CHARACTERISATION OF BRUCELLA ISOLATES FROM HUMAN AND ANIMAL PATIENTS IN NAROK DISTRICT OF KENYA

By

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DECLARATION.

This thesis is my original work and has not been presented for examination in any other university.

Dr. Joseph. T. M. Mugambi.....

This thesis has been submitted with my approval as a university supervisor.

1. **Dr. Lilly C. Bebora**.....

2. **Prof. Samuel M. Arimi**..... 19.11.01

DEDICATION.

This thesis is specially dedicated to the memories of my late father, Mr. Philip M'Mugambi and late brother John Kiriinya; my beloved wife Roselyn Nelima, my mother Sarah Ncurubi; my daughter Jemimah Karamuta and sons, Mutwiri, Mwenda and Muritani and my young brother Edward Bundi 'Mugambi. Lastly to the welfare of the poor, pitiable, suffering patients for whom I deeply feel.

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ABSTRACT.

Brucellosis is a cosmopolitan zoonotic disease that afflicts man, his domestic animals and wildlife. The smooth members of the genus are the main culprits worldwide. The incidence of the disease in humans, and which directly relates to that in animals, is highly dependent on animal husbandry practices, animal population, animal population density and inter-group interactions. Disease in humans is related to the interaction between humans and animals, living standards, hygiene and food customs. Owing to these reasons, Narok, the district where this study was undertaken, has the full potential for high occurrence of brucellosis both in animals and humans. It is for this reason that this study was undertaken with the aim of recovering *Brucella* pathogens from man and animals; to gain an insight into the prevalence in animals through sero-survey and where possible to establish the sources of infections to the human patients.

The study was carried out in three overlapping phases in which human patients clinically suspected of brucellosis, in the opinion of the clinician, had two 10ml blood samples collected from the radial vein into vacutainer tubes. One sample was collected and preserved in a vacutainer tube containing potassium oxalate while the other sample, for serum harvesting, was collected into a tube without the anticoagulant. Hemoculture of the preserved blood was done into diphasic medium for not less than eight weeks. The human samples for culture were

chosen on the basis of a quick Rose Bengal plate test (RBPT) on serum, carried out at the point of sampling. This led to the culture of 397 patients who tested positive and 134 (one in five) of the negative ones, all totalling 531. The total human serum samples were 957.

The selection of animal herds was random, using multistage computer methods, and also specific i.e. home herds in which human patients' blood had shown signs of growth or the serum was positive on RBPT. From each home herd, a random sample of 20 cattle, 10 sheep and 10 goats were chosen and each animal had 10ml of jugular blood collected into a vacutainer tube. Animals with signs implicative of brucellosis such as lameness, abortion or hygroma had a second 10ml of blood taken for hemoculture as in the human suspects. In all, cattle sera were 1,620, caprine 1,257 and ovine 557. The cultured animal blood were 388 comprising 132 cattle, 196 goats and 50 sheep; ~~out~~ of which 54 cattle, 73 goats and 34 sheep had a history of abortion.

The human and animal sera were simultaneously screened at the central Veterinary Investigation Laboratories (Vet Labs), Kabete; using Rose Bengal plate test (RBPT), Serum agglutination test (SAT) and Complement fixation test (CFT). Thus, the human serum was tested on RBPT twice; at the sampling points and at Vet Labs Kabete.

From the 531 cultured human blood samples, *Brucella abortus, Yersinia* and *Campylobacter* species were isolated three, eight and 11 times, respectively. From the cultured animal blood, only *Campylobacter* species were realised in one cow, eight goats and 11 sheep. The 957 human sera yielded a cumulative total of 78 (8.2%) reactors with individual test results being; RBPT 62 (6.5%), SAT 40 (4.2%) and CFT 23 (2.9%). From the 1620 cattle sera tested, 177 (10.9%) cumulative reactors were realised with RBPT picking 75 (4.6%), SAT 136 (8.4%), and CFT 53(3.3%). The 1257 caprine sera had 106 (8.4%) reactors with RBPT picking 38(2.7%), SAT 69(5.5%) and CFT 39(3.1%), while the ovine reactors were 45(8.1%) with RBPT picking 23(4.1%), SAT 19(3.4%) and CFT 16(2.9%), respectively.

From the serological results, it is clear that brucellosis is widely distributed in Narok District both in human beings and in animals. Many human cases were misdiagnosed both clinically and serologically probably due to poor interpretation of RBPT results and also due to cross-reactivity as a result of infections by other organisms such as *Yersinia* species. Clinical misdiagnosis could be due to inadequate understanding of the possible differentials, while poor interpretation of RBPT readings could be a result of inadequate training. This poor interpretation of RBPT results led to prolonged antibiotic treatment on patients, who otherwise were without brucellosis. Isolation of *Yersinia* species was accompanied by positive RBPT results, while that of *Campylobacter* species showed no cross-

reaction. Yersiniosis, campylobacteriosis and probably typhoid, may be a big menace especially in place and times when human resistance is compromised by malnutrition as in this case. These other diseases are directly linked to lack of clean drinking water, poor personal hygiene and absence of toilets.

The serological results tend to imply that sheep and goats aborted due to brucellosis as flocks with a high rate of abortion also had higher reactor rates. Also, the serological reactors in the flocks with a high rate of abortion tended to have higher titres than the titres realised in the reactors from flocks with low abortion rate or those that had no abortions at all. Also, the three serological tests used, tended to agree more in groups with high abortion rates than in the groups in which no abortions had been noticed.

Campylobacteriosis is another common cause of abortion particularly in small ruminants in which it was isolated only from sero-negative sheep and goats with abortion.

1.0. INTRODUCTION.

Brucella are a coherent assembly of closely related organisms, separated from one another by differences in metabolic characteristics and sensitivity to dyes (Jones, *et al.*,1963). They cause the disease brucellosis, which is one of the most important zoonoses in many parts of the world. The importance of this disease in humans dates back to 1887 when David Bruce first isolated the causative agent from livers of British soldiers dying in Malta (Wilson and Miles, 1975). It was found to be a zoonosis by Zammit and Harrocks in 1905, and is to date one of the most important zoonosis (Young, 1989). Since then, the disease has been associated with domestic animals i.e. bovine, caprine, ovine, cameridae, swine and canidae (Acha and Szyfres, 1989; Jubb *et al.*, 1993). It is more of a veterinary problem occurring in the domestic animals although it has also been diagnosed in wild ones, with significant antibody levels being recorded in the latter (Binminger *et al* 1980; Paling *et al.*, 1988; Randostits *et al.,* 1994; Waghela and Karstand, 1986). The disease in all animals is remarkably similar, it being characterised by relapsing bacteraemia that becomes intermittent to chronic in later stages and which may sometimes have recurrences for upto 2 years in 5-10% of the animals (Waghela, 1978).

Cattle, goats and sheep are mainly infected by *B. melitensis*, *B. abortus* and sometimes by *B. suis*. Apart from the above, sheep are the main hosts to *B. ovis,*

while pigs are transiently infected by *B. suis* and to a lesser extent by *B. abortus*. Horses may be infected by *B. abortus, B. melitensis, B. suis and B. ovis*. The canidae are preferentially infected by *B. canis* and also by *B. abortus* or *B. melitensis*, while camels are largely infected by *B. melitensis* (Jubb *et al.,* 1993). The disease in most animal species is mainly confined to the reproductive system, in which it is marked by the accompanying signs.

The disease is still the most important zoonosis with man, who is an accidental dead end host, playing no role in its maintenance. Man is infected by all smooth *Brucella* species except *B.neotomae,* and presents clinically with highly divergent signs, symptoms and severity. Of the rough species, he is infected by *B canis* but not *B. ovis*. The human disease is an occupational hazard being commonest among animal handlers, veterinarians, farmers, butchers and those consuming raw animal products especially from goats and sheep. The infection rate in humans is markedly lower than that in the source animals (Arnow *et al.,* 1984). However, there is a direct relationship between the incidence in both hosts, with the disease pattern in people being highly influenced by animal husbandry practices, food customs and standards of hygiene (Oomen, 1975). Pastoralists are high in the order of the victims, especially those in close proximity with animals or those who share shelter with the animals (Philpott and Auko, 1972; Oomen, 1975). Whole families may be infected through sharing shelter with animals (Elberg, 1981; Oomen, 1975). The disease situation is worse in developing countries where there is low

living standard and poor hygiene (Zimmerman *et al.,* 1990). In some such developing countries, the disease is thought to have reached epidemic proportions, especially among the nomads and pastoralists (Young, 1989). In many such countries the disease is either on the increase or more cases are reported due to better diagnostic methods (Kolar, 1987). The disease is also high in people associated with bacteriology in the laboratory, those handling infected material or travellers into areas where the animal disease is endemic (Acha and Szyfres, 1989; Arnow *et al.,* 1984, Oomen, 1975).

Animal infections are acquired by ingestion of contaminated pastures and water and by direct contact or coitus. The establishment and development of disease is similar in all animals with the susceptibility being dependent on the animal species, its age, reproductive stage and resistance and the infecting dose of the organism. Aborting animals remain asymptomatic carriers for prolonged periods during which time organisms are shed in milk, vaginal secretions and seminal fluids, thereby contaminating the environment and consequently facilitating spread of the disease. Community shared pastures and watering points are main sources of spread for nomadic flocks or those on poor management. Domestic carnivorous animals may acquire *B. abortus* and *B. melitensis* mainly by consuming contaminated foetuses, placentas and milk (Prior, 1976).

In Kenya, the animal disease is endemic, whereby in some parts of the country the prevalence may be high. Many cases have been reported in the various annual reports of the Ministry of Agriculture and elsewhere (Anon 1947, 1955 and 1965; Kagunya, 1977; Waghela, 1978). Serological tests for 10 years (1978 to 1987) at Veterinary Laboratories (Vet Labs) Kabete, gave the following results: 16,273 cattle, 2,761 (17.08%) positive; 2,851 goats, 113 (4%) positive and 1,374 sheep, 529 (38.5%) positive. From 1,112 human sera screened in the same Laboratory for the same period, 378 (32.75%) were positive. The human sera were mainly from the districts of Narok and Turkana (Vet Labs annual reports, 1978 -1988). In Narok alone, the human disease has been found to constitute 13.6% of febrile conditions (Muriuki, 1994). Other past human brucellosis cases were reported by Oomen and Waghela (1974) and Maichomo (1996). Frequently, *B. melitensis* has been the one isolated (Oomen, 1975).

Narok district is largely inhabited by the Maasai. The Maasai homes are built around the circumference of a circular Kraal in which cattle are penned at night. The sheep and goats (shoats) are housed in a small partition of the manyatta or in a hut adjacent to the family one. The manure collects just outside the doorstep. Outside the Kraal, there is a clean area where milking and aided suckling of animal orphans is done. The women, assisted by the children, undertake this chore. The children graze the shoats. During the course of such operations, people come into direct contact with the animals, their secretions and manure. It is from such

operations that people most likely get infected. The men who are in less contact with the animals are at less risk. This may account for the higher numbers of reactors in women and children than in men (Maichomo, 1996). The very young children are at a risk of infection from milk.

The area where the Maasai live lacks clean water and therefore the standard of hygiene is very low. Most people are readily exposed to infected animals that are shedding organisms or, indirectly, to contaminated water and the environment, as the arrangement of the housing units makes contact with possible sources unavoidable. Thus, based on the mode of life, food customs and lack of proper hygiene, the Maasai are at a very high risk of zoonotic and water borne diseases. It is for this reason that the study was undertaken with the following objectives:-

i). To isolate *Brucella* pathogens from patients manifesting and clinically diagnosed for brucellosis.

ii). To establish serologically whether patients had had contact with the pathogen.

iii). To establish the reactor rate of randomly sampled animals with a view to defining the potential risk to the human population.

iv). To establish the infection rate, both serologically and bacteriologically in animals from homesteads of confirmed brucellosis patients.

2.0. LITERATURE REVIEW.

2.1. Brief history.

According to the account given by Wilson and Miles (1975), *B. melitensis,* the first member of the genus *Brucella,* was isolated by David Bruce in 1887 from livers of British soldiers dying from a febrile disease in Malta. Its source was found to have a link to milk from goats and sheep. This species is the most virulent for man among the *Brucella* species. *B. abortus* was isolated by Bang in 1897 from cows showing infectious abortion. It is parasitic to cattle and is also infective to all other domestic animals and man. *B. suis* whose natural host are the swine was isolated by Traum in 1914 from foetuses of affected sows. The last smooth species, *B. neotomae,* was encountered by Stoenner and Lackman from desert rats (*Neotoma lopidus*) in 1957. According to the same authors, two stable rough species; *B. ovis and B. canis* were reported by Bundle and Boyes from the genital tract of infected sheep for the former, while the latter species was described by Carmichael and Brunner in 1968, as a cause of widespread abortions in dogs in the USA.

The disease caused by these organisms has been referred to by various names. In man, the names are brucellosis, undulant fever, Malta fever and Mediterranean

fever. In cattle it is called infectious abortion, enzootic abortion and Bang's disease, among others (Randostits *et al.,* 1994).

2.2. General characteristics of Brucella organisms.

The genus *Brucella* consists of six species of small non-motile, non-spore forming, gram-negative aerobic cocco-bacilli. Each has specific biotypes that differ from one another on the basis of slight biochemical activity, resistance to aniline dyes and phage. They have an aerobic respiratory type of metabolism and a cytochrome based electron transport system with oxygen or nitrate as the final electron acceptor. Many strains require supplemental carbon dioxide for growth especially on first isolation (Corbel and Morgan, 1984). Each species has a predilection for a specific host, but will also infect a wide range of other animals and man: *B.abortus* is for cattle, *B. melitensis* for goats and to a lesser extent sheep, *B.suis* for swine and *B. canis* for canidae. *B.ovis* causes epididymitis in rams. *B.neotomae* has only been isolated once from desert rat in Utah in the USA and whether it is pathogenic is not known for sure (Wilson and Miles, 1975). The type species is *B.melitensis.* All pathogenic members of the genus target cells of the host's reticulo-endothelial system, into which they lounge (Corbel and Morgan, 1984).

2.2.1. The organisms' resistance and survival.

These organisms are destroyed by heat (60^0C for 10 minutes), phenol (15 minutes exposure) and low temperature e.g. at 0^0C for a month. *B. abortus* can however, survive for 6 months in sealed culture tubes on agar and is culturable from sterile water for 3-4 months. *B. melitensis* is reputed to survive for 6 days in urine, 6 weeks in dust, 10 weeks in water or soil and up to 8 months in liquid manure. In fermenting milk, both organisms are rapidly destroyed (Corbel and Morgan, 1984, Acha and Szyfres, 1989).

2. 2. 2 Cellular and colonial morphology and staining characteristics of *Brucella* organisms.

Brucellae are short straight rods or cocci with sides that are parallel or convex outwards. They are shorter than all the other gram-negative bacteria with yet shorter forms that may appear oval, coccal or diplococci when about to divide (Wilson and Miles, 1975). *B. melitensis* is considered more coccal than *B. abortus* with the latter being more capable of changing to bacillary forms especially in rich media like blood agar. On smear the cells appear singly, in pairs, short chains with end to end, in small groups or clusters. When in chains the long axis is in the

direction in which they are lying, unlike gram negative diplococci whose axis is at right angles (Corbel and Morgan, 1984). The short chains predominate when grown in liquid medium. All brucellae have fairly constant morphological features.

The colonial forms when viewed in reflected light are smooth, intermediate, rough or mucoid. Smooth colonies are easily emulsifiable to form stable saline solution while rough forms are granular (Alton *et al.,* 1975, Corbel and Morgan, 1984). Ammonium oxalate and crystal violet greatly help to discern the different colonial forms, which in clear media may appear raised with entire edges and transparent with a smooth shiny surface. They may appear pale honey colour when viewed by penetrating light. The smooth forms are more pathogenic than the rough, and are encountered more frequently. The smooth species also have non-smooth variants that are antigenically different, less virulent and unstable. The change from smooth to rough involves genetic deletion and production of incomplete lipids (Corbel and Morgan, 1984). Brucellae are not truly acid fast but stain red on Machiavello's and modified Ziehl Nielsen (ZN) stains. The cell wall is responsible for the gram negativity and antigenic composition.

2.2.3. Growth requirement.

Brucellae are slow growing fastidious organisms with complex nutritional requirements. Most strains require a rich medium with several amino acids such as thiamine, nicotinamide, calcium pantothionate and magnesium ions. On primary culture they do not grow in an aerobic atmosphere but require added carbon dioxide. The CO_2 is incorporated directly into pyramidines, glycine and alanine. Their optimum growth temperature is 37^0C, although they will grow in temperature ranges of 20-40^0C. The most suitable pH is 6.6-7.4 (Corbel and Morgan, 1984).

Some species like *B. abortus* are metabolisers of mesoerythritol instead of glucose. This sugar alcohol (erythritol) that is abundant in pregnant uteri of some animals is thought to improve *in-vivo* growth (Jubb *et al.,* 1993). *In vitro* growth is improved by addition of serum, blood or tissue extracts. Serum dextrose broth and agar are ideal (Alton, *et al.,* 1975). The serum serves as a source of nutrients and neutralises inhibitors present in peptone (Corbel and Morgan, 1984). Also essential for growth are iron and magnesium ions, which stimulate growth and have a regulatory role.

In solid media, colonies are rarely visible in 48 hrs (Alton *et al.*, 1975). Only vigorous strains of *B.abortus, B.suis* and *B.melitensis* may grow on MacConkey's medium, while all the rest are inhibited (Wilson and Miles, 1975). Growth in liquid media rapidly dissociates to the rough forms but this can be prevented by vigorous aeration of the medium.

2.2.4. Biochemical characteristics.

The organisms yield energy by an oxidative process through the pentose monophosphate pathway. The rate of oxidation is a characteristic of some strains (Corbel and Morgan, 1984). Brucellae are catalase and nitrate positive and are usually oxidase positive except for a few strains. They produce hydrogen sulphide at rates that are biotype specific. Most strains from almost all species are reported to have urease activity but the rates of hydrolysis vary. *B. suis* splits urea fastest, changing the colour of Christensen's medium almost immediately upon inoculation. *B. abortus* especially the reference strain B544 (NTCC), takes longer than *B. suis,* with *B. melitensis* taking even longer. *B. ovis* has no urease activity at all. The rate of urea hydrolysis is a useful characteristic for speciation (Corbel and Morgan, 1984). From the accounts of Alton *et al.*, 1975; Meyer and Cameron (1961) demonstrated that each *Brucella* species has a specific and definitive pattern of oxygen utilisation on selected amino acids and carbohydrates as

measured by Warburg apparatus and expressed as oxygen co-efficient. *Brucella* organisms do not produce acid from carbohydrate in conventional media except probably *B. neotomae* (Wilson and Miles, 1975). Members of the genus are negative for methyl red (MR), citrate, indole and do not produce acetylmethylcarbinol. They do not lyse erythrocytes but may turn blood agar medium greenish (Alton *et al.,* 1975).

2.2.5. Suitable material and culture conditions for Brucella isolation.

The most suitable human specimen for culture is blood. This is because the disease condition tends to manifest itself in acute cases as bacteraemia. Three blood samples per patient taken in 24 hours and cultured in 10% CO_2 has shown a success rate of 17-84.5% (Diaz and Moriyon, 1989). In animals, the best materials for culture from the dam are vaginal mucus secretion just before abortion or parturition, semen from the bull and stomach contents from the aborted foetus. The organisms are also recoverable from the udder, uterus, infected foetal membranes, seminal fluid of bulls, meconium, liver and lung from the aborted foetus (Jubb *et al.,* 1993). Milk may be an excellent material for culture. Majority (90%) of infected cows will have infected udders. This is evident from a few days to parturition in 85% of acute and 15 % of chronically infected animals, which therefore shed organisms in milk (Cordes and Carter, 1979). The voiding of the

organisms disappears rapidly after parturition. From animal carcasses, the best materials for culture are lymph nodes and testicular tissues. When culturing liquid material liquid medium is most suitable. Serum dextrose broth, as described by Casteneda in 1942, is the most preferred liquid medium (Alton *et al.,* 1975). When using such liquid media, vigorous aeration by agitation minimises dissociation of the organism.

When it is suspected that the culture material is contaminated, selective medium with antibiotics is recommended, especially on primary isolation. *Brucella* can also be isolated by animal inoculation. The organism may not always be isolated from infected animals and some infected ones may not show clinical signs and hence are not likely to be sampled for disease diagnosis. Calves born to infected mothers are nearly always infected. This aspect of the disease is important particularly in females since they may remain latently infected up to the time of first calving when the disease manifests itself (Plommet, 1976b).

