ROLE OF BLOCKING ANTIBODY AND COOMBS ANTIGLOBULIN TESTS IN THE DETECTION OF BRUCELLOSIS IN SHEEP

THESIS

SUBMITTED TO THE ORISSA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY, BHUBANESWAR IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY SCIENCE (BACTERIOLOGY AND VIROLOGY)

1984

BISWANATH ACHARYA, B.V.Sc. & A.H.
Department of Bacteriology, Faculty of Veterinary Science and Animal Husbandry
BHUBANESWAR - 751 003

Dr. S.N.Panda,
Professor and Head,
Department of Bacteriology and Hygiene,
Faculty of Veterinary Science and Animal
Husbandry, OUAT, Bhubaneswar -751003.

C E R T I F I C A T E

This is to certify that the thesis entitled " Role of Blocking antibody and Coombs antiglobulin tests in the detection of brucellosis in sheep" submitted in partial fulfilment of the requirements for the degree of Master of Veterinary Science in Bacteriology and Virology of the Orissa University of Agriculture and Technology, Bhubaneswar is a faithful record of bonafide research work carried out by Sri Biswanath Acharya, B.V.Sc. & A.H. under my guidance and supervision.

It is further certified that no part of the thesis has been submitted for any other degree or diploma.

(S.N.Panda) 7.5.84

This is to certify that I have no objection for supplying to any Scientist only one copy or any part of this thesis at a time through reprographic process, if necessary, for rendering reference services in a library or documentation centre.

B Mukherjee
7.5.84

AUTHOR

A C K N O W L E D G E M E N T S

The author is deeply indebted to Dr. S.N.Panda, G.V.Sc., A.I.V.R.I., M.I.P.H.A., Head, Department of Bacteriology and Hygiene, for his constant guidance, valuable advice and help in preparation of thesis.

Grateful acknowledgement is due to Dr. B.C.Nayak, B.V.Sc. & A.H., M.V.Sc., F.R.V.C.S., Ph.D., Dean, Faculty of Veterinary Science and Animal Husbandry for providing adequate facilities to carry out this study.

Grateful acknowledgements are also due to Government of Orissa and the Director of Animal Husbandry, Dairy and Veterinary Services, Orissa for deputing me to continue post-graduate studies.

Thanks are also due to Dr. N.K.Dutta, B.V.Sc. & A.H., M.S., Ph.D., Reader, Department of Bacteriology, Dr. B.C.Kar, B.V.Sc. & A.H., M.V.Sc., Epidemiologist and Dr.N.Hotta, B.V.Sc. & A.H., M.V.Sc., Junior Epidemiologist, ICAR Coordinated Project on Epidemiological studies on F.M.D. for their continued interest and constructive suggestions.

Thanks are also due to Dr. M.C.Nayak, Manager, Sheep Breeding Farm, Deogan; Dr. S.D.Nayak, Manager, Sheep Breeding Farm, Chiplima; Dr. N.Sahoo, V.A.S., Veterinary Hospital, Berhampur and Dr. D.N.Bisoal, J.V.O., Baripada and their staff for their ungrudging cooperation during collection of serum samples required for this investigation.

He acknowledges sincere thanks to P.R.Mishra, R.C.Das, P.K.Sahu and R.B.Patnaik for their help in carrying out this work.

The cooperation and assistance rendered by Sri R.C.Patnaik and Sri B.Mishra, Laboratory Technicians and other staff members of Bacteriology Department are gratefully acknowledged.

