPTER 3. EXPERIMENTAL INOCULATION OF CATTLE WITH KILLED BRUCELLA ABORTUS

Introduction

Data collected and evaluated in chapter 7 indicated that animals BCT positive yet negative to the CFT were more prevalent in infected herds than in non-infected herds. A possible explanation for the phenomenon is that animals exposed to the antigen but which resist infection, develop a transient BCT titre but do not react to the CFT.

Cullen and Corbel (1970) inoculated adult milking cows, that had been vaccinated as calves, with various doses of living and dead strain 19 Br. abortus. A rapid rise in titre was noted. Titres achieved maximum levels after two weeks and began to fall again after one month. A dose/response effect was noted suggesting that the vaccine was acting partly as a non-infecting antigen. Beck et al. (1964) conducted similar experiments with similar results and stressed the importance of using properly cleaned vaccination equipment.

The objective of the experiment reported in this section was to examine the serological response to killed Br. abortus by cows which had been vaccinated as calves but which did not have any detectable titres to the SAT, CFT or BCT at the commencement of this investigation.

Materials and Methods

Animals: Nine cull dairy cows were available for use, these animals were all more than eight years of age and had all been vaccinated with strain 19 Br. abortus as calves. They were all negative to the SAT, CFT and BCT.

Inoculation: The contents of a vial of commercial strain 19 Br. abortus vaccine* were reconstituted with distilled water and diluted with the aid of Wellcome opacity tubes+ to final cell concentrations of 1×10^9 and 1×10^6 organisms/ml. The diluted

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* I.C.I. Tasman Vaccine Ltd., Upper Hutt, New Zealand.

\+ Wellcome Research Laboratories, Beckenham, Kent.

organisms were then heat killed in a water bath at 80 °C for 60 mins and checked for sterility by inoculating onto blood-agar.

Two cows were each given 1×10^9 killed organisms by subcutaneous injection and one further cow received 1×10^6 organisms s/c.

Three other animals were each dosed by spraying 1 ml of a 1×10^9 organism suspension into one eye. This was achieved by forcing it through a 26G syringe needle which had been flattened to cause a fine spray.

The final three cows each received organisms intra-nasally by spraying 1 ml of a 1×10^9 organisms/ml suspension onto and into the external nares.

Animals were bled at approximately weekly intervals following inoculation and a second dose, by the same route as the first, was given after eight weeks.

Sera were collected and stored at -20 °C until the last blood collection was made. All sera were then tested together as one batch. Card, serum agglutination and complement fixation tests were performed as described in chapter 3. Both warm and cold fixation CF tests were carried out.

Results

BCT, SAT and CFT titres for the nine cows before and after inoculation with killed strain 19 vaccine are shown in Figures 3a, 3b and 3c.

As indicated by the histograms, agglutination titres tended to be readily produced whereas complement fixation and card test titres were rather low and transient. Titres to all tests reached peak levels within 14 days of the initial inoculation. Following the repeat dose titres again rose but generally did not exceed those obtained after the initial inoculation. Highest titres were stimulated by subcutaneous inoculation. Conjunctival and nasal routes of administration resulted in only low titres.

Figure 3 Histogram of titres given by cattle inoculated with killed Brucella abortus strain 19.

(a) Cows inoculated subcutaneously

Nos. 57 & 70	1×10^9 organisms
No. 92	1×10^6 organisms

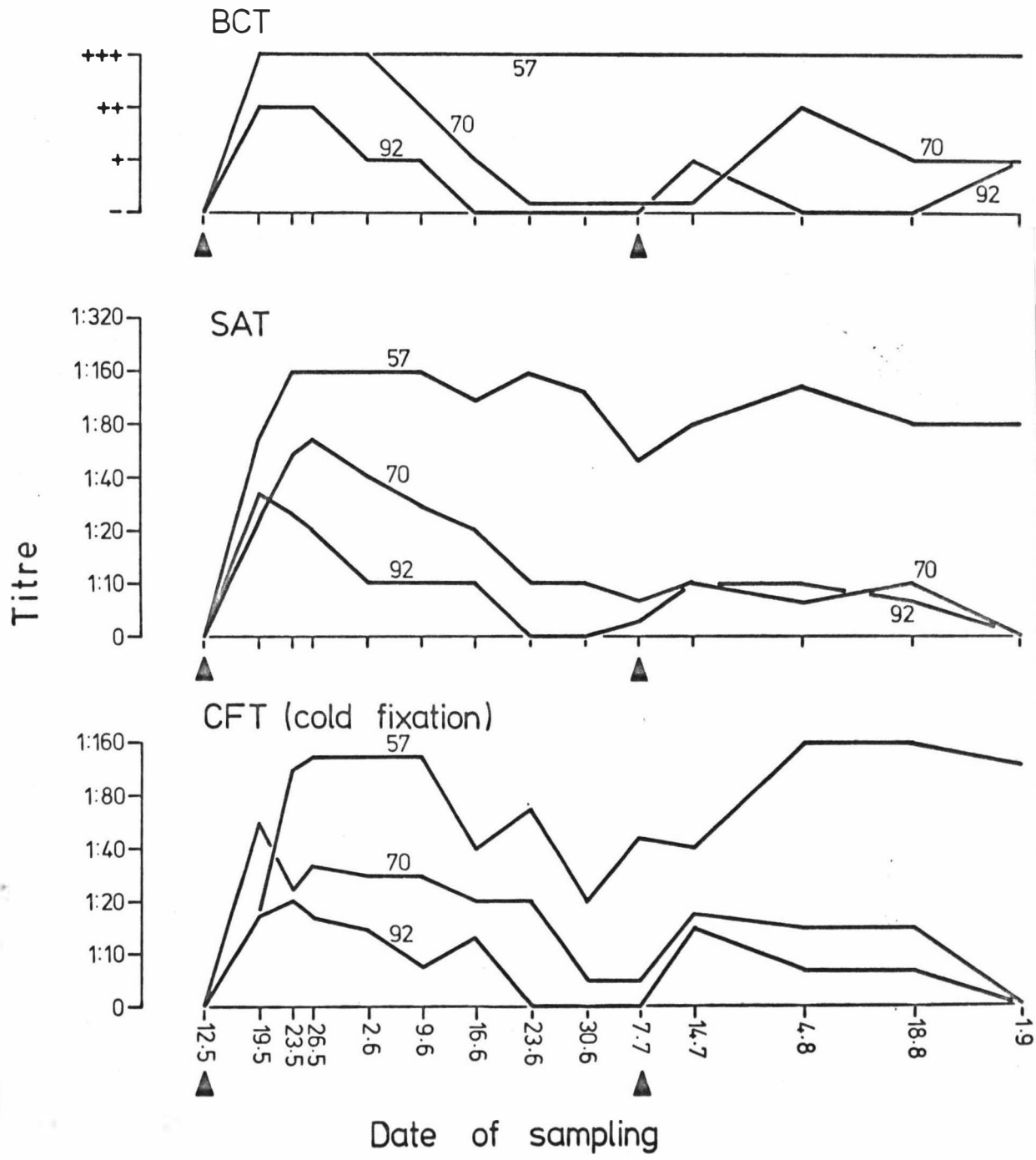

BCT
+++
++
+
-
57
70
92
70
92
SAT
1:320
1:160
1:80
1:40
1:20
1:10
0
57
70
92
70
92
CFT (cold fixation)
1:160
1:80
1:40
1:20
1:10
0
57
70
92
12.5
19.5
23.5
26.5
2.6
9.6
16.6
23.6
30.6
7.7
14.7
4.8
18.8
1.9
Titre
Date of sampling

Figure 3 Histogram of titres given by cattle inoculated with killed Brucella abortus strain 19.

(b) Cows inoculated via conjunctiva.

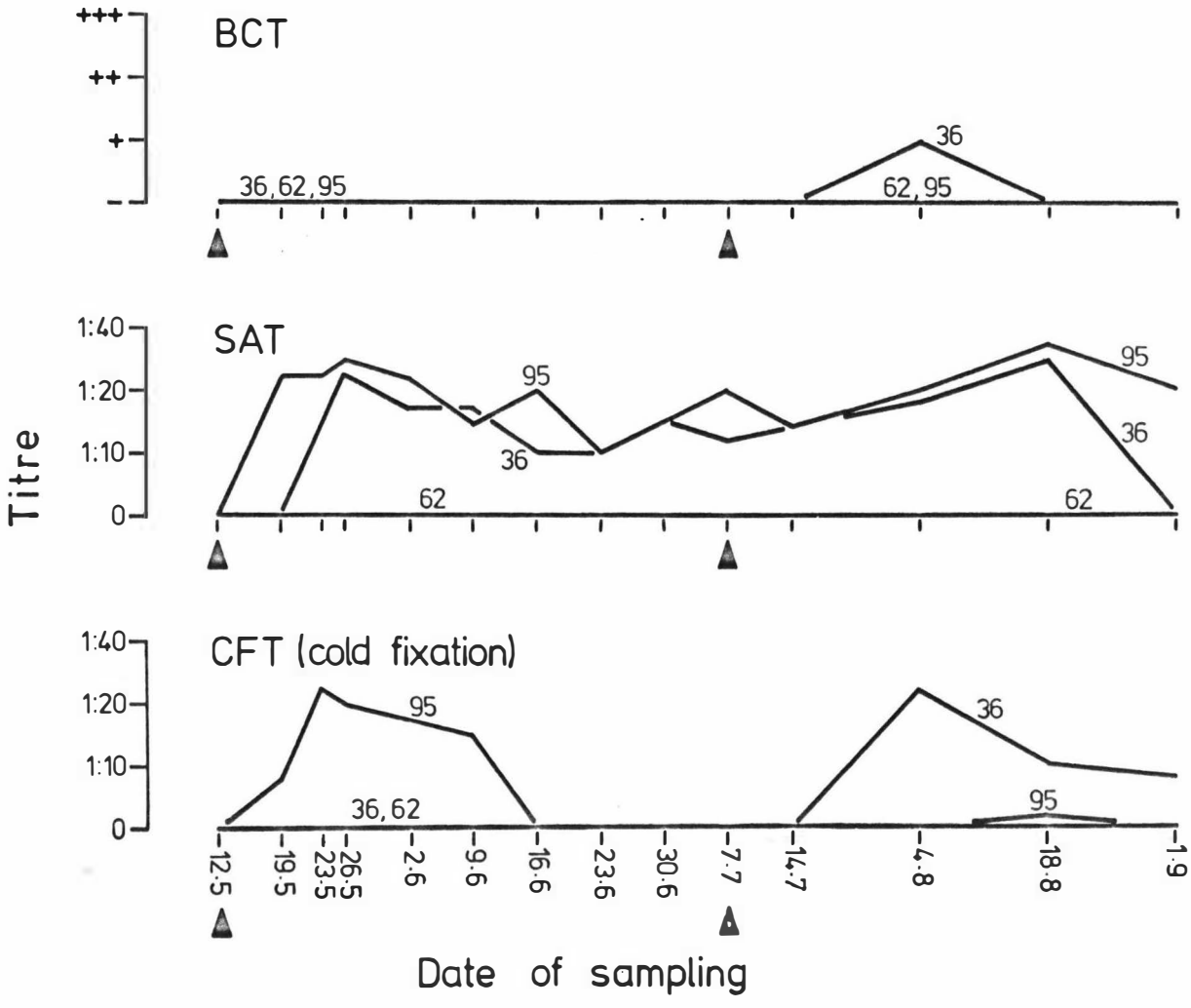
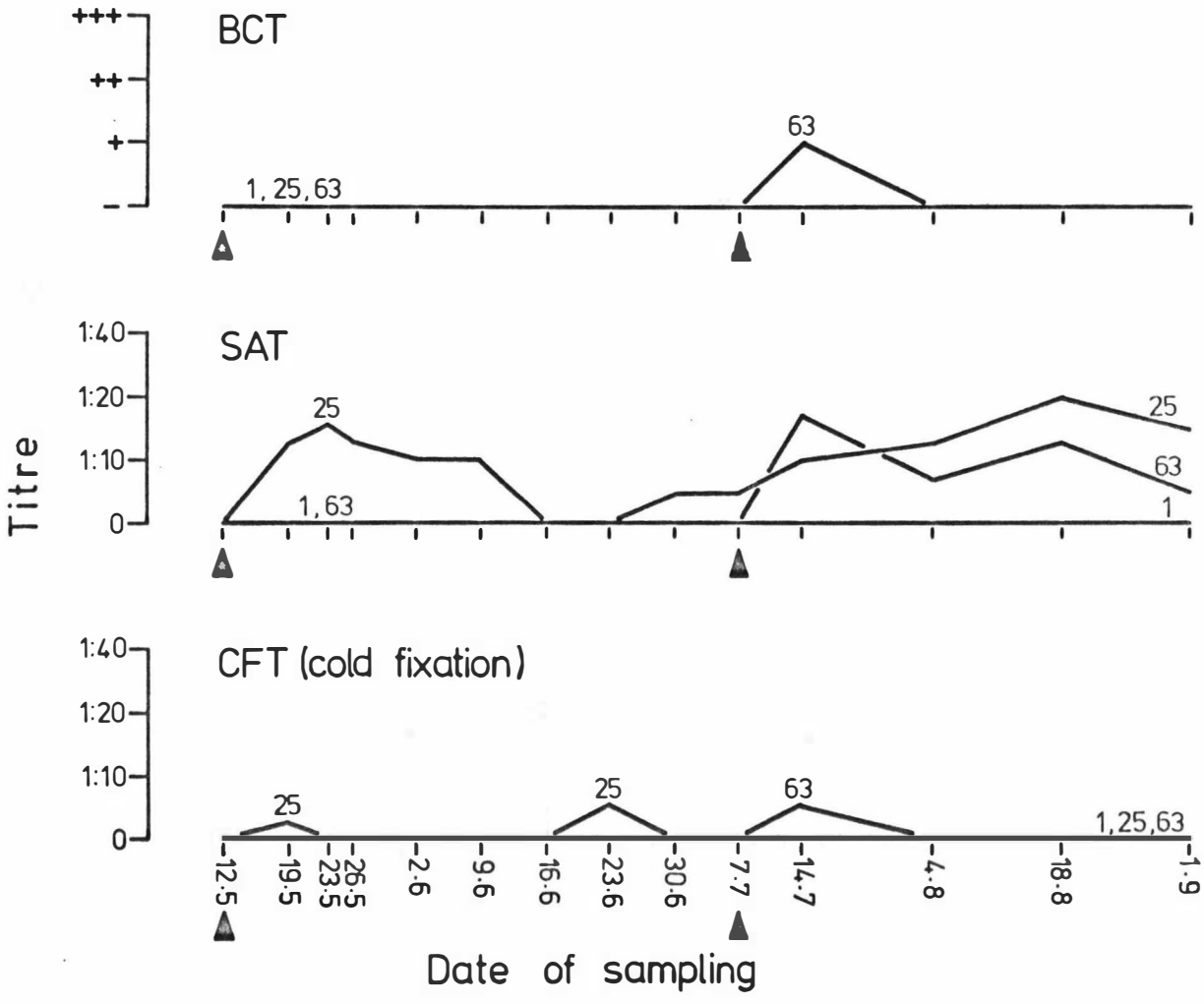

BCT
+++
++
+
-
36,62,95
36
62,95
SAT
1:40
1:20
1:10
0
95
36
62
95
36
62
Titre
CFT (cold fixation)
1:40
1:20
1:10
0
95
36,62
36
95
12.5
19.5
23.5
26.5
2.6
9.6
16.6
23.6
30.6
7.7
14.7
4.8
18.8
1.9
Date of sampling

Figure 3 Histogram of titres given by cattle inoculated with killed Brucella abortus strain 19.

(c) Cows inoculated via nasal route.


BCT
+++
++
+
-
63
1, 25, 63
SAT
1:40
1:20
1:10
0
25
25
63
1
1, 63
Titre
CFT (cold fixation)
1:40
1:20
1:10
0
25
25
63
1, 25, 63
12.5
19.5
23.5
26.5
2.6
9.6
16.6
23.6
30.6
7.7
14.7
4.8
18.8
1.9
Date of sampling

Discussion

Although the administration of organisms subcutaneously was satisfactorily achieved the same could not be said of the conjunctival and nasal routes. When spraying of the suspension onto the conjunctiva was attempted, the cows tried to avoid the spray by turning their heads and closing their eyelids. Attempts at intra-nasal spraying resulted in similar resentment with the added complication of fierce expiration. Consequently somewhat less than the full dose of 1×10^9 organisms was received by animals inoculated by these routes.

Since all animals had been vaccinated as calves with live strain 19 Br. abortus vaccine the rise in titre following inoculation with dead organisms was not altogether unexpected.