2.2.6. Isolation, identification and speciation by biochemical reaction and resistance to Phage.

For initial culture, the test material is incubated in the presence and absence of carbon dioxide in order to determine whether special gas tensions are essential for

growth. The recommended carbon dioxide concentration is 10%. The results are most reliable when the tests are carried out just after the isolation before development of independence to carbon dioxide (Alton *et al.,* 1975). The organisms are identified on the basis of gram, modified Ziehl Nielsen's or modified Koster's stain reactions (Alton *et al.,* 1975). It is important to note that the organisms do not utilise carbohydrates nor do they produce acid from them except probably *B. neotomae* (Wilson and Miles, 1975). Therefore, any organism that ferments carbohydrates in conventional media, is not *Brucella* (Alton *et al.,* 1975, Corbel and Morgan, 1984). The fermentation test is carried out in glucose broth dosed with an acid indicator. Organisms that conform to *Brucella* on physical observations and biochemical tests can then be speciated using phage lysis and the ability to grow in varied specific aniline dye concentrations. Known positive control organisms are normally run alongside the test ones for comparison of results.

All *Brucella* bacteriophages belong to the same phage family. For identification of *Brucella* using phage, the culture is inoculated with routine test dilution (RTD) of the virus; this is the minimum concentration that produces complete lysis of the propagating bacterial strain for that particular phage. These phages belong to five classes. The group *Tbilisi (Tb)* that was isolated in the USSR has been most widely used and is designated reference phage1 (group1). It is lytic to both smooth and intermediate growth phases of *B. abortus* but has limited lytic activity

to *B. neotomae*. It has action on *B. suis* only at a concentration lower than RTD. Group 2 is made up of the *Firenze* phage strains that replicate in and lyse smooth, semi-intermediate and intermediate forms of *B.abortus, B. neotomae* and *B.suis*. Group 3 is typified by *Weybridge(wb)* which replicates in and forms plaques on smooth, semi-intermediate and intermediate cultures of *B. abortus, B. neotomae* and *B.suis*. Phages in this group do not produce plaques on cultures of B. *melitensis*. Group 4 are the *Berkeley* phage, Bk0, Bk1 and Bk2. Only Bk2 replicates in, and causes lysis of *B. abortus, B. melitensis, B. neotomae* and *B. suis* but has no lytic activity on *B. canis* or *B. ovis*. Group 5 is derived from phage R which are genetic mutants from a mixture of other phages. They are genetically unstable and have no lytic activity on non-smooth cultures of any species of *Brucella*. They are lytic to *B. ovis,* non-smooth phases of *B. abortus* and *B. suis*. There is a close relationship between susceptibility of *Brucella* culture lysis by standard suspensions of these phages and the bacterial species as defined by oxidative metabolic tests against some selected amino acids, their urea cycle intermediates and carbohydrates (Corbel and Morgan, 1984; Corbel, 1987).

2.2.6.1. Typing Brucella species using mono-specific sera.

For most *Brucella* cultures, agglutination with homologous mono-specific serum specific against *B. abortus* and *B. melitensis* is confirmatory. The mono-specific

serum is prepared against FAO/WHO reference strains 544(NTCC) for *B. abortus* and 16M(NTCC) for *B. melitensis*. The two mono-specific sera are prepared by cross-absorption between *B. abortus* and *B. melitensis*. Some cultures are agglutinated to varying degrees with both mono-specific sera (Alton *et al.*, 1975).

2.3. The Brucella cell wall molecular structure and its antigenic composition.

The cell wall is very important for maintaining the cell structure, for host attachment and colonisation. It is the part of the cell that is in direct contact with the environment and to which the host responds. It is made up of an organised triple layered molecular organelle whose outermost part is a 9 nanometer (nm) lipopolysaccharide-protein structure (Corbel and Morgan, 1984). These cell wall layers of lipopolysaccharide-protein, mucoprotein and lipoprotein are arranged in a unique manner. The layers of lipopolysaccharides (LPS) and proteins are linked to an electron dense inner layer of 3-5 nm. The dense part corresponds to the cross-linked muramic acid that contains peptidoglycans (Corbel and Morgan, 1984). The peptidoglycans are located just below the LPS and immediately above the periplastic space. The electron dense triple layered periplastic space is thought to harbour the enzymes that digest the cell wall in preparation for binary fission. The LPS covers most of the bacterial surface, whereby its side chain gives colonies their phenotypic smoothness. The phenotypic smoothness or roughness of the

colonial forms of the organism is in turn related to the quantity of the LPS. Gas liquid chromatography shows that *B. canis* has a unique LPS profile (Corbel and Morgan, 1984).

The LPS, which are a diverse group of molecules, are made of lipids bound to the polysaccharide (PS). The PS is the outermost part of the LPS, and so it is exposed to the exterior of the cell. These PS molecules are more prominent in *Brucella* than in *E. coli* (Corbel and Morgan, 1984). Long chain fatty acids attached to the PS distinguish *Brucella* from other gram-negative organisms. The overall fatty acid composition in the *Brucella* cell is significantly distinctive and is of value in identification, classification and taxonomy at the genus and subgenus levels. It constitutes 17.5% of total cell wall weight. Among the LPS of *B. melitensis* is a unique lipid A that is linked to Keto-d-6 glutaric acid (Kdo) at C-6. This lipid and its sister polysaccharides may be attached to the cytoplasmic membrane (Moreno *et al.,* 1987). The cell wall accounts for 21% of the total smooth bacterium's dry weight and 14% in the non-smooth strains, since the latter lacks LPS in their walls. From this weight, about 6.2% in the rough LPS and 1.3% in the smooth cellular forms is due to proteins. The proteins contribute much less to the quantity of the cell wall and are less prominent serologically. Their remnants during purification may be responsible for the mitogenic properties of the LPS.

2.3.1. Lipopolysaccharide (LPS) composition and activity.

These are a group of complex molecules on the cell surface consisting of lipid A and polysaccharides (PS) that are also bound closely to protein moieties. Lipid A from different bacteria may have different biological activity among which, is mitogenic activity (Zygmunt *et al.*, 1994). The LPS though of near similarity to PS is not identical to it. The LPS consists of five blocks of N formyl perosamine molecules. Four of these blocks have a molecular arrangement with alpha I-2 linked and the other one is alpha 1-3 linked residues. The residues are made of core oligosaccharides such as glucose, mannose, 2 amino 2, 6 dideoxy D glucose (glucosamine) and Keto-D-glutaric acid (KDO). The residues of *Brucella* share some activities with those of *Enterobacteria* and *Yersinia enterocolitica,* despite the latter having a distinctly different fatty acid composition (Moreno *et al.,* 1987). This means *Brucella* will remove antibodies against LPS of *Y. enterocolitica* from serum on absorption, due to the closeness of these molecules. This near identity for both is an attribute of PS, which consists of a side chain (O chain) that is exposed to the surface. This PS accounts for 1-9% of the cell's dry weight. It bears the two major immunodorminant antigens A and M in *B. abortus* and *B. melitensis* (Aragon *et al.* 1996). The 'O' chain in the rough *B. melitensis* is exposed intracellularly (Cloeckaert *et al.* 1992). A and M which are present in both smooth and non-smooth colonial forms of *Brucella* are responsible for the bacteria's main serological activities. They account for the structural similarities

of the smooth cell wall LPS and amino hydroxy compounds (Vizcaino *et al.*, 1996). These antigens are carbohydrate in nature and are responsible for the cross reactivity between the two *Brucella* species.

Antigen A is dorminant in *B. abortus* and M in *B. melitensis* with each imparting its characteristics to the particular species. They are responsible for the immunological activities of both species and account for the cross-reactivity between the two (Moreno *et al.* 1987; Aragon *et al.,* 1996). The M antigen is a complex O chain pentasaccharide homopolymer, made up of core oligosaccharides, that are in turn made of glucose, mannose, 4, 6 dideoxy 2 methyl 4 (N methylamino) D mannose and 4,6 dideoxy 3- O methyl 1-4 (N methyl amino D mannose) (Bundle *et al.*,1989). It has five N formyl perosamine blocks in the ratio of 1:4 for a 1-2 and a 1-3 linked to the sugars 4,6 dideoxy-4- formamido D mannose as shown by nuclear magnetic resonance. The one alpha 1-2 and four alpha 1-3 linked sugars are formyl derivatives (Meckle *et al*, 1989). The M antigen also has intermediate forms with varying ratios between alpha 1-2 linked and alpha 1-3 linked forms as occurs in *B. suis* biotype 3 (Bundle *et al.*,1987). Although the two species have both A and M antigens, the epitope densities for the two antigens in both species are variable. The alpha 1-2 linked components are on a tetrasaccharide segment which provides the structural basis for a common A antigen type determinant (Aragon *et al.* 1996). Apart from the cell wall antigens, all members of the genus possess characteristic intracellular ones too.

Antigen A is a formamido homopolymer of alpha 1-2 and alpha 1-3 linked 4, 6 dideoxy 4 formamido D manopyranose residues as monomeric linear unbranched, repeating units that have functional isomerism. It is difficult to separate it from M (Bundle *et al.*,1987; Meckle *et al.*, 1989). Both have extensive serological cross-reactivity based on polyclonal antisera due to their shared epitopic features involving alpha 1-2 linked aminoglycosyl residues. It has also been observed that antibodies with equal affinity for A and M epitopes can be effectively inhibited by alpha 1-2 linked tri and tetrasaccharides (Bundle *et al.*, 1989). These authors also suggested that quantities of the two antigens may be similar but their epitope densities may be different. There are common epitopes *on* A and M antigens that are responsible for the cross-reactivity between *B. abortus* and *B. melitensis* antisera. These epitopes are denoted by c and y. The formamido entities of both antigens are similar with rotational isomerism E and Z. Monoclonal antibody test (MAB) have shown that A and M are on the same molecule (Bundle *et al.*, 1989, Meckle *et al.*, 1989). The cross reactivity between A and M antigens of *Brucella* O chain and those of other gram negative bacteria is associated with the M amyl derivative at 4 amino 4-6 dideoxyl D mannose pyranose units (Bundle *et al.*, 1989). Therefore the difference between smooth *B. abortus, B. suis* and *B. melitensis* are quantitative and not qualitative.

The 'O' chain of *Brucella* and that of *Y. enterocolitica* are identical, with the latter's resembling those of *B. abortus* 1119-3 and *E. coli* 0:157. This similarity is due to the occurrence of the 2-O glycosylated residues of 4 acetamido 4-6 dideoxy a D mannose pyranosyl units. The cross reactivity of *Brucella* and *Yersinia* is either due to tetrasaccharide determinants or their terminal oligosaccharide components that form part of the cross-reacting antigen polymers for A and M and which in turn are responsible for the cross-reactivity between *B. abortus, B. melitensis* and *Y. enterocolitica* 0:9 (Bundle *et al.*, 1987). The A antigen of *Yersinia* is 10 times less reactive than that in *Brucella* (Meckle *et al.*, 1989). This difference in the reactivity is because the antigen A of *Yersinia* lacks alpha 1-2 linkages. Antiserum to *B. abortus* removes sLPS of *Yersinia enterocolitica* 0:9. The *Vibrio cholerae* O chain also has quite a lot of resemblance to that of *B. abortus* though its LPS has quite a distinct fatty acid composition and structure. Between them, there are seven similar fatty acid components that account for 85% of the dry weight in both (Moreno *et al.*, 1987). Both have lipid A as part of the LPS.

A and M antigens stimulate production of IgM and IgG2 while IgG1 production is stimulated by protein antigens (Berman and Kurtz, 1987). *B. abortus* and *B. melitensis* induce production of antibodies that react in immunoprecipitation test and also against the native hapten. It is thought that the native hapten is different

but identical to the PS of the LPS. Its basic structure is identical to the PS in *B. melitensis*.

2.3. 2. Proteins.

These are the second most important cell wall constituents from the point of view of protection to the host. Those on the cell wall are referred to as the outer membrane proteins (OMP). The proteins are less exposed to the surface than the LPS and so they are not as accessible to host antibodies. As the organisms become rough, the OMP become more exposed to the surface (Schurig *et al.*, 1981). *Brucella* have three groups of major OMP and some minor ones, all being closely associated with peptidoglycans (Pg) that are in turn strongly associated with the cell wall but which can be unbound from each other by lysozymes (Cloeckaert *et al.*, 1992). The *Brucella* Pg has a composition like that of *E. coli* (Cloeckaert *et al.*,1992). Each species also has a characteristic reproducible protein with unique qualitative and quantitative attributes (Hill and Cook, 1994). The chromatography pattern of the OMP revealed that all species of the genus have low or high quantities with minor qualitative and quantitative differences. These patterns are reproducible (Hill and Cook, 1994). The OMP are classified on the basis of molecular weight as; group 1 of 89-94 kda, group 2 or porins of 35-40 kda and group 3 of 25-30 kda respectively (Moriyon and Berman, 1983, Corbel and

Morgan, 1984). Group 1 is more exposed than the others (Cloeckaert *et al.*, 1992). The porins are trimmers linked to LPS. They occur in varying quantities in the cell and show a lot of homology within the smooth *Brucella* species and *E. coli*. The quantity in *B. ovis* and *B. suis* is lower than in *B.melitensis* and *B. abortus* (Winter, 1987, Tibor *et al.*, 1996). Group 3 proteins are strongly bound to the peptidoglycans and have a lot of heterogeneity with group 2 and resemble OMPA of *E. coli* on the basis of amino acid sequence (Winter, 1987). The total protein of group 2 and 3 for the smooth and rough *Brucella* are quantitatively equal. The two proteins form the cell wall matrix with its hydrophilic pores that allow passage of low molecular weight hydrophilic substances. Both have a common antigen; b (Verstreate and Winter, 1984). Apart from the two matrix proteins there is also a 38 kda protein bound to the peptidoglycan which also contributes to the matrix (Moriyon and Berman, 1983; Moriyon *et al.*,1987). Apart from the three OMP proteins, there are minor ones of 10, 16.5, 19 and 89-94 kda among which are glycoproteins and lipoproteins. These minor proteins have little biological activity though probably different immunogens (Hill and Cook, 1994). One of the minor OMP is a 62 kda protein that was detected in pigs infected with *Brucella*. It is a homologue of a 65 kda protein of *Mycobacterium leprae* and has also been recognised in *Y. enterocolitica* 0:9 (Spencer *et al., 1994)*. This protein may be a common cross-reactivity antigen as elucidated by immunoblot using *B. melitensis* antigen (Santos *et al.*, 1984). Important antigenic components of OMP are denoted a, b, c, d, and e. d is the most predominant, while b is most widespread

within the 3 protein groups, having been demonstrated in *B. abortus, B. melitensis, B. ovis* and *B. canis*. Antigen a, is on group 2 and 3 only, while c, d and e are only in Group 3. These antigens may have relevance to the protective immunity as some of the organism's proteins, especially the minor ones, have been traced in the plasma of infected animals even after bacteraemia has ceased (Carmichael *et al.*, 1989).

Antibodies to OMP are mainly targeted to group 2 and 3 and to a lesser extent on minor proteins of 55-62, 70-74 and 89- 94 kda. A protein denoted OMP 31 (31-34 kda) is among the major ones in *B. melitensis* but minor in *B. abortus*. It has been said to be protective in mice. If indeed it is protective, then it may be a good candidate for development of subcellular vaccines against sheep and goat brucellosis due to both *B.melitensis* and *B.ovis* (Dubray, 1987; Vizcaino *et al.*, 1996, Zygmunt *et al.*, 1994). It has shown some promise as a candidate for such vaccine development (Santos *et al.*, 1984). Antibodies against this minor protein were found in cattle vaccinated with *B.abortus* s19. Its protection is derived from a 36 amino acid region (Vizcaino *et al.*, 1996). A cytoplasmic protein in *B. canis* and *B. abortus* is similar to the minor one stated above. It may also have some relevance in protective immunity and in distinction of vaccinates from infected animals on immunoblot (Verstreate and Winter, 1984). Vaccination with protein antigens produced effects similar to infection of animals that had been vaccinated when sexually mature. Infected sheep produce antibodies against minor, major

OMP and LPS molecules (Zygmunt *et al.*, 1994). The reaction in the vaccinated ones was more intense in immunoblot based test than in infected ones (Zygmunt *et al.*, 1994). If OMP are protective, they would yield ideal vaccines since they lack the LPS to which serological results are interfered (Santos *et al.*, 1984). Immunity based on OMP is cell mediated. This by itself may not be adequate for protection.

2.4. Disease conditions caused by Brucella organisms.

2.4.1 Human disease.

The human disease has been known in the Mediterranean region and the Middle East since biblical times. It has continued to be prevalent in the Near East and South America. It is the cause of the third highest fevers in Cairo (Araj *et al.*, 1986). In some nations the disease has reached alarming proportions (Young, 1989). The main sources of human infection in these places are cattle, sheep, goats and swine; with camels playing a minor role (Araj *et al.*, 1986). However, camels may be a major source of human epidemic in Kuwait and the Mediterranean region (Sultan *et al.*, 1991).

Man is infected by *B. abortus, B. melitensis, B. suis* and *B. canis*. *B. melitensis* is the most pathogenic, followed by *B.suis, B. abortus* and *B. canis,* in that order,

with cases due to the latter being reported since 1972 (Young, 1989). More cases of undulant fever are from *B.suis* for equal numbers of exposed persons to *B. abortus* (Hayes and Calif, 1934).

The incubation period is insidious and of variable duration possibly due to differences in the organism's virulence. The disease is acute and very rarely sub-acute, ranging from self-limiting fever of unknown origin, to complicated and eventually fatal disease. Persons of all ages are affected but more so those in the working stages of life. Patients will be seen to have abnormalities linked to reticulo-endothelial system with most of them manifesting with acute or chronic disease that is characterised by fever of 40^0C, chills and profuse sweating often with malodour at night, headaches, lethargy, joint swellings and pains, anorexia and myalgia. Occasional rare signs are weakness, easy fatigue, malaise, mental disorders and depression among others,.depending on organs in which localisation takes place (Young, 1989).

Complications are common, mainly being osteo-articular and therefore patients will present with spondylitis and sacroillitis as shown by radiographic abnormalities mainly at the axial site. Sacroillitis is the commonest complication whereby some patients may require surgical treatment to relieve the pressure on the spinal cord. Pain of the gluteal and lower lumber therefore are frequent

clinical features (Colmenero, *et al.*, 1992; Solera *et al.*, 1992). In infections involving the liver, the patient will have mild neutrophenia.

Most persons are exposed to low constant doses that might either sensitise them or give them immunity leading to serologically positive reactions (Young, 1989; Plommet *et al.*, 1987). Migration workers who contract the disease present with non-specific symptoms. In such persons it may be difficult to diagnose the disease (Plommet *et al.*, 1987). Some patients may fail to develop serological reaction as shown in a 16 year old girl who developed multiple subcutaneous abscesses, osteomyelitis and severe colitis but was serologically negative and was positively diagnosed on hemoculture. She was positive only on lymphocyte proliferation assay (Potasman *et al.*, 1991; Stermer *et al.*, 1991)). Infection is acquired through skin abrasions, aerosols, mucosa of the gastrointestinal tract, and to a minor extent through the conjuctiva and the upper respiratory tract. Among affected communities the poor are most afflicted (Elberg 1981). Human patients on treatment with antibiotics may become serologically negative as the immune complexes dwindle upon such therapy.

2.4.2. Animal diseases.

Almost all domestic animal species are infected. The establishment of disease depends on age, productive status, host resistance, infecting dose and virulence of the particular strain. The disease starts as septicaemia with relapsing bacteraemia and later localisation to various organs. This tendency to localise is highest with *B.suis* (Blood *et al.*, 1985). Young animals, though susceptible, retain infection up to puberty whence they tend to shed the organisms only for a short while. In sexually mature animals the infection tends to persist indefinitely.

Infection is acquired through the mucus membranes of the gastrointestinal and upper respiratory tracts, abraded skin, congenitally and by transplacental route in cattle and swine. In the latter, infection may also be acquired through coitus. In cattle, high doses of the pathogen are required to induce disease and so in most cases such as artificial insemination and through transplacental routes, focal infections do occur (Plommet, 1976b).

From these portals of entry, the organisms drain into lymph nodes of head or the local ones, where they invade the lymphocytes leading to acute regional lymphadenitis. The organisms have a carbohydrate virulent factor on their cell walls, that helps them to bind to a protein receptor on B lymphocytes (Campbell et al., 1994; Jubb *et al.*, 1993). From the lymph nodes the organisms are spread by haematogenous route to the lymphoid organs from where they are then released to the blood stream leading to recurrent bacteraemia or persistent chronic infection.

The organisms then infect macrophages in which they multiply and are spread to various organs. Multiplication in the lymphoid organs or tissues such as the liver, spleen and bone marrow leads to granuloma formation. The granulomas may later develop into abscesses. Virulent strains survive and multiply in macrophages (Bounous *et al.,* 1993; Cheers, 1985, Cheville, *et al.,* 1996). The multiplication is particularly high in the thoracic duct (Jubb *et al.,* 1993). Infected lymph nodes are infiltrated by neutrophils and eosinophils; leading to proliferation of the cellular components of the germinal centres.