(B.N.Acharya)

C O N T E N T S

***

C H A P T E R - I

INTRODUCTION

C H A P T E R - I

INTRODUCTION

Brucellosis in sheep has been recognised since long as an important disease entity not only as a serious hazard in the development of sheep industry but also as one of the most important zoonotic diseases. In western countries, where sheep contribute substantially to animal husbandry economy in the form of mutton and wool, brucellosis has attained special significance as by far the most important bacterial disease. In France, Dubois (1910) described " Malta Fever" in sheep. Bruce (1930) in British Columbia recorded a small outbreak of brucellosis in ewes which had aborted. Similarly, Anonaides and Miaoulis (1931) described the disease in Greece and Huddleson (1943) in U.S.A. Stonner (1951) isolated Brucella aborrus from the milk of an ewe followed by large-scale survey by other workers in U.S.A. (Marsh, 1960). Ovine brucellosis appears to be widespread and has been recorded from most of the countries of the world. There are several publications on ovine brucellosis in U.S.S.R. since 1935 onwards (Pyatkin and Krivoshein, 1960). The disease has also been reported from Germany, Hungary, Switzerland, Italy, Africa, Mongolia, Scotland and virtually from almost all parts of the globe (Stableforth and Galloway, 1959). In most of these countries, the main causative organism of ovine brucellosis was Brucella melitensis and in

rare cases Br. abortus. However, in Australia and Newzealand Brucella ovis is the predominant organism causing epeddymitis leading to infertility in rams and perhaps abortion and neonatal mortality in ewes (Dennis, 1972).

In so far as India is concerned, brucellosis in sheep has not attracted the same amount of attention of research workers as compared to the voluminous literature available in the field of bovine brucellosis.

Indeed, most of the workers who have investigated brucellosis in domesticated ruminants other than cattle have tested sheep and goats simultaneously in view of the prevailing animal husbandry practices in our country where these two species of animals are invariably kept and maintained together, except in special conditions where different farms existed for sheep and goats. Mathur (1968) had found that maximum number of reactors among sheep were in ewes than animals of younger age. Since mutton is a major source of animal protein in this country, brucellosis can cut down the mutton output by lesser lamb crop and lesser carcase weight. This may also hamper wool production substantially in chronically infected sheep thereby resulting in economic loss. The earliest reference to occurrence of brucellosis in sheep is of Polding (1943, 1947 a, 1947 b and 1948) followed by Rao (1952) and according to them abortion in sheep and goats might be due to causes other than brucellosis. Infected sheep and goats showed agglutination titre for a

short period and such were likely to be missed, a feature which is different from bovine brucellosis. Subsequent investigations have confirmed the presence of brucellosis among sheep in almost all the states of India (Nilakantan and Pande, 1948; Pathak, 1965; Satyaprakash et al. 1967; Ganguleo et al. 1967; Mathur, 1968; Sen, 1968 and Zaki et al. 1975).

In Orissa, brucellosis in cattle is known to be endemic with high incidence in village cattle particularly of the coastal districts as reported by Polding (Op.cit.); Panda and Das (1965) and Pat and Panigrahi (1966). The incidence in cattle in organised farms is also equally high almost throughout the state (Panda and Das, Op.cit). Limited work carried out on the incidence of Brucella reactors among goats and sheep in Orissa has confirmed the presence of the disease in these animals, although the reactor percentage was relatively low (Das et al. 1961). However, no systematic work has been carried out on brucellosis in sheep.

The state of Orissa has a sizable sheep population and is one of the states in India where it forms an important asset and source of income of the weaker sections of the society. As has been mentioned earlier, the usual practice under village condition is to raise sheep and goats together along with cattle and all these three species live in intimate contact with each other with every chance of infection spreading from one species to the other and

vice versa. The total number of sheep present in Orissa as per the census report of 1982 and their districtwise distribution is as follows:

Name of Veterinary District		Sheep Population
1. Balasore	...	1,395
2. Bargharh	...	55,893
3. Bolangir	...	2,50,559
4. Cuttack	...	2,00,758
5. Dhenkanal	...	1,12,167
6. Ganjam	...	2,63,777
7. Jajpur	...	68,109
8. Kalahandi	...	2,14,352
9. Keonjhar	...	94,415
10. Koraput	...	1,52,689
11. Mayurbhanj	...	1,44,808
12. Phulbani	...	61,956
13. Puri	...	23,07,949
14. Rayagada	...	23,597
15. Sambalpur	...	42,999
16. Sundargarh	...	17,375
		19,89,798