Animals inoculated subcutaneously gave the most significant titre enhancement, as might have been expected. There was a suggestion of a dose-response effect in that animals 1 and 2 which received 1×10^9 organisms gave higher titres in all tests than animal No. 3 which received only 1×10^6 organisms.

Animals treated intra-nasally and intra-conjunctivally did not respond well serologically but the observation that some animals did in fact respond, particularly to the SAT, supports the hypothesis that sensitised animals naturally exposed to dead organisms, may develop titres. Titres rose rapidly to achieve maximum levels after two weeks; following the repeat dose of organisms most fell to pre-inoculation levels within two months. The response following repeat inoculation was no greater than that seen after the first dose which initiated the anamnestic response.

Beck et al.(1964) and Cullen and Corbel (1970) inoculated cows to test the effect of using syringes contaminated with strain 19 Br. abortus on subsequent titre development. They were concerned with the SAT and CFT response and did not investigate the post-inoculation development of BCT titres. This experiment has demonstrated that BCT titres may be stimulated by killed Br. abortus organisms and maintained even after significant SAT and CFT titres have disappeared. Further investigation of this aspect using a field strain of Brucella and dosing via the oral route is required.

CHAPTER 4. STUDIES ON BRUCELLA-SPECIFIC SERUM ANTIBODY FRACTIONS

Introduction

The "Prozone" phenomena which occurs in Brucella complement fixation tests has been described in chapter 2.

Because the automated complement fixation test, on which the New Zealand Brucellosis Eradication Scheme is based is essentially a one dilution test, it has been criticised on the grounds that it may fail to detect prozoning sera which do not react at the particular dilution of the automated test system. One of the objectives of this thesis is to justify the use of the automated CFT and to show that it can and does detect such prozoning sera.

Antibody produced in response to Brucella abortus infection or Br. abortus strain 19 vaccination is found in the IgG_1, IgG_2 and IgM immunoglobulin classes (Rice and Boyes, 1971; Beh, 1974). The relative proportions of each of these immunoglobulin classes in the sera of infected and vaccinated animals may vary (Beh and Lascelles, 1973). It has also been shown that the various serological tests used in the diagnosis of brucellosis vary considerably in their ability to detect antibody of a particular immunoglobulin class (Beh 1973; Allan et al., 1976).

The experiments outlined in this section were undertaken to provide an understanding of the immunological basis of prozoning in the CFT. This was achieved by using the procedure of Nash and Heremans (1969) for the quantitative determination of antibody belonging to the various immunological classes and relating findings to complement fixation titres of sera under test. Further understanding of the prozone phenomenon was gained by conducting complement fixation tests on serum fractions and mixtures of serum fractions derived from prozoning sera.

Materials and Methods

Ion Exchange Chromatography

A 30 cm x 1.5 cm diam. column of diethylaminoethyl cellulose

(DEAE)* was prepared and equilibrated with 0.2M phosphate buffer pH 8.0. Two mls serum or bovine colostrum which had previously been dialysed against this buffer for two days was introduced. The column was eluted with starting buffer and then with a gradient to 0.6M phosphate buffer pH 8.0 using a Varigrad Chamber+. The type of elution gradient obtained is presented in Figure 4.

Protein elution was monitored by continuously recording the absorbance, at 254 nm, of the eluate. A typical trace is shown in Figure 5. Fractions of eluate were collected, pooled where required, and concentrated by dialysis against polyethylene glycol (M.W. 20,000).

Gel Filtration

Sephadex G-200 beads were swollen, degassed and poured into a 60 cm x 2.5 cm diam. column. Following equilibration with 0.015 M phosphate buffered saline pH 7.2, 1 ml of previously dialysed serum in which 0.2 g sucrose had been dissolved was layered onto the top of the gel. Protein elution was monitored by recording the absorbance of the buffer at 254 nm and fractions were concentrated by dialysis against polyethylene glycol. A typical absorbance pattern is shown in Figure 6.

Immunoelectrophoresis (IEP)

IEP agar was prepared as described in appendix I. Immuno-electrophoresis was performed against rabbit anti-bovine whole serum and against rabbit anti-bovine immunoglobulin. A typical IEP plate is shown in Figure 7.

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* DE-52 Whatman, England.

+ Varigrad. Bachler Instruments, Fort Lee, N.Y.

Figure 4 Elution gradient of phosphate buffer used in DEAE cellulose column

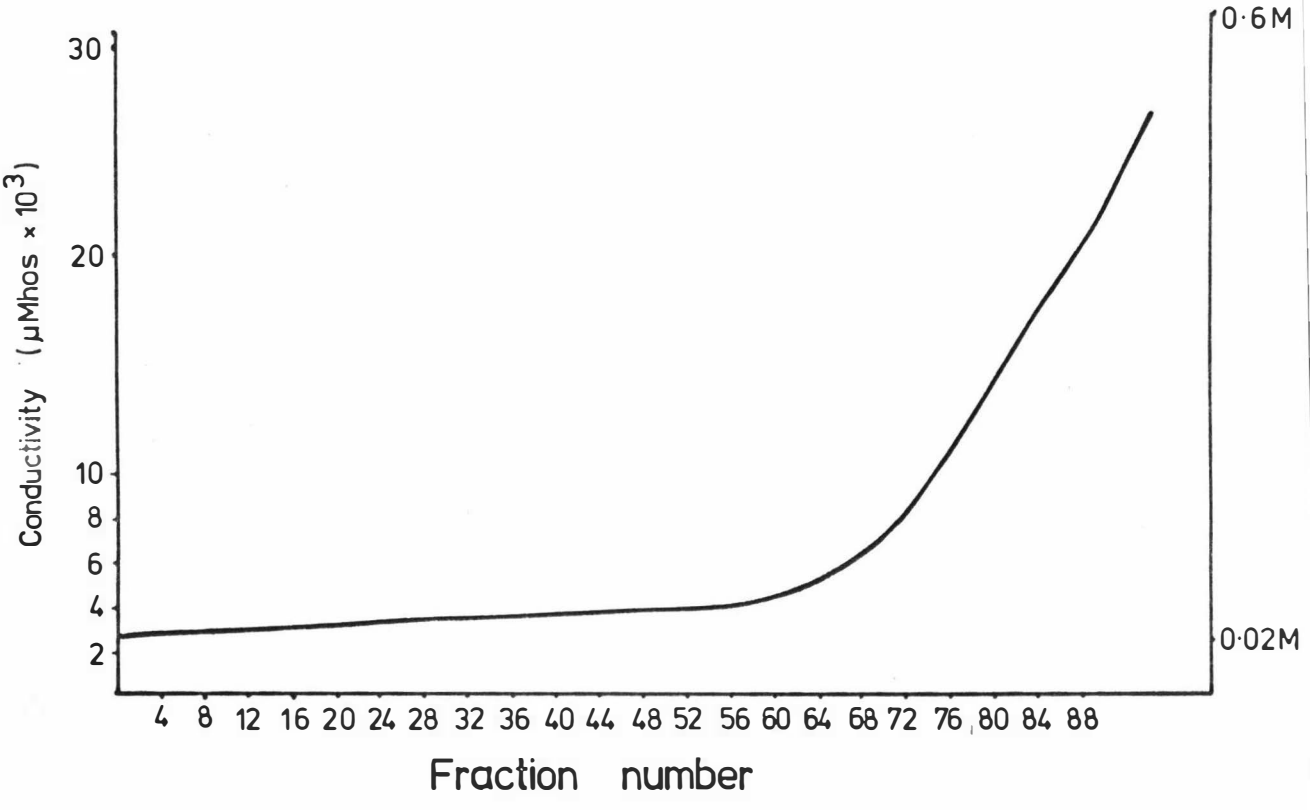

0·6M
30
20
10
8
6
4
2
0·02M
Conductivity (μMhos × 10³)
4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88
Fraction number

Figure 5 Typical trace of absorbance at 254 nm of eluate of DEAE cellulose column

Figure 6 Typical trace of absorbance at 254 nm of eluate from sephadex G200 column.

Fraction numbers indicate location of eluate fraction taken for electrophoresis as displayed in figure 7.

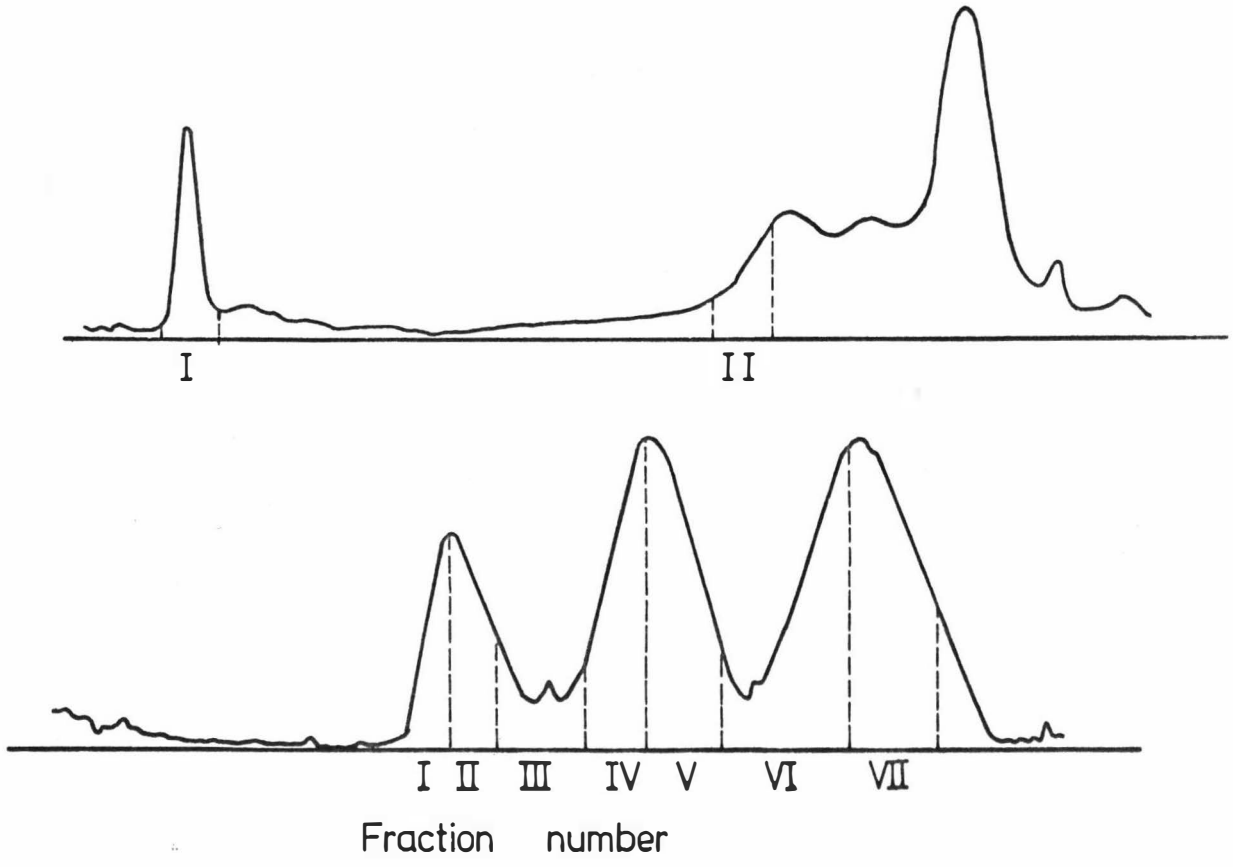
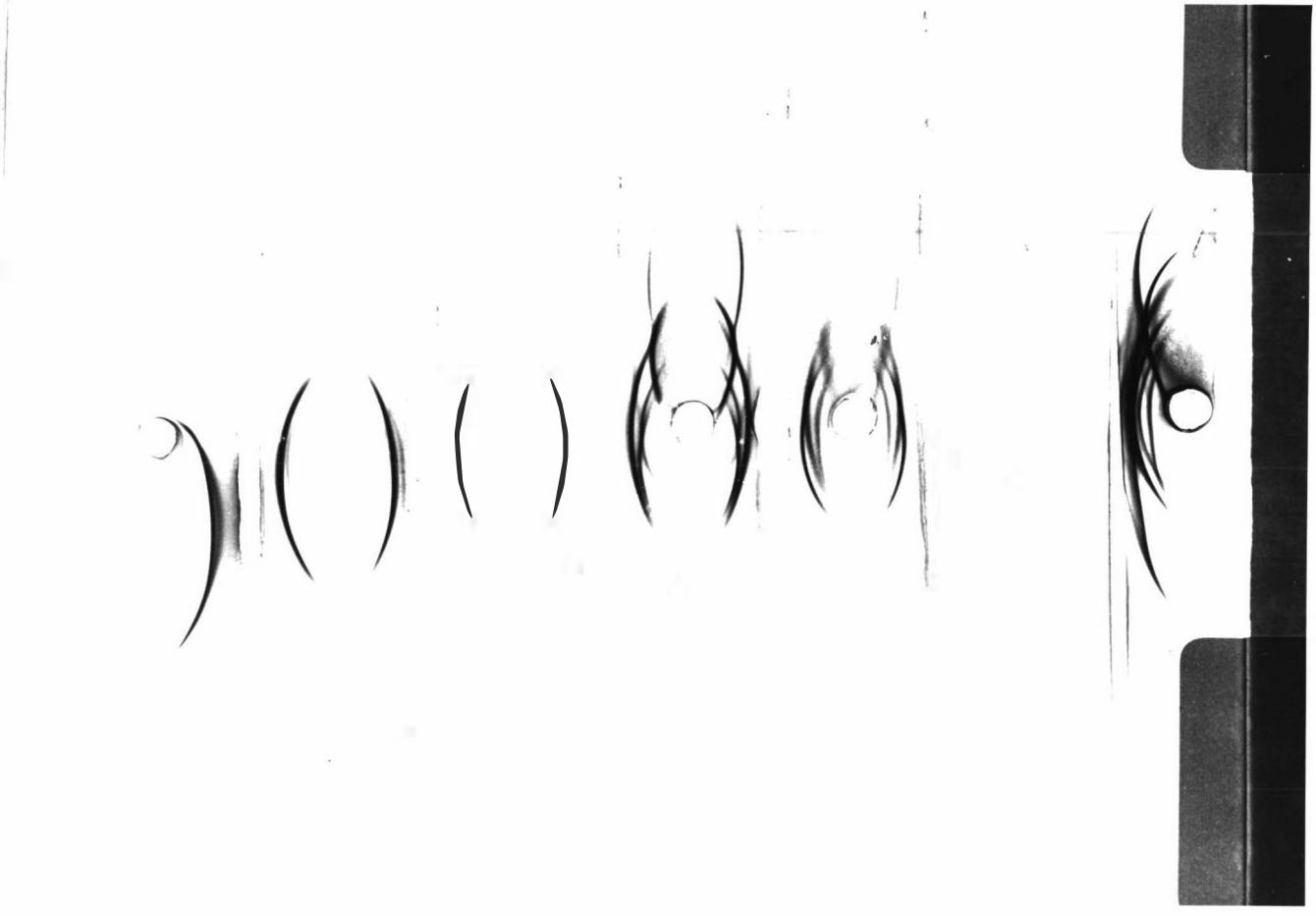

I
II
I II III IV V VI VII
Fraction number

Figure 7 Typical electrophoresis plate for serum fractionated on DEAE cellulose



I II III IV V VI VII

Numbers refer to fractions taken as indicated in figure 5.
Troughs contain rabbit anti-bovine whole serum, wells contain sample of appropriate fraction.

Immunodiffusion

Immunodiffusion agar was prepared as described in Appendix I, this agar was identical to that used for IEP. Immunodiffusion was used where appropriate to detect contamination with unwanted classes of immunoglobulin. It was particularly useful in monitoring cross adsorption of specific antisera.

Preparation of Antisera

Solutions of approximately 1-2 mg/ml of specific immunoglobulins were prepared. 1 ml of each of IgG_1, IgG_2 and IgM was emulsified with 1 ml Freund's complete adjuvant* and injected in two equal doses into each hind leg of each of three rabbits.

Further inoculations of immunoglobulin were given two and six weeks later. Freund's incomplete adjuvant was used to emulsify the immunoglobulin preparation at these later treatments. Rabbit serum was harvested at two, four and six weeks following the final inoculation and preserved with 0.1% sodium azide.

Anti IgG_1 serum was absorbed by slowly adding IgG_2 until no Anti IgG_1-IgG_2 precipitin line could be detected on immunodiffusion, likewise Anti IgG_2 serum was absorbed with IgG_1 and Anti IgM was absorbed with IgG_1. In each case 0.005 mg of specific immunoglobulin per ml of antiserum was added approximately every hour until absorption was complete. Approximately 0.05 mg of specific immunoglobulin was required per ml of antiserum in each case.