Brucellae have special affinity for the ungulate pregnant endometrium, foetal placenta and fluids and to a lesser extent testis in the male; possibly due to attraction by the little erythritol that these tissues contain. In the pregnant uterus, the organisms are carried by blood to the periphery of the caruncles from where they spread to and invade foetal chorionic villi. Here they are phagocytosed by erythrophagocytic trophoblasts of chorion leading to haematomas in foetal caruncle especially late in gestation. Localisation in the uterus may lead to abortion if the lesions are severe while that in the testis leads to epididymitis and orchitis. If infection is less severe, premature birth or stillbirths arise. Calves so borne may be viable or not. About 1/3 of infected animals abort (Jubb *et al.,*1993). It takes months before the slow spreading lesions lead to abortions that normally occur in the 7-8th months of pregnancy in the bovine female. Less frequently in cattle, signs of spondylitis, arthritis and mastitis that is accompanied by nodular

udder lesions are seen. There may also be carpal bursitis (Hygroma). Lesions remain for sometime after abortion or birth. Foetal membranes are thickened, necrotic with the inter-cotyledonary spaces variously involved. Affected areas are thickened by gelatinous opaque fluid or toughened by coagulation of inflammatory exudate. The necrotic cotyledons are soft yellow-grey and covered by a sticky odourless brown exudate. Their stroma are oedematous, with increased leucocytic mononuclear cells, while the chorionic epithelial cells are staffed with bacteria. The amniotic fluid may be viscoid (Jubb *et al.,* 1993).

Lesions in the bovine foetus include necrotic focal areas and granuloma in the lymph nodes, liver, spleen and kidneys. Calves born to infected mothers especially female ones are usually infected but the disease usually wanes in them before they reach puberty (Naggy and Hignett, 1967). In the male calves the organism may be retained to adult hood (Rankins, 1965).

Cattle experience higher reactor rate than pigs and so the pigs may be more resistant to *B.suis* than cattle are to *B.abortus*. Pigs acquire infection through alimentary system, per vaginum and through the broken skin. In this host, the disease is marked by low bacteraemia, arthritis and abscesseating spondylitis. Infected swine manifest disease by storm abortions, under-developed piglets, stillbirth or weak neonates and a high incidence of embryonic death. Abortion occurs at 2-3 months. The incidence of placental retention is lower than in the

bovine female. Infected swine may show lymphadenitis and bacteraemia for years with some sows shedding organisms all along. Localisation occurs in many organs especially the genitalia, skeleton including vertebral synoviae, mammary glands, lymph nodes, spleen, liver, kidneys, bladder and even brain. The organism has a high affinity for the skeleton and joints where it leads to granuloma formation. The uterus may develop nodules or false plaques in the mucosa with thickening of the wall that may lead to luminal stricture or occlusion resulting in probable pyosalphinx and granuloma of the supporting ligament. The uterus may accumulate mononuclear cells or leukocytes enmeshed in strands of mucin or amorphous globes of mucus. Bacteria remain in granulomatous foci in the non-pregnant endometrium leading to necrosis and calcification. Articular lesions begin as synovitis, fibrinopurulent vertebral osteomyelitis especially in the lumbar region leading to paravertebral abscess. In males, the organism is shed in semen from the accessory genitalia and so coitus can transmit the disease ((Jubb *et al.*, 1993; Randostits *et al.*, 1994)

In equine, the disease may be marked by chronic bacteraemia that becomes intermittent with a tendency to recur during pregnancy. The disease may be accompanied by recurrent equine ophthalmitis. The organisms localise at parturition in spleen, mammary gland, lymph nodes, pregnant uterus and lymphoid tissue. In the male they localise in the accessory sex glands. The most common

manifestations in equine are bursitis and fistulas withers. Abortion and hygroma are rarely encountered signs (Jubb *et al.,* 1993).

In camels *B. melitensis,* biovar 1 was isolated from milk and aborted foetii, with cases being reported in Egypt, Saudi Arabia, Sudan, Kenya, Somali, Ethiopia and Libya (Gamaal *et al.,* 1993; Kagunya and Waiyaki, 1978, Waghela *et al.*, 1978). *B. abortus* also affects domestic buffaloes and yaks with signs similar to those of cows. It also infects camels, lama and hares among other mammals (Randostits *et al.,* 1994). Fowls have been reported to harbor *B. abortus* organisms but show no symptoms (Araj *et al* 1986; Jubb *et al.,* 1993; Randostits *et al.,* 1994).

B. abortus affects sheep mainly through the oral route (Shaw, 1976b). Infected flocks may show storm abortion, sterility and neonatal death in the dam and orchitis and sterility in the ram; while some sheep may show no signs at all. Affected flocks may have many sero-reactors with few among them delivering under-developed foetuses. Ewes infected in the last 1/4 of gestation give birth to live lambs while those infected early may have abortion or weak neonates. On post-mortem, the neonates show fibrinous deposits on the liver, heart and lungs with *B. abortus* being recovered from foetal stomach. Some shedding ewes give birth to live lambs that may be free of brucellae. Some aborting ewes in flocks known to be infected by *Brucella* organisms were serologically negative for brucellosis on RBPT (Chartier, 1992). The organism is recovered from milk of

infected ewes (Shaw, 1976a). In flocks infected by *B. melitensis* the organism is isolated for periods extending to 2 years which is longer than the period in which *B. abortus* is recoverable from this species (Waghela, 1978). Goats may be culturally positive on 2nd pregnancy with different flocks showing different aftermaths of abortion storms. Some flocks may show complete recovery (Waghela, 1978).

Canine brucellosis is characterised by abortion, embryonic death and infertility while males show testicular atrophy, epididymitis, prostitis, scrotal dermatitis and infertility. Lymphadenitis and splenitis are infrequent signs. Dogs and cats when infected by *B. abortus* or *B. melitensis* manifest with fever, emaciation, orchitis, anaestrous, arthritis and sometimes abortion (Forbes, 1990, Jubb *et al.,* 1993). Also, occurring are disk spondyilitis, inter-vertebral disk infection and chronic lymphocytic endophthalmitis. This may be an immunologically mediated reaction (Jubb *et al.,* 1993). In such, aqueous humor may have agglutinating titre that could be more extensive than that in the serum (Jubb *et al.,* 1993). Dogs acquire *B.abortus* from cattle, as shown in 10 farms in which the same organism was isolated from the infected cattle and the dogs. Infection in dogs occurred through ingestion of infected milk and foetal membranes or aborted foetii (Forbes, 1990). Dogs may have the potential to infect cattle and could pose a danger for a longer duration of time though the risk of transmission is small (Crawford *et al.*, 1988a; Forbes, 1990). Serological diagnosis in dogs is made difficult by extensive cross-

reaction between rough cell envelope antigens of *B. canis* and heterospecific antibodies (Carmichael *et al.,* 1989; Flores and Baer 1979). Sero-conversion was not directly related to culture positive cases.

The disease causes economic losses through abortions, infertility marked by prolonged calving intervals, retained placentas, weak unthrifty neonates that are prone to high mortality and loss of draught power due to lameness subsequent to hygromas (McDermott *et al.,* 1987; Jubb *et al.,* 1993). It also causes economic losses due to restriction on international trade.

2.5. Diagnosis of brucellosis in humans and in animals.

Clinical diagnosis of human brucellosis presents a difficult challenge due to the protean nature of the disease, its multiple system involvement, divergent clinical manifestations and asymptomatic individuals (Abramson *et al.,* 1991). Laboratory diagnosis is based on blood culture results, high or persistent serum antibodies or by measuring anti-protein humoral responses (Diaz and Moriyon, 1989; Goldbaum *et al.,* 1992, Moreno, *et al.*, 1992; Solera *et al.*, 1991,). Three human blood samples taken in 24 hrs and cultured in 10% CO_2 has shown a success rate of 17 - 84.5% (Diaz and Moriyon, 1989). In endocarditis, bacteraemia is continuous. This increases the chances of recovering the organism from the blood. On

serology, caution needs to be exercised as cross-reactivity occurs with cholera, tularaemia, and yersiniosis (Kittleberger *et al* 1995I; Diaz and Moriyon, 1989). Culture is recommended where cross-reactivity is suspected.

Diagnosis of the disease in animals is by direct culture, serology of the suspect's serum or milk and allergic skin reaction. Isolation of the causative bacteria or identification of bacterial antigens in plasma is the surest mode of diagnosis (Carter and ChengGappa, 1985; Plommet, 1987). Material for culture from the dam are blood, milk, cotyledons, and foetal membranes, while from the foetus, suitable materials are stomach contents, lungs, liver, spleen and meconium (Alton *et al.,* 1975, and Corbel and Morgan, 1984). From carcasses, lymph nodes are the material of choice. In males seminal vesicles and testicular material are useful as sources of brucellae (Jubb *et al.,* 1993).

The organisms are easily recovered from culture specimen by innoculating the appropriate media such as liquid medium for liquid material, solid media such as serum dextrose agar for solid material or by use of laboratory animals. The latter are particularly suitable if the culture material is heavily contaminated (Alton *et al.,* 1975; Blood *et al.,*1985; Olascoaga, 1976). In case the material is contaminated, selective media with antibiotic is preferred for primary culture. The isolates are identified on the basis of carbon dioxide requirement, acriflavine dye test, and

colony staining with crystal violet and typing by biochemical reactions, monospecific serum, dye resistance and phage susceptibility.

Serological methods of diagnosis are the most extensively used mode of diagnosis of brucellosis in both animals and humans. They have a lot of uncertainties but they are more successful than the definitive isolation of the pathogen. A wide range of tests is used in disease surveys, epidemiological studies and in eradication. None of the methods, however, is without its peculiar limitations (Diaz and Moriyon, 1989). This necessitates use of more than one method to confirm diagnosis (Davis *et al.,* 1990). In field testing, RBPT gives results that are more consistent with CFT than SAT (Wood & Corbel, 1973). Its results are most prolonged and persistent (Hayes and Chappel, 1982). Carrying out RBPT is so easy that persons with minimal training, especially where laboratory facilities are lacking, can do it (Agorreta *et al.,* 1991). It is, however, recommended that confirmation of diagnosis be sought in suitable laboratories (Oomen and Waghela, 1974). None of the tests nor a combination of them can detect all cases of brucellosis and as such their results are only pointers to the disease state in the herd. A combination of the tests minimises the shortcomings of the individual test (Olascoaga, 1976). CFT in conjunction with RBPT are considered most accurate tests. The two tests and SAT are used as basic operative techniques (Diaz and Moriyon, 1989).

Being indirect methods, serological tests do not identify the individual immunoglobulins involved in the reaction but only give an indication of the dominant ones. The uncertainties in serology based diagnosis is complicated by many factors such as low agglutinin levels, latency, very early infection, chronicity, prozone phenomenon, blocking antibodies and failure by the test to distinguish active infection, recovery, vaccination or cross reactivity. Good serological methods should be able to classify animals as reactors or vaccinates. This is frequently difficult or uncertain. Non-specific reactions due to rough antigen suspensions do occur (Diaz and Moriyon, 1989). Positive results do not have much weight for single animals but have more significance on herd basis (Kulshreshtha and Ramchandran, 1979). The methods in common use are Rose Bengal Plate test (RBPT), milk ring test (MRT), Serum Agglutination test (SAT) and Complement fixation test (CFT). Other available tests are Card Test, Rivanol test, Coombs antiglobulin test, 2 Mercapto-ethanol (ME) and Enzyme linked immuno assays, among them Enzyme linked immunoabsorbent assay (ELISA) (Alton *et al.*, 1975; Araj *et al.,* 1986; Diaz and Moriyon, 1989). These tests are an indirect indication of the presence of homologous antibodies (Olascoaga, 1976). Some less important methods are indirect fluorescence assay and haemagglutination test (Kulshreshtha and Ramchandran 1979). Some of the tests such as RBPT and MRT are good for herd screening as these can be carried out at the sampling point. The results so obtained can then be confirmed using a quantitative method. RBPT and CFT have also been found useful together as

screening tests (Stemshorn *et al.,* 1985). CFT and ME have always been used as supplemental tests. The most used antigens for polyclonal antibody detection are *B. abortus* s99 or 1119-3.

CFT may be negative due to progressive changes in concentration of serum antibodies, their antigenic structure, specific isotype composition and avidity and prozone phenomenon which is linked to the ratio of IgG1 to IgG2, all of which affect the serological results (Williams *et al.,* 1991). These characteristics differ from animal to animal. The persistent reactions could be due to non-specific antigens and IgM (Chappel *et al.*, 1982b; Cullen and Corbel, 1970). CFT and SAT may persist at the rates of 0.5% to 1.5% in different animals, while RBPT may persist at 2%. ELISA readings may remain positive long after shedding of organisms has ceased (Diaz and Moriyon , 1989).

The screening test results are confirmed using complementary tests that are considered more reliable (Olascoaga, 1976). They distinguish heterospecific reactions while detecting incomplete antibodies. They are also useful for differentiating vaccinates from infected animals. These tests are of particular importance in problem herds. Problem herds are those with persistent reactors despite culling of all animals testing positive by MRT but which are negative on serology. Such animals require to be tested by serology and bacterial culture (Jones *et al.,* 1963). As control enters terminal stages, conventional tests are

limited in their ability to detect all infected cattle in herds with persistent infections (Jones *et al.,* 1963). CFT, used in conjunction with ME, is useful at differentiating vaccinates from infected animals (Alton, 1978).

None of these tests can confirm freedom from disease. The specificity and sensitivity to antibody assay for diagnosis of brucellosis are limited by the cross-reactions between *Brucella* antibodies and those against other bacteria. Some infected animals may fail to show clinical disease or develop antibodies, hence their failure to be detected (Lapraik *et al.,* 1975). Kolar (1987), observed that it was impossible to detect all infected animals at any one time using whatever method or even to do so on repeat examination. He suggested that a flock be declared infected even if it has only one detectable reactor. Some tests are less efficient as regards some animal species.

It was observed and is agreed that agglutinins appear earlier than complement fixing antibodies (Hayes and Calif, 1934). They also disappear faster (Waghela, 1978). The level of antibodies is not influenced by stress on the infected animal (Cullen and Corbel, 1970). At initial stages, SAT has a significantly higher titre, while CFT may be negative. However, negative cases on SAT when CFT is positive do occur, particularly in chronic illness or on recovery from the disease. There are changes in antibody titre, composition, antigenic specificity, isotype composition and avidity as disease progresses to chronicity. These characteristics

vary from animal to animal (Chappel *et al.,* 1982b). CFT titre may persist and remain positive for up to 8 months. Persistent reactions could be due to non-specific antigens reacting with IgM. CFT and ME detect IgG. Mercapto- ethanol breaks down IgM. In RBPT, reaction to IgM is very sensitive (Allen *et al.,* 1920). Precipitation reactions are due to O chain of LPS. When serum becomes negative on SAT and CFT, it may signify recovery.

Immunoglobulins and their classes are quantifiable using radioimmunoassay (RIA), ELISA and immunofluorescence (Chappel *et al.*, 1982a; McNaught et al., 1977). Studies by Corbel (1972) have shown that 19s immunoglobulin which is basically IgM has no RBPT activity. The 7s or IgG are produced in infection and vaccination although serological responses vary with the stage of disease. Both IgG and IgM are detected by SAT (Corbel, 1972). In aborting ewes, SAT detected the highest number while diagnosing chronic clinical brucellosis (Corbel, 1972, Mahajan and Kulshreshtha, 1991). Presence of IgG is likely to correlate with active or chronic infections (Nicolleti, 1971). These responses are directed against antigens A, M and the proteins. A drop in their titre may indicate vaccinal titre (Acha and Szyfres, 1989). No cross-reactions occur against protein antigens that are responsible for the humoral and delayed type hypersensitivity reactions.

The commonly used antigens for polyclonal serological tests; *B.abortus* s99 and 1119-3, are suitable for brucellosis due to smooth *Brucella;* they cannot diagnose

brucellosis due to *B.canis* that is in rough phase (Alton *et al.,* 1975, Olascoaga, 1976).

2. 5.1. Rose Bengal plate test (RBPT).

This is a qualitative screening test with a high sensitivity and specificity in which a positive reaction is an indication of disease, not only in the individual animal, but in the whole herd. It is cheap, rapid, sensitive and allows for testing many samples in a short time. In a report by Diaz *et al.,* (1968), it was most sensitive, diagnosing 98.3%. It misses very few infected animals whether early or later in the course of disease. In field cases, it was found to be a better indicator of brucellosis than SAT. It is however, not adequate on its own since it has false reactions (Corbel, 1972). False positives occur due to antibodies against other bacteria. The test is based on whole cell phenol inactivated *B. abortus* s99 stained with Rose Bengal dye and buffered at pH 3.65. The test is carried out by thoroughly mixing equal volumes of antigen and serum (Alton *et al.,* 1975). The antigens detect agglutinins to all smooth brucellae. Antigens A and M of the smooth lipopolysaccharides are the important surface molecules in this test. Employing antigens A and M together increases reactor rates by 27% in infections by biovars where M is the dorminant antigen, like in *B.melitensis* or *B. abortus* biovars 4, 5 and 9. The test is sometimes over sensitive with cattle sera and it does not distinguish vaccinates

from infected animals nor chronic from acute and also does not pick latent infections (Corbel, 1985). In field cases, it gives results that are consistent with CFT but not SAT (Wood and Corbel, 1973). Although it correlates well with CFT, it may fail to detect some goats reacting at titres of 1:5 on CFT but negative on SAT (Philpott and Auko, 1972; Alton, 1978).

Eluted RBPT active antibodies are IgG, which are also active in CFT and Coomb's test but which show weak activity on SAT. The active fraction is IgG1, which according to Corbel (1972), is the only one detected by RBPT. Diaz and Moriyon (1989), however, reported that IgG1, IgG2 and IgM are all agglutinating immunoglobulins, with IgM producing very strong reaction on RBPT. The presence of IgG2 only, may not be accompanied by positive results because it is destroyed by the low pH of 3.65.

RBPT showed reactions in infected animals, more so among those of over two years but the younger ones had a higher infection rate (Turkson and Boadu, 1992). It gives negative results in adult animals that were vaccinated during calfhood, although a few animals may persistently show false positive reactions (Chappel *et al.,* 1982b). It is positive alongside CFT and SAT especially in cattle but not quite so in sheep and goats in which it was found less useful. Results obtained are confirmed by SAT and CFT (Alton *et al.,* 1975).

2.5.2. Milk ring test (MRT).

Milk ring test (MRT) is a very convenient brucellosis screening test for potentially infected lactating dairy animals (Morgan *et al.*, 1975). It is particularly suitable for prevalence studies especially at the start of a control program. Sampling of pooled milk; the test material of choice, is carried out at milk collecting centres. The test is carried out by mixing whole milk with a heat-inactivated smooth *Brucella* antigen stained with hematoxylin (Tetrazolium has also been used instead of hematoxylin) (Gregory, 1953). In this test, the antigen aggregates globular agglutinins to form a cluster. The resultant globular complexes combine with the fat in the cream to form a ring. The ring formed in a cow's milk rises to the top while that in goat milk settles to the bottom of the tube (Kolar, 1987). Attempts have been made to standardise the test and to estimate the titre but the idea has not gained much in popularity (Gregory, 1953). The results are affected by the sampling methods that lead to scant or excess cream: heating of the milk above 110^0C for 5 minutes and the duration of the milk storage. Storage of milk at 4^0C will yield results for up to two weeks. False positive results may be obtained in fresh milk but these may disappear once the milk is chilled for a time (Herr, 1982)

The antibody in milk associated with agglutination is IgA, which is produced locally in the udder and is heat labile (Beh, 1974). Not all *Brucella* infected animals have udder infection. All those that are without udder infection will yield

negative results at all times on MRT. MRT gives a good correlation with udder infection and animals so infected will shed the bacteria in milk for a long time (9-21 weeks) post partum (Zowghi *et al.,* 1990). A high proportion of these animals may have *Brucella* isolated from the milk despite being MRT negative. In a herd with more reactors on MRT than RBPT, Zowghi *et al.,* (1990) isolated the bacterium in 119 of 397 MRT negative milk samples. From such animals mechanical spread of disease is very likely.

Serum from animals whose milk produces positive results on milk ring test is tested using serological tests in order to identify the individual infected animals (Gallagher, 1973). Agglutinin titres in milk and those in serum of the same animal do not usually correlate. The best results are produced when sampling is done three times a year as in an eradication programme, and the reactors are repeat tested serologically in order to unearth all false negatives and those that pick the infection with time (Carter and ChengGappa, 1985).