(Source - Bureau of Statistics, Orissa)

Although the isolation, identification and typing of Brucella organisms afford correct appraisal of infection status in animal herds, in a developing country like India, serological tests have been playing an ever-increasingly significant role in this regard, in views of the limited

laboratory facilities available in most parts of the country. Huddleson (1943) developed a quick (plate) agglutination test for the diagnosis of brucellosis and since then a large number of serological tests have been developed. These include the tube agglutination, complement fixation, milk ring, mucous agglutination, blocking antibody, mercaptoethanol, the brucellin, whey agglutination and Coombs antiglobulin tests. The plate and tube agglutination tests, as standardised by the FAO/WHO Expert Committee on Brucellosis (Report, 1964; Gangulee et al. 1967), have found the widest usage in the hands of research workers both under field and laboratory conditions. Not withstanding the simplicity of these tests, they suffer from certain draw-backs, the most important among which is their failure in detecting hundred per cent of infected animals in a herd. This has led to the deploying of more sensitive serological tests such as the complement fixation, blocking antibody and Coombs antiglobulin tests. The failure of the routine agglutination tests viz. plate and tube, is more significant in human beings, sheep and goats due to the fact that complete antibodies do not regularly appear in the blood following infection in these species (Report, op.cit.) . It has been repeatedly confirmed by several workers, both in India and abroad , that in a significant number of sheep and goats, infection due to Brucellae gives rise to the formation of incomplete antibodies which are specific to the causative organism(s) but fail to result in visible agglutination even when performed under

stringent conditions. Such animals, though infected, go undetected and remain in the herd thereby perpetuating infection and coming in the way of complete identification of the individual animals and total eradication of the disease.

Different mechanisms may operate in various situations to block agglutination even in the presence of specific antibodies. One explanation is the presence of monovalent antibodies. Since this antibody has one combining site, it cannot bridge between antigens to form clumps. Another mechanism may be the close location of antigens on the surface of the cells, so that combination of one antibody may physically interfere with combination of another (Merchant and Packer, 1966).

Since brucellosis is a chronic disease, this situation assumes special significance in the epidemiology of the disease. In view of this, the application of ordinary plate and tube agglutination tests had limited value particularly in sheep and goats and it is imperative that serological technique(s) capable of detecting blocking antibodies must be deployed to detect all the infected animals in a given population. Perusal of available literature reveals that the Coombs antiglobulin tests is by far the most accurate sero-diagnostic method currently available to detect incomplete antibodies against brucellosis as compared to complement fixation and blocking antibody tests (Nanjan, 1963; Lepennec and Boyson, 1965 ; Argottee et al. 1977). Whereas in human beings the Coombs test has been increasingly used in

recent years for detection of undulent fever, large-scale application of this test for detection of ovine and caprine brucellosis has not yet been initiated although substantial progress has been made in this regard with bovine brucellosis.

Taking all these facts into consideration, it was decided to undertake a small-scale survey on the incidence of brucellosis in sheep in Orissa using the standard agglutinati test (both plate and tube) and also the blocking antibody and Coombs tests.

The main objectives of this investigation are to find out the reactor percentage among sheep to the ordinary agglutination tests, ascertain how many of the negative samples reacted to blocking antibody test and independently to the Coombs antiglobulin test.

It is hoped that the results of these investigations would throw some light on the problem of brucellosis among sheep in Orissa in its totality and stimulate interest for further investigations on the problem. The ultimate objective however is to assess the magnitude of the problem of ovine brucellosis in Orissa and to evaluate the efficacy of the Coombs test as a tool for future control and eradication programmes.

Last but not the least, any investigation on ovine brucellosis particularly with regard to its incidence has a direct bearing on human health in as much as the predominent aetiological agent of ovine brucellosis is Br. melitensis.