Quantitation of Immunoglobulins

Approximate estimations of immunoglobulin concentrations were obtained by measurement of absorbance of solutions at 280 nm in an ultra violet spectrophotometer and reading against a standard curve.

Standard solutions of immunoglobulins were prepared by taking a pure solution of immunoglobulin of unknown concentration and dialysing for five days against frequent changes of 0.05 M ammonium carbonate. The immunoglobulin preparation was then

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* Difco, Detroit.

distributed into pre-weighed vials and freeze-dried. Following lyophilisation the vials were again weighed and the quantity of dried immunoglobulin material calculated. This material was then reconstituted with phosphate buffered saline to the required concentration.

Single Radial Immunodiffusion (SRID) tests

Quantitative single radial immunodiffusion tests were performed according to the method of Mancini et al.(1965). Plates were incubated for two days and then washed for three days in saline and for a further day in distilled water. They were then dried and stained with Amido black and decolourised before ring diameters were read (see Appendix I). Each plate contained four standards from which a standard curve was prepared. If any sample gave a ring diameter which fell outside the range of the standards it was concentrated or diluted and retested.

Adsorption of Brucella Specific Antibody

A standardised concentrate of Brucella abortus antigen (strain 99) was centrifuged at 4000 g for 20 minutes to deposit cells. These were resuspended to one-tenth of the original volume of concentrate in phosphate buffered saline (PBS). This super-concentrated antigen suspension was used to adsorb test sera.

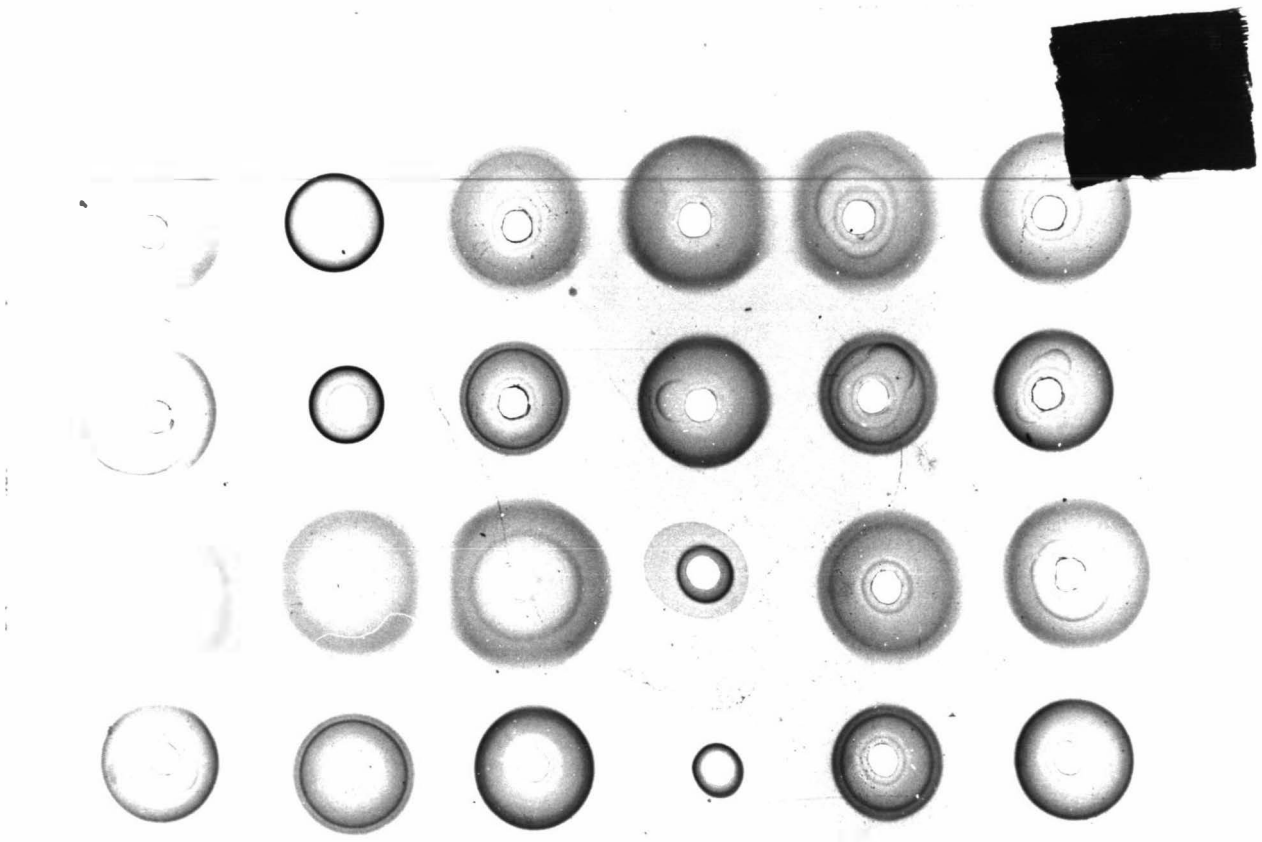
For each serum a series of four tubes was prepared as follows:

	Serum (ml)	Antigen (ml)	PBS (ml)
T.1	0.2	0.2	0.6
T.2	0.1	0.2	0.7
C.1	0.2	-	0.8
C.2	0.1	-	0.9

After incubation at 4 ^{o}C for two days tubes were centrifuged at 4000 g for 20 mins and the supernatants removed and tested by SRID.

The amount of specific immunoglobulin measured by each test was calculated by plotting the concentration of each standard

Figure 8 Typical single radial immunodiffusion plate



immunoglobulin against the square of its corresponding precipitin ring diameter, concentrations of unknown samples were read directly from this chart. Five sets of four samples (T_1, T_2, C_1, C_2) and four standards were included on each plate.

After determining the immunoglobulin concentration for each sample, Brucella specific percentages were calculated as follows:

$$\text{Ig conc. of absorbed serum } [T] = \frac{2[T_2] + [T_1]}{2}$$

$$\text{Ig conc. of control serum } [C] = \frac{2[C_2] + [C_1]}{2}$$

$$\text{Brucella specific \% of serum} = \frac{[C] - [T] \times 100}{[C]}$$

Inhibition of Specific IgG_1 Complement Fixing Activity by Specific IgG_2

Aliquots of IgG_1 and IgG_2 fractions of a strongly prozoning serum, were taken from a DEAE column as described in previously and were each adsorbed with Br. abortus antigen using the method given in above.

Protein concentrations of adsorbed and unadsorbed aliquots were measured by the Folin-Ciocalteu method (Lowry et al., 1951). The concentration of specific IgG_1 and IgG_2 was then determined by calculating the difference between test and control samples.

A series of test samples was prepared such that each contained a known quantity of anti-Brucella specific IgG_1 and IgG_2. Complement fixation tests were then performed on each sample to determine the extent to which prozoning occurred.

Results

The quality of the IgG_1, IgG_2 and IgM preparations recovered from the DEAE cellulose and sephadex columns was monitored by immunoelectrophoresis and immunodiffusion. IgG_1 and IgG_2 preparations were generally of a high quality and after cross

adsorption to remove anti-light chain antibody were rendered sub-class specific. IgM preparations were also adsorbed with IgG_1 to remove anti-light chain antibody. Most IgM preparations were found on electrophoresis to be contaminated with α_2 macroglobulin.

Only pure preparations were used to stimulate antiserum production.

Immunoglobulin Standards

Dialysis against ammonium carbonate followed by lyophilisation proved to be a very satisfactory method of estimating the protein concentration of various immunoglobulin fractions. All vials into which a control sample of dialysed PBS was placed showed no weight gain thus indicating that dialysis had been complete.

SRID Tests on Adsorbed and Unadsorbed Sera

Table X indicates IgG_1, IgG_2 and IgM concentrations for each of fourteen sera tested. Brucella specific percentages for IgG_1 and IgG_2 are also given. The "prozone" index number indicates the number of dilutions in which prozoning occurred in the warm fixation CFT. The figure in the titre column indicates the number of doubling dilutions in which reactions were obtained, thus a figure of four means the titre is 1:32 (where 1:4 is the serum dilution in the first tube of the series and in the fourth doubling dilution the titre is 1:32.

An assessment of the degree of error occurring within the SRID test system was made by calculating the correlation coefficient (r) of the sets of results obtained for $[C_1]$ $[C_2]$ and $[T_1]$ $[T_2]$ where $[T_1] = 2[T_2]$ and $[C_1] = 2[C_2]$

For $[T_1]$ $[T_2]$ $r = 0.88$

For $[C_1]$ $[C_2]$ $r = 0.93$

Table X. Immunoglobulin concentration and Brucella specific antibody percentage related to degree of prozoning of warm CFT titre

Serum No.	Immunoglobulin Conc. (mg/ml) IgG_1	IgG_2	IgM	Brucella specific % IgG_1	IgG_2	Prozone Index	Titre
1	10.6	10.2	2.4	10.8	12.2	4	10
2	10.9	10.5	1.5	1.0	8.5	-	2
3	11.7	10.9	2.7	.9	3.7	-	4
4	11.4	11.8	1.8	13.6	13.5	*	9^+
5	11.4	12.6	4.6	8.1	0	-	9
6	8.2	8.7	2.5	0	0	-	3
7	10.6	12.5	2.0	9.0	0	-	-
8	14.1	18.6	2.6	3.6	10.8	-	6
9	6.3	6.8	1.6	24.8	33.6	*	10^+
10	13.2	16.6	2.4	10.4	9.3	*	9^+
11	10.8	16.0	3.0	8.3	5.5	3	9
12	9.3	15.5	3.3	3.5	2.2	2	7
13	7.5	7.8	3.1	0	0	-	5
14	8.2	4.75	2.2	0	0	-	2

* Sera gave no titre at all to warm fixation CFT - very strong prozone

\+ Titre given is for cold fixation CFT, and indicates the number of doubling dilutions to which complete fixation was given

Serology on IgG_1 and IgG_2 Fractions and Combinations of IgG_1 and IgG_2

Table XI shows concentrations of total and specific IgG_1 and IgG_2, prepared as described previously and estimated by the method of Lowry *et al* (1951).

Table XII gives typical results for warm fixation CFT's with varying proportions of specific IgG_1 and IgG_2.

Increasing the proportion of specific IgG_2 induced prozoning and eventually inhibited all complement fixation. Cold fixation tests were not as sensitive to IgG_2 in terms of prozone formation but were nevertheless affected.

Reactions of immunoglobulin aliquots to the SAT and card test are shown in Table XIII.

Table XI. Specific and total immunoglobulin concentrations in aliquots of IgG_1 and IgG_2

	Total Ig conc. mg/ml	Specific Ig conc. mg/ml	% specific Ig
IgG_1	5.8	.67	11.6
IgG_2	6.4	.82	12.8

Table XII. Results of warm fixation CF tests for mixtures of specific IgG_1 and IgG_2

IgG_1 µg/ml	IgG_2 µg/ml	1:4	1:8	1:16	1:32	1:64	1:128
250	0	4	4	4	4	4	-
200	50	-	2	4	4	4	-
150	100	-	-	2	3	-	-
100	150	-	-	-	-	-	-
50	200	-	-	-	-	-	-
0	250	-	-	-	-	-	-

Table XIII. Reaction of IgG_1 and IgG_2 to serum agglutination and card tests

Immunoglobulin	wt. specific Ig mg/ml	SAT* i.u.	SAT* titre	Card Test
IgG_1	.67	372	$4^{4}3$	+++
IgG_2	.82	536	$4^{5}1$	+++

* SAT = serum agglutination test

i.u. = international units. Second International Standard Anti-*Brucella abortus* serum by definition contains 1 000 i.u. per ml.

Discussion

The existence of the prozone phenomenon in the complement fixation test has received little attention until recently. Although the effect was undoubtedly recognised by early workers it was not described or investigated in any depth. This may have been because of its transient appearance or simply that it was not thought significant or understood. Alton and Jones (1967) in their standard monograph on Laboratory Techniques in Brucellosis do not mention the effect although its importance is recognised in the second edition (Alton et al., 1975a).

Alton et al.(1975b) in a study of cultural and serological relationships, obtained a positive culture from an animal which also gave a very strong prozone to the CFT. They drew attention to the danger of using insufficient dilutions when performing the test in case such "prozoners" were not detected.

Plackett and Alton (1975) and McNaught et al.(1977) have examined various aspects of prozoning. Both groups of workers have noted that specific IgG_2 is capable of inducing prozoning and can eventually block complement fixation of specific IgG_1 and IgM entirely if present in sufficient quantity.

In the course of the present study a number of strong "prozoning" sera were collected from known infected animals. One very strong prozoning sample was taken from an animal which despite extensive culture attempts could not be proved to be infected. These sera formed the basis of this series of experiments designed to investigate the respective roles of Brucella specific IgG_1, IgG_2 and IgM. By using the technique of radial immunodiffusion it was hoped to quantify specific IgG_1, IgG_2 and IgM in various sera and relate these findings to serological titres.

Quantitative measurements of Brucella-specific antibody in various immunoglobulin classes have previously been made on immunoglobulins following fractionation. The only recorded study of this nature is that of Allan et al.(1976) who after adsorbing the various serum fractions with Br. abortus measured initial and post-adsorption protein levels by the method of Lowry et al.(1951) using Folin-Ciocalteu reagent. They also used a radio-iodination method to measure adsorbed immunoglobulin directly and concluded that this was a superior procedure to that of protein measurement.

Beh (1974) used a method similar to the one described in this thesis to measure Brucella specific antibody concentration changes in cattle sera following strain 19 vaccination or infection.

The single radial immunodiffusion test (SRID) is one of the few methods available for directly measuring specific classes or sub-classes of immunoglobulins. Its sensitivity is claimed to be capable of detecting microgram quantities of immunoglobulin (Mancini, 1965), but it is also difficult to prepare and standardise. In this series of experiments it was found that preparation for the test including serum fractionation, preparation of antisera and standardisation of plates and standardised antigen was indeed time consuming. It was also noted that the test could be very sensitive and considerable time was spent adjusting reagents so that it eventually gave precipitation rings of 0.5-1.5 cm for the range of samples used. A disappointing aspect of the use of this test was its poor precision. Although suitable for estimating total immunoglobulin content of a sample, when estimating the Brucella specific percentage by a process of subtraction of adsorbed from unadsorbed values, the errors were compounded to a degree that made results suspect. For IgG_1 and IgG_2 the large differences between adsorbed and unadsorbed values for prozoning and strong CFT positive sera meant that for these sera results were more meaningful. However, for estimations of IgM no significant differences were found between adsorbed and unadsorbed sera. This was more likely due to the lack of precision of the test than the real absence of specific immunoglobulin.

Pfeiffer et al. (1977) estimated the error of the SRID test to be in the order of 5-10% for measuring bovine immunoglobulin levels. In this series of tests it was estimated to be more like 10-20%. When the difference between two tests, each with errors of this magnitude is taken the error of the difference can easily become quite unacceptable. With further experience in the technique and refinement of some of the methods used it is expected that the magnitude of errors could be reduced somewhat. However, the problem remains that unless the tests are very precise and are repeated a number of times, an accurate measure of the small amounts of Brucella specific antibody in serum made directly, without prior fractionation, will be extremely difficult.

In the light of experience gained in this study it is considered that the SRID test is able to offer sufficient accuracy for measurement of small amounts of specific antibody only after the experimenter has become thoroughly familiar with the technique and experienced in its use. It does, however, offer considerable sensitivity coupled with the use of very small volumes of serum - as little as 20 µl per determination.

Mancini et al.(1965) in their initial evaluation of the SRID test claimed that the standard deviation from the mean of antigen measurements was only 2%. Nash and Heremans (1969) in a study of specific mouse immunoglobulin levels claimed an error level of 3% for each measurement. It appears that with more careful attention to detail, error levels for detection of bovine immunoglobulins may be able to be reduced to levels approaching these.

Pfeiffer et al. (1977) also evaluated commercial SRID kits and found that errors were somewhat greater than in tests performed on their laboratory prepared plates.

Despite the test limitations, results obtained for percentages of specific IgG_1 and IgG_2 in the sera tested concur with the findings of McNaught et al.(1977) and indicate that in prozoning sera there is a higher proportion of specific IgG_2 than in non-prozoning sera. These results are supported by the findings that addition of specific IgG_2 to specific IgG_1 will induce prozoning.