2.5.3. Serum agglutination test (SAT).

On account of Wilson and Miles (1975); Wright and Smith (1897) described agglutinins and also developed the serum agglutination test (SAT). SAT is based on sedimentation of agglutinin-antigen complexes. The test with its modifications

that use macro and micro volumes is used as a supplemental test for RBPT (Alton *et al.,* 1975). It is based on multiple serial double dilutions with equal volumes of standardised antigens. The antigens are heat inactivated, smooth whole *Brucella* s99 or 1119-3 bacterial cells. The micro method, used more in America, is based on strain 1119-3 dyed cells and is carried out in micro titre plates (Alton *et al.,* 1975; Olascoaga, 1976). The test is standardised internationally against international standard anti-*Brucella abortus* serum (ISABS) and its results are expressed as international units or a reciprocal of the dilution (Olascoaga, 1976). The numerator represents the degree of agglutination and denominator the dilution. The recommended cut off point is 100 IU for infected and 200 IU for vaccinated animals respectively (Alton *et al.,* 1975). The antigen used for SAT is not suitable for diagnosis of brucellosis due to *B. canis* (Alton *et al.,* 1975). SAT can be used initially in an eradication programme since it has been found useful against animals vaccinated with s19 at calf-hood and reactors. The reliability of the test can be increased by use of CFT alongside it, for detecting carriers in the herd. It is important to carry out SAT and CFT whether RBPT is positive or not (Kagunya and Waiyaki, 1978). SAT has a high correlation with RBPT but it is not very useful in detecting early infection; so it is of no value where RBPT has failed. A retest is advised after 30-60 days for SAT seroreactors because it has high non-specific reactions and is also less accurate than CFT (Alton *et al.,* 1975; Olascoaga, 1976). It does not distinguish recently infected animals from those with chronic disease nor does it detect infection by rough strains when *B. abortus*

s99 is used as antigen. In chronic cases agglutinating titre may be low while the disease is still active. It has a low accuracy in problem herds since it does not detect all infected animals or those in which the disease has localised in the genital tract (Jones *et al.,* 1963; Nicolleti 1967). In the latter case, spermato-agglutination or vaginal mucus agglutination are more useful. Though SAT has found a lot of use, it has limitations of low accuracy in problem herds. False positives and negatives do occur. These false reactions are due to what was later associated with rough strains or due to cross-reaction between *Brucella* antigens and antibodies against unrelated organisms like *Y. enterocolitica* 0:9 and some other gram negative bacteria (Diaz and Moriyon, 1989). These reactions could also be due to non-specific agglutinins distinct from antibodies present in certain bovine sera. The activity of these agglutinins is inhibited at pH 4 (Corbel, 1972).

The antibodies detected in SAT are agglutinins (IgG and IgM). IgM is produced very early in the infection and dwindles rapidly leaving IgG, which is present in all active cases. IgG1 is the fraction responsible for the test. The two antibodies (IgG and IgM) are in detectable levels in serum from day 4 post infection and are commonly present in active infections (Waghela, 1978). IgM may be the only one present when the titre is low and this is where CFT becomes useful alongside SAT (Waghela, 1978). At this stage, tests specific for IgG, such as CFT and ME, would be most appropriate. SAT titre tends to rise in normally lambing ewes that had previously aborted due to brucellosis, although some may abort with no

serological titre but with organisms being isolated (Shaw, 1976a). Serological results in sheep may be at great variance with the disease states. Corbel, in 1972, observed more SAT reactors among aborting ewes than in RBPT positive ones and yet the two tests pick more positives than CFT. High numbers of infected sheep may fail to sero-convert on all tests. The results from a wide range of the tests, when combined, show more of the reactors than for individual tests. In porcine, if there is a single reactor or a suspicious reaction, then all must be culled. This is because antibody titres decline very fast in infected animals. The agglutinin in swine is IgM and so SAT is highly recommended for the diagnosis of individual animals. It differentiates the naturally infected animals from those vaccinated with s19. Sera with hemolysed red cells is not suitable for SAT because phenol interferes with the free antigen that may lead to false agglutination.

In human patients, SAT is inferior to CFT, because in the latter, IgG2 fixes complement but lacks agglutinating power. This leads to poor results when the disease is chronic or when the agglutinin concentration is low. Thus, negative results may be due to very low or non-agglutinating antibodies. SAT and CFT will become negative at different times in the course of the infection and when they do the patient has probably recovered (Young, 1989).

2.5.4. Complement fixation test (CFT).

Complement fixation test (CFT) is a very useful supplemental test especially with sera that has low agglutination titre. It can be automated and used as a confirmatory test following RBPT screening. On use in several herds, it detected reactions before the other tests and its results were more specific than the agglutination test ones. However, a better picture is got when CFT is carried out together with other tests (Jones *et al.*, 1963; Plommet., 1976a). It is highly recommended for the diagnosis of human and animal brucellosis. In man, it diagnoses acute and chronic disease and in animals it is also useful for differentiating natural infection from vaccination with s19. In both cases, it relates best with clinical disease as it classifies them as reactors or non-reactors (Rice *et al.*, 1966, Diaz and Moriyon, 1989). Human CFT titres are higher than agglutinins at advanced stages of the disease. The test also gives accurate results in chronic infections when other tests have become negative, thus establishing chronicity (Diaz and Moriyon, 1989). In animals vaccinated with s19 in their adulthood, high CFT titre is associated with field strains, since its titre is known to recede rapidly in vaccinated non-infected animals.

The complement fixation test does not detect latent infections and the results may be affected by the prozone phenomenon. Poor quality sera, i.e sera that is contaminated by microbial organisms or hemolysed sera are difficult to test with

CFT. The test can be undertaken on cold fixation at 4^0C for 14-18 hrs or at 37^0C for 2 hours. Cold fixation yields better results than warm fixation but it is only applicable with few samples. The test results depend a lot on the accuracy of complement titration (complement is the most fragile component of the test reagents). However, this test is complex and laborious, involving many steps and therefore requiring trained experienced persons. The test is not standardised internationally and interpretation of results varies from country to country. CFT gives more false negatives than radioimmunoassay (RIA) and indirect haemagglutination test (IHT) (Williams *et al.;* 1991). It also has poor correlation with bacterial isolation, especially in unvaccinated animals, in early stages of the disease (Jones *et al.,* 1963, Hayes and Chappel, 1982). CFT and RBPT remained positive for long in infected animals while CFT showed the fewest false negatives (Waghela, 1978). Burki (1954) reported that CFT failed to detect 9% of cattle positive on culture or those with abortion, while SAT failed to diagnose 12.5%. It has also been found to miss some infected animals in some herds when the disease is in chronic stages (Cordes and Carter 1979, Sutherland *et al* 1982). Some animals secreting the causative pathogens may not be detected by CFT for some time (Sutherland and Hollander, 1986).

Agglutination tests tend to have a high percentage of false negatives compared to CFT, probably due to the type of antigen in use, prozone reaction or the stage of disease. It has been observed that sera with similar agglutinating activity can

differ markedly with CF activity because the different tests measure different immunological activity (Davidsen and Herbert, 1978). This is true with other tests. Prozoning sera usually show reaction on SAT and RBPT because IgG2, a player in SAT, is good at agglutinating but does not fix complement. In CFT, it can block fixation by IgG1 and IgM leading to prozone phenomenon. This phenomenon can be overcome by cold fixation or by use of excess antigen to mop up the excess IgG2 (Corbel, 1985).

The reactions in CFT are based on large antigens; A and M that have multivalent binding sites and IgG1 as the major serum component (Corbel, 1972; Allan *et al*, 1976). These authors observed that the immunoglobulins reactive in CFT are IgM and IgG1 with IgM being more reactive. IgM is partially inactivated by heating to 60^0C and so it may not be as important as IgG1. Antibodies raised against non-agglutinogens persist longer than those against agglutinogens (Cullen and Corbel, 1970). However, sometimes the concentration of IgM produced is inadequate to cause positive agglutination, in which case only CFT may yield some titre (Stemshorn *et al*, 1985b). Antibodies detectable by CFT start showing from day 10 post infection; at this time the test will become positive simultaneously with SAT, but as the disease advances to chronicity, its titres rise while the SAT ones decline (Waghela, 1978). Burki (1954) recommended that CF titre of 25 units per millilitre (++ at 1/25) be taken as suspicious while higher titre be taken as positive. Philpott and Auko (1972) recommended a titre of 1/5 as a positive cut-off point for

brucellosis for sheep and goats. Titres of 1/10 are regarded as positive (Waghela, 1978). CFT gives less false positives than other tests in early infected non-vaccinated animals and detects most animals that are positive on RBPT while producing better results than SAT. The complement fixation test is the test method of choice in sheep and goats although some infected sheep may show low or negative titre, hence the need for strict culling criterion. When compared to bacteriology the test showed a sensitivity of 99.3% and a specificity of 89.7% (Corbel, 1985). In vaccinated non-infected and in recovered animals, CFT titres recede faster than SAT ones.

Titres on CFT tend to remain higher and more persistent in infected animals than SAT ones (Rice *et al.*, 1966). It has been found to produce better results than RBPT i.e. detect more positive animals. Differentiation of serum agglutination test titre due to vaccination from that due to non-specific infection remains a problem. Sera from vaccinated animals that react on RBPT can be verified with CFT. Though CFT has some success in differentiating reactions due to vaccination and infection, the accuracy of the distinction is frequently flawed. CFT is useful in animals vaccinated at 3-8 months. In calfhood infection IgG tend to disappear within 8 months of age (Alton, 1978). Some limitations of CFT such as low sensitivity can be overcome by use of ELISA as the latter detects low antibody concentrations (Chad *et al.*, 1989).

2. 5.4.1. Prozone phenomenon.

This phenomenon refers to a situation where a high concentration of serum antibodies prevents any reaction in CFT. It is common with bovine serum due to IgG2 and probably IgA (Corbel, 1985). When IgG2, which is good at agglutination, is in high ratio to IgG1, it may lead to negative CFT results, especially with warm fixation, due to its blocking effect. This phenomenon is exacerbated by the handling method (Herr, 1982). She observed more of this phenomenon in sera taking two or more days on transit. Prozoning sera usually show reactions on SAT and RBPT. The phenomenon is frequent in several sera of animals vaccinated with strain H45/20 and challenged just before participation in CFT. Effects of vaccination with H45/20 were minimal on CFT, with titres being transient. In such sera, RBPT and SAT had lower specificity and had higher false positive reaction rates (Sutherland, 1983). The phenomenon could also be due to alteration of antigenic sites, due to enzymatic action or due to contaminants (Olascoaga, 1976). Such reactions are characterised by low SAT titre of 1:20 and some such infected animals may be passed as free of brucellosis due to this phenomenon. Increasing the antigen concentration reduces this effect (McNaught *et al.,* 1977). A ratio of antigen to antibody of 1:20 produces optimal results and in the process reduces the sensitivity of the test though erasing the phenomenon (Gaultney *et al.*, 1971). When this happens, indirect haemagglutination test (IHT) is a better alternative (Cordes and Carter 1979).

2. 5.5. Enzyme linked immunoabsorbent assay (ELISA).

Enzyme linked immunoabsorbent assay (ELISA) is a simple, specific enzyme based primary binding assay with a superior performance over conventional tests. It has a high degree of accuracy and a wide range of modifications for diagnosis of brucellosis among other diseases. With appropriate modification it can be used to test sera from all animal species, while also increasing the effectiveness of the control programme. It is a rapid, sensitive and specific test that is useful for both mass screening and individual animal diagnosis. This test is also useful for identifying immunoglobulin profiles in serum and in cerebrospinal fluid (Araj *et al.,* 1986). It is the more recent of the serological tests and has been considered the method of choice for diagnosing brucellosis (Nielsen *et al.,* 1987). It favourably deals with the shortcomings of CFT, RBPT and SAT such as sensitivity and specificity and also, it can be modified to use monoclonal antibody techniques and radioimmunoassay by raising antisera against IgG. The antisera so raised will incriminate IgG1 and IgG2 but not IgM. Anti-light chain murine monoclonal antibody is conjugated to horseradish peroxidase for use as the conjugate. Use of monoclonal antibodies and radioimmunoassay helps to circumvent the problems of standardisation with polyclonal antibodies (Nielsen *et al.,* 1987). The test is also useful in detecting *Brucella* antigens in plasma (Limet, 1987). Antigenaemia

relates favourably with *Brucella* in spleen of infected mice but there are factors in plasma that may interfere with the results. These factors are destroyed by heating the sera before testing. Antigens for use in ELISA are bacterial polysaccharides (PS), lipopolysacchrides (LPS) or O chain of the LPS immobilised on polystyrene wells. Use of O chain as antigen discriminates between vaccinated and infected animals (Bulgin, 1990; Lord and Cherwonogrodozky, 1992). The results are read as optical density and are expressed as spectrophotometric absorbance values (SAV) based on the choice of a suitable threshold. The choice of the threshold is dependent on the prevalence of the disease, vaccination practice and the desired result. Serum with a high SAV is considered positive (Corbel and Morgan, 1984). Results are plotted as a graph where they appear as a continuous optical density curve unlike those for polyclonal serology which are dichotomous and therefore do not reflect small changes in titre, as they are based on double dilution (Williams *et al.,* 1991). The sensitivity for ELISA, which normally stands at 95%, is equal for all the dilutions. This high sensitivity plus its high specificity of 99.7% makes the test results easy to analyse (Caravano *et al.,* 1987). It detects early focal or latent non-clinical human infections and is also useful in diagnosis of patients with endocarditis (Caravano *et al.,* 1987; Serre *et al.,* 1987). Thus, it is useful for mapping out problem areas, as its readings remain positive even when shedding of the organism has ceased (Dargatz *et al.,* 1990). ELISA results compare favourably with those for culture, and the test also produces reasonable results on sera that produce anticomplementary reactions on testing with CFT. It is more dependable

than mercapto-ethanol on polyclonal serology as it identifies the reacting immunoglobulins as IgG, IgM, IgA and IgE; thereby classifying the disease as acute or chronic (Magee, 1980). However, it has not been accepted to replace the conventional methods because it is more expensive and requires specialised laboratories (Young and Edward, 1986). It has variously been shown to have a high correlation with RBPT and SAT in acute cases and that it produces positive results longer than CFT in chronic cases while detecting small changes in antibody levels (Nielsen *et al.*, 1987).

The sera of vaccinated animals do not react with O chain of the PS. Post vaccinal titre levels decline to very low but may remain positive for some time in a few animals. When using O chain of the PS as antigen, ELISA shows a higher intensity of reaction in vaccinates than in infected animals but fails to detect vaccinated, infected animals at very early stages and may also classify 2% of healthy human controls as positive (Araj *et al.*, 1986). Its results are affected by the bacterial culture phase and sometimes plates may absorb antigen and thereby affect results. Titration should be done in duplicate and repeating sera with more than 5% variation between the two tests. The test reagents are fragile and liable to spoilage on freezing.

2. 5. 6. Allergic skin test.

Allergic skin test is useful for diagnosis. Guinea pigs sensitised with LPS produce erythemas on challenge with protein antigens. The reaction is the same whether the proteins used are from rough or smooth *Brucella* forms, and can be used to differentiate brucellosis from infections due to *Y. enterocolitica*. The role of LPS in allergic reaction is not known (Jones, 1974). Positive skin test is indicative of past or present infection but not necessarily an active one. Weak or strong skin test reaction is associated with high serological titres but the test is not suitable for routine diagnosis because the antigen used may induce a rise in antibody production, thereby confusing the diagnosis (Alton *et al.,* 1975).

2. 6. Host immunity to infections by Brucella.

Animals infected by *Brucella* organisms accord themselves protection by production of humoral antibodies: IgG and IgM among others, and cell mediated immunity (CMI), or both (Hoffman and Nicolleti, 1979). Vaccination also offers protection by production of humoral antibodies and probably by stimulating cell mediated immunity. CMI is taken as the more effective arm of immunity (Stevens *et al.,* 1995a). Some infected animals with very little or no antibodies do occur (Hayes and Chappel, 1982). At the site of entry into the host; the organism is

destroyed by neutrophils' brucicidal activity that is probably facilitated by peroxide and oxygen dependent mechanisms (Cheers, 1985). The complement cascade has also been shown to mediate killing of *B. abortus,* though some strains may be resistant. This resistance is associated with the pathogen's virulence particularly the smooth forms, with the avirulent strains being highly sensitive to serum mediated killing by the complement cascade (Eisencheck *et al.*, 1995). Complement cascade is sometimes activated by antibody independent mechanism though not by alternative pathway. The brucellae that survive killing thus, invade and lounge in macrophages where they are either destroyed or are offered protection (Parma and Sautisteban, 1984). Those that survive within macrophages are transported through the circulatory system to various organs. In this way macrophages contribute both to immunity and pathogenesis (Campbell *et al.*, 1994; Cheers, 1985).

2. 6. 1 Immunoglobulins produced in *Brucella* infections.

Cells infected with *B. abortus* produce interferon, which activates monocytes to transform to immunoglobulin producing monocytes (Cheers, 1985). Complement fixing and haemagglutinating antibodies appear before agglutinins. IgM is the first one to be produced, followed shortly by IgG1 that rises and reaches peak by day 32 (Waghela, 1978). These and IgG2 appear within a short time of one another in

infected animals and are later followed by IgE that has been detected in 76% of acute human cases using Indirect fluorescent antibody technique (IFA) and radio immunoassay (RIA) (Serre *et al.,* 1987). Secondary response of IgM is like in initial stimulus. Once production has reached peak, it stabilises and the immunoglobulins then persist for sometime before they start to disappear slowly. The peak of IgG1 is three times that of IgM; it persists longer and declines more slowly than IgM and so it accounts for most positive cases as the residual antibody in the seroreactors (Beh, 1974). Its level is related to the strength of the antigenic stimulus, thus serving as an indication of active disease, while its persistence is associated with progressive acute infection, chronic disease or repeated infection. Repetition of antigenic stimulus causes a more rapid, abundant and persistent production of IgG. When IgG disappears, it probably signifies an end of infection (Carter and ChengGappa, 1985)

The distribution of IgG and IgM is different in chronically infected animals and vaccinated ones (Beh and Lascelles, 1973). IgM characterises very early or persistent infection following vaccination. In vaccinated animals where only IgM may be produced, agglutinins appear earlier than in infected animals. They disappear fastest depending on the infecting dose, route of inoculum and animal species. In animals vaccinated with s19 at 8 months of age, the level of IgG is usually higher than that in animals vaccinated during adulthood (Beh, 1974). These immunoglobulins are responsible for serological reactions. CFT is a result

of both IgG and IgM, though the latter is measured more efficiently by SAT and RBPT than the former (Allan *et al.,* 1976). In vaccinated animals RBPT is less reliable than both CFT and SAT (Kapur *et al.*, 1979). Swine do not have a similar immunological response as cattle (Carter and ChengGappa, 1985).

2.7. Control of brucellosis.

2. 7.1. Control of the disease in animals.

Knowing that human brucellosis is an occupational hazard, being commonest in animal handlers, and that man is a dead end host; its control should be through eradication of the disease in the source animal, though this is a difficult task. The aim in an eradication programme is to eliminate the disease in the shortest possible time, using the most economical methods (Dolan *et al.*, 1979). The mode of achieving this should be resource efficient, logistically feasible and locally sustainable (McDermott *et al.,* 1987). One common approach involves educating people on the proper herd management, extensive strategic use of vaccination programs and an enlightened application and interpretation of serological test results (Dolan *et al.*, 1979). Test and slaughter is effective and quick but it is expensive and sometimes impractical when the prevalence is high. It may also be unthinkable in some management set-ups (Alausa, 1980). It has however been noted that where all animals in an infected herd have been slaughtered, brucellosis

does not recur. In areas of low prevalence, slaughtering the entire herd is the most effective strategy since it helps to contain or limit the excretion of the pathogens thereby halting its spread. This cuts out the source of human infections and reduces the economic losses.

Vaccination is a vital tool in an eradication programme as it increases the number and proportion of resistant animals with a concomitant elimination of reactors. Any serological suspect can then be removed and slaughtered. Removal of infected cows prior to their abortion or parturition reduces the exposure and consequently new infections and therefore increases the effectiveness of vaccination with *B. abortus* s19 (Crawford *et al.,* 1988a). It has been observed that the stage of gestation and parturition are more important factors in herd plan than early removal of post parturient infected cows following vaccination with s19 (Crawford *et al.,* 1988a). Post parturient cows shed organisms and also give birth to calves that are nearly always infected. Some of these calves shed infection while others may retain it as latent carriers up to the time of sexual maturity when they start shedding; thus leading to the breakdown in the vaccination programme (Crawford *et al.,* 1988a). This means vaccination alone cannot lead to eradication because of continued contamination of the environment (Kolar, 1987). The best way of preventing exposure to clean animals is by vaccination followed by separation of calves from cows and backed by test and slaughter. It has, however, been doubted whether removal of infected cows by frequent testing and slaughter

is of much value, as it has been argued that retention of reactor cattle did not increase spread of brucellosis in vaccinated beef herds (Manthei 1959; Crawford, 1988b).

Two types of vaccines have found practical use in the control of animal brucellosis. The most extensively used ones are the live attenuated vaccines like *B.abortus* strain 19 (s19) and *B.melitensis* rev1. Schuring *et al.*, (1981), claim that a laboratory mutant of *B.abortus* strain 2308 code-named RB51; a rough live vaccine, is effective. Live vaccines have the best immunogenicity for prolonged periods of time, whereby a single dose may be adequate for life. However, they produce long-standing serological responses that interfere with diagnosis. This adverse effect is reduced by vaccination through the conjuctival route (Alton, 1978).