C H A P T E R - II

REVIEW OF LITERATURE

C H A P T E R - II

REVIEW OF LITERATURE

In 1887, Bruce, an english bacteriologist, isolated the causative agent of Malta fever. Bang in Denmark established the aetiology of contagious bovine abortion in 1896. Brucella suis was isolated in 1914 by American investigator, Traum . Br. ovis which causes genital disease in Australia and Newzealand was discovered by Buddle and Boyes in 1953. Other species such as Br. neotomae and Br. canis were discovered in 1957 and 1967 respectively by Stoenner and Lackman and Charmichael. The latter three species viz. Br. neotomae, Br. ovis and Br. canis have not been recorded in India.

The literature of brucellosis is so vast that it is not possible in this thesis to cover all aspects of this disease or the organisms respobsible for brucellosis in animals and man. Indeed, there are now several comprehensive text books available in english literature (Huddleson, 1943; Spink, 1956; Dalrymplechampneys, 1960 and Crawford and Hadalgo, 1977). Besides text books, there are numerous monographs on different aspects of brucellosis such as the one in India by Ganguloe et al. (1960). Since the work reported in this thesis mainly relates to serological diagnosis of brucellosis in sheep, the review of literature has been restricted to incidence of the disease in sheep, epizootiology of the disease, pathogenesis, serological diagnosis,

immunisation and such other topics which have relevance, in one way or the other, to the work actually carried out by the author. Accordingly, for the sake of convenience, the review has been split into pertinent sub-headings.

Aetiology:

Brucellosis in sheep is mostly caused by Br. melitensis although Mathur (1968) has isolated Br. abortus from cases of ovine abortion.

Brucellae are coccal or ovoid shaped organisms measuring 0.5 to 0.7 micron in size. Under electron microscope, Br. abortus and Br. melitensis appear as coccal or coccobacillary, whereas Br. suis appear rod-shaped. They are gram-negative, nonmotile, without spores, generally noncapsulated but in some strains capsules have been demonstrated. The DNA of brucella contains 56 to 58 per cent G + C. The organisms normally appear in singles, pairs, clumps or small chains of 4 to 6 and stain fairly well with ordinary dyes, irregular staining being a characteristic feature in old cultures. Surprisingly, Huddleson (1947) demonstrated the presence of a capsule for the first time on brucella cells by a procedure which showed capsule on smooth and intermediate forms of Brucella. Virulence of a strain can be determined by the presence of a capsule and capsular material in it. Unstable L form of several brucella strains have been obtained by Khristoforov et al. (1960) who treated recently isolated and laboratory strains of three classical Brucella species with penicillin and lysine. L form were regularly formed from

Br. suis but not all strains of Brucella produced L forms. These forms obtained with three International Units of penicillin per ml of the medium reverted when reinoculated to the medium without a transforming factor.

The electron microscopical study of the Brucella organisms, as reported by Kessel et al. (1963), revealed no morphological differences between the smooth and rough types. Neither there was morphological difference between the organisms grown on tryptone agar or within the monolayer culture of monocytes. The outer wall was triple-layered and inner wall single layered. Intracellular peripheral bodies were seen which had triple layered membranes. Progeny of phagocytosed bacteria remained within the original ingestion vacuole with remarkable preservation of the architecture of host cells. The Brucella organisms inside the host cells, normally remain separated from other ingested material. In contrast to above studies, Grund (1964) studied the organisms in dead culture and found coccoid forms more plentiful in old cultures. Very small forms measuring 0.2 micron in diameter were also commonly found in cultures of 8 days old which grew like bud from coccoid mother cells. Reduction in size is a result and expression of a reduction in metabolic intensity.