Brandon et al.(1971) and Williams and Green (1976) studied the manner in which bovine serum IgG_1 and IgG_2 levels change at about the time of parturition. Since IgG_1 is actively drawn from serum into the udder (Brandon et al.1971, Mach and Pahud,1971), serum levels fall and the IgG_1:IgG_2 ratio can change quite dramatically. Serum IgG_1 levels can fall to one quarter of preparturition levels whilst IgG_2 remains relatively constant (Williams and Green,1976). Such a change in ratio may induce prozoning in serum from a Brucella infected animal which has otherwise only low serum levels of specific IgG_2. Further detailed study of the way in which Brucella specific immunoglobulin levels vary at about the time of parturition are required to assess the significance of prozoning during this period. In the infected animal the rapid proliferation of the organism and consequent major stimulation to the hosts immune system combined with the changing IgG_1:IgG_2 ratio at this time should generate an interesting and challenging study.

CHAPTER 5. APPRAISAL OF THE AUTO-ANALYZER ADAPTATION OF THE COMPLEMENT FIXATION TEST

Introduction

Auto-Analyzer adaptations of the CFT for brucellosis have previously been described by Joubert et al. (1967) and Miller et al. (1973). The system at present used in New Zealand for routine testing of sera submitted as part of the New Zealand Brucellosis Eradication Scheme, has been described by Te Punga (1971), Elliot and Pullan (1973) and Timbs et al. (1978b). None of these authors have fully described the dynamics of the system nor have they directly related results to those obtained by use of classical manual CF tests. In his initial evaluation of the automated CFT Te Punga (unpublished) found that the sera of 80 of 82 culture positive cattle reacted to the automated CFT, the two sera that were negative were also negative to the manual CFT.

The experiments outlined in this section were designed to critically examine the effectiveness of the automated CFT. The dynamics of the test system were first investigated to enable an understanding of the reaction process, then various prozoning sera were tested to fit their observed pattern of reaction with the expected result.

Materials and Methods

System Description

Schematic diagrams of the equipment configuration for the automated CFT are shown in Figures 9 and 10. The automated CFT is essentially a warm fixation CFT carried out at a single dilution while flowing along a tube. Serum which has previously been inactivated at 58 ^{o}C for 50 minutes is aspirated from the sample cup and diluted with buffer, complement and antigen: an initial incubation lasting 14 minutes then takes place. Sheep erythrocytes and haemolysin (rabbit anti-sheep erythrocyte

Figure 9 Schematic diagram of automated complement fixation test

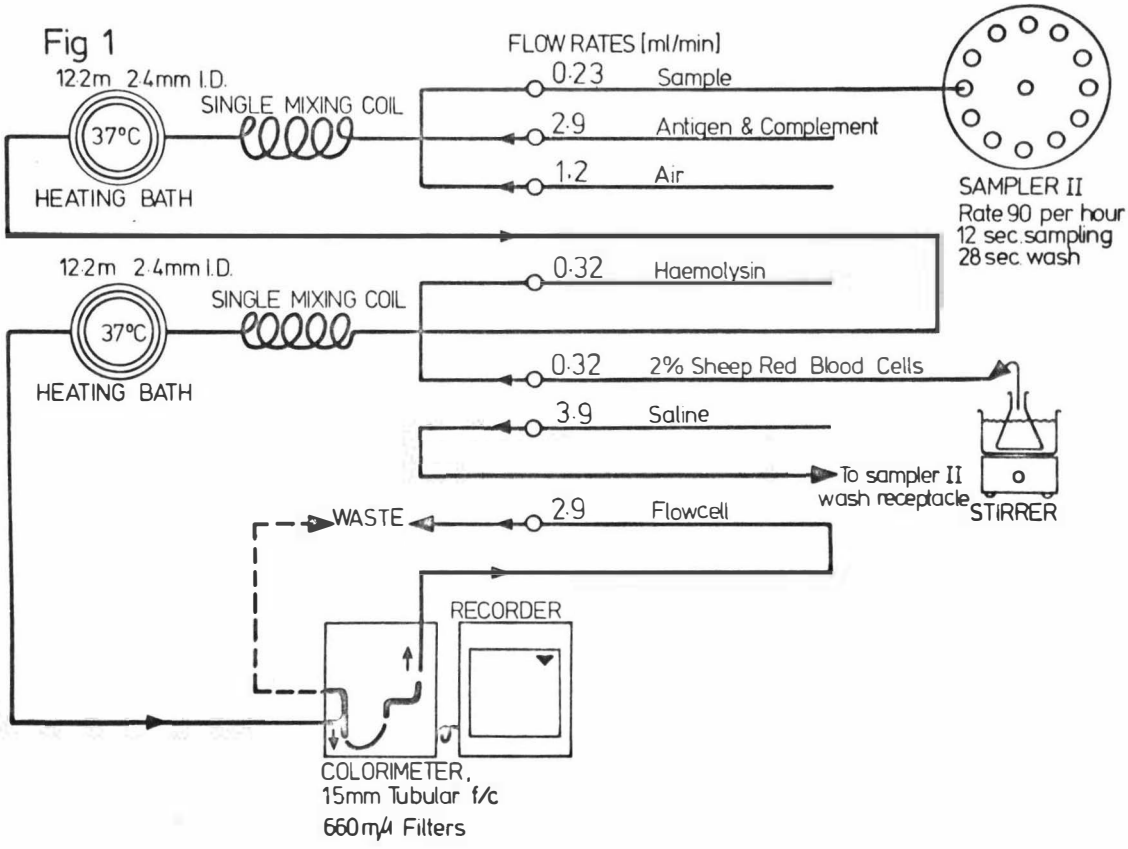
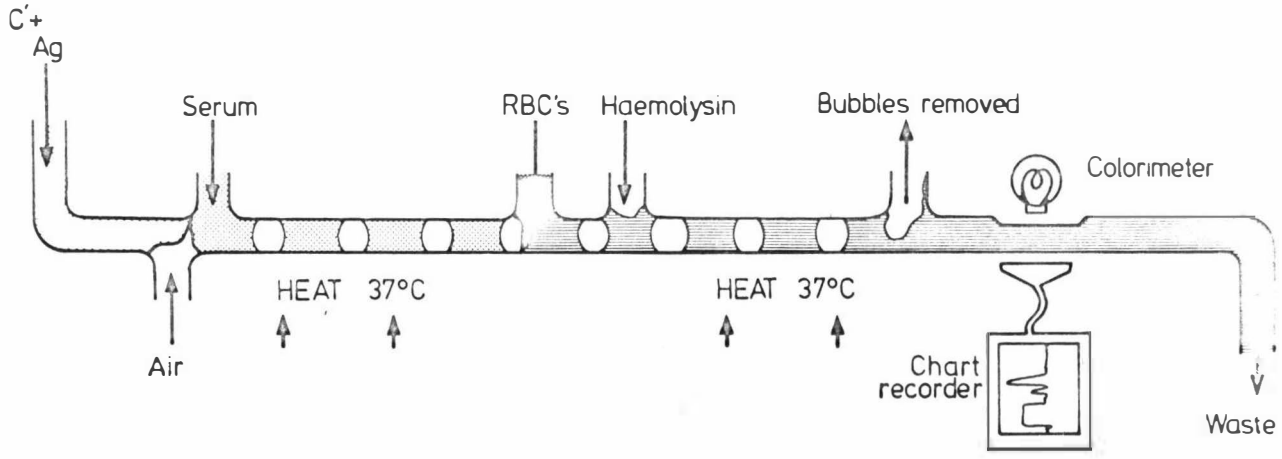


Fig 1
12·2m 2·4mm I.D.
SINGLE MIXING COIL
37°C
HEATING BATH
FLOW RATES [ml/min]
0·23 Sample
2·9 Antigen & Complement
1·2 Air
SAMPLER II
Rate 90 per hour
12 sec. sampling
28 sec. wash
12·2m 2·4mm I.D.
SINGLE MIXING COIL
37°C
HEATING BATH
0·32 Haemolysin
0·32 2% Sheep Red Blood Cells
3·9 Saline
To sampler II
wash receptacle
STIRRER
WASTE
2·9 Flowcell
RECORDER
COLORIMETER,
15mm Tubular f/c
660mμ Filters

Figure 10 Principle of continuous flow analysis as adapted to the complement fixation test



C = Complement

Ag = Antigen

RBC's = Sheep red blood cells

serum) are then added and after a further 10 minutes incubation the degree of haemolysis of the sheep erythrocytes is measured in a colorimeter and recorded on a chart recorder.

According to Beer's law the quantity of monchromatic light absorbed by a solution is directly proportional to the concentration of the solute, within certain limits, and the absorbance or optical density (OD) is related to the transmission (T) of light through the solution by the expression

$$O.D. = \log \left(\frac{1}{T}\right) = - \log T$$

In the system described, the presence of particulate erythrocytes in the flow stream prevents the direct measurement of soluable haemoglobin for the determination of the degree of haemolysis of the cells. Instead, an indirect method must be used whereby the transmittance of erythrocytes in suspension is used.

Thus, when there is no haemolysis, the presence of a high concentration of erythrocytes prevents transmission of light and when there is 100% haemolysis, the absence of erythrocytes allows light to pass unrestricted. The effect of a varying haemoglobulin concentration - due to partial or complete lysis of cells - is minimised by using a 660 nm filter.

Since the incident light is largely dispersed by reflection from particulate erythrocytes rather than being truly absorbed by a solute, Beer's law cannot be strictly applied. However, in contrast to most applications of colorimetry where a uniform quantitative result is required, a simple chart showing only the relative number of erythrocytes in suspension for a given sample, is all that is required from this system.

Reagents

Reagents used for the automated CFT were the same as those used for the manual test except that titrations were carried

out on the Auto-Analyzer and dilutions were therefore different from those used in the manual test.

Titration of Reagents

Sheep erythrocytes were collected and preserved in Alsever's solution as described by Alton et al. (1975a). After washing three times in barbital buffer, pH 7.4, cells were standardised at 2% by centrifuging at 2000 g for 10 mins.

In the presence of excess complement a minimal haemolytic dose of rabbit haemolysin* was determined by introducing decreasing concentrations of haemolysin until the steady state representing 100% lysis of the cells was disrupted. The concentration of haemolysin which marked the beginning of incomplete lysis was termed the "minimal haemolytic dose". Routine working strength haemolysin was prepared to three times the minimal haemolytic concentration. The haemolysin titre obtained by this method was generally similar to that given by the manual method of Alton et al. (1975a).

Antigen was titrated by sampling a weak positive serum and varying the antigen concentration. The antigen concentration giving the highest peak for the sample was taken as the optimal concentration for use in the test.

Complement+ was supplied as lyophilised guinea-pig serum preserved by a modification (Cruickshank et al., 1972) of the method of Richardson (1941). After sheep red blood cells, haemolysin and antigen had been titrated the optimal complement concentration was determined with reference to a "standard" serum. The

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* Commonwealth Serum Laboratories, Melbourne, Aust.

\+ Becton Dickinson and Co., Cockeysville, Maryland.

complement concentration was adjusted so that the "standard" serum, when sampled, gave a peak height equivalent to 50% lysis of the sheep erythrocytes. In practice it was difficult to keep the standard height at exactly this level so a tolerance of between 25% and 75% lysis was usually allowed. Once the standard height moved outside this range the complement concentration was adjusted to return it to within these limits.

Preparation of Standard Serum

A feature of the Auto-Analyzer adaptation of the CF tests is that all test sera are compared with a predetermined standard and are classed as being positive or negative with respect to this standard. The FAO/WHO Expert Committee on Brucellosis (Anon., 1971) suggests that a serum containing more than the equivalent of $\frac{1}{30}$ i.u. of the complement fixing activity of the 2nd International Standard Anti-Brucella abortus Serum (2nd ISAbS) should be classed as positive.

A "standard serum" was prepared by taking a large volume of serum from a cow previously shown to have a high CFT titre. This was then diluted with Brucella negative serum until a serum with equivalent CF titre to the second ISAbS was obtained. For convenience 1 ml aliquots of this serum were lyophylised for use as a laboratory "standard serum".

Continuous Dilution Method of Titration

Titration of reagents for use in the Auto-Analyzer adaptation of the CFT is usually accomplished by preparing a series of discrete concentrations of the reagent under test and applying each reagent to the system in order of increasing or decreasing concentration.

To investigate details of the effects of changes in concentration of reagents, the Auto-Analyzer lends itself to the use of a continually changing concentration gradient. This gradient is achieved by aspirating a concentrated solution of the reagent under test from a beaker and at the same time diluting the remaining reagent with buffer or other suitable diluent. As long as the flow into and out of the reagent receptacle is the same, and the contents are kept well mixed, the reagent

concentration at any given time can be calculated by use of the expression:-

$$C_t = C_o e^{-kt}$$

where C_t = concentration at time t

C_o = original concentration

$$k = \frac{\text{flow rate into or out of receptacle } (f)}{\text{Volume of reagent in receptacle } (V_t)}$$

t = time

The derivation of this equation is given in the caption to Figure 11.

This continuous dilution gradient method was used in investigations of antigen and complement interaction in the automated CFT.

Determination of Von Krogh's Equation Characteristics for the Automated Complement Fixation Test

(a) Introduction

In the past, haemolytic activity of complement has been estimated usually in terms of the smallest amount of fresh serum which will produce complete lysis of a specified number of sensitised red cells. However, during recent years there has been recognition of the advantage of using a 50% haemolysis end-point. The relationship between the amount of complement used and the proportion of cells lysed is not linear, but follows a sigmoidal curve. Relatively large amounts of complement are required to initiate and to complete lysis but in the central region the degree of lysis is sensitive to small changes in complement concentration. Von Krogh (1916) developed a mathematical description of the observed data on haemolysis and the Von Krogh equation has been commonly employed to quantify various components of the complement fixation tests system.

Figure 11 Continuous dilution method of titration

A beaker or conical flask is placed on a magnetic stirrer. A measured volume (V) of reagent with concentration (C_o) is placed in the flask and continually stirred while a diluent is added at constant flow rate f. At the same time, reagent is withdrawn from the flask at constant flow rate f.

Thus :

Concentration of substance in initial reagent

$$C = \frac{M}{V}$$ where M = mass

$$\frac{dM}{dt} = M = -f \times c$$ M = change in mass

$$\therefore \quad c = \frac{}{V} = -\frac{f}{V} \cdot c$$

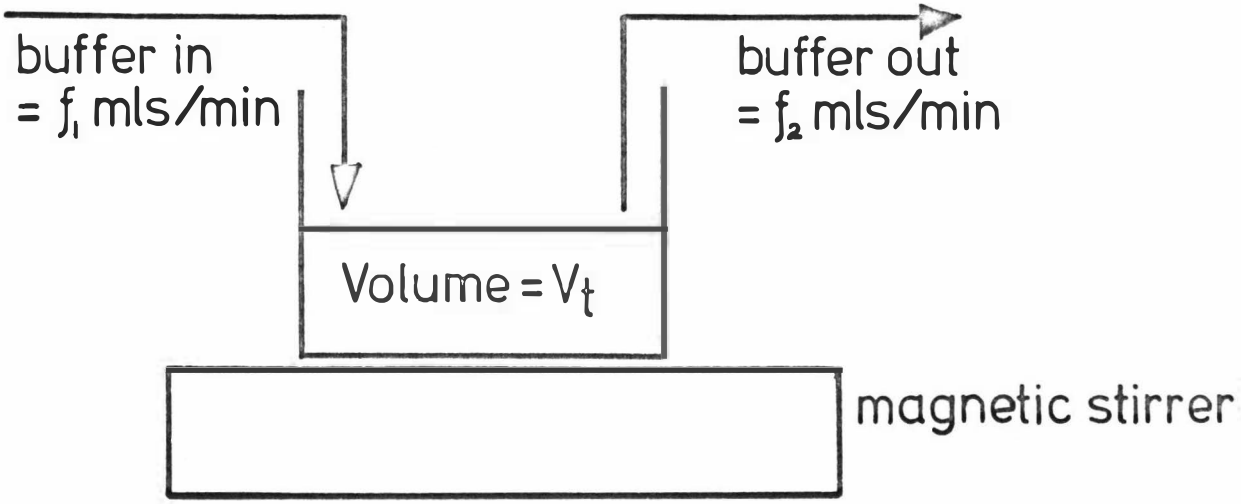
which has the solution

$$c = Ae^{-kt}$$

where $A = C_o$ = c at time $t = 0$

and $K = \frac{f}{V}$

$$\therefore \quad = C_t = C_o e^{-(f/V)t}$$


buffer in
= f_1 mls/min
buffer out
= f_2 mls/min
Volume = V_t
magnetic stirrer

$$\chi = k \left(\frac{y}{1-y}\right)^{\frac{1}{n}}$$

where χ = amount of complement (mls)

y = degree of haemolysis (i.e. 100 y = % haemolysis)

k = 50% unit of complement (CH_{50} unit)

when y = 0.5 (i.e. 50% haemolysis), then $\frac{y}{1-y}$ = 1 and therefore x = k. Thus the CH_{50} unit is expressed in mls of complement required to give 50% haemolysis. The magnitude of the exponent, $\frac{1}{n}$, depends on the experimental conditions but is usually 0.2 $\pm$ 10%; its value determines the shape of the sigmoidal curve (Kabat and Mayer, 1961). A knowledge of the value of $\frac{1}{n}$ for the automated complement fixation test system will therefore lead to a greater understanding of the dynamics of the reaction.