B.abortus s19 produces best results when it is used during calf-hood. It has been tried against infections by all *Brucella* species but more so against bovine brucellosis due to *B.abortus*. A higher dose of s19 increases the serological activity and protection (Crawford *et al.*, 1990). Its efficacy is modified by degree of vaccination cover, the level of the risk of exposure, the dose of infecting organism, the colonial state of the vaccine seed, its viability and virulence (Crawford *et al.*, 1990). In adults, the normal dose of 10^9 organisms is protective but may also be infective when administered by normal route and so a reduced

dose is recommended (Alton, 1978). Conjuctival route of inoculation is more efficient than the subcutaneous one for vaccination (Plommet *et al.,* 1987). *B.abortus* s19 vaccination of adult animals is risky because it may cause disease in some, especially those in late stages of pregnancy. Some calfhood vaccinated animals were found infected at slaughter (Kolar, 1987; Sutherland *et al.,* 1982). Strain 19 may also produce side effects such as epididymitis and epiphysitis in rams and may occasionally establish chronic infection in calves or cause persistent specific antibody titres. It is pathogenic to man (Alton *et al.,* 1975). Some erythritol resistant, strains 19 mutant cell lines may appear in all vaccine cultures. These mutants are more pathogenic and may persist and cause disease as animals mature, with possible abortion, therefore leading to a breakdown in the control programme (Sangori *et al.,* 1996). The s19 vaccine can be cleaned of these mutants by growing vaccine seed material in a minimal medium whose source of carbon is glycerol. The glycerol inhibits the growth of the mutants. This method of purification is simple and cheap (Zygmunt *et al.,* 1994).

B.melitensis rev1 has been found to be protective to sheep and goats against *B. melitensis* and *B.ovis* and is the best against ram epididymitis due to the former. It can be used in adults by applying a reduced dose of 10^4 or 10^5 colony forming units (CFU) as a single dose about 1 month before service (Crowther *et al.,* 1977). The protection is dependent on the residual virulence of the vaccine strain and route of inoculation. The effective dose; 10^{5-7} CFU for rev1 is less than the 10^9

vaccine that was previously thought to be ideal. This reduced dose helps to cut on cost of production, and consequently that of controlling the disease (Kolar, 1987). Rev1 is more protective than *B. suis* strain 2, for sheep on challenge with virulent strain 53 H38 (Blasco *et al.,* 1993). It, however, evokes a strong serological response that interferes with diagnosis of infection due to *B. melitensis*. It is virulent to humans and may cause abortion in vaccinated animals. Use of conjunctival route of inoculation interferes with serological diagnosis more than use of subcutaneous route. Early vaccination leads to low agglutination and CFT titres. In such cases residual antibody titres diminish rapidly and so sero-diagnosis is little affected when complementary diagnostic tests are used. Vaccination of adult animals with reduced doses is nearly as effective and safe (Alton, 1978). However, neither live vaccines nor the inactivated ones offer 100% protection.

Rb51 induces CMI response that is associated with protection. It does not produce antibodies against LPS "O "chain antigen or lipid A and so does not interfere with diagnosis using whole cell antigens (Stevens *et al.,* 1995b). It is less virulent than strain 2308. However, resistance to challenge is lower than that due to s19, probably because it is less persistent in the body than s19 or due to the lack of antibodies against strain 2308. Strain 19 vaccinated animals had a higher proliferation of spleen cells when challenged with 2308. Protein fractions of 2308 and Rb51 were not protective in rams when challenged with *B.ovis* since they do not induce CMI (Sangori *et al.,* 1996). Rb51 is not protective at all against *B. ovis*

infection (De-Bangues *et al.*, 1995). The failure of protection is based on the fact that they have identical antigens that are extractable in soluble fraction (Diaz *et al.*, 1967).

Killed cell vaccines are *B.abortus* 45/20 and *B. melitensis* H38 oil adjuvant vaccine. These have been tried in different countries with low successes. *B.suis* biotype 2 has been developed and used in China (Blasco *et al.*, 1993). It is administered to sheep in drinking water but it has not been accepted widely. Fraction vaccines based on LPS, Ps and proteins have been tried. LPS ones have been shown to evoke antibody production but not CMI that is most important in protection against intracellular pathogens (Santos *et al.*, 1984).

There are no human vaccines since it is difficult to ascertain quality. Such vaccines should be innocuous. So human disease is best controlled by effective control of disease in animals. Also, most underdeveloped countries have no brucellosis control programmes due to lack of adequate information on disease situation, ineffective animal health education, poor animal husbandry practices and unavailability of financial resources (Kolar, 1987).

2. 7. 2. Control of brucellosis in humans.

Control of human brucellosis is mainly through the use of empheric treatment. Treatment should commence after blood for culture has been collected. Successful therapeutic combinations for brucellosis are tetracycline and streptomycin, doxycycline and streptomycin, doxycycline and rifampin, with treatment by tetracycline and streptomycin still being most reliable and effective (Lambea *et al.,* 1992). Rifampin combined with cotrimoxazole has also shown quite a bit of success (Kutty *et al.,* 1991). Treatment combinations involving rifampin are said to be less effective than those of tetracyclines. Some of the shortcomings are: lower effectiveness and relapses, especially in severe cases (Diaz-Entre Sotos *et al.,* 1992; Solera *et al.,* 1991). Cases with complications require more intensive treatment. When spondylitis is the complication, surgical intervention to relieve pressure on the spinal chord or to drain paravertebral abscesses may be essential (Colmenero *et al.,* 1991, Kutty *et al.,* 1991). Some of the treated patients may show a high frequency of relapses (Moreno *et al.,* 1992). The relapses are best diagnosed by hemoculture, specific symptoms such as prolonged fever, spondylitis, arthritis, orchitis and high persisting serological titre after the treatment (Moreno *et al.,* 1992).

3:0. MATERIALS and METHODS

3:1. The area of study.

This study was carried out in Narok district in the Rift valley province of Kenya. Narok is in the southwestern part of Kenya; to it's southwest is the district of Transmara; to its west are Bomet, Kisii and Nyamira; to its north is Nakuru; to its east is Kajiado and to its south is the Republic of Tanzania. This expansive district has an area of 16,000 square kilometres and a human population of 400,000 people. It is rich in wildlife among which the herbivores exceed two million. The domestic livestock are about one million, with cattle making 50%, sheep 30% and goats 20%. The world famous Maasai Mara game park with its wealth of a wide range of wildlife and a retinue of lodges is located here. The district occupies the upper half of the rift valley from the floor to the western escarpment. Topographically it consists of large stretches of flat plains and, occasionally, areas rising sharply to the escarpments. The general altitude is 1,500-2,100 M. The escarpments may rise from 2,100 M towards the Mau that towers to about 2,700 M above sea level.

Most of the district is warm the year round with maximum daily temperatures of 20-30^0 C, except for the higher areas, which are cool with the temperature frequently falling to around 10^0C. The rainfall, that is generally scanty (between 500-800mm annually), follows a similar distribution pattern to the temperatures. The high altitude

areas may receive around 1300 mm of rain. Most of this rainfall is received during the long rains' season from March to July. The vegetation too follows the rainfall and weather patterns with the lower parts to the east and south being savannah grassland interspersed with shrubs and acacia. The higher grounds mainly to the Northwest are shrub-lands that give rise to thick forests.

Droughts are not uncommon. When they come, they leave a lot of devastation in their wake, thereby forcing migration of the people, their stocks and even game. At the start of this study, in July 1994, the area was just starting to recover from one such calamity that had claimed large numbers of livestock and game. People showing signs of outright malnutrition were a common feature. A large part of the district was still reeling from this impact.

The bigger part of the human population is the Maasai, who are to a large extent nomadic. This mode of life is fast dying out due to restrictions imposed by private land acquisition. The Maasai live in a manyatta. A close family lives in a cluster of such manyatta that are fenced together into large kraals that may form a small village. As the people start to settle, some are far flung from water sources, forcing them to dig shallow surface dams that trap run-off water during the rains. This water is used both by animals and people. Most families have no sanitary facilities and hence are at great risk of waterborne diseases. This area was chosen for study because of the following reasons:

1. Human sera sent for brucellosis testing at the Central Veterinary Investigation Laboratory at Kabete, recorded a high reactor rate.

2. A follow-up retrospective study of data from four health outlets in the district showed that diseases presenting with flu-like symptoms account for over 75% of all human ailment in this district and that brucellosis ranked high among them (Muriuki, 1994).

3. People in this area have a pastoral mode of life. Studies elsewhere have shown that such a life style has a high relationship with occurrence of brucellosis (Acha and Szyfres, 1989; Wereney *et al.*, 1979).

3.2. Sampling.

Test samples were taken from people with fever, chills, sweating, joint pains and headaches among other symptoms, and from domestic animals with signs and symptoms that incriminated brucellosis, such as lameness, hygroma, abortions, retained placentas, and infertility. Some animals in homes where a patient was suspected to have brucellosis were also sampled.

3.2.1. Sampling of the human patients.

3.2.1.1. Sampling points

Sampling of human patients was done at the Narok District Hospital (NDH) and the dispensaries of Olasiti, Siayapei and Mararianta. The dispensaries are run by Christian Mission Foundation (CMF) and have quite a high degree of autonomy from the district hospital. The district hospital is in the heart of Narok town. It receives most of its patients from the town and referral cases from the dispensaries. Olasiti and Siayapei dispensaries are along the Narok-Nairobi road about 55 and 10 km from Narok town, respectively. Mararianta is about 110 km to the south of Narok town, just outside the game park and close to the Kenya-Tanzania border. The area around Mararianta is purely pastoral while those around Olasiti and Siayapei are largely settled.

3.2.1.2. Mode of sampling.

Two blood samples from each patient with flu-like symptoms were collected, from the radial vein, using 21-gauge needles, into vacutainer tubes (BBL), after disinfecting the respective area with methylated spirit. Blood for culture was collected into the tube with the anticoagulant (potassium oxalate), while that for serum harvesting was collected into the tube without the anticoagulant. From the

tube without the anticoagulant, and which was filled first, serum was harvested. On filling, the tube with the anticoagulant was then rolled between the palms for thorough mixing. The blood for culture was chilled until the time of media inoculation. From the other tube, serum was harvested after 24 hours of chilling and thereafter tested with Rose Bengal dyed *Brucella* antigen, at the collecting point. The blood of patients whose serum tested positive for brucellosis and one out of every five of the negative, were cultured into a di-phasic brucella medium, using Casterneda`s method (Alton *et al.,* 1975). The rest were destroyed. In all, 1117 human serum samples were collected and tested at the points of sampling using RBPT. From this number, 957 were retested at the Central Veterinary Investigation Laboratories at Kabete (Vet Labs) using RBPT, SAT and CFT. Blood of 531 of these patients was cultured for *Brucella* pathogens in addition to their corresponding sera being screened for anti-brucella antibodies.

3. 2. 2. Sampling of animals.

3. 2. 2. 1. Sampling points

The animal sampling was undertaken in the administrative division of Ololunga, Mau and Osupuko. Location and then sub-location were chosen randomly using computer methods. This led to the eventual sampling in the administrative sub-locations of

Koyiaki, Megwara, Olorte, Elang`ata-Enteriti, Entasikira, Majimoto, Enara marti-shoriki, Enelerai, Elmesutie and Nkorrkori. All the animal sera from the random sampling and from the matched sampling were 1,620 cattle, 1,257 goats and 577 sheep. Blood samples from 132 cattle, 196 goats and 50 sheep were cultured. Of the animals whose blood was cultured, 54 cattle, 73 goats and 34 ewes had a history of abortion. All the animal sera, like the human ones, were tested at Vet Labs using Rose Bengal plate test (RBPT), serum agglutination test (SAT) and complement fixation test (CFT).

3.2.2.2. Sampling procedure.

The animal blood was collected from the jugular vein after making it bulge by applying pressure onto the jugular fallow. The samples were treated in the same way as those of the human beings. Sampling was done for 2-3 weeks before the specimens were delivered to the laboratory for the processing, owing to the big distance between the area of sampling to the laboratory and the distance between the homes. The samples were chilled from the time of collection up to the time of culturing.

3.2.3. Sampling of animals from homes of brucellosis suspect human patients.

Homesteads of human patients suspected of suffering from brucellosis, either by isolation or from positive reaction on RBPT at the sampling points, had their respective animals screened. The sampling procedure was as in 3.2.2.2.

3:3. Culturing blood for bacterial isolation.

The quick Rose Bengal plate test (RBPT) done on sera at the collecting point was used to determine which human blood samples to be cultured. Both human and animal blood samples for culture were chosen as follows:

i). All those blood samples from human patients whose sera tested positive for brucellosis on RBPT.

ii). One out of every five human samples from among the lot that tested negative for brucellosis on RBPT was randomly chosen for culture.

iii). Blood collected from animals with a history incriminative of brucellosis such as abortion or hygroma.

The blood samples were initially cultured in a diphasic medium containing serum dextrose broth and serum dextrose agar using Casterneda's method (Alton *et al.*, 1975). The blood was injected into the medium bottles and shaken to mix the blood

and the serum dextrose broth. The liquid phase was then rolled onto the solid agar to flood it completely. The bottles were incubated at 37^0C in a standing position for a period of up to eight weeks and checked for growth on the solid phase every third day. Reflooding of the solid agar was done wherever there was no growth. Once growth appeared, the date was noted and reflooding was stopped, but the incubation continued up to the eighth week. At the end of this primary incubation, cultures with growth were streaked onto blood agar plates in duplicate; one plate was incubated in 10 % CO_2 and the other without the CO_2. Slants without growth after the eighth week were destroyed by autoclaving. Those that were discoloured and rendered opaque due to decomposition of the blood were regarded as positive until proven otherwise on subculture. In order to comprehensively classify all the bacteria recovered on blood culture, the colonies were purified and subjected to the following battery of tests: gram and modified Ziehl Nielsen stain reactions, urea hydrolysis, production of hydrogen sulphide and indole; methyl red, Voges-Proskauer and citrate (IMViC) reactions; fermentation of sugars, nitrate reduction, growth on dyes, growth at 25^0 C and 42^0 C, and motility at 22^0 C and 37^0C. Wherever there were acidifying bacteria, sugar alcohol fermentation test was done. The setting and reading of the tests were done according to Alton *et al.,* 1975, Cowan (1977), Corbel and Morgan (1984) and Smibert and Kapat (1984). A known *Brucella melitensis* (16M, NTCC) isolate was subjected to the same tests to serve as a model strain. The sugars used in the tests included glucose, maltose, lactose, sucrose, arabinose, trehalose, mannose, mannitol and xylose.

3.4. Serological tests.

The serological tests carried out to screen the collected sera for brucellosis were: Rose Bengal plate test (RBPT), serum tube agglutination test (SAT) and complement fixation test (CFT). The dyed antigen for the RBPT was obtained from the Central Veterinary Investigation Laboratories, Ministry of Agriculture, Kabete. It is derived from smooth *Brucella abortus* strain 99. This and that for SAT were prepared according to recommendations of the FAO/WHO Monographs by Alton *et al.* (1975). The RBPT antigen consisted of phenol inactivated, whole smooth *Brucella abortus* cells, while the SAT antigen was the same strain, inactivated by heating. The CFT antigen was *Brucella abortus* from the Max Von Potten Institute of Berlin in Germany.

3.4.1. Rose Bengal plate test (RBPT).

Rose Bengal plate test (RBPT) was carried out using the dyed *Brucella* antigen as described above. To carry out the test, twenty five microlitres of test serum were placed in a white porcelain tile well to which an equal volume of the antigen was added, and the contents mixed with an applicator stick. The tile was then placed on

an electric shaker, rocked for 4 minutes and checked for agglutination by rocking it gently from side to side. Known positive and negative sera were always included in the test as controls (Alton *et al.*, 1975).

3.4.2. Serum tube agglutination test (SAT).

Glass tubes 15 x 85 mm (Pyrex No 9820 USA) were used. The tubes were arranged on a rack in several rows; three tubes per row. Three dilutions per sample were carried out thus: 1ml of phenol saline (0.5% phenol in 0.85% saline solution) was put into the first tube and 0.5 ml into each of two other tubes. To the 1ml in tube one, 0.2ml of the test serum was added and the two were then mixed thoroughly, using a 1ml pipette fitted with a rubber teat. The extra 0.2 ml of the diluted serum was removed and discarded. Using the same pipette, 0.5 ml was transferred to the second tube. The contents of tube two were mixed and 0.5 ml was transferred to the third and last tube. From the last tube, 0.5 ml was removed and discarded. This means that each tube eventually had 0.5 ml of serially double diluted serum to the concentrations of 1/6, 1/12 and 1/24. Positive and negative control sera were diluted in the same manner as the test ones in eight tubes instead of the three used in the test serum. Positive and negative control sera were diluted to the same specifications as the test ones in eight tubes instead of the three used in the test serum. All test sera showing a positive reaction were later retested to the same dilution as in the controls,

to establish its final titre. For the controls, the final dilution was 1/768. To each tube of the test serum and those of the control, 0.5 ml of the working dilution (1/100) of the antigen prepared earlier was added. The innoculum was incubated at 37^0C for 24 hours. A positive result was represented by a button of sediment and a clear supernatant. The last tube showing partial or complete clearing was declared the end point and its titre noted. The clarity was graded from + for 25%, ++ for 50%, +++ for 75% clearing and ++++ for complete clearing.

3.4.3. Complement fixation test (CFT).

The antigen, *Brucella abortus,* from Max Von Potten Institute in Berlin and complement were standardised before use, to the Weybridge standards (Alton *et al.*, 1975). The complement and hemolysin were titrated against each other to establish a working concentration of each. The working concentration of complement was a 1/80 dilution in veronal buffered diluent (VBD). Details of how VBD was prepared are given in appendix 13.

Before carrying out the microtire CFT, the test sera were individually diluted at the rate of 50μl serum to 200μl of pre-diluted (1:5) veronal buffered diluent (VBD), using Pyrex glass tubes of 15x85mm, as recommended by FAO/WHO (Alton *et al.*, 1975). The tubes were properly labelled with respect to each sample. Known

positive and negative sera were also set up to serve as controls. The diluted serum samples were then incubated in a water bath set at 56^0C for 30 minutes (for bovine serum) or 62^0C for 30 minutes (for human, goat and sheep sera) in order to inactivate the natural complement. These samples were later transferred individually to plastic microtitre plates (Limbro USA), each sample per well. Taking the standard plate of 8 rows A-H, with each row having 12 wells, 8 serum samples were processed per plate. Before transfer of the above samples, 25μl of working concentration of VBD were placed in each of wells, 2-6 and 8-12 of each row of the plate, so as to run dilutions in duplicate. Twenty five microlitres of the inactivated test serum was added to wells 1 and 2 and from the latter well a double dilution was made through to well 6. The process was repeated for wells 7-12. The exercise of diluting was hastened by use of a hand multi-channel dispenser (Titretek) fixed with 8 tips. Twenty five microlitres of standardised antigen was added to each of the first 6 wells of the row (i.e. 1-6) for the entire plate. To wells 7-12, an additional 25μl of VBD was added to equate the volumes; these were to serve as antigen controls. Titrated complement, at volumes of 50μl was then added to each well of the plate. The plates were then taped shut to stop spillage during thorough mixing through shaking. The plates were then incubated at 37^OC for one hour. After incubation, 50μl of 2% sensitised sheep red blood cells (rbcs) were added to each well, and the mixture incubated at 37^OC for another 30 minutes. The plates were then spanned at 1500g for 10 minutes before reading the test as follows: in positive cases (i.e. where the serum contains homologous antibodies) a button of rbcs was formed at the bottom of the well. In a

negative case (i.e. where the serum does not contain homologous antibody), there was hemolysis of rbcs, hence no button. This hemolysis could be of various intensities, ranging from trace to complete hemolysis. The positives were graded as: +4 for wells with complete clearing of the diluent above the cells, +3, for 75% sedimentation (i.e. 25% hemolysis), +2 for 50% sedimentation/ hemolysis and +1 for 25% sedimentation (i. e 75% hemolysis).

4.0. RESULTS.

4.1. Human results

4.1.1. Bacterial isolates from the cultured human blood.

Out of the 957 human patients serologically tested for brucellosis, 531 had their blood cultured for bacterial pathogens. *Brucella*, *Yersinia* and *Campylobacter* species were isolated. *Brucella abortus* was isolated from three patients who were positive on RBPT, SAT and CFT. Isolates that conformed to the genus *Yersinia* were recovered from eight patients who were positive on RBPT. *Campylobacter* species were isolated from 11 patients. The sera of these patients were negative to all the brucellosis serological tests. The characterisation details of the respective isolates are given on appendix 13.1; 13.2 and 13.3, respectively.

Brucella abortus was isolated from two patients from Olasiti and one from Narok town. *Yersinia* was isolated from three patients in Olasiti, three from Siayapei and two from Narok town. *Campylobacter* was isolated from two patients from Mararianta, two from Siayapei, six from Olasiti and one from the district hospital. The two patients from Mararianta were from a single home in Lolgorian in Transmara district.

4.1.2. Serological results.

A total of 957 (628 females and 329 males) human patients' sera were screened using RBPT, SAT and CFT. Overall, the three brucellosis serological tests run at Kabete had a total of 78 (8.2% of 957) reactors, with the 329 males having 33 (10.03%), while the 628 females had 45(7.2%) (Table1). RBPT with 62 reactors was most sensitive, followed by SAT with 39 and then CFT 21 (Table1). Sera of the 531 patients whose blood was cultured yielded a total of 70 (13.18%) reactors for the three tests. The other 8 reactors, all female and positive only on RBPT, were from patients whose blood was not cultured. RBPT and SAT realised a percentage reactor rate in males that was one and half times that in females, while CFT picked twice the female reactor rate (Table 1). Looking at the results per test or combination of tests (Table 2), the importance of using the three tests on each serum sample was highlighted. Its advantage being that the exercise detects more positive sera, than when a single test is used. Majority (76%) of the reactors were 15 years or below with the 33 male and 20 of the 45 female reactors being in this range, while the other reactors were distributed as shown in (Table 3). Only 11 patients (4 female and 7 male) were positive on all three tests (Table 4).