Production and characterization of Brucella sporoblasts appear to be some what related to the production of L forms. Hines et al. (1964) observed that Brucella cells were converted to spherical forms by exposure to penicillin.

glycine or a combination of the two for 48 hours which were osmotically sensitive, failed to absorb specific bacteriophage, did not revert to bacillary form and did not possess endotoxin. Cellular antigens were still present and no surface antigens were uncovered. Differences were seen between penicillin and glycine sporoblasts in as much as penicillin sporoblasts were not osmotically sensitive and were capable of reversion to bacillary forms, whereas glycine sporoblasts were sensitive to lowered osmotic pressure and reverted only slightly.

The process of dissociation from smooth to rough Brucella cells was the subject of intensive study by Vysotskii et al. (1967) and Vysotskii (1968). They made submicroscopic studies of the ultrathin sections of rough Brucella cells and found that rough variants of Brucella differed significantly from smooth cells by possesing capsule-like substance, a more developed system of intracytoplasmic membrane structure and thicker cytoplasm. Morphological polymorphism was the characteristic of rough cells and there was no species difference in the R cell of Brucella, the process of dissociation being associated with cytoplasmic density.

Antigenic structure and serological diagnosis:

The Brucella organisms contain four antigens naemly A,M,G and R. The M. antigen is predominent among the Brucellae of sheep and goats and the A antigen in the other species. At present, it is known that all the three Brucella species contain seven antigen (1,2,3,4,5,6 and 7) which are arranged

in the form of a mosaic on the cell surface (Pyatkin and Krivoshein, 1960).

Substances, polysaccharide in character, with no type-specificity have been extracted from all the three classical species of Brucellae. Recently, thermolabile Vi antigen has also been discovered in Brucella. Simultaneous immunisation with both O and Vi antigen gave better protection against infection(Pyatkin and Krivoshein, Op.cit.) The antigenic structure is one of the major criteria for classification. The cross-agglutination and agglutination absorption tests are based on the principle of their antigenic structure. These methods, besides defining the species of organisms, give information regarding their genetic interrelationships.

The various components of the Brucella cells have been long since studied. The lipid content was 4 to 5 per cent of the whole dried cell as reported by Stahl (1941). This acetone soluble fat gave on saponification fatty acids and glycerol, lipids being biologicallyinactive and were probably a constituent of the capsule of the organism.

Foster and Ribi (1962) prepared cell walls and protoplasms seperately by disrupting organisms in a refrigerated condition and cell walls were purified by sedimentation in a linear glycerol gradient. Antigens capable of protecting mice against infection and reacting against usual antiserum were present chiefly within cell walls including substances responsible for primary inflammation of the skin of rabbits. As per their findings,

the protoplasm had very limited antigenic activity. Poluzek et al. (1963) found that some antigens similar to cell wall antigens were also associated with DNA of cells. These DNA complexed antigens were, however, only revealed after heating of DNA preparations. When the DNA preparation was fractionated into dense and less dense fractions by gradient centrifugation, the antigens did not appear to be associated with anyof the fractions. The authors believed that association of some of the surface antigens to nucleic acids of cells might be a fairy common phenomenon in nature.

A biochemical differentiation of Br. abortus, Br. melitensis and Br. suis was attempted by several workers (See Ramchandran, 1964). He found that these three species could be differentiated by RNA-protein and DNA protein ratios including some differences in composition of other constituents such as non-proteins, purines, reducing sugars and glucosamin. Serologically, antigenic relationship was shown between Br. melitensis and Br. ovis by Dias et al (1967) There were lot of cross reactions between the antigens and homologus sera of Br. melitensis and Br. ovis which substantiated inclusion of Br. ovis in the genus Brucella. The polynucleotide sequences of DNA of Br. canis was compared with other species by Meyer (1958) and it was found that the sequences mostly tallied with that of Br. abortus, Br. melitensis and [illegible] Br. suis. Br. ovis lacks some of the polynucleotide sequences of Br. suis and as such it appeared to be a deletion mutant of Br. suis. Surface

antigens of Brucella were also studied by Diaz et al. (1968) with regard to their diffusability in agar gels. From the surface of Brucella cells, protein, lipopolysaccharid and polysaccharide protein antigens were extracted and it was found that several protein antigens extracted from smooth Brucella cells were also associated with rough cells. Br.melitensis antigens showed greater diffusability in agar than Br. abortus antigens but mild acid hydrolysis of Br.abortus antigens also increased their diffusability in agar.