(b) Method

A continuous dilution method of complement titration was used. Values of % transmission given by the chart recorder were transformed into % haemolysis and the complement concentration required for a given degree of haemolysis of red cells calculated. The colorimeter and chart recorder were balanced so that distilled water gave 100% transmission and a complete obstruction to the light path gave 0% transmission. The % haemolysis (H) figure for any given % transmission (T) was calculated by the expression:-

$$\%H = 1 - \frac{OD_{\chi} - OD_{100}}{OD_{o} - OD_{100}} \times 100$$

where $OD_{\chi} = - \log T_{\chi}$

$OD_{100} = - \log T_{100}$

$OD_{o} = - \log_{o}$

and $T_{\chi} = \frac{\%\ \text{Transmission at point } \chi}{100}$

$T_{100} = \frac{\%\ \text{Transmission for 100\% haemolysis}}{100}$

$T_{o} = \frac{\%\ \text{Transmission for 0\% haemolysis}}{100}$

The relationship between this degree of haemolysis and % transmission was also obtained by substituting appropriate values in the simultaneous equations.

$$Y_o = M.\ OD_o + C$$

and $$Y_1 = M.\ OD_{100} + C$$

then solving for M and C

where Y_o = degree of haemolysis of 0 (i.e. 0% haemolysis)

Y_1 = degree of haemolysis of 1 (i.e.100% haemolysis)

OD_o = optical density at 0% haemolysis

OD_{100} = optical density at 100% haemolysis

M = slope of line

Values of y could then be obtained for any values of OD_{χ}, represented by $OD_{\chi} = -\log T_{\chi}$, taken directly from the particular Auto-Analyzer chart by substitution in the equation $y = M.\ OD_{\chi} + C$.

Two methods were used for the calculation of CH_{50} unit.

1. A graph of complement concentration [C] plotted against $\frac{y}{1-y}$ was prepared and the complement concentration required for 50% haemolysis ($\frac{y}{1-y} = 1$) was read directly from the graph. By this method the neatness of fit of the straight line (when drawn on Log/Log paper) could be readily observed and gave an indication of the accuracy of the method.

2. The %T figure corresponding to 50% haemolysis was read directly from the chart and the complement concentration required to give this value was then calculated directly.

$$\text{OD for 50\% lysis} = \frac{OD_{100} + OD_o}{2} = OD_{50}$$

$$\text{and} \quad \%T \text{ for 50\% lysis} = 10^{-OD_{50}} \times 100$$

The value of the exponent, $\frac{1}{n}$, in the Von Krogh equation was determined directly from the graph of $[C]$ v $\frac{y}{1-y}$ where $\frac{1}{n}$ is represented by the slope of the straight line, or by transformation of the equation to

$$\frac{1}{n} = \frac{\log \chi - \log K}{\log \frac{y}{1-y}}$$

When $y = 0.5$ (i.e. 50% haemolysis), then

$\frac{y}{1-y} = 1$ and $\log \frac{y}{1-y} = 0$, also $\log \chi = \log K$ so that

$\log \chi - \log K = 0$.

Thus to use this equation a value of y other than 0.5 must be used with its corresponding value of χ.

Investigation of Resultant Serum Dilutions in the Automated Complement Fixation Test

(a) Introduction

In the automated CFT sample is aspirated at 0.23 ml/min for 12 seconds and injected into the antigen/complement flow stream. This is followed by aspiration of buffer until the next sample is taken. At the point of introduction into the main flow stream the sample is diluted 0.23: 3.13 or 1: 13.6 (sample line 0.23 ml/min, antigen/complement line 2.9 ml/min). However, because of carry-over in the flow stream the sample will not stay at this concentration but will become progressively more dilute. To test the extent of this dilution - carry-over effect radio-labelled serum was used and traced through the flow-stream.

(b) Method

An aliquot of serum containing I^{125} labelled bovine gamma globulin was prepared. Three 0.25 ml samples were taken and the gamma

emission of each was counted in an auto-gamma scintillation spectrometer*. Sample (1) was aspirated by the sampler for the standard time of 12 secs and the reagent mixture reaching the end of the system after the addition of red blood cells and haemolysin and final incubation was collected in small aliquots. These aliquots were then placed in the scintillation spectrometer and the number of counts/min for each was measured. Samples (2) and (3) were aspirated in a similar way except that the collection of aliquots was made directly after the initial incubation and prior to the addition of haemolytic system. In this way the dilution gradient of the sample was estimated at about the time of the primary antigen-antibody reaction rather than at the end of the haemolytic reaction as with sample (1).

Investigation of the Effect of Antigen Concentration on Complement Fixing Ability

(a) Introduction

The continuous flow nature of the Auto-Analyzer adaptation of the complement fixation test lends itself to use as a tool to investigate various aspects of the kinetics of the test. Antigen titrations are carried out as standard practice in the performance of complement fixation tests with the objective of finding the optimal antigen concentration for routine use in the test. The standard manual antigen titration such as that given by Alton et al.(1975a) calls for the use of a known positive serum against which the antigen is titrated. It has been noted that different "positive" serums may have different optimal antigen requirements for the CFT. The Auto-Analyzer proved to be a convenient tool for investigating the extent of these differences.

(b) Method

By choosing suitable serum and complement concentrations and using a continuous dilution method of altering antigen concentration, the trace obtained on the Auto-Analyzer chart recorder directly

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* Packard Gamma-scintillation spectrometer.

reflected the complement fixing ability of various antigen/antibody ratios. A number of different sera and serum dilutions were tested.

The Ability of the Automated CFT to Detect Prozoning Sera

(a) Introduction

The automated CFT is essentially a one dilution warm fixation type test. As such, its principle has been criticised on the grounds that it will fail to detect prozoning sera. A previous section in this chapter explained how an experiment was designed to show how the sample within the Auto-Analyzer did not retain a discrete dilution level but rather formed a continuous dilution gradient. Thus the automated CFT measures the degree of complement fixation on a wide range of serum dilutions for each sample.

(b) Method

A series of prozoning sera were tested at various antigen dilutions by the automated complement fixation test.

Results

Determination of Von Krogh's Equation

A typical trace obtained by continuously diluting complement in the absence of samples is shown in Figure 12. The spike labelled "A" represents an air bubble introduced to indicate the start of the continuous dilution of complement. The curve between B and C represents the change in haemolysis of cells from 100% to 0%. By calculating the % transmission at which 50% of the cells are lysed and measuring the distance of this point from the spike A the dilution of complement required to lyse 50% of the red cells may be determined.

A graph plotted from the chart in Figure 12 is shown in Figure 13. Values of $[C]$ and $\frac{y}{1-y}$ were derived for this graph as follows.

Figure 12 Auto-Analyzer trace given by continuously diluting complement in the absence of samples

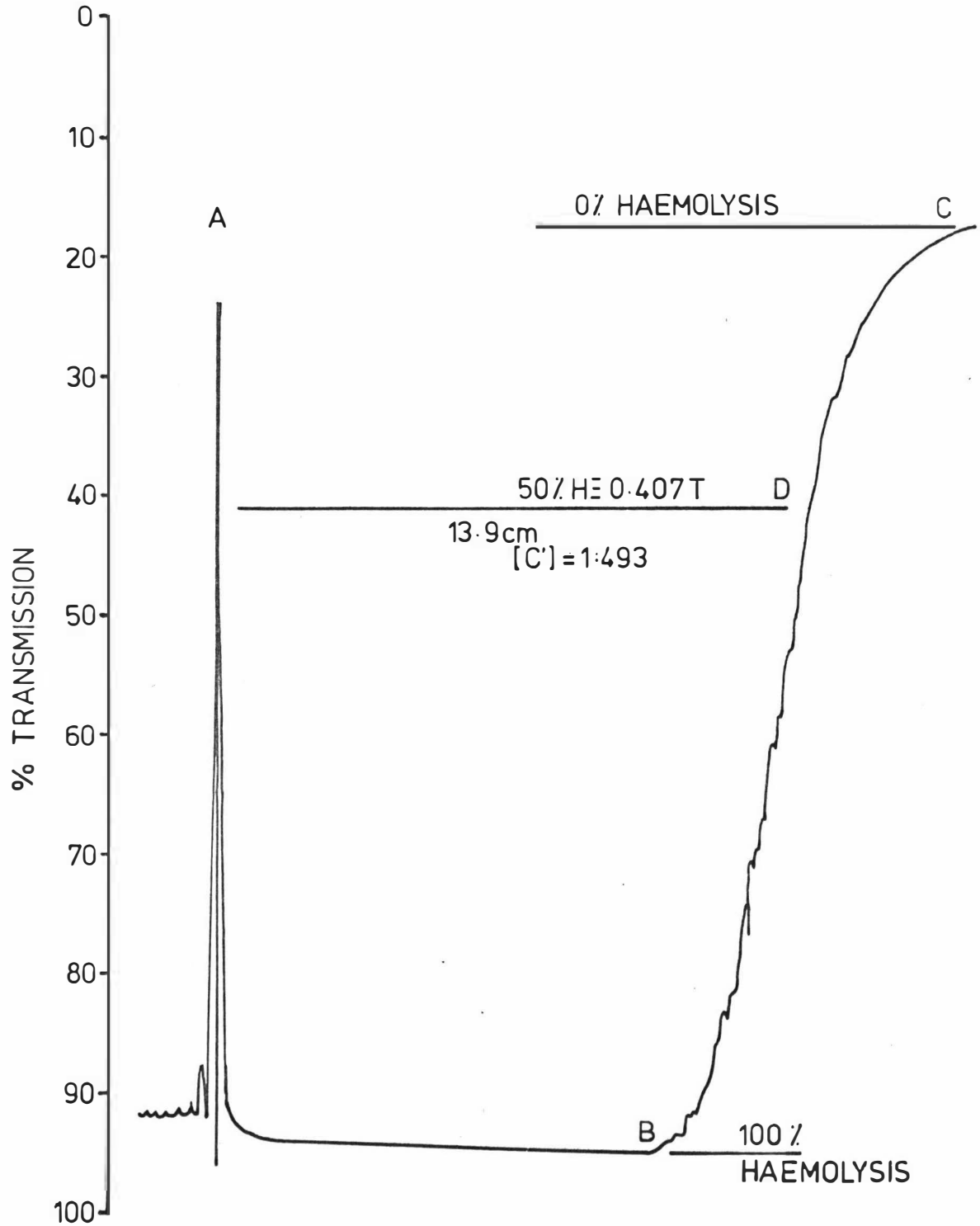
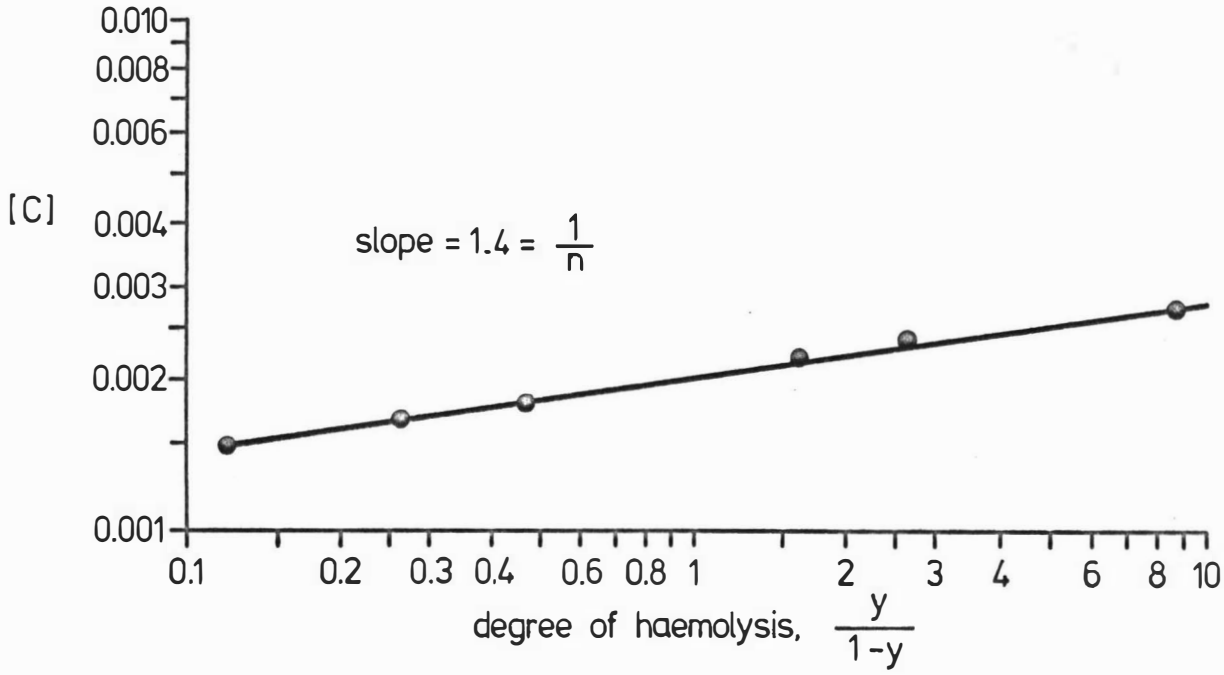

0
10
20
30
40
50
60
70
80
90
100
% TRANSMISSION
A
0% HAEMOLYSIS
C
50% HE 0·407 T
D
13·9cm
[C'] = 1:493
B
100 %
HAEMOLYSIS

Figure 13 Graph drawn by plotting values derived from chart shown in figure 12. By measuring the slope of this line a value for $\frac{1}{n}$ in the von Krogh equation is obtained.


0.010
0.008
0.006
[C]
0.004
0.003
0.002
0.001
slope = 1.4 = 1/n
0.1
0.2
0.3
0.4
0.6
0.8
1
2
3
4
6
8
10
degree of haemolysis, y/(1-y)

The distance (d_χ) from the origin (spike A in Figure 12) to the point on the chart representing a particular % transmission is converted into a measure of time (t_χ) by

$$t_\chi = \frac{d_\chi}{s}$$

where s = chart speed in cm/min (= .762 cm/min)

now $C_t = C_o \, e^{-kt}$

where C_t = concentration at time t

C_o = original concentration

k = $\frac{\text{flow rate into or out of receptacle } (f)}{\text{Volume of reagent in receptacle } (V_t)}$

For this series of experiments f = 2.51 mls/min

$$V_t = 20 \text{ mls}$$

To find degree of haemolysis (y)

$$y = 1 - \frac{OD_\chi - OD_{100}}{OD_o - OD_{100}}$$

where $OD_\chi = - \log T$

and $T_\chi = \frac{\% \text{ Transmission at point } \chi}{100}$

Thus the series of points for the graph shown in Figure 13 are:-

T_χ	[C]	$\frac{y}{1-y}$
0.21	0.00146	0.119
0.25	0.00166	0.226
0.3	0.00179	0.468
0.5	0.00217	1.638
0.6	0.00235	2.676
0.8	0.00271	8.804

The slope of the curve can be measured and found to be 0.14 which is the value of $\frac{1}{n}$ in the Von Krogh equation.

Alternatively for any particular point on the chart in Figure 10

$$\frac{1}{n} = \frac{\log \chi - \log k}{\log \frac{y}{1-y}}$$

where χ = amount of complement for degree of lysis y
and k = amount of complement for 50% lysis (i.e. CH_{50} unit)

From the chart in Figure 12 the CH_{50} unit was measured directly and found to be 1:493 or 0.00203. From the graph Figure 13 the point of intersect obtained when $\frac{y}{1-y} = 1$ represents the CH_{50} unit and is seen to be 0.002. Thus for a value of T = 0.5 for example:

$$\frac{1}{n} = \frac{\log 0.00217 - \log 0.00203}{\log 1.638}$$

$$= .1351$$

For a series of experiments using the same batch of reagents, values of the CH_{50} unit and of $\frac{1}{n}$ were calculated as:-

CH_{50} (dilution of C)	$\frac{1}{n}$
1:493	.14
1:498	.138
1:493	.137
1:495	.14

When antigen was added to the system values for $\frac{1}{n}$ remained at 0.138-0.14 but the CH_{50} dilution of complement reduced to approximately 1:485 for a 1:1000 dilution of antigen and to 1:470 when a 1:500 antigen dilution was used. Thus the degree of anti-complementary activity in the antigen could be quantified in terms of CH_{50} units. Values of the CH_{50} unit also varied slightly according to the batch of red cells used but again the value of $\frac{1}{n}$ remained constant at about 0.138.