Human patients were sampled from 13 sublocations with only 7 sublocations accounting for 54 of the seropositive patients. The number of seroreactors per sublocation were one patient from Narosura, three each in Lolgorian, Suswa and

Ntulele, eight in Narok town, 12 in Siayapei and 25 in Olasiti. The health outlets recorded reactors thus; Olasiti 39, NDH 16 and Siayapei 19, with 21 (7 male and 14 female) of the reactors from Olasiti dispensary being from Olasiti sublocation. The other reactors were one each from Narok town and Narosura, two each from Lolgorian, Suswa, Siayapei, and three from Ntulele.

Olasiti Dispensary had the closest agreement between the patient's clinical signs and the serological results. The other two outlets diagnosed and treated for brucellosis some patients who had only one or two clinical signs common for brucellosis. Though the source of 20 reactors is not known, it is clear that Olasiti sublocation with 21 reactors, Siayapei with 15 and Narok town with 11 were highly infected (Table 5).

4.1.3. Clinical signs of the sero-positive human patients.

Joint pains was the most common clinical sign being recorded in 37 patients, headache in 19, body pain and weakness each in 10 patients, fever in 9, sweats in 3, chills in 6 and abdominal discomfort in 5, either alone or in various permutations (Table 5). Headache, joint pain, body pain and weakness were together recorded in 7 patients.

***Table 1:* The cumulative serological results for all the human patients; male and female and their respective percentages for the three serological tests used.**

Serological test used	Cumulative number of male serological reactors for the specific test	Percentage reactor rate for the 329 males for the specific test.	Cumulative number of female serological reactors for the specific test	Percentage reactor rate for the 628 females for the specific test	Total Human serological reactors for the specific test for both sexes	Percentage serological reactor rate for the 957 human sera for the specific test.
RBPT	25	7.6	37	5.9	62	6.5
SAT	18	5.5	20	3.2	38	3.9
CFT	11	3.3	10	1.6	21	2.2

TABLE 2: A breakdown of the serological results with respect to the different tests or a combination of the tests for female and male patients and the corresponding percentage reactor rates for the 957 patients tested serologically.

Serological test used	Number of seropositive males for the specific test.	Percentage reactor rate for males for the specific test.	Number of sero-positive females for the specific test	Percentage reactor rate for the females for the specific test.	Total sero-positive patients for the specific test.	Human percentage reactor rate for the specific test.
RBPT only	11	1.14	23	2.4	34	3.55
SAT only	6	0.62	4	0.41	10	1.04
CFT only	2	0.2	1	0.10	3	0.31
RBPT+SAT	5	0.52	8	0.84	13	1.35
RBPT + CFT	1	0.10	1	0.10	2	0.20
SAT+CFT	1	0.10	4	0.42	5	0.52
RBPT+SAT +CFT	7	0.73	7	0.73	14	1.46
Total	**33**	**3.44**	**45**	**4.7**	**78**	**8.2**

TABLE 3. The relative reactor rate for male and female reactors as a percentage of the total (78) seroreactors for different age intervals .

Age in Years.	Number of seropositive males for the specific age interval	Number of sero-positive females for the specific age interval.	Total sero-positive patients (males and females) for the specific age interval.	The human cumulative percentage reactor rate for the specific age interval
0-1	20	16	36	46.1
2-5	3	3	6	53.8
6-10	3	5	8	64.1
11-15	7	1	8	74.4
16-20	0	5	5	80.8
21-25	0	4	4	85.9
26-30	0	4	4	91.0
>30	0	7	7	100.00
Total	**33**	**45**	**78**	**100.00**

Table 4. A breakdown of the serological results with respect to the different tests or a combination of the tests for female and male patients for different age intervals for the 78 seropositive human patients.

The human seroreactors for specific age intervals for male and female patients for the specific test.

	Female reactors for the specific age intervals for the specific test								Male reactors for the specific age intervals				
Test(s)	0-5	6-10	11-15	16-20	21-25	26-30	>31	**Total**	0-5	6-10	11-15	**Total**	**Grand Total**
RBPT only	13	1	0	1	2	2	4	**23**	9	0	2	**11**	**34**
SAT only	1	0	0	0	0	2	1	**4**	1	3	2	**6**	**10**
CFT only	1	0	0	0	0	0	0	**1**	2	0	0	**2**	**3**
RBPT+SAT	1	2	0	4	1	0	0	**8**	2	1	2	**5**	**13**
RBPT+CFT	1	0	0	0	0	0	0	**2**	1	0	0	**1**	**3**
CFT+SAT	2	1	0	0	0	0	0	**3**	1	0	0	**1**	**4**
RBPT+SAT+CFT	0	1	1	0	1	0	1	**4**	4	2	1	**7**	**11**
TOTAL	**20**	**5**	**1**	**5**	**4**	**4**	**6**	**45**	**20**	**6**	**7**	**33**	**78**

Table 5. The distribution of human seropositive patients for the various sublocations and the frequency of the clinical signs recorded for the eropositive atients.

Number of patients versus the clinical sign recorded for the particular sublocation per gender versus the patient's clinical signs.

Location of source	Sex	Reactors	No clinical signs	Fever	Body ache	Joint pain	Body pain	Weakness	Sweats
source unknown	M	16	18	-	-	-	-		-
	F	10	10	-	-	-	-	-	-
Lolgorian	M	2	-	1	0	2	-	-	-
	F	1	-	1	0	1	-	-	-
Ntulele	M	1	-	0	0	2	-	-	-
	F	2	-	0	0	2	-	-	-
Narok town	M	1	-	0	1	1	-	-	-
	F	6	-	2	4	5	2	3	-
Suswa	M	0	-	0	0	0	-	-	-
	F	3	-	0	0	2	1	-	-
Narosura	M	0	-	0	0	0	-	-	-
	F	1	-	0	0	1	-	-	-
Siaypei	M	3	-	1	3	2	1	2	-
	F	9	-	0	1	2	-	-	-
Olasiti	M	10	-	3	4	7	1	2	2
	F	13	-	0	5	9	5	3	1
Total	**M**	**33**	**18**	**5**	**9**	**16**	**2**	**4**	**2**
	F	**45**	**10**	**4**	**10**	**21**	**8**	**6**	**1**
Grand Total		**78**	**28**	**9**	**19**	**37**		**10**	**3**

4. 2. Animal results.

4.2.1. Bacterial isolates from the cultured animal blood.

Fifty four cows from 25 herds, 98 does from 30 flocks and 38 ewes from 17 flocks, all with a history of abortion, were among the 132 cattle, 196 goats and 50 sheep whose blood was cultured for bacterial pathogens. From the cultured animal blood, *Campylobacter* species was isolated from 11 sheep, eight goats and one cow; they were all serologically negative for brucellosis.

4.2.2. Serological Results.

Table 6,7 and 8 give a breakdown of serological reactors with respect to different tests for cattle, goats and sheep, respectively, both for animals with a history of abortion and those without. A total of 1,620 cattle sera, 1,257 goat sera and 557 sheep sera were tested for brucellosis. The cattle results were 177(10.9%) reactors consisting of 132 female and 45 male. The goat results were 112(8.4%) reactors, comprising of 83 females and 29 males, while the sheep results were 45(8.1%) reactors consisting of 34 females and 11 males.

The 177 cattle reactors were from 56 different herds, with the individual test results being RBPT 75, SAT 133 and CFT 53, either alone or in various combinations (Table 6). Twenty (37.03%) of the 54 cows with abortions from 9 herds were serologically positive; with RBPT picking nine reactors, CFT eight and SAT 13. The three tests agreed in six cases (Table 6). The 106 goat reactors were from 30 flocks. They comprised of 90(8.5%) from the 1062 random samples and 16(8.2%) from the 195 from household with seropositive human reactors (matched human animal sampling). The 90 reactors from random sampling were picked by the three tests thus: RBPT 50, SAT 43, CFT 37, while from the matched sampling the three tests' results were RBPT 8, SAT 8 and CFT 3. From the 98 goats that had aborted, 19 (19.4%) were serologically positive for brucellosis with RBPT picking 12 reactors, SAT 13 and CFT 11 (Table 7). The three tests agreed more in goats with abortion; 6 times and once in goats without abortion. The 557 sheep samples tested serologically at Vet Labs, Kabete, had 45 reactors (11 male and 34 ewes) from 12 random and 7 follow-up flocks with RBPT picking 23: SAT 8 and CFT 25. From the 39 ewes with abortion, 8(20.5%) were serologically positive for brucellosis (Table 8). Four female and one male seroreactors were lame. In this species RBPT and CFT were positive together in 8 cases.

4.3. Results for humans and animals sampled from the same homes.

Matched human-animal sampling was done in 2 homes in Lolgorian, 8 in Siayapei and 11 in Olasiti in which 26 human, 206 cattle, 195 goats and 179 sheep were screened. The samples per area were: Lolgorian 3 humans, 40 cattle, 52 goats and 24 sheep; Siayapei, 9 humans, 67 cattle, 75 goats and 48 sheep and Olasiti 14 humans, 99 cattle, 68 goats and 109 sheep. From these, there were 27 cattle, nine goats and 11 sheep reactors. A herd in Lolgorian with five cows that had a history of abortion and another in Siayapei with nine similarly affected cows recorded four and seven seroreactors among those with abortion, respectively. The home in Lolgorian, had two human patients from whom *Campylobacto*r species was isolated. The two were serologically negative for brucellosis. A home in Siayapei had one seropositive human patient. Seven homes in Olasiti each had one seropositive human patient with only four homes having seropositive animals.

The animal reactors in Siayapei were seven cows from a single herd and a reactor each per home for the other one cow, three goats and two sheep. The seven cattle reactors from the one herd had all aborted. The number of animals from Olasiti that were tested for brucellosis were 99 cattle from seven herds, 68 goats from eight flocks and 109 sheep from 11 flocks. From the above tested sera there were seven cattle, six goat and eight sheep reactors. Abortions in animals in this sublocation were reported in one cow, seven goats and 19 sheep, at the rates of one

cow, six goats and eight ewes; nine ewes, one sheep each in two flocks and one goat per home. The two groups with high abortion rates in shoats had three cattle, three goats and two sheep reacting in one group and a sheep in the other. The other reactors were one cow, three goats and two sheep in a group, one cow and two goats in another group and lastly one cow and one sheep. Two seropositive sheep had also aborted.

The sample sizes per sublocation for the three animal species and their respective sero-reactors are summarised in Table 9. The reactors per sublocation were Entasikira 28 cattle, two goat and six sheep, with two homes having particularly high reactor rates of six cattle, 11 goats and four sheep in one group and one cow and 3 sheep in another. Abortions in animals in this sublocation were reported in four cows, 20 goats and three sheep, that had five goats and one sheep reactor.

In Elang'ata Enteriti abortions were reported in six cows, 20 goats and three sheep. These were five cows, 10 goats and three sheep in one group, one cow and four goats and lastly six goats per group. The respective reactors per group were five goats and one sheep from the first group. Other reactors per sublocation were 18 cattle, one goat and two sheep from Koyiaki, six cows, 14 goats and six sheep in Majimoto, 14 cattle, five goats and three sheep from Megwara, eight cattle and six goats from Nkorrkori, five cattle reactors and one sheep from Mararianta and six cattle and five goats from Olorte (Table 10).

The number of groups of cattle, sheep and goats sampled per sublocation were: Enaramartishoriki three groups each with cattle, sheep and goats; Enelerai, 17 cattle herds, 14 goat and 10 sheep flocks; Entasikira, 10 herds, nine goat flocks and seven sheep flocks; Elangata Enteriti, 15 cattle, 14 goat and six sheep groups; Majimoto, 14 cattle, 11 goat and seven sheep groups; Megwara, 10 cattle, 11 goats and 10 sheep groups; Nkorrkori, 16 cattle herds, nine goat flocks and six sheep flocks; Elmesutie, one group with cattle goats and one sheep; Mararianta had two matched and two random samples groups and Olorte had six groups, each with cattle goat and sheep (Table 10).

A total of thirteen cattle herds in Elang'at Enteriti, Entasikira, and Majimoto among the randomly sampled groups and a herd each in Siayapei and Mararianta among those of matched sampling had high abortion rates in cattle. In Lolgorian a manyatta had abortions in 13 cattle, five goats and one sheep and in Siayapei one herd had nine abortions in cattle. Among the matched human-animal home herds in Olasiti there were high abortions in two groups that had nine and eight abortions in sheep. The latter group also had abortions in five goats and one cow among those sampled. Cattle and sheep for matched homes had a lower reactor rate of 7.8% and 6.1% respectively to the 11.4% and 9% the random samples.

Table 6: A breakdown of serological reactions with respect to different tests; for cows with histories of abortion and those without.

Male and female with and without abortion as tested by the relevant tests.

The number of serological reactors for the respective test or a combination of tests for cattle for random and matched sampling.

	RBPT	SAT only	CFT only	RBPT and SAT	RBPT and CFT	CFT and SAT	RBPT, CFT and SAT	Total
Female bovine without abortion	19	35	1	27	2	**29**	0	113
Female bovine with abortion	3	7	3	0	1	0	5	20
Total female bovine reactors	**22**	**42**	**4**	**27**	**3**	**29**	**5**	**132**
Male reactors	7	16	5	10	0	6	1	45
Total bovine reactors	**29**	**58**	**9**	**37**	**3**	**35**	**6**	**177**

Table 7. A breakdown of serological reactions with respect to different tests; for goats with a histories of abortion and those without.

Male and female; with and without abortion as tested by the relevant tests.	The number of serological reactors for the respective test or a combination of tests for goats from random and matched sampling summed together.							
	RBPT	SAT	CFT only	RBPT and SAT	RBPT and CFT	CFT and SAT	RBPT, SAT and CFT	TOTAL
Females without abortion	**13**	19	5	3	4	19	1	64
Females with abortion	**2**	5	1	1	3	1	6	19
Total female reactors	**15**	**24**	**6**	**4**	**7**	**20**	**7**	**83**
Male reactors	4	18	0	2	5	0	0	29
TOTAL	**19**	**42**	**6**	**6**	**12**	**20**	**7**	**112**

***Table 8:* A breakdown of serological reactions with respect to different tests; for ewes with histories of abortion and those without..**

Male and female; with and without abortion as tested by the relevant tests.	RBPT only	CFT only	SAT only	RBPT and CFT	SAT and CFT	RBPT, SAT and CFT	Total
Females without abortion	8	2	9	5	2	1	26
Females with abortion.	3	2	1	2	0	0	8
Total female reactors	**11**	**4**	**10**	**7**	**2**	**1**	**34**
Male reactors	4	1	5	0	1	0	11
TOTAL	**15**	**5**	**15**	**7**	**3**	**1**	**45**

The number of serological reactors for the respective test or a combination of tests for sheep from random and matched sampling.

Table 9. Geographical distribution of the reactors from among the random sampled animals. Number of reactors and as a percentage of the total number of samples for the corresponding species.

	Cattle, number of reactors and as a percentage			Goats: number of reactors and as percentage			Sheep, number of reactors and as percentage		
Sublocation of source	Total	reactors	percentage	Total	reactors	percentage	Total	reactors	percentag
Enelerai	226	28	12.38	142	2	1.4	58	4	6.89
Enaramarti	23	0	0.00	62	2	3.22	13	0	0
Elang'ata Enteriti	172	22	12.79	124	33	26.6	63	7	11.11
Entasikira	210	23	10.9	132	11	8.33	72	9	12.5
Majimoto	136	10	7.4	147	20	13.6	59	4	6.77
Megwara	119	14	11.67	62	5	8.06	38	3	7.89
Nkorrkori	201	14	6.9	102	8	7.83	64	2	3.13
Olorte	72	6	8.3	80	1	1.25	10	0	0
Olmesutie	11	0	0.00	11	2	18..2	10	0	0.00
Koyiaki	212	19	8.96	44	7	15..9	25	2	8
Mararianta	82	7	8.5	38	6	15.7	34	4	11.67
Olasiti	99	7	7.07	73	6	8.2	109	8	7.34
Siayapei	67	9	13.44	75	3	4.0	48	2	4.17
Total	**1620**	**177**	**10.93**	**1161**	**106**	**9.06**	**577**	**45**	**8.43**

Table 10. Herd and flock numbers sampled from the various sublocations and the number of infected herds/flocks from each sublocation..

Sublocation of source	Total cattle herds	Total infected herds	Total goat flocks	Infected goats flocks	Total sheep flocks	Infected Sheep flocks
Enelerai	17	10	14	1	10	4
Enaramarti	3	0	3		3	0
Elang'ata Enteriti	15	3	14		6	
Entasikira	10	4	9		7	3
Majimoto	8	4	6	4	7	2
Megwara	10	3	11	1	10	1
Nkorrkori	16	8	9		6	0
Olorte	4		9	1	4	0
Olmesutie	1		1	0	1	0
Koyiaki	14		11		7	
Mararianta	6	2	6	2	6	1
Olasiti	7	4	8	3	11	4
Siayapei	8	3	6	3	5	2
Total	**125**	**41**	**102**	**15**	**73**	**17**

5.0 DISCUSSION.

Brucellosis is a zoonotic disease whose pattern in humans follows the pattern in the source animals. In this study the disease was diagnosed in humans both on culture and on serology and only on serology in animals. No relationship was established between the disease in humans and that in animals. Also, maladies that were not expected at the start of the study were encountered in both humans and animals. In these other diseases, as in brucellosis, no link was established between the diseases in humans and those in animals. The sources of the causative pathogens were also not established, nor were the links between the various host species, if any, elucidated. The study also showed that there is a problem with the ability to diagnose the various human diseases encountered and also that there is a shortage of medical facilities.

From the cultured human blood, three, eight and 11 isolates of *B.abortus*, *Yersinia* species and *Campylobacter* species, respectively, were recovered. From the cultured animal blood *Campylobacter* species were isolated from 11 sheep, eight goats and one cow. The three *Brucella* isolates took basic fuchsin stain on modified Ziehl Neelsen stain test, did not acidify sugars and sugar alcohols and grew on dyed media. On the basis of these reactions, the organisms closest resembled *B.abortus* biotypes 2 and 4 (Alton. *et al.*, 1975). Biotype 2 has been

isolated in domestic and wild ruminants in the same ecosystem before (Kalinear and Staak, 1973). *B.melitensis,* which has been linked to past human cases (Oomen and Waghela, 1974), was not recovered. There were no *Brucella* isolates from any of the blood of the three animal species tested. This could mean either failure to recover organisms present in the blood or that the animals did not have bacteraemia due to brucellosis at the time of sampling.

In humans, *Brucella* is isolated from blood only when there is bacteraemia, which is normally intermittent. Continuous bacteraemia occurs early in the infection or in complications with endocarditis (Young, 1989). However, it has been observed that even in patients with bacteraemia, the chances of isolating the organism when blood is cultured is less than 6% (Lambea *et al.* 1992). Young (1989), reported successful isolation in 17-85% when three samples are taken from a patient in one day. In this study, therefore, the recovery of 3 isolates from a single sample per person per day from 21 CFT positive patients is within limits.

Some of the samples, both in humans and animals were chilled at $4^{O}C$ up to the time of culture because they could not be processed immediately upon collection owing to the long distances between the place of sampling to the laboratory and in animals, the distance between the homes. However, looking at the serological results, the delay may not have had much effect on the results and possibly no more isolates would have been realised even with prompt processing. Also,

considering that *Yersinia* species and the more fastidious *Campylobacter* were isolated in humans for both organisms and in humans and animals for the latter pathogen, the failure to recover more brucellae was probably because no more patients had bacteraemia at the time of sampling. This is supported to some extent by the high variability of the serological results, as in active disease all three tests are usually positive together (Young, 1989). Zimmerman *et al.,* (1990), reported that migratory workers in contact with brucellosis present with non-specific symptoms. Such persons may be difficult to diagnose clinically. Most Maasai people, particularly men, have a migratory mode of life. This mode of life contributes to poor diagnosis as such patients present with vague signs. Recovery of organisms from such patients is highly unlikely due to low or absent bacteraemia at the time of diagnosis even in persons with active disease. So the recovery of the three *Brucella* organisms was quite a success. *Yersinia* rather than *Brucella,* was isolated in eight RBPT positive patients. The fact that *Yersinia* was isolated and the fact that yersiniosis resembles brucellosis clinically, there is a possibility that it was unsuspectedly missed, whereas it may have been more common than brucellosis. As stated earlier, antibodies to it, are picked by *Brucella* antigens.