Leong (1968) reported that the endotoxin of smooth Brucella cells lay in the slow diffusing component identified as the biologically active endotoxin. Destruction of the slow diffusing component by acid hydrolysis resulted in a corresponding loss of toxicity. Chromate 51 became attached almost exclusively to the slow diffusing, biologically active component and hence was a valid label for endotoxin derived from smooth Br.abortus. Brucella organisms were also fractioned by Shukla (1979) to find out the toxic fraction as well as the fractins that could be used as immunogen but devoid of producing complement fixing and agglutinating antibodies. He named the fractions F_0, F_1, F_2, F_3 and F_4. Out of these F_2 did not produce sensitisation in guineapigs and also did not give rise to agglutinating and complement fixing antibodies. The protection it offered to mice might be due to incomplete antibodies as well as cellular immunity. Br.ovis appeared

to be antigenically more stable than classical Brucella strains from the work of Orlov et al. (1976). Eight strains of Br.ovis were passaged many times through guineapigs and sheep and were found to retain their antigenic stability and agglutinogenic properties. Br. melitensis might infect sheep in rough form but reverted to smooth on isolation. Cattle is the true host for Br.abortus, pig for Br.suis and sheep and goats for Br.melitensis but each natural host may be infected with other two species. Br.melitensis is as pathogenic to swine as Br. suis. In horse, infection is usually associated with fistulous withers and Pollevil. However, in all cases of infection or vaccination, complement fixing antibodies develop earlier and agglutinating antibodies later. Different species of animals show different degree of susceptibility to brucellosis. Contrary to general rule, young animals are less susceptible and clear off the infection more readily than aged animals. Therefore, calves are vaccinated at the age of 4-8 months with 5 ml of strain-19 vaccine subcutaneously. Following infection, agglutinins accumulate in the blood sufficient for their detection from 10th to 12th day of the disease. Other humoral factors such as production of opsonins, complement fixing substance, incomplete antibodies play definite role in immunity. Specific phage also produces insusceptibility to brucellosis.

To differentiate between vaccinal and infection titres in bovine species Kerr (1955) described vaginal mucus agglutination test which was found to be negative in

cows vaccinated with strain-19 vaccine. The antibody was IgA which was secretary type and was only present in the vaginal mucus of animals infected with virulent organisms.

Glenchur (1959) demonstrated blocking antibodies in experimental rabbits. Six rabbits were infected intravenously by massive doses of Br.melitensis out of which three were treated with antibiotics and rest kept untreated. In the untreated rabbits, the increase in gamma globulin and blocking antibodies was noted by 29th day whereas in the treated rabbitsrise of gamma globulin and blocking antibodies could not be noted upto 59th day of infection. Zinneman and Glenchur (1959) working further on blocking antibodies reported that blocking antibodies appeared later than agglutinating antibodies and persisted after the agglutinating antibodies had subsided. The blocking antibodies originally appeared in the gamma globulin fraction of the serum but soon shifted to beta globulin. For testing blocking antibodies, Rasaschino and Redailli (1959) used Coombs test in bovine brucellosis and observed that even though the Coombs test corresponded to agglutination titre in majority of cases, it revealed few positive animals which had the history of abortion. The Coombs test became positive earlier and remained longer than agglutination test. The antiglobulinswere usually species specific but Dolezal et al. (1959) have shown that human antiglobulin serum could be used in the Coombs test for diagnosis of brucellosis in bovine. Besides cattle,

the Coombs test has also been used in pigs and goats by Cedro et al. (1959) and the animals which were negative or reacted only at low titre became positive by this test. Bovine brucellosis was also diagnosed by Schuster (1961) using the slide modification of the antiglobulin test. This modification appeared even superior to complement fixation test. The Coombs test was compared to agglutination and complement fixation tests in bovines by Buchmeiser (1961). He examined 402 samples by all the above three tests and concluded that routine examinations should be conducted by agglutination test and confirmation by complement fixation and Coombs tests.