Determination of the Dilution Gradient of Serum in the Automated CFT

Figure 14 illustrates the dilution gradient for each radio-labelled sample after travelling through the Auto-Analyzer system. Sample 1 traversed the complete system whilst samples 2 and 3 were retrieved following the initial incubation and prior to the addition of haemolytic system. For sample 1 aliquots were each of 0.7 mls whereas for samples 2 and 3 aliquot volumes were 0.25 ml each.

Figure 15 indicates the relative serum concentration in each aliquot and compares it to the serum concentration immediately following aspiration and with the serum concentrations in a set of tubes in which a manual fixation CFT is being performed. The initial serum concentration in the Auto-Analyzer during the period which serum is aspirated is 1:13.6 (0.0734).

$$\text{Sample conc. in aliquot retrieved} = \frac{\text{counts/min/aliquot}}{\text{Total counts/min recovered}} \times \frac{\text{initial vol. of sample introduced}}{\text{vol. of aliquot retrieved}}$$

Table XIV summarises counts per minute recorded at the various stages of the experiment. The number of counts was fairly constant between samples and would have more nearly approximated the number of counts aspirated if more aliquots had been collected and counted for each sample.

Figure 14 Dilution gradient given by serum containing I^{125} labelled bovine gamma globulin.

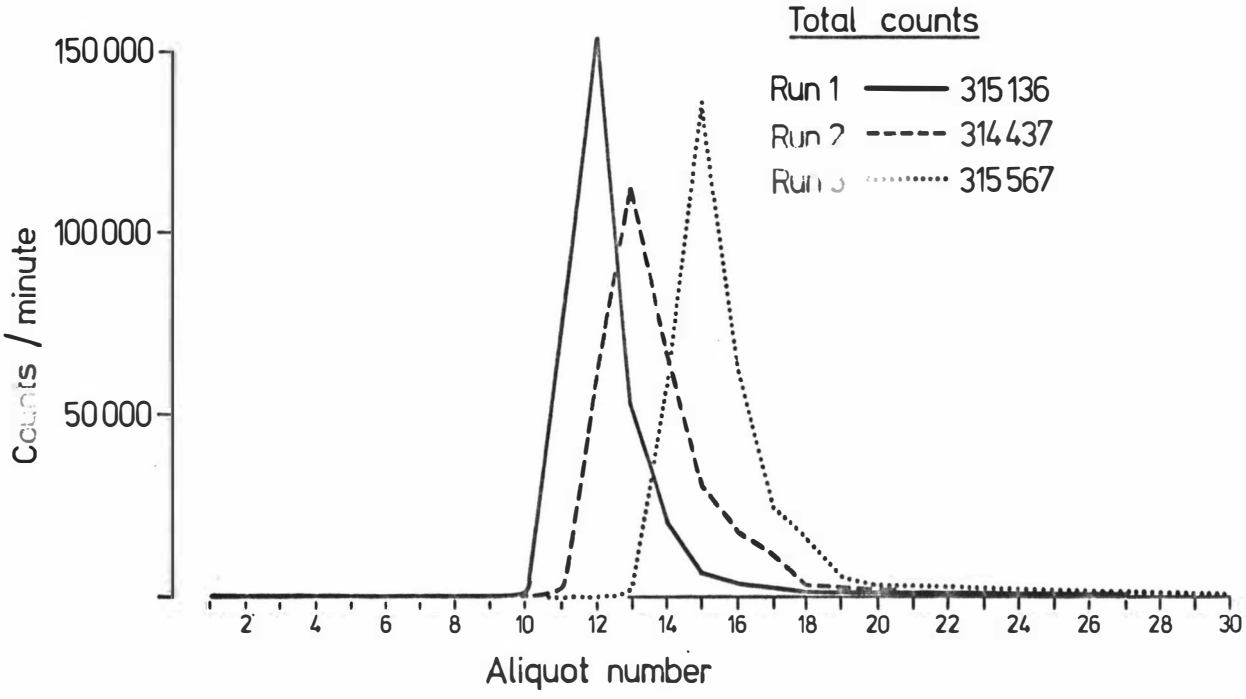

Total counts
Run 1 315136
Run 2 314437
Run 3 315567
150000
100000
50000
Counts / minute
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Aliquot number

Figure 15 Histogram of relative serum dilutions in each aliquot taken from Auto-Analyzer

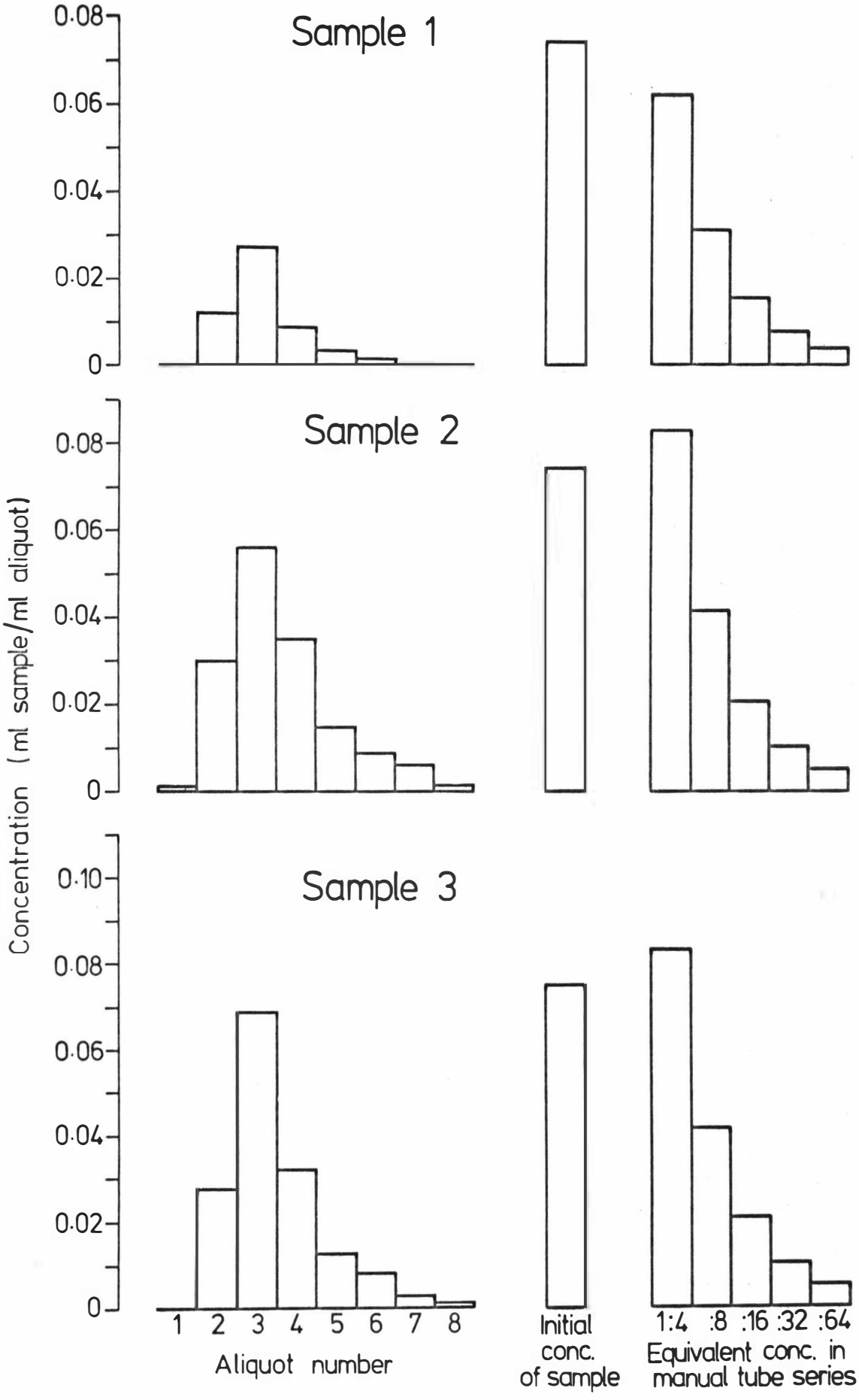

Sample 1
Sample 2
Sample 3
0·08
0·06
0·04
0·02
0
0·10
Concentration (ml sample/ml aliquot)
1 2 3 4 5 6 7 8
Aliquot number
Initial conc. of sample
1:4 :8 :16 :32 :64
Equivalent conc. in manual tube series

Table XIV. Summary of levels of radioactivity recorded before and after dilution of samples by Auto-Analyzer

Sample No.	1	2	3
Initial counts per min (cpm)	1267606	1267606	1267606
Counts remaining after sample aspirated (cpm)	898702	897164	897045
∴ counts aspirated (cpm)	368904	370442	370561
Total counts retrieved (cpm)	315136	314437	315569

Effect of Antigen Concentration on *Brucella abortus* Complement Fixation Test

Figure 16 illustrates a series of typical antigen titration charts obtained by using a continuous dilution method of antigen titration with the complement concentration adjusted to give mid-range peak heights. The corresponding antigen dilution for each peak height has been calculated and noted on the chart. Whereas sample C requires an antigen dilution of about 1:3000 for optimal complement fixation, sample A requires in the region of 1:1000 antigen. At antigen dilutions of 1:3000 or more sample A fails to show evidence of significant complement fixation.

Figure 17 shows how different dilutions of the same serum also have different optimal antigen concentrations. The serum used for this set of curves was a strongly prozoning serum and it can be seen that in general the concentration of antigen required is relatively high, i.e. less than 1:750.

Figures 18 and 19 chart optimal antigen dilutions for different dilutions of positive non-prozoning sera. In these cases, although less antigen is required as the sample is diluted, an antigen more concentrated than 1:1000 is not required.

The Characteristics of Auto-Analyzer Chart Traces Given by Prozoning Sera

A number of sera known to prozone in manual complement fixation tests were tested on the Auto-Analyzer. Figure 20 illustrates a series of typical peaks for prozoning and non-prozoning sera at various antigen dilutions. It can be seen that while the highest standard peak height is obtained when the antigen dilution is at 1:1000, prozoning serum peak heights are greatest at [Ag] = 1:500. Table XV gives manual CFT results for each of these sera.

The most notable peaks are those given by sample numbers 6, 7 and 10. These very strongly prozoning sera each have a characteristic diphasic peak.

Figure 16 Series of typical antigen titration charts from Auto-Analyzer; figures represent the reciprocal of the effective dilution of antigen at tip of peak.
Each chart was constructed by repeatedly sampling a positive serum while the antigen was being continuously diluted.
The series of peaks represent the strength of reaction given by the serum at each antigen dilution.

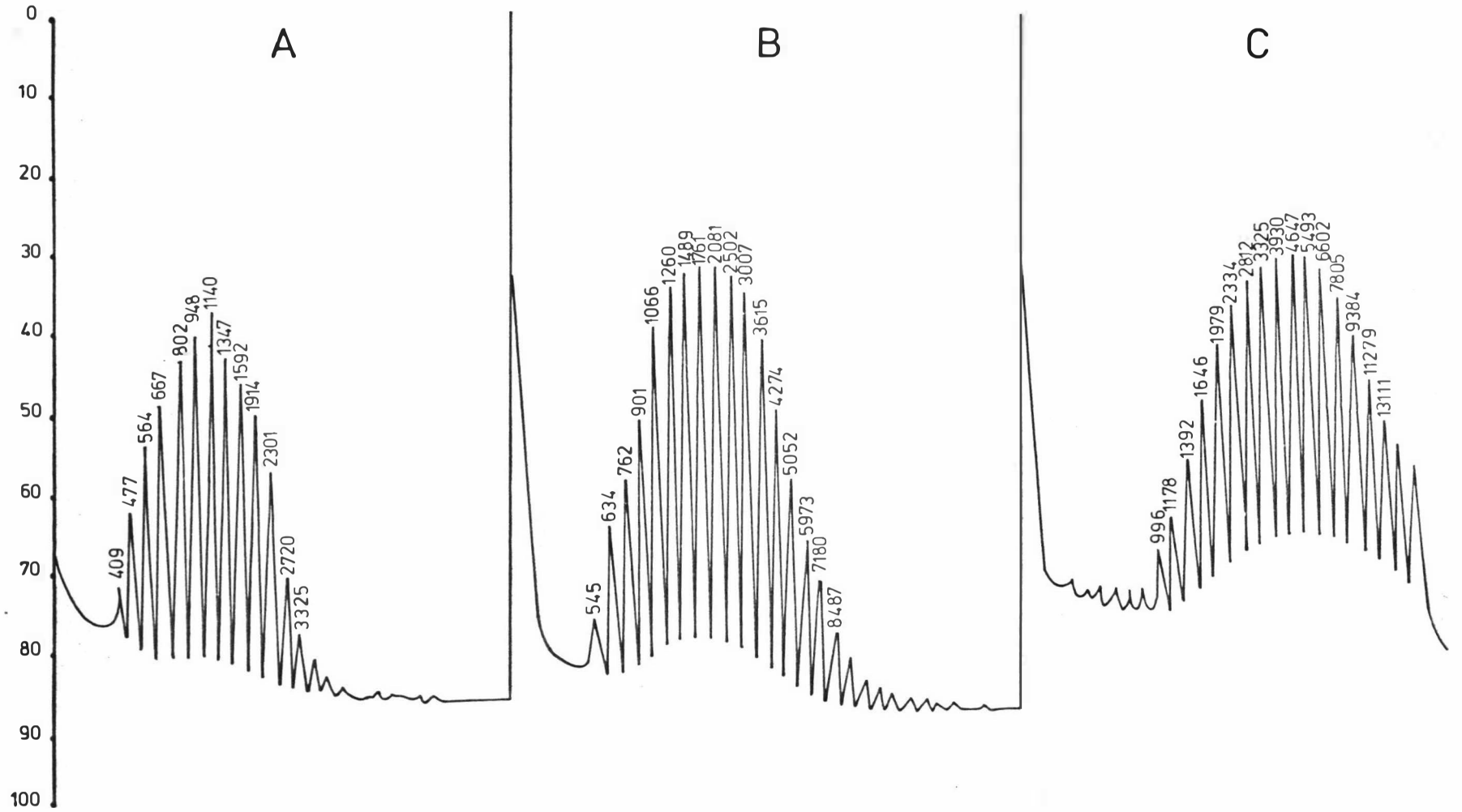

A
B
C
0
10
20
30
40
50
60
70
80
90
100
409
477
564
667
802
948
1140
1347
1592
1914
2301
2720
3325
545
634
762
901
1066
1260
1489
1761
2081
2502
3007
3615
4274
5052
5973
7180
8487
996
1178
1392
1646
1979
2334
2812
3325
3930
4647
5493
6602
7805
9384
11279
13111

Figure 17 Antigen titration with varying serum dilutions of a prozoning serum. Curves were constructed from a series of charts similar to those displayed in figure 16.