Hemoculture for *Brucella,* was successful in only 0.73% in humans and zero in animals, as there were no *Brucella* isolated from any of the animal blood for the three domestic species. The results from the animals are unusual especially among

the aborting ones, although culturing for *Brucella* is known to be time consuming and has a low success rate (Olascoaga, 1976; Diaz and moriyon, 1989). The length of the period between the time of sampling and that of abortion may account for this in cattle. In sheep and goats, however, sampling was done within two months of abortion. It has been reported that *Brucella* organisms are more easily recovered in animal blood in early stages of infection for a short period only (Waghela, 1978). By culturing only seroreactors Musa *et al.* (1979), isolated *Brucella* from milk, lymph nodes and hygroma fluid but rarely from blood. The same authors also reported that about 80% of infected cattle excreted *B.abortus* through vaginal discharges for only 4 weeks post partum, while most chronic carriers shed organisms irregularly and intermittently through the same route. *Brucella* organisms remain localised in tissues or milk in very small numbers and in such cases isolation using laboratory animals helps to concentrate the organisms, thereby enabling recovery of the organism (Alton *et al.,* 1975).

Yersinia species was isolated from human blood eight times, which was more frequent than the three *Brucella;* no *Yersinia* isolates were realised from animals. These results imply that this organism may be a common human pathogen in this district and probably some of the patients earlier reported to have had brucellosis due to positive RBPT results may actually have had yersiniosis only. *Brucella* and *Yersinia* organisms share antigens that cross-react readily (Corbel, 1975; Meckle *et al.*, 1989).

Isolation is the most reliable mode of diagnosis but the incidence of successful culture from blood is usually low. On serology, paired sera collected after 2 weeks and tested with homologous antigen to check for changes in titre is advised. A rise in titre is an indication of active disease. This is the most used mode of diagnosis although antibodies may decline rapidly with symptoms still persisting (Granfors and Viljanen, 1978).

Campylobacter species were found to cause septicaemia in human patients and septicaemia with abortion in sheep, goats and cows as the organism was isolated from the blood of 11 persons, 11 sheep, eight goats and one cow. There could have been more similarly infected animals, since some seven other isolates from sheep fizzled out before being characterised. This organism was isolated from two patients from the same home. The two patients had been sampled six weeks apart. This home had numerous sheep and cattle that had aborted and which were serologically negative for brucellosis.

The factors that favoured isolation of *Campylobacter* species were proper enrichment of the medium with blood and the gaseous partial pressures of 85% nitrogen, 10% carbon dioxide and 5% oxygen. The organisms are heterotrophic, requiring microaerophilic environment for growth, with rigid nutritional requirements (Dekeyser 1984; Smibert and Kapat, 1984). They will grow in most

blood-enriched media including brucella agar base enriched with RBC, particularly sheep RBCs (Smibert and Kapat, 1984). The blood enhances aero-tolerance and improves survival due to its having catalase and superoxides (Dekeyser, 1984). The material was initially cultured in diphasic medium (serum Brucella agar and serum dextrose broth). The blood contained in the culture specimen may have contributed to the enrichment required for the growth of the organism, while the time given in this study was much more than the essential two weeks needed for growth to occur. For subculture, blood agar base enriched with 10% defibrinated blood, in an atmosphere of 10% carbon dioxide, 85% nitrogen and 5% oxygen, was used. All these conditions favour the growth of *Campylobacter*. Incubation at 37^0C favours growth of all species in the genus, as 42^0C inhibits that of *C.fetus fetus* and 25^0C that of *C.jejuni* (Smibert and Kapat, 1984).

The various morphological forms of the isolates were as observed by Smibert and Kapat (1984), who reported that the cells might be short, straight, curved, helical or spiral. The spirals are formed when the helical cells are joined together. The morphology is highly influenced by stage of growth. In old cultures, the medium becomes alkaline and this forces the cells to become coccoid before they die and fizzle out fast (Smibert and Kapat, 1984; Skirrow and Benjamin, 1980). In this study, four of the seven isolates that rapidly fizzled out started doing so by sticking onto the blood agar.

Patients with persistent bacteraemia due to brucellosis tend to have high serological titre and agglutinating antibodies except when the disease is very recent (Diaz and moriyon, 1989). While isolation of the organism is the sure method of diagnosis, it is less used than serological tests because it is time consuming and with less success. However, of the serological tests used, none can detect all cases, while differentiating latent, acute and chronic patients, due to the complexity of *Brucella* antigens. The tests used in this case were RBPT, SAT and CFT. The cut-off point for SAT was 1/50, that for CFT was 1/10. These are WeyBridge standards as adopted in Central Veterinary Investigation Laboratories, Ministry of Agriculture, Kenya. These standards were recommended by Olascoaga (1976) and Waghela (1978). The human reactor rate of 8.2% from 957 sera was a lot higher than the 4.8% observed for a six year period in the same District using the same test method (Muriuki, *et.al.*, 1994). The possible reasons for the higher reactor rate are; (i) an upsurge of brucellosis, (ii) reactors due to cross-reactivity due to infections by *Yersinia*, (iii) high infection rate due to reduced human resistance, (iv) higher infection rate arising from increased infection in the source animals or (v) changes in diagnostic strategy. *Yersinia* that causes a related disease and is serologically picked by RBPT may have contributed to some of the cases reported earlier. It may be a bigger problem than brucellosis. RBPT and, to some extent SAT, reactions were probably due to cross-reaction with *Brucella* antigens. Yersiniosis has not been reported in this area before but

the current isolation rate shows that it needs to be given more prominence in future diagnosis. Also, considering that the reactor rate of 8.2% is way above the 4.8% average for six years for brucellosis infections reported earlier (Muriuki, 1994), the difference this time may be due to entry of *Yersinia* in the flay.

Rose Bengal plate test results of the human sera did not show consistency with those of the other tests. It has been reported as a very sensitive test as it misses very few infected animals (Diaz and Moriyon., 1989), and that it is a better field indicator of brucellosis than SAT (Corbel, 1972). Hosie *et al.*(1985) reported that it is relatively less sensitive in sheep, with Hayes and Chappel (1982) reporting that it had failed in 33 sera that were positive on SAT. RBPT and CFT become positive earlier than other tests, with RBPT reactions persisting longer. The two tests are said to show a lot of correlation, but in this study this was not the case. RBPT has been found to detect infections by some other bacteria, such as *Yersinia* (Corbel, 1972).

SAT, which is not in use for diagnosis in the institutions in Narok, where the studies were carried out, detected the second highest human reactor rate. It is known to pick false positives due to cross reactivity and also due to re-infections, which means it has a lower specificity than CFT (Kulshreshtha and Ramachandran, 1979). Foz *et al* 1971, observed in a concurrent study, that CFT and SAT together pick 91.7% of positive cases but in this case, the two tests

agreed only in 16 of the 78 human seroreactors, while the titres for both tests were found to be low or moderate. Hayes and Chappel (1982) reported significant CFT titres with SAT being negative in chronic or recovered cases. They also observed that SAT is sometimes positive when CFT is negative in 4.6% of sera in initial days of disease. In the latter case, one would expect a high success rate with isolations but this was not the case.

In humans in this study RBPT realised more reactors than SAT that in turn had more reactors than CFT. CFT gave the lowest reactor rate of 3.3% and since it detects more active infections, this indicates that the number that may have had bacteraemia was low. In animals there were a lot more reactors on SAT than RBPT, that was in turn about $1^1/_2$ more than CFT. The cut off point for CFT of 1/10 adopted in this case may be too strong, leaving out some positive animals. Philpott and Auko (1972) suggested a cut-off point of 1/5 that definitely would have produced more reactors, that would probably have levelled off with the reactors on RBPT but not SAT. The very high SAT reactor rate could be a result of a reactant in a lot of sera that was detected to a lesser extent by RBPT, but which does not fix complement. The same observation has been claimed to occur when the disease is at late stages. SAT titres have been shown to rise in relapses or in re-infections and that it has also been found to pick reactors when the other tests are negative (Shaw, 1976a). SAT picked twice as many cattle reactors as RBPT that had picked one and half times more reactors than CFT. A small

proportion of cattle showing false positives on SAT due to persistent antibodies do occur (Chappel *et al.*, 1982b).

The three tests have been reported to detect most of the infected animals (Hayes and Chappel, 1982; Waghela, 1978). The cumulative reactor rate of 10.9% for the three tests in cattle is high. Those with abortion had a cumulative reactor rate of 20.5%. Some herds particularly had very high reactor rates. For example, two herds from which 9 and 13 animals with abortion were sampled had corresponding serological reactor rates of 6/9 (66.7%) and 7/13 (53.01%). The sera had titres that were above those got in the rest of the sampled population. This observation indicates that most animals in the two herds may have had active disease although no causative organism was isolated. The seronegative cattle with abortion were either free of brucellosis, in chronic state of disease, or failed to sero-convert or were infected by a non-crossreacting organism like *Campylobacter*.

The respective reactor rate in goats was 9.7% for those without abortion and 19.4% for those that had aborted. Some mixed flocks of sheep and goats had more abortions in sheep though the goats had more sero-reactors. The reactor rates for animals with abortion was a lot lower than would be expected in infected animals aborting due to brucellosis only.

The respective reactor rates in sheep was 8.4% for those without abortion, while those that had aborted had a reactor rate of 21.05%. The reactors in sheep were rarely detected by more than one test. Shaw (1976a) observed that only SAT gives significant titres in some infected flocks but the contrary was true in this study in which this test picked 19 out of the 45 reactors (Table 8). This test that has a fairly low sensitivity in sheep compared to the other animals is less useful for screening sheep than in cattle (Hosie *et al.* 1985). This could be true in this case, as it has been reported that infected sheep, though aborting, may sometimes fail to sero-convert while still shedding organisms per *vaginum* (Shaw 1976a). Those that seroconvert will have a rise in titre in lambings subsequent to the abortion, with recovery of brucellae (Hayes and Chappel, 1982). SAT is said to be less accurate than CFT and in this study, the number of sero reactors on SAT among those with abortion was 1/39 to 4/39 for CFT (Table 8).

Serum agglutination test picks non-specific agglutinins in sheep due to *Yersinia enterocolitica*, that causes reactions with high titre to the three *Brucella* species; *B.suis, B.abortus and B.melitensis* (Corbel, 1972). A lot of animals that were sero-positive for *Brucella* on a single test could have been infected by *Yersinia* species whose antibodies cross react with the antigen used and which were found in significant numbers in human patients. The cross reactivity is due to similitude of the LPS of *Brucella* to that of *Yersinia* and is based on both IgG and IgM. Most positive animals with active infection will have IgG1 in their sera.

If any cases were due to *Yersinia,* then the reactors due to *Brucella* were lower than the serological results imply. This could be one reason for the failure to isolate *Brucella* from the animals. *Yersinia* has been linked to abortions in sheep (Adesiyun *et al.*, 1986).

Laboratory diagnosis of yersiniosis is based on bacterial culture and detection of antibodies. RBPT was positive in all patients from whom *Yersinia* was isolated, SAT was positive in only a few while CFT was negative in all. Infections by *Yersinia* are associated with strong antibody responses but detection of these antibodies by *Brucella* antigens elicits a weak reaction especially with CFT. The cross-reactivity is the same for all smooth *Brucella* species (Granfors *et al.*, 1980). Serological cross-reactivity in all tests when using whole cell antigens is commonest with *Yersinia enterocolitica* due to shared LPS epitopes. *Yersinia enterocolitica* strain O:9 is antigenically very similar to *Brucella* and so it is the commonest cause of false positives in *Brucella* agglutination tests and CFT (Meckle *et al.,* 1989).

The human serological results obtained in this study are very confusing as 46.1% of the 78 reactors were up to 1 year of age, 76% of the 78 including all male reactors were up to 15 years of age. Only 14 % of the reactors were above 25 years (Table 3). This implies that the immunogenic state of the children may have

been suspect. The source of brucellosis in children is mainly milk, but it being a staple food of the Maasai, means most children would be at near equal risk. Also, considering that the proportion of infected herds is moderate (Table 10), the source, if it is milk would follow the pattern in animals and so the infection in children would be less widespread. This gives some credence to water as a probable source of infection. *Yersinia* is more of a water borne problem than *Brucella* and so its role in human ailments looks highly probable. The very high RBPT reactors in comparison to the other tests also point to the same direction.

The *Campylobacter* isolates were, 12 *C. fetus*, 1 *C.jejuni/coli* and 7 not classified. The *C. fetus* was isolated from 6 sheep and 6 goats. In addition, most of those that fizzled out before typing were from sheep. Although in all, there were 39 sheep to 98 goats, with abortion, sampled, the general tendency in flocks comprising sheep and goats was that more sheep than goats would be affected. The abortions in both species were occurring at about the same stage of gestation. This means the higher number of infected ewes in mixed flocks cannot be ascribed to the stage of gestation but probably to higher susceptibility in sheep. It probably means that more sheep than goats were infected by *Campylobacter* species. Cattle in herds mixed with aborting sheep and goats had either no abortion or only a case per herd. The serological results for animals with abortion show some correlation between abortions and serology as an indication of brucellosis, but fail to account for all the abortions, as it has been found that sensitivities of the three tests

combined is near 100% (Hayes and Chappel, 1982). However, since no *Brucella* was isolated, it is possible that most animals had chronic disease. There were more affected goat flocks to the sheep ones and hence the higher number of goats with abortion sampled. Observing that these two animal species were mixing freely and that the number of sheep aborting (39 sheep to 98 goats) was less than half that of goats, but having a higher number with bacteraemia, this difference may be pure chance or a difference in infectivity, with sheep being more easily infected. Looking at the feeding habits, goats are browsers and sheep grazers that feed much longer, hence more readily exposed. *Campylobacter* was isolated only from sero-negative, aborting sheep, goats and a cow from homes that were far from one another.

Campylobacter species were isolated from human patients, a cow and small domestic stock in three of the five sampled regions of the district. This means it was widely distributed in the district though it does not seem to have been known at the start of this study. This organism was responsible for a significant proportion of the abortions and so it could be a big problem in the district. It seems to be present in a way that is highly related to the sero-converting pathogen. That only a single isolation was made from a seronegative cow with abortion could mean that either no more cattle had septicaemia due to either *Brucella* or *Campylobacter* or, if any, they were very few indeed.

The culture and the serological results together show that in this district, animals are variously infected by *Campylobacter* and *Brucella* species. The isolation of *Campylobacter* species and the serological results, when looked at together, show that there was no cross reactivity between the *Brucella* antigen used and *Campylobacter*, assuming that all the infected animals developed antibodies to the infecting organism. The culture results also show that *Campylobacter* is responsible for part or all the sero-negative animals that had aborted. The serological results show that some abortions in cattle, sheep and goats were due to *Brucella,* while in others there was little or no relationship at all. The abortion in caprine, unlike in the other animal species, showed a stronger relationship with the serological results, accounting for half of the abortions. Among those with abortion, 12 were positive on RBPT, 14 on SAT and 7 on CFT with a number being positive on a combination of tests (Table 7).

The three *Brucella* isolated from human patients were got from a single patient from Narok town and two from Olasiti. The two places had 7 and 25 human seroreactors, with Siayapei accounting for another 12; the other human reactors were thinly distributed in the rest of the district. The proximity to the health facility seems to have a relationship with the reactor frequency. This therefore means there could be patients who did not seek medical help due to its inaccessibility.

The eight *Yersinia* isolates were four from Olasiti, three from Siayapei and one from Narok town. This distribution pattern resembles that for the serological results in which Olasiti was leading followed distantly by Siayapei, with Narok town further off and a nominal number being distributed in the rest of the district.

Campylobacter species was isolated from 20 of the animals sampled. Of the 20 isolates, 18 were from samples collected in Maji-Moto sublocation. The one cow was from Enara Martishoriki and the one ewe from Olasiti. In one home in Olasiti, abortions were reported in over 30 sheep and goats. From among them, six goats and eight sheep had their blood cultured for bacterial pathogens. Out of all these samples, *Campylobacter* species was isolated from one ewe, with two other spiruroid organisms fizzling out before characterisation and were therefore disregarded.

The animal results show that some homes, concentrated in some specific areas, had high abortion rates both among the randomly sampled animals and the matched sampling. A high proportion of homes in which matched sampling was carried out seem to have had a high abortion rate in animals, more so in cattle in Mararianta and Siayapei and in sheep and goats in Olasiti. The numbers of *Campylobacter* isolates were; 4, 3, 2 per flock in 3 flocks and 1 per flock in 7 flocks. Some of the matched homes had different human ailments that may also have afflicted the animals, since some humans in these homes complained of flu

like symptoms while the animals had high abortions. However, human and animal serological results from such homes are not much different from those among the randomly sampled animals, though some of these homes had a genuine problem as manifested by seroreactors in both humans and animals, with the latter having numerous abortions.

It has been observed that improved management and sanitation diminish reactor rates in animals (Kolar, 1987), but the local conditions are the opposite and are therefore conducive to the spread of the disease. Currently there is no control programme and the disease prevalence could be higher. To institute a control programme involves test and slaughter, an act that is unthinkable in some management set ups (Alausa, 1980), and this is true in this case. Therefore, it will take a while before control measures are effected here.

Diagnosis of human brucellosis is based on a high index of clinical suspicion (Araj *et al.*, 1986; Cespedes, 1992). Brucellosis has clinical similitude to malaria, flu, Rift Valley fever (RVF), rheumatic fever and Q fever among others (Diaz and Moriyon, 1989). Serologically it is confused with yersiniosis, among others. From this study there is ample evidence that most of the personnel did not have any knowledge about some of these other diseases. This lack of awareness resulted in a lot of clinical misdiagnosis of the human ailments. Misdiagnosis of brucellosis is mainly due to its diverse manifestations, chronicity and

complications. Inadequate history taking has been found to contribute heavily in failure to diagnose brucellosis especially in non-enzootic areas (Diaz and Moriyon, 1989). Brucellosis is misdiagnosed in Narok district because most of the personnel may not have been aware of some of the diseases like yersiniosis and campylobacteriosis that were encountered. In some instances patients presenting with a single clinical sign were treated for brucellosis. Also, patients presenting with signs infrequent of brucellosis, such as abdominal pain, were diagnosed for brucellosis, and although a patient with unique colitis and bleeding due to brucellosis has been reported, this is a very rare complication indeed (Potasman *et al.*, 1991; Stermer *et al.*, 1991). Diseases of the digestive system like typhoid, cramps, etc would have been more realistic diagnosis.

At the beginning of this study, it was believed that the area around Mara-Rianta had an outbreak of what was thought to be brucellosis. From 30 blood samples collected in this area, this belief was disqualified, as none of them was positive for brucellosis, neither on serology nor on culture. All these cases had been treated for brucellosis. Instead, two patients from the same home, in which there were abortions in sheep and cattle, had *Campylobacter fetus* isolated from them. The blood of the two had been taken six weeks apart. This and the fact that *Campylobacter* was isolated in three of the five sampled regions in small domestic stock with abortion, means the problem may rank higher than brucellosis but that it is not known. Diagnosticians do not seem to have been aware of other diseases

like campylobacteriosis, yersiniosis and to some extent typhoid, which cause abdominal pain. Yersiniosis presents with fever and arthritis but it also does not seem to feature among diseases reported in the area before (Muriuki, 1994).

Olasiti dispensary, though manned by not so well trained personnel, had the best perception of the disease as numerous human seroreactors were closely matched by incriminating clinical signs. The district hospital that is manned by among the better qualified personnel in the district had the best sample: reactor ratio but most patients were diagnosed on the basis of one or two clinical signs. This manifests a poor approach to disease diagnosis and this is a pointer to the fact that most diagnosticians are not properly equipped to deal with the many human ailments in this district. However, although the personnel in the district hospital unlike those in the dispensaries diagnosed typhoid frequently, their perception of brucellosis was not much different. In some cases, patients with only abdominal pain were diagnosed and treated for brucellosis in all the three health outlets. Lack of awareness of diseases like campylobacteriosis, yersiniosis and to some extent typhoid, all of which cause abdominal pain and pyrexia, and failure to interpret RBPT results well, were the main causes of misdiagnosis. It has been claimed that RBPT can be used by untrained persons (Agorreta *et al.,* 1991), but from this study, it was noted that this needs to be taken with a lot of caution.

There are no laboratory facilities for diagnosing most of the diseases encountered in this study and this is another factor that contributed to poor results. The nomadic way of life, the high cost of seeking medical treatment, cultural beliefs, poor personal hygiene and lack of clean water, all which influence the disease history and clinical picture, make it very difficult to accurately distinguish most of the possible maladies on the basis of clinical presentation. The clinical diagnosis also hinges a lot on the severity of the disease thereby giving malaria a lot of prominence (Muriuki *et al.* 1995; Maichomo, 1996). This tended to preclude or delay the more likely diagnosis.

Yersinia and *Brucella* share the same antigenic structure where the common components are the functional alpha 1-2 and alpha 1-3 linked formyl groups of the lipopolysaccharides. The formyl derivatives of *Brucella* are x10 more reactive than the *Yersinia* ones and therefore yield higher titres on serological test (Santos *et al.,* 1984). This accounts for the disparity seen in cross reactivity where the strength of reactivity is not equal to the homologous antigen. Thus, a lot of *Yersinia* infections will be detected as weak brucellosis when *Brucella* antigen is used; this tends to agree with findings in this study in which most positive humans and animals had low SAT and CFT titres. The cross-reaction is based on IgM and IgG against bacterial PS and LPS and probably protein agglutinogen complexes (Corbel and Urray, 1975). Cross-reactivity between *Brucella* and *Yersinia* has

been linked to A antigenic determinant (Kittleberger *et al.*, 1995 II). This means less cross-reactivity is expected in *B. melitensis* based infections.