With a view to evolving an easier agglutination test for routine use, Murty and Kausik (1963) tried a rapid whole blood agglutination test in rural areas of U.P. Heparinised or oxalated microslides and coloured antigen were used for this purpose . Comparison of this test with blood serum plate, blood serum tube, milk ring, whole milk plate, whey plate and whey tube tests indicated that the former was quite suitable for screening purposes in feild survey.

An intensive study of 80,000 cattle sera in Czechoslovakia was done by modified Coombs test (Hajdu,1963). Blood serum and whey were also tested by agglutination, complemet fixation and ring tests. The Coombs test was found highly specific and was recommended for confirming the results of agglutination tests. Another modification of the Coombs test was found very useful by Jontisch (1964). Antigen

antibody mixture in which no agglutination occurred was centrifuged and bacterial sediment washed and resuspended. From this suspension smears were prepared and stained with 1 to 2 drops of fluorescein isothiocyanate conjugated antiglobulin serum for 30 minutes. Antiglobulin serum of the conjugate was specific for tests on bovine serum because antibovine globulin serum was used. On microscopic examination under ultraviolet light, presence of incomplete antibody was shown by yellow green flurescence of Brucella. This test required much less time than the agglutination tests but with the help of fluorscent microscope.

In Orissa, bovine and caprine brucellosis have been surveyed by agglutination, milk ring and blocking antibody tests. By using agglutination and milk ring tests, Panda and Das (1965) found reactor percentage among cattle in farms to be varying between 10 to 47 per cent and 26 percent in village cattle. Similarly, Panda and Pat (1967) subjected sera of 271 goats to blocking antibody test and found that the percentage of reactors, which was 4 to 5 per cent by ordinary agglutination test, increased to 12 to 13 per cent . Ovine brucellosis was extensively studied by Le Pennec (1967). Agglutination, complement fixation and Coombs tests were carried out on sheep with or without brucellosis. In sheep, the agglutination test was unreliable but the complement fixation and antiglobulin tests were equally effective. The tube agglutination test

was also performed by the tetrazolium stained antigen (Pat and Panda, 1968) with complete agreement with standard agglutination test except in one case. The test was performed as usual but instead of plain antigen, the A.B.R. antigen was added at the rate of 0.05 ml per tube and the titre could be read after 5 hours of incubation instead of 18 - 24 hours. Encouraged by the utility of complement fixation test particularly in early infection phase of brucellosis, Gulrajani et al. (1966) tried conglutinating complement absorption test for detecting antibodies from natural cases of bovine brucellosis. Seventy one known positive sera samples were tested by the above method and 69 of them were positive for conglutination test; hence, the test was recommended for sero diagnosis of brucellosis. This test took less time than tube agglutination test and complement fixation tests. Pathak (1968) has rightly pointed out that heat inactivation (56°C for 30 minutes) test was supplemental to standard tube test, so also acidified plate test was supplemental to rapid plate test. In a farm where Brucella positive and negative animals were kept together, the standard tube test was not very much useful to detect all animals. The application of heat inactivation test could detect 9 per cent more reactors than with standard tube test. Specific Brucella agglutinins were searched in milk of goats by Sivdeokar and Pathak (1969) who conducted combined milk and blood tests in goats slaughtered at Jabalpur abbattoir. Capillary

method of M.R.T. could cut short the time of the tests to 2 hours, hence milk ring test could be conviniently used in goats to test individual animals. By centrifugation method the agglutination result could be known in 15 minutes. Capilary MRT was found to avoid false positive and false negative reactions due to discrepancy of fat quality and quantity in milk.