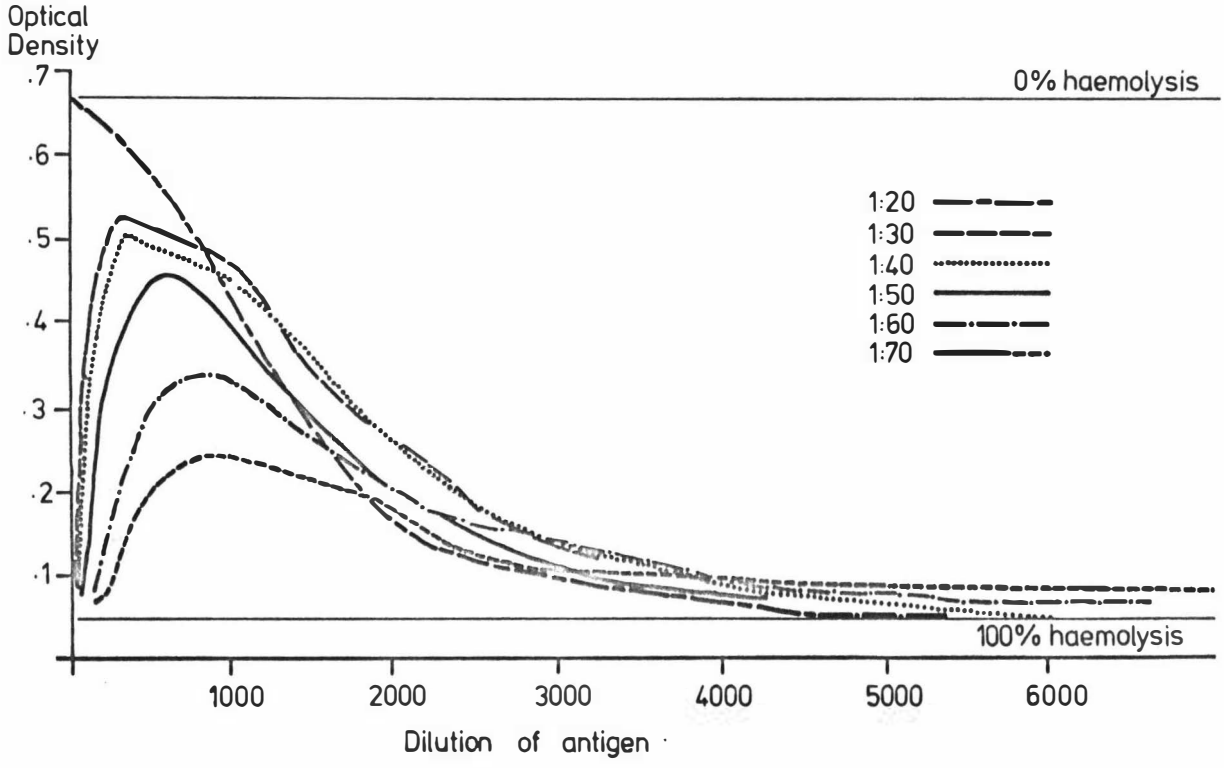

Optical
Density
.7
.6
.5
.4
.3
.2
.1
0% haemolysis
100% haemolysis
1:20
1:30
1:40
1:50
1:60
1:70
1000
2000
3000
4000
5000
6000
Dilution of antigen

Figure 18 Antigen titration with varying serum dilutions of a non-prozoning serum

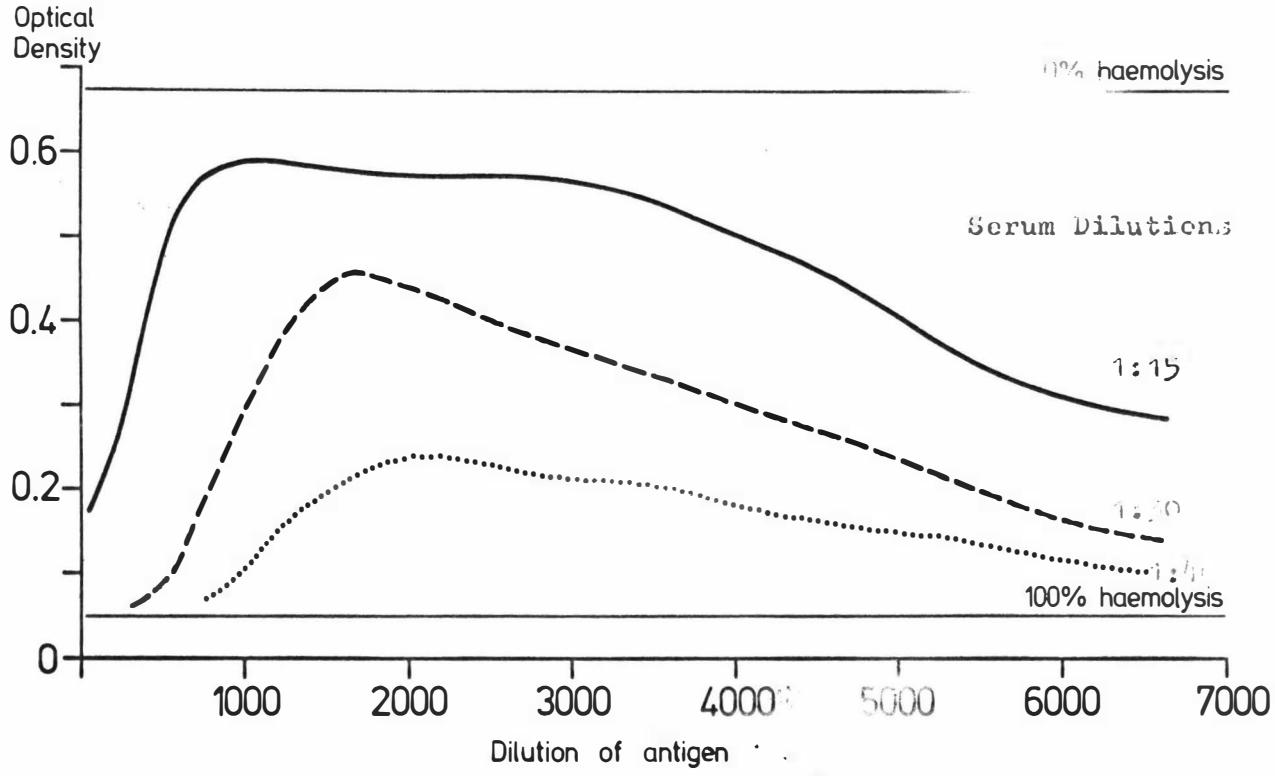

Optical
Density
0% haemolysis
0.6
Serum Dilutions
0.4
1:15
0.2
100% haemolysis
0
1000
2000
3000
4000
5000
6000
7000
Dilution of antigen

Figure 19 Antigen titration with varying serum dilutions of a non-prozoning serum

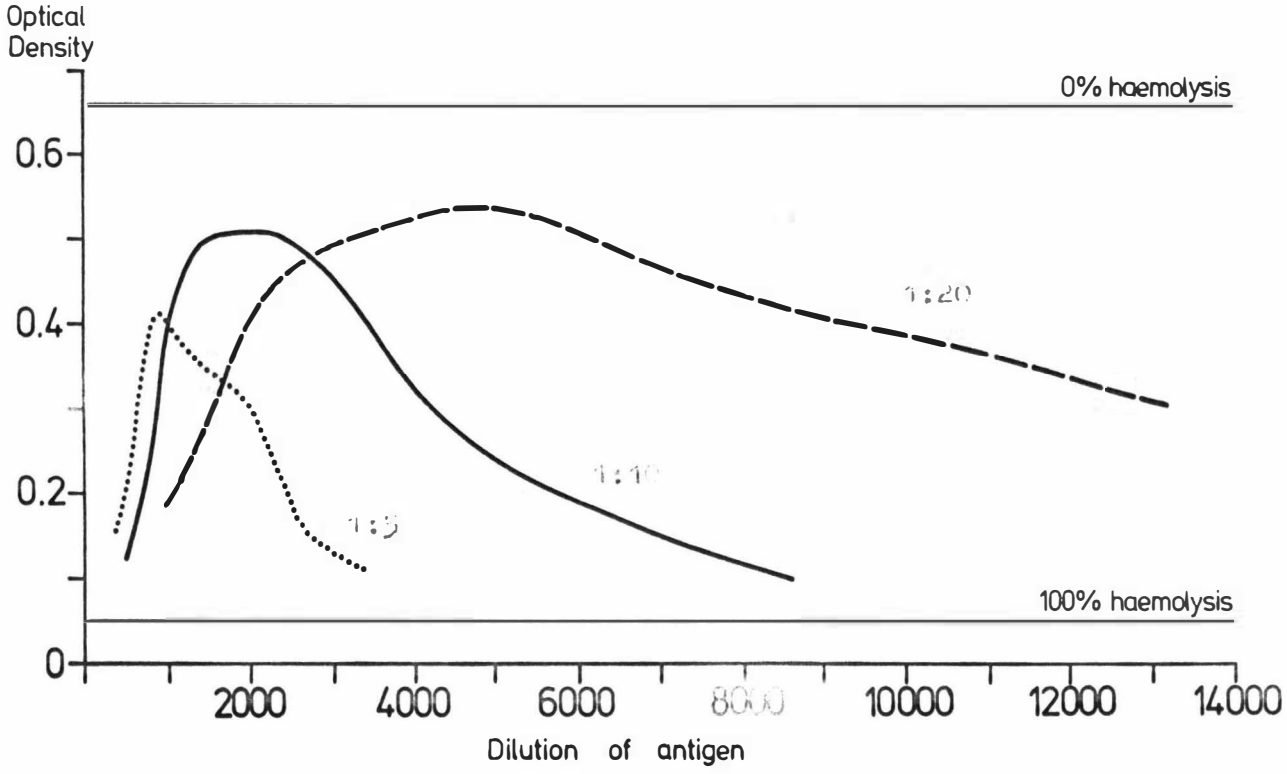

Optical Density
0% haemolysis
0.6
0.4
0.2
1:20
1:10
1:5
100% haemolysis
0
2000
4000
6000
8000
10000
12000
14000
Dilution of antigen

Figure 20 Effect of variation in antigen concentration on prozoning sera in automated complement fixation test

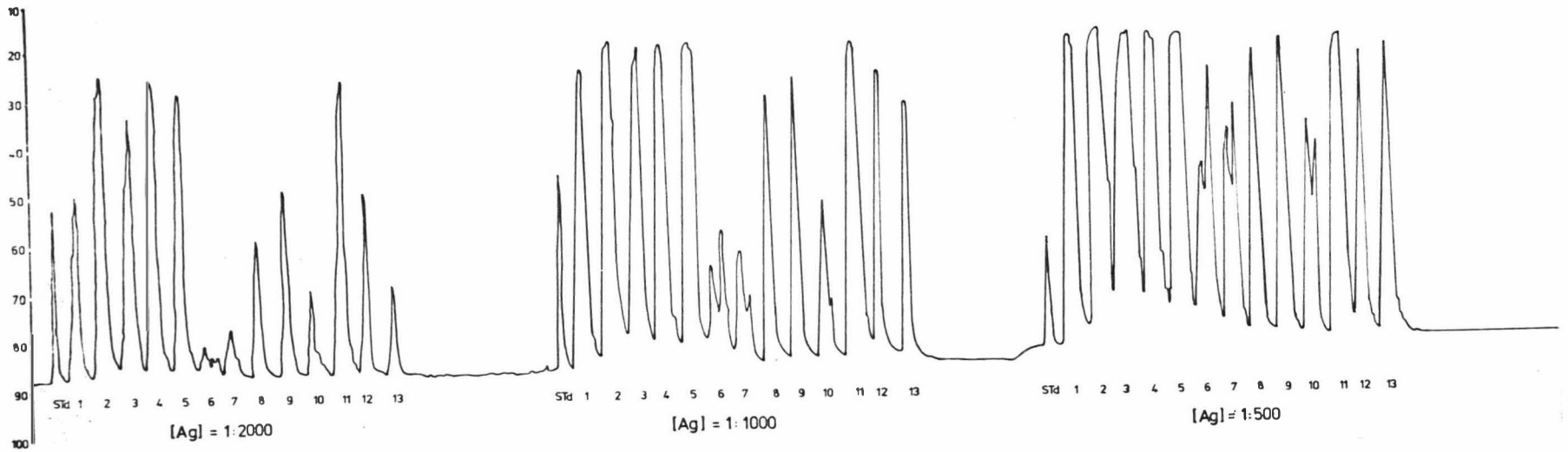

10
20
30
40
50
60
70
80
90
100
STd 1 2 3 4 5 6 7 8 9 10 11 12 13
[Ag] = 1:2000
STd 1 2 3 4 5 6 7 8 9 10 11 12 13
[Ag] = 1:1000
STd 1 2 3 4 5 6 7 8 9 10 11 12 13
[Ag] = 1:500

Table XV. Manual CFT results for sera giving peaks shown in Figure 20

Sample No.	CFT titre	
	Warm fixation	Cold fixation
1	$-^{3}234^{2}2$	$34^{5}3$
2	4^{9}	4^{10}
3	$-^{4}234^{5}$	$234^{7}3$
4	$34^{6}1$	$4^{6}2$
5	4^{6}	4^{6}
6	$-^{20}$	$-^{5}34^{3}$
7	$-^{20}$	$-^{6}24^{3}$
8	4^{2}	4^{2}
9	$4^{2}2$	432
10	$-^{4}123^{3}2$	$4^{7}2$
11	24^{6}	4^{7}
12	4^{3}	4^{4}
13	$-^{2}24^{2}2$	234^{4}
Standard	42	42

Titres are given in the notation explained in section 3.2 where $4^{2}2$ indicates that a reaction of 4(++++) was seen in the first two tubes of the series and a reaction of 2(++) was seen in the third tube. $-^{20}$ = No reaction seen in any of a 20 tube series.

Discussion

Use of the Auto-Analyzer adaptation of the complement fixation test for *Brucella abortus* antibodies has enabled the New Zealand brucellosis eradication scheme to proceed at a rate beyond that which would have been attainable if manual testing systems had been employed.

Sobota and Gillen (1965), Vargues *et al.*(1965) and Gaillon *et al.*(1966) have described Auto-Analyzer adaptations of the complement fixation test particularly with respect to the sero-diagnosis of syphilis. Joubert *et al.*(1967) later described an automated complement fixation test for brucellosis but did not elaborate on its practicality or properly evaluate it against manual tests or known infected animals.

Te Punga (1971) first described the automated CFT as used in the New Zealand eradication scheme. He described the mechanics of the test but did not detail the epidemiological studies that led to its acceptance as a definitive test. In the unpublished studies associated with the development of the automated test, Te Punga concluded that the cold fixation CFT related better to the automated test than did the warm fixation method. It was also observed that the cold fixation test was more efficient at detecting infected animals than was the warm fixation test, this advantage of the cold test has also been noted by Zeissig and Mansfield (1930), Trilenko (1957) and Isayama (1961).

Minor modifications to the original (Te Punga 1971) manifold layout and testing methods have been made and are detailed by Timbs *et al.*(1978a).

Of utmost importance in the performance of the automated CFT is the use of a "standard" serum with each group of 39 samples (each Auto-Analyzer carousel holds 39 unknown, plus one "standard", a total of 40 samples). The titre of this "standard" is set according to accepted international opinion and for this series of experiments was used at a complement fixation titre equivalent to 1/30 th that of the Second International Standard anti-*Brucella abortus* serum.

The continuous dilution method of titration has been used in studies of the kinetics of complement fixation systems by Vargues

and Ayera (1963), Vargues (1965) and Vargues et al. (1966). In the present study it proved to be a most useful means by which antigen and complement affinity relationships could be accurately measured.

It has been shown that various prozoning sera can produce peak heights below that of the "standard" despite being positive to manual tests. The choice of antigen concentration is most important in this regard. Experiments outlined in this chapter have shown how sera have different optimal antigen concentrations and how a particular concentration chosen after a titration with one serum may not be entirely suitable for another serum. The effect of an increased concentration of antigen in suppressing prozoning is often seen when performing manual antigen titrations but has only recently been formally described (McNaught et al., 1977).

In manual tests a wide latitude of antigen concentration is tolerable provided sufficient serum dilutions are made (e.g. 1:100-1:400 Ag). However, in the automated test the use of a sub-optimal antigen concentration cannot be compensated for by other factors. Unfortunately, one particular concentration will not be optimal for all sera so a compromise must be reached. It is thus important to choose for antigen titrations, a serum which is known to require a mid-range optimal antigen concentration and to err on the side of a more concentrated reagent when in doubt. In practise, the serum developed for use as the laboratory standard should be chosen with this particular quality in mind. From the results shown in this section and those of McNaught et al. (1977) it is apparent that prozoning sera require high antigen concentrations to effect optimal complement fixation, and the extension of this argument is that sera with high concentrations of specific IgG_2 require more antigen than do those with lower amounts of IgG_2 antibody.

Von Krogh (1916) applied mathematical formulae to various chemical reaction rates. Among the systems he investigated was that of sensitised erythrocyte lysis by complement. According to Mayer (1961) the value of $\frac{1}{n}$ in the Von Krogh equation $[\chi = K(\frac{y}{1-y})^{\frac{1}{n}}]$ determines the shape of the sigmoidal curve and is usually $0.2 \mp 10\%$.

Manual complement titrations performed in connection with this project invariably gave this value for $\frac{1}{n}$. On the other hand the automated complement fixation test system was found to give a consistent value of $\frac{1}{n} = 0.14$. The value of $\frac{1}{n}$ can be raised by either increasing the concentration of erythrocytes, or increasing the calcium ion concentration $[Ca^{++}]$.

Since the same buffer was used in both manual and automated test systems it could not be a change in $[Ca^{++}]$ that affected the value of $\frac{1}{n}$. In the manual CFT a 2% erythrocyte suspension is used and upon dilution in the test system equates to a final concentration of 0.25%. In the automated test the final erythrocyte concentration is calculated by dividing the erythrocyte pump tube flow rate by the total of the reagent pump tube flow rates and multiplying by 2/100 (Initial red cell conc. = 2%).

Thus the final concentration of erythrocytes in the automated test is

$$\frac{0.32}{2.9 + .23 + .32 + .32} \times \frac{2}{100} = 0.17\%$$

which is somewhat less than the 0.25% concentration obtained in the manual test and explains the relatively low value of $\frac{1}{n}$.