Yersinia biotypes O:2, O:3, O:5, O:8 and O:9 are pathogenic to man and animals (Randostits *et al.*, 1994). Yersiniosis and brucellosis have a fairly similar clinical picture and so patients of the former will easily be mistakenly diagnosed and treated for the latter. As Mandal *et al.*, (1984) noted, *Yersinia* is one agent that should be sought more and when it is realised, such results be given a lot of prominence. Though not isolated in animals, it has a high likelihood of being present in Narok in both humans and animals since it is known to infect and cause abortion in sheep and goats (Randostits *et al.,* 1994).

Yersinia pseudotuberculosis is associated with sporadic pyaemia in sheep and may cause sporadic abortions, occasional mastitis in goats and orchitis in rams. *Y.pseudotuberculosis* and *Y.enterocolitica* have been linked to enterocolitis in sheep, cattle, pigs and goats. The young sheep, particularly those with debilitating conditions or under stress, are most susceptible (Randostits *et al.,* 1994). This organism was found to have infected between 1-90% of sheep within flocks in Nigeria, while 36% of the animal population had antibodies to it (Adesiyun *et al.*, 1986). He thought the disease might be on the rise. The situation in Narok could be the same, as the animals with abortions cannot be explained by the realised results. It has also been reported that dual infections (brucellosis and yersiniosis)

cannot be ruled out (Kittleberger *et al.*, 1995II). This situation is also likely to be in play in Narok.

In man, yersiniosis is basically a visceral problem but sometimes this may be accompanied by arthritic disease. Patients manifest with fever, athrological pain and septicaemia; signs that are also present in brucellosis. So, both diseases are not distinguishable clinically and to some extent, serologically. Septicaemia and severe arthritis are serious and long-standing complications of *Y.enterocolitica* infections, particularly in debilitated people. Abdominal pains preceded arthritis. Joints of the knee, ankle, elbow and wrist are usually the most commonly affected. Patients also, frequently have back pain (Granfors *et al.*, 1980). This progression of disease may easily fail to be appreciated, a situation likely to be real in Narok where most persons are known not to seek medical help early. This may account for the many patients for whom the medical personnel did not record any clinical signs. Most *Yersinia* infections were in children; majority of them less than one year. This is probably due to their lower immune status, resulting from malnourishment, and close contact with the source, which most probably was water. Yersiniosis is known to originate from food or through contaminated water (Randostits *et al.* 1994). The *Yersinia* could also be from animals contaminating water in the *siranga,* although milk is also a likely source.

Campylobacter has been recovered from the blood of debilitated human patients. Infected patients may manifest with either systemic (extra gastro-intestinal infection) or localised gastro-intestinal form. The systemic form may be characterised by fever, septic arthritis, meningitis and endocarditis (Mandal *et al.* 1984). *C.fetus fetus* is the main cause of extra gastric form, particularly on debilitated persons, as an opportunistic pathogen. This species has also been recovered from patients with purulent pleurisy and subcutaneous abscesses of the thorax. Most such patients had underlying problems like alcoholism, liver cirrhosis, diabetes mellitus, arteriosclerosis and rheumatic heart disease, among others. The gastro-intestinal form is mainly caused by *C. Jejuni.* It is more common in children, in whom it causes diarrhoea, probably with rigor or abdominal peri-umbilical cramps (Mandal *et al.,* 1984). The stool is watery, foul smelling; sometimes accompanied by fresh blood, inflammatory exudate, polymorphonuclear cells and bacteria. Prolonged signs are gastro-enteritis, enteritis, enterocolitis with abdominal pain, pussy mucoid or bloody diarrhoea. In this way it may resemble acute appendicitis (Mandal *et al.,* 1984). The findings in this study seem to agree with those of Devlin and McIntyre (1983), that *Campylobacter* species may be a common cause of human sepsis in the tropics, but that its isolation has been impeded by lack of suitable methods or prohibitive costs of antibiotics for selective media. The organism is also a frequent cause of human diarrhoea or extra gastro-enteric infections (Butzler, 1984; Newell, 1984). *C. fetus.venerialis* has variously been isolated from faeces of normal animals,

diarrhoeic calves, babies and kittens and has also been cultured from blood of pregnant women (Taylor and Al-mashat, 1984).

Apart from abortions and low fertility, *Campylobacter* is associated with low mortality and diverse morbidities. It causes bacteraemia that results in placentitis, that in turn leads to fetal anoxia and death, culminating in non-venereal enzootic abortion in sheep and sporadic abortions in cattle (Dekeyser, 1984). The abortions occur in the first 1/3 of the gestation in cattle and late in pregnancy in shoats (Jubb *et al.*. 1993, Randostits *et al.*, 1994). *C.fetus venerialis* contributes 95% of enzootic infertility in infected cattle while C.*fetus fetus* contributes 5% (Dekeyser, 1984). Infected bulls remain carriers for long periods with spermatozoa being affected. *C.fetus fetus* may cause diarrhoea in milk-fed or ruminating calves (Taylor and Al-mashat, 1984). It also causes mild vaginitis or endometritis and is transmitted in semen (Dekeyser, 1984). It is significant due to losses it causes in human treatment and due to infertility and abortions in animals (Salman *et al.*, 1991).

*Campylobacte*r was isolated from patients in most corners of Narok district in both humans and animals. Spread and sustenance of campylobacteriosis in Maasailand is favoured by lack of toilets in a large part of the district, sharing of water between humans, domestic and wild animals, and the domestic arrangement in which manyatta open into accumulating mounds of manure with all the animal

waste. It goes without saying that most children defecate into and play in the manure. This may account for the very high numbers of infected children. The lack of toilets generates a permanent source of contamination for the water preserved in the *siranga.* The source of infections was not established but like has been reported elsewhere, it may have been from animals through milk, contaminated water or food. Milk, and possibly direct contact with animal secretions, manure or aborted foetii may have contributed the rest. Human excreta, pig gut, packaged beef, pork, lamb and chicken meat or offal and sewerage have been incriminated but not proven (Jenes,. 1984; Blaser et al., 1983). The foods and sewerage which have been implicated in other places, have no role in this case as they are not available.

Rivers are contaminated by human and animal waste especially from those with acute enteritis (Bolton *et al.*, 1985, Arimi *et al.*, 1988). The *Siranga* may be contaminated the same way. Other incriminated sources are improperly pasteurised milk, water and probably raw food (Hutchinson *et al.,* 1985). So milk, a staple food of the Maasai, water and probably direct contact are the most probable sources of infection to the people.

In 1993-1994 the area of study had drought that ravaged most of the district, occasioning poor nutrient supply that may have complicated immunogenic status, making the people and animals highly vulnerable to disease. The little water

available must have been heavily contaminated through sharing between humans, livestock and wild animals. As cattle are affected more by drought than shoats, some families may have depended on goat milk to feed the children. The Goats had the highest number of abortions, thus may have been the main source of this pathogen. Goats produce milk in quantities too small for boiling; hence further magnifying the danger. This shows the complexity of the health problem, which is made worse by the lack of appropriate medical services, especially laboratories. The nature of the disease is also not easy for clinicians to unravel. It is therefore, possible that most diseases are not reported in the area under consideration due to lack of diagnostic facilities. *C. jejuni*, which has variously been reported in water, has full potential to rampage into most populations that lack clean drinking water. However, the *Campylobacter* isolated in this case is mainly *C. fetus*, which was not only recovered from humans but also from sheep and goats. It is a lesser water contaminant than the other *Campylobacter* species and so direct contact cannot be ruled out. This calls for more extensive studies encompassing waterborne diseases and the epidemiology of each.

6.0. CONCLUSIONS

This study has shown that brucellosis and other related diseases like campylobacteriosis and yersiniosis are present in humans and animals in Narok

district. No direct linkage was established between human and animal disease because where follow-ups were undertaken, the findings were different and so no concrete conclusions can be drawn. Although the study was set up to investigate the occurrence of brucellosis, it is *Campylobacter* that was isolated from goats, sheep and a cow that had aborted and from septicaemic human patients all who were sero-negative for *Brucella*. *Yersinia* species was isolated from human patients who were positive for brucellosis on RBPT and for some on SAT but not CFT. *Brucella* and *Yersinia*, and to some extent *Salmonella typhi* (the causative agent for typhoid fever in man), share antigens and hence the serological cross-reactions. The fact that cases diagnosed as brucellosis, and treated as such, ended up being something else, i.e. campylobacteriosis or yersiniosis shows that there is need for follow-up studies in the area for example:-

1. To carry out a study on water-borne diseases, their importance in human ailments, and their probable zoonotic nature.

2. Survey the incidence of yersiniosis, which tends to show clinical signs more in immuno-compromised human patients, probably due to lack of adequate food. Investigations should also be extended to animals to establish whether it may be contributing to the abortions.

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APPENDIX I. Diphasic Medium.

1.a. Preparation of bottles for diphasic media.

The metal caps of 200 millilitre flat medical bottles had their rubber linings removed and circular holes of about 6mm diameter punched off the centre. When the rubber linings were replaced, the central part was exposed where the hole was bored. The exposed part was used for injecting the required carbon dioxide and blood sample into the air space or into the diphasic medium, respectively.

Appendix 1.b. Diphasic medium of Serum dextrose agar and Serum dextrose broth.

Forty three grams of Brucella agar (BBL) plus 10 gms of technical agar no 3 (oxoid) were weighed and dissolved in 1Lt of distilled water to make a 2.5% concentration of agar. The mixture was heated to dissolve and 40 millilitre volumes were distributed into 200 mls flat medical bottles, prepared as above. This was sterilised in an autoclave at $121^{O}C$ for 15 minutes, cooled in a water bath to $56^{0}C$ before 4 cc of sterile inactivated horse serum was injected aseptically. The bottles were then made to lie on the side for slants to form.

Fifteen grams of dehydrated dextrose powder (BBL) was weighed and dissolved in a litre of distilled water. It was sterilised in an autoclave at 15 lbs/in^2 for 15 minutes, cooled and enriched as above before 20mls was injected aseptically into the slant media prepared above. The diphasic medium so formed was incubated at 37^{0}C overnight to test for sterility ready for use.

Appendix 2. Preparation of blood agar.

Forty grams of blood agar base (Oxoid) was weighed and dissolved in a litre of distilled water by heating and then sterilised by autoclaving at 121^0 C for 15 minutes. It was cooled to 56^0 C and sterile defibrinated bovine blood added to make a 10% blood concentration. After thorough mixing it was poured aseptically into pre-sterilized glass petri-dishes at the rate of 15 ml per plate. Bubbles were blown off the media by flaming before it cooled to set. The media was dried in an incubator at 37^0 C to ascertain sterility before use.

Appendix 3 Preparation of dyed Serum *Brucella* Agar media.

Two dyed media, in respect of Thionin and basic fuchsin were prepared thus: 0.5 grams of the respective dye was weighed out to the nearest 3 decimal places. Each was heated in 99.5ml of distilled water to dissolve. Part of this concentration of

1/200 was double diluted twice in series to make stock dye solutions of 1/400 and 1/800. Measured volumes of the three stock solutions were added to *Brucella* agar base; prepared as in appendix 1. 2. To 114 mls of the *Brucella* agar base, was added 1ml of the dye stock solution and repeated for each stock solution. This resulted in concentrations of 1/25,000, 1/50,000 and 1/100,000. The process was repeated for the other dye. The dyed media were sterilised at 121^0C for 15 minutes, cooled to 56^0C in a water bath and serum added to make a 10% serum concentration. Fifteen mls was dispensed into pre sterilised petri-dishes. The media was allowed to set and sterility ascertained before use.

APPENDIX 4. Hydrogen Sulphide Indicator medium.

Serum enriched working *Brucella* agar base was constituted as in Appendix 1.2 above. Fifteen ml was aseptically dispensed into sterile culture tubes. By laying the tubes on the sides at an angle, a butt and slant were made. Sterility was ascertained by incubating the media at 37^0C overnight.

Appendix 5. Urease Detection medium.

Fifteen grams of urea agar base was weighed and dissolved in 100 mls of distilled water. The solution was sterilised by filtration through a 0.22micron millipore Seitz

filter mounted onto a plastic filtering cup. Twenty grams of technical agar no3 was dissolved in 1Lt of distilled water. The media was sterilised in an autoclave at 121^{O}C for 15 minutes. When it had cooled to 56^{O}C, sterile urea base was added and the mixture aseptically distributed into sterile culture tubes at the rate of 15mls. Butt and slant were made as already described and sterility was ascertained before use.

Appendix 6. MR-VP (OXOID).

Fifteen grams of dehydrated media is weighed and dissolved in distilled water. Four ml of the solution was dispensed into bijoux bottles using oxoid pipetter and sterilised at 15lbs/inch^2 for 15 minutes. Sterility was ascertained by incubating at 37^{O}C overnight and checked for turbidity, hence contamination.

Appendix 7. Tryptone water

Fifteen grams tryptone was weighed and dissolved in 1 litre of distilled water. Four ml of this solution was dispensed into bijoux bottles and sterilised at 15lbs/inch^2 for 15 minutes. Sterility ascertained as described above, before use. The media was inoculated in duplicate for MR and VP test.

Appendix 8. Simmon`s citrate.

Simmon`s citrate dehydrated media was weighed out at the rate of 24.2 gm per litre and dissolved by heating. Four ml was dispensed into bijoux bottles and sterilised at 121^0 C for 15 minutes. Slants were allowed to form by laying the bottles on their sides. Sterility was ascertained before use.

Appendix 9A. Nitrate Reduction Test media.

Nine grams of tryptone broth powder was dissolved in 0.9 Lt of distilled water. It was sterilised by autoclaving and enriched with 100 mls of sterile inactivated horse serum. Four ml was distributed aseptically into bijoux bottles and incubated overnight to test for sterility, before use.

Appendix 9 B. Nitrate Reduction indicator system

1. Eight 8 grams of sulfanilic acid reagent was weighed and dissolved in 1 Lt of 5N acetic acid to make solution A.

2. Five grams of alpha naphthlamine was dissolved in 5N acetic acid to make solution B.

Appendix 10. Bacteriological Sugars.

All the bacteriological sugars were prepared in the same way, each in an appropriately labeled bijoux bottle. Aldrade`s peptone (oxoid) at the rate of 29 gram was weighed and mixed with 10 grams of the particular sugar. They were dissolved together in a litre of distilled water. The media was warmed to dissolve and distributed into bijoux bottles at the rate of 4mls each. A Durham's tube was dipped into the media before it was sterilised by steaming at 100^0C for 30 minutes each day for three days. Sterility was ascertained by incubating at 37^0 C overnight. Each bottle was inoculated with a colony of the suspect bacteria and incubated overnight. Growth was indicated by turbidity and acidity by colour change from clear to pink.

Appendix 11. Oxidase reagent.

Fifteen grams of tetramethyl-p-phenylene diamine dihydrochloride was weighed and dissolved it 100 ml of distilled water. Blotting paper strips of 0..5 cm by 4 cm were dipped and retained in the solution for one hour to soak. The paper strips were dried in a freeze drier overnight. They are removed and stored in opaque bottles ready for use. Testing for oxidase reaction entailed touching the test colony with a corner of the impregnated blotting paper strips. A positive reaction was denoted by colour change to purple at the point of contact.

Appendix 12. Veronal buffered Diluent for CFT Testing

Stock solution of concentrated Veronal buffered diluent (VBD) was constituted by mixing three solutions thus:

Solution 1.

Nine and half (9.5) grams of anhydrous magnessium chloride and 3.7gm calcium chloride were dissolved into a litre of distilled water to make a 1 mol and 0.3 mol solution in respective of each salt.

Solution 2.

Three point seven five (3.75) grams of diethylbarbiturate was dissolved into 1400ml of normal saline.

Solution 3.

To make this solution 5.75gm of diethylbarbituric acid was dissolving in 500 mls of distilled water.

To make a stock solution of Veronal buffered diluent, 5 ml of solution1 was added to the mixture of solution 2 and 3 above. The resulting stock solution is stored in a refrigerator. Just before a test on each working day, a 1:5 working stock solution of VBD is prepared by mixing 1 part of stock solution to 5 parts of distilled water.

Appendix 13 Criteria for classifying Organisms as *Brucella, Yersinia* and *Campylobacter* Species.

A battery of tests was used for characterising the isolated organisms thus:- Cellular and colonial morphology, gram reaction, modified Ziehl Nielsen staining, the ability to grow in 1/25,000, 1/50,000 and 1/100,000 dye concentrations for Thionin and Basic Fuchsin. Catalase, Oxidase and IMViC (indole, methyl-red, Vorges-Proskaer) reactions, production of urease, hydrogen sulphide and growth and motility at 22, 37 and 42^0C, nitrate reduction and fermentation of Sugars (glucose, lactose, Maltose, Mannitol, mannose, trehalose, Xylose, arabinose, salicin and Dulcitol).

Appendix 13. 1. Characterisation of *Brucella* Isolates

The gram negative tiny rods or cocco-baccilli that took red stain on modified Ziehl Nelsen stain, were catalase and oxidase positive, turned lead acetate stripes black at the margin for hydrogen sulphide test and turned urea medium slightly pink; hence doubtful for both tests. Two of these isolates were resistant to a concentration of 1/25,000 for both thionin and basic Fuchsin, while one was sensitive. They were resistant to the other concentrations of the dye used. They

reduced nitrates only in serum enriched broth and failed to ferment carbohydrate substrates.

The three were from the blood of a lad and a young woman from Olasiti and a baby girl from Narok district hospital. The three patients were serologically were positive for brucellosis on the three tests used, with low SAT and CFT titres. The three organisms conformed to the characteristics of the genus *Brucella*.

Appendix 13. 2 Characterisation of *Yersinia* Isolates.

These were organisms that were gram negative short rods with occasional bipolar staining cells that grew at 22^0C, 25^0C, 37^0C and 42^0C and motile only at 22^0C and doubtful at 25^0C. They were catalase positive. Urease and hydrogen sulphide negative. They were all negative on lactose and dulcitol. They fermented and produced acid from glucose, maltose, trehalose, xylose, mannitol, mannose, sucrose, salicin and for some in arabinose. They grew in and were therefore resistant to all concentrations of Basic Fuchsin and grew poorly in 1/25,000 Thionin showing only scant growth on incubation overnight, improved growth in 1/50,000 after incubation overnight and quick growth in concetration of 1/100,000. These organisms are consistent with *Yersinia enterocolitica* and remotely with

Yersinia pseudotuberculosis. The SAT titre in the human patients from whom they were isolated were low in the range of 1/50-1/200.

Appendix 13.3. Characterisation of Campylobacter Isolates.

Organisms with small round raised clear colonies with a pinkish tinge from the rear surface or irregular dew drop-like colonies that spread along the line of streak or were joined by thin pencil-lines into a continuos mass. The cells were short to medium rods with various sizes that on serial subcultures changed to comma or spiral forms and later to spherical forms before fizzling out. All developed the spiral forms at some stage. Some would develop round bodies before the spiral forms. The cells were gram negative and would or would not take red colour in modified Ziehl Nielsen stain and only grew in a special gaseous environment of 85 % nitrogen 10 % carbon dioxide and 5 % oxygen. They failed to ferment any of the sugars and would not grow without special gas atmosphere. Seventeen were Oxidase positive with 6 being weakly catalase positive. All those that fizzled out fast were catalase positive. Fizzling out seemed faster on subculture in blood agar plates than in the biphasic medium. For nitrate reductase, the organisms would not grow in media without added serum and so these results were ignored. Only a few were motile on dark field microscopy but this may be attributable to long delay before carrying out the test.

They grew in serum enriched dye media in 1/50,000 Thionin or weaker dye concentration but not at 1/25,000. They were not inhibited by any of the concentrations of Basic fuchsin. However they failed to grow in solid serum *Brucella* agar slants without dye for hydrogen sulphite detection, probably due to lack of the correct partial gas tension. In serum enriched broth they all reduced nitrates. In the other broth media they failed to grow.

On culture at temperatures of 25, 37 and 42^{0}C, they all grew at 37^{0}C, 12 at 25, 3 at 42^{0}C and 2 at all temperature levels. Those that grew at 25^{0}C and 37^{0}C but failed to grow at 42^{0}C were regarded as C. fetus, while those that grew 37^{0}C and 42^{0}C but not 25^{0}Cwere regarded as *C.Jejuni/coli* group. Six human isolates grew at 25^{0}C and 3 at 42 ^{0}, while 2 failed to grow in both conditions. This means there were 6 *C. fetus* and 3 *C.Jejuni/coli* group and 2 failing to be classified. All those that grew at 42^{0}C had dew drop-like colonies and were regarded as *C. Jejuni/coli* group This characteristic was completely absent in *C.fetus*.