When $\frac{1}{n}$ is small, a small change in complement concentration will result in a large change in the degree of lysis. Thus the automated test as described is relatively sensitive to small differences in the titre of samples which have approximately the same antibody concentration as does the standard serum, i.e. when they achieve sufficient fixation of complement to give 20-80% haemolysis of erythrocytes.

The I^{125} radio-labelled protein method proved to be a useful way of measuring the dilution gradient of the sample within the Auto-Analyzer system. Earlier attempts to measure this sample dilution gradient with dyes generally proved to be unsatisfactory due to difficulties in balancing the colorimeter/recorder to accurately measure a sufficiently large gradient of dye colour, and due to dyes tending to adhere to the internal walls of the tubing. It has been stated that the main problem with the automated CF test is the difficulty in choosing a suitably representative serum dilution to use (Miller, 1971a). With a knowledge of the serum dilution gradient eventually achieved - as shown in figures 14 and 15 - the way in which a given reaction is represented on the chart

recorder is more readily comprehended. It is the overall balance of the system and the way in which reagents are titrated as well as the serum dilution chosen that eventually determines the validity of results for a wide range of samples.

Prozoning samples present a peculiar problem. It has been shown by McNaught et al. (1977) and in the experiments outlined in chapter 5 that specific IgG_2 is capable of inhibiting complement fixation by specific IgG_1. The nature of this blocking effect is unknown, but its intensity can be reduced by increasing antigen concentration. Results illustrated by Figures 17 and 18 show how increasing antigen concentrations will also inhibit the effect of prozoning in the automated test.

Figures 17, 18 and 19 illustrate how different sera have different optimal antigen concentrations for complement fixation. Although it has not been conclusively proven, it appears that the mechanisms responsible for prozoning are the same as those mediating the unusually high antigen binding capacity, for optimal complement fixation, in some sera, as the degree of prozoning exhibited by a serum is antigen concentration dependent. Sera that do not normally show prozoning in manual tests may in fact still have Brucella-specific IgG_2 present, the amount of which may well determine the optimal antigen concentration for best complement fixation.

Thus the concentration of specific IgG_2 within a sample appears to determine its characteristics for:-

(i) Prozoning

(ii) Optimal antigen concentration for complement fixation.

It also appears that "prozoning" is simply a visual manifestation of a serious antigen/antibody imbalance, within a test system, which is mediated by specific IgG_2. A less serious imbalance may not necessarily be visible but it may well cause differences in titre between two similar sera when different antigen concentrations are used in the CF test. This effect was often seen when two sera of apparently identical titre were found to have differing titres when tested using a different antigen concentration. Generally titre differences within acceptable antigen dilution ranges were small enough to be of little consequence but occasional sera, particularly those that prozoned

in manual tests, did show larger titre variations. Figure 20 illustrates how the sensitivity of the automated test varies with antigen concentration and in particular how the degree of prozoning of a serum influences differences in peak heights obtained. By using sufficiently strong antigen the peak heights of prozoning sera can be elevated. However, this may be at the expense of suppressing peak heights of normal sera if unduly high antigen concentrations are used.

It is important therefore, to choose an antigen concentration sufficiently high to enable detection of prozoning sera yet not so high that normal - particularly low titre - peaks are suppressed.

Following from these observations it can be appreciated that a simple direct interpretation of chart traces is not all-together possible. Although all peaks above that of the standard may be taken as positive it cannot be said that all peaks below the standard are negative. Some of these "small" peaks may be due to prozoning sera. Usually such peaks, especially those given by strongly prozoning sera, are recognised by the trained observer. Prozone peaks are generally proportionally wide and often have a ragged top c.f. Figure 20. Peaks due to low titred normal sera are fine and have a sharp spike. To properly interpret charts it is therefore necessary to repeat manually any sample giving a definite peak below the standard peak height: fortunately the prevalence of such peaks is normally very low.

CHAPTER 6. ANALYSIS OF FIELD DATA

Introduction

The experiments and concepts developed in this thesis have largely evolved in response to the fundamental question "what is the significance of animals which are repeatedly card test positive and complement fixation test negative, in the New Zealand brucellosis eradication scheme?" To enable a proper appreciation of the relationship between the card and complement fixation tests national and regional data was analysed to assess the way in which the two tests interacted as the scheme progressed. Timbs et al. (1978a) investigated the existence and eventual fate of animals that were repeatedly BCT+ and CFT-. This study examines the prevalence of BCT+/CFT- animals in herds with varying initial CFT reactor rates.

Materials and Methods

Certain basic information concerning details of all tests carried out during the N.Z. brucellosis eradication scheme is stored within a central computer file.

Until August 1977 all sera from initial dairy herd tests were card tested and card test positive sera were then complement fixation tested. Initial beef herd tests were treated in this way until August 1975. At tests other than these the card test was not necessarily performed and the computer files did not differentiate herds which were card tested and had no BCT positives from those which were not card tested. Thus data from all repeat herd tests and some initial beef tests could not be used.

Results

Table XVI lists details of BCT and CFT reactor rates for dairy and beef herds at initial herd tests.

(a) For dairy herds

Linear regression $y = a + bx$

$$y = \frac{\text{No BCT+} - \text{No CFT+}}{\text{No animals} - \text{No CFT+}} \times 100$$

$$x = \frac{\text{No CFT+}}{\text{No animals}} \times 100$$

$a = 2.5$ $b = 0.225$

$F_{1,5} = 60.71$ ** (1%) $R^2 = 92.4\ \%$

(b) For beef herds

$a = 3.7$ $b = 0.346$

$F_{1,5} = 18.10$ ** (1%) $R^2 = 78.4\%$

Thus a significant correlation exists between the overall herd infection rate and the proportion of animals within herds that are BCT+ but CFT-.

Table XVI. BCT and CFT reactor rates at initial herd tests

(a) Dairy herds

Herd CFT reactor rate	No. herds	No. animals	No. BCT+	No. CFT+	% CFT- that are BCT+
0	4895	520137	17903	-	3.44
0-1	1435	244045	5924	1586	1.79
1-5	4147	533992	27927	14173	2.64
5-10	2439	343042	38341	24977	4.20
10-15	1305	183006	31496	22401	5.66
15-20	608	83122	18898	14291	6.69
> 20	418	49090	15231	12357	7.82

(b) Beef herds

Herd CFT reactor rate	No. herds	No. animals	No. BCT+	No. CFT+	% CFT- that are BCT+
0	6207	832184	33904	-	4.07
0-1	829	277155	9434	1198	2.97
1-5	1274	220418	16188	5319	5.05
5-10	406	80729	11427	5779	7.53
10-15	155	30945	5878	3673	8.09
15-20	58	8371	1842	1426	6.00
> 20	63	5010	1854	1318	14.52

Discussion

Following the inability to culture significant organisms from 20 repeat BCT+/CFT- cows the theme of this project switched to an investigation of the means by which animals might react to the card test but not to the complement fixation test.

Possible explanations for the occurrence of BCT+/CFT- animals are:-

(i) If the animal is infected an unusually high specific IgG_1:IgG_2 ratio may cause the complement fixation test to prozone to such an extent that it is erroneously considered to be negative.

(ii) An animal, previously sensitised by calfhood strain 19 vaccination, may come into contact with killed Br. abortus organisms or with low doses of live organisms, such that an immune response is generated without infection becoming established.

(iii) A chronically infected animal may have only low levels of specific IgG_1 and IgG_2. Such low levels may cause the card test to react but may not cause a complement fixation reaction either because of the very low antibody levels or because of an unsuitable IgG_1:IgG_2 ratio.

(iv) In the early stages of infection an IgM response generally precedes that of IgG. The greater sensitivity of the card test to specific IgM (Allan et al 1976) may result in a card test reaction in the absence of any complement fixation titre.

In Case (iv) it is to be expected that at a subsequent sampling the animal would have become BCT+/CFT+ whilst in cases (i), (ii) and (iii) it is conceivable that an animal could continue to be BCT+/CFT- for some time.

In this thesis an attempt has been made to investigate some of the circumstances leading to the existence of such BCT+/CFT- animals particularly those which may be explained by points (i) and (ii) above.

Analysis of the data presented in Table XVI illustrates that animals in heavily infected herds are more likely to be BCT+/CFT- than animals in non-infected or lightly infected herds. Thus it is evident that the card test reactivity may be stimulated by exposure to - but not necessarily infection by - *Brucella* organisms.

It has been observed by some workers that a card test reaction develops earlier than a CFT titre in infected animals (Nicoletti, 1967; Morgan *et al.*,1969; Davis,1971; Fensterbank,1973; Pietz, 1977). It could be that some of the BCT+/CFT- animals investigated above belong to this group. Results of experiments reported by Fensterbank (1973) and Pietz (1977) indicate that the average period between development of a positive BCT reaction and a positive CFT reaction is of the order of 10-20 days. It is unlikely therefore that this phenomena contributes significantly to the overall numbers of BCT+/CFT- animals detected. Timbs *et al.*(1978a) found that approximately 16% of BCT+/CFT- animals, in infected herds, detected at any particular herd test might be expected to become CFT+ at a later test. Included in this number would be animals in the early stages of titre development which had developed a BCT titre prior to producing a CFT titre.

CHAPTER 7. GENERAL DISCUSSION

In choosing a test on which to base a bovine brucellosis control scheme it has been customary for each country to carry out pilot studies to determine not only the prevalence and distribution of the disease but also to investigate the relative merits of the various diagnostic tests. Until recently it has been the SAT that has invariably been chosen for use in routine testing whilst various modifications of this test such as mercapto-ethanol, rivanol, heat-labile and Coombs tests have been used as supplemental tests. Among the developed countries New Zealand was relatively late in formulating an eradication programme. Technological improvements did, however, allow the development of an automated complement fixation test which was chosen as the definitive test for use in the scheme.

A particular problem met with in justifying the use of the Auto-Analyzer adaptation of the CFT was the theoretical argument that as the test is essentially carried out at one dilution, it may not detect sera which cause a strong prozone reaction. Investigation of this aspect has shown that in practice such sera are in fact detected, and examination of the serum dilution gradient within the test system has explained the ability of the automated test to detect such sera.

Examination of prozoning sera recovered from known infected animals revealed that it is the *Brucella* specific IgG_1 : IgG_2 ratio that determines the degree of prozoning of a serum in the CFT. It was also shown that a high antigen concentration can suppress the extent to which such prozoning occurs. The results obtained confirm the findings of Plackett and Alton (1975) and of McNaught *et al*. (1977) that sufficient quantities of *Brucella* specific IgG_2 can cause prozoning and abnormal reactions in the CFT or prevent complement fixation by IgG_1 altogether.

Although a serious imbalance of the *Brucella* specific IgG_1 : IgG_2 ratio will lead to obvious prozoning, a less serious imbalance will not be manifest in this way but may be reflected in sub-optimal complement fixation leading to a reduced titre. The extent to which a titre may be reduced is dependent upon the degree to which the antigen concentration used varies from the optimal antigen concentration required for the particular serum.

To ensure that the complement fixation test system is made as sensitive as possible to prozoning sera, it is essential that adequate care is taken in the titration of antigen. A series of known positive sera must be used in antigen titrations to enable an appreciation of the relative range of optimal values likely to be obtained for a wide range of sera. This principles applies equally to titrations for the automated or manual tests.

The existence of prozoning sera can explain why some sera are BCT+ yet apparently CFT-. Such sera can be those with an unusually high proportion of specific IgG_2 which completely blocks any complement fixation. The frequency with which such sera occur and their likely significance in an eradication programme is unknown. Animals yielding prozoning sera have occasionally been re-bled after 2-6 weeks and the prozone effect has usually been very much reduced - and often non-existent - in the repeat sample. Brandon *et al*. (1971) and Williams and Green (1976) found that at about the time of parturition serum IgG_1 levels fall as this immunoglobulin is selectively concentrated in the udder. The serum IgG_1 : IgG_2 ratio therefore undergoes considerable change. A reversion to a more normal ratio is achieved some 4-6 weeks after parturition. Because of the repetitive nature of testing in an eradication programme the existence of a small number of cattle with low IgG_1 : IgG_2 ratios at any particular time is unlikely to unduly affect testing progress. However, care must be taken in the interpretation of test results from recently calved or aborted cows, and the existence of potentially false negative CFT results should be recognised.

Investigations of the way in which the IgG_1 : IgG_2 ratio changes in bovines are practically non-existent. Because of the effect a change in the *Brucella* specific IgG_1 : IgG_2 ratio may have on results of the CFT, examination of the physiological changes in this ratio particularly during the incubation phase of infection and also at about the time of parturition, is required.

Upon examination of scheme records, it was apparent that more animals were likely to be BCT+/CFT- in infected herds than in non-infected herds. This lead to the hypothesis that animals previously vaccinated with living strain 19 *Br. abortus* as calves, then exposed to, but not infected by, the virulent organism as

adults, may develop a BCT titre in the absence of any titre to the CFT. Although studies using light challenges of virulent organisms were not carried out, inoculation with killed organisms did stimulate serological responses and proved that such exposed animals could in fact be BCT+/CFT-. On the other hand it was also shown that other animals similarly exposed could be BCT-/CFT+.

Another mechanism by which the BCT may produce false positive reactions but which has not been investigated in this thesis is the presence of residual strain 19 vaccination titres. As IgM is the principle immunoglobulin stimulated in response to Br. abortus strain 19 vaccination, it has been postulated that BCT+/CFT- reactions in non-infected cattle may be due to the effect of a residual vaccination titre (Alton et al., 1975b). Allan et al. (1975) demonstrated that specific IgM antibody is up to ten times more efficient on a weight basis at producing a BCT reaction than is either IgG_1 or IgG_2.

Strain 19 vaccination of all female calves was compulsory in New Zealand between 1966 and 1976. The present voluntary vaccination programme will eventually be phased out when the prevalence of the disease is reduced to an acceptable level. As cows which were not vaccinated as calves begin to form a significant proportion of all cattle being tested, it is expected that the prevalence of unusual serological reactions will decline.

APPENDIX I - REAGENTS

(i) Barbital buffered saline (Kabat and Mayer 1961) pH 7.3)

Sodium chloride	85 g
Sodium 5,5-diethyl-barbiturate	3.75 g
5,5-diethylbarbituric acid	5.75 g
Magnesium chloride (anhydrous)	0.475 g
Calcium chloride (anhydrous)	0.185 g

Make up to 2 litres with distilled water to prepare a concentrated stock solution. Dilute stock solution 1:5 (1 ml stock plus 4 ml water) to prepare working strength buffer.

(ii) Alsever's solution

Glucose	18.66 g
Sodium chloride	4.18 g
Sodium citrate	8.00 g
Citric acid	0.55 g
Distilled water to	1000 ml

The solution placed in screw-capped bottles and autoclaved.

(iii)

Phosphate buffered saline	1 ml
Di-sodium hydrogen phosphate Na_2HPO_4	12 g
Sodium di-hydrogen phosphate $NaH_2PO_4 2H_2O$	2.5 g
Sodium chloride NaCl	85 g
Distilled water to	1000 ml

(iv) Phosphate buffer 1 M

Solution A:

Sodium dihydrogen phosphate
$NaH_2PO_4\ 2H_2O$ 31.202 g/l

Solution B:

Di-sodium hydrogen phosphate
Na_2HPO_4 28.39 g/l

Adjust solution A to pH 8 with solution B.

(v) Phenol saline

Sodium chloride	8.5	g
Phenol	5.0	g
Distilled water to	1000	ml

(vi) Immunoelectrophoresis (IEP) agar
Radial immunodiffusion (RID) agar
Gel diffusion (GD) agar

(a) Prepare barbiturate buffer pH 8.6

Sodium diethylbarbiturate	9	g
0.1 M Hydrochloric acid	65	ml
Sodium azide	0.5	g
Distilled water to	1000	ml

(b) Add 3 g Ion agar or Noble agar to 100 ml barbiturate, heat to dissolve.

Distribute in suitable volumes into screw capped bottles.

When required for use add an equal volume of barbiturate buffer and heat in a boiling water bath until dissolved, then pour onto glass plates. For RID agar add anti-serum at suitable dilution to buffer, heat buffer and agar to 58 ^{o}C, mix quickly and pour plate.

(vii) I.E.P., R.I.D., G.D. Stain

(a) Rinse solution

Methanol	5 parts
Distilled water	5 parts
Glacial acetic acid	1 part

(b) Stain

Dissolve 1 gm Amido black in 1 litre rinse solution.

(c) Procedure

After development of reaction in agar plate rinse plate for 3 days in frequent changes of 1.5% NaCl. Wash for a further day in distilled water.

Cover plate with filter paper and dry at 37 ^{o}C overnight.

Stain by immersing in stain solution for approx 30 mins then transfer to rinse solution for 15-20 minutes to decolourise. Dry.

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