A modification of the standard agglutination test was performed on the filter paper by Lazarev et al. (1969). One drop of bovine serum and one drop of coloured Brucella antigen were mixed on a glass plate and one drop of mixture was placed on filter paper. Different patterns of absorption in filter paper depended on the presence or absence of microagglomerates of stained organisms. A dark blue central spot with a colourless surrounding was considered positive and homologus light blue spot negative. The results of this method were in full agreement with those of the complement fixation and agglutination tests. With the filter paper, the results were available in 1 to 2 minutes and one technichan could examine 600 serum samples daily. As reported by Pathak (1969) a precipitation reaction is also possible in brucellosis. For this, heat stable water soluble antigen can be isolated from Br. abortus strain -99 for gel precipitation reaction. This serves as an additional method for differentiation of specific and nonspecific agglutinins.

A study of the sequence of appearance of Brucella agglutinins in calves was conducted by Kondaurov and Chekishev (1969) who vaccinated some groups of calves and infected other groups with virulent Br. abortus. Blood samples were collected once a week for 8 weeks from each group. The thermolabile agglutinins reached the peak in two weeks and thermostable agglutinins reached peak in six to nine week. Kulshrestha and Ramachandran (1970) found that agglutinins, haemagglutinins and complement fixing antibodies appeared together in infected animals but agglutinins appeared earlier in vaccinated animals. All the three types of antibodies also persisted longer in infected animals. The indirect haemagglutination test conducted by them consisted of sheep erythrocytes with a trichloracetic acid extract of dried Brucella cells and then allowing them to react with serum at double dilution. Surface fixation test has also been tried on whey by Enchev and Khristoforov (1970) and was found to be equally reliable as whey agglutination test.

Kiss (1970) has observed that incomplete antibodies appeared within 12 hours of antigenic stimulation and persisted four to five years after immunization. These antibodies could be detected by antiglobulin test. Antiglobulin test could also be applied to milk whey as reported by Singh and Pathak (1970). However, the results of this test varied from quarter to quarter and from time to time. Whey antiglobulin test has also been performed by Khadi (1971) who compared whey Coombs test with

whey complement fixation and whey agglutination tests. The whey Coombs test was definitely superior to whey agglutination test in sheep. A correlation between the card test and antiglobulin test was also studied by O'Reilly and Cunningham (1971). The serum samples positive to card test were also positive to tube agglutination test but not to the antiglobulin test, thereby indicating that incomplete antibodies did not take part in the card test. The whey tests, such as agglutination and complement fixation in ovine, were specific for brucellosis as found by Enchev and Khristoforov (1971), but they detected fewer number of reactors in comparision to blood serum tests. The same authors along with Ivanov (1971) also performed ring test on the blood serum of sheep. By performing the test on 210 naturally infected sheep and on 296 experimentally infected sheep, they came to the conclusion that it was superior to agglutination test but inferior to complement fixation test. The same authors (1971) also studied surface fixation test on ovine serum with ring test antigen. This test (Castaneda Surface Fixation) detected fewer number of reactors than complement reaction and ring tests but had the advantage of simplicity of performance. The serum ring test was also compared to serum agglutination test by Giannakoulas and Xenos (1971) who found that both were almost of equal value. Of the two tetrazolium and haematoxylene stained antigens available for ring test, the tetrazolium antigen was found to be superior by Tanwani

and Pathak (1971). Comparison of agglutination and complement fixation tests were also done with Rose Bengal test and Coombs antiglobulin test by Pilet et al.(1972) and Lewkowicz (1972) and the latter tests were reported to be superior. Besides superiority, the antiglobulin test could be an useful tool for differentiating vaccinated and naturally infected animals. In vaccinated animals, the antiglobulin titre waned away after 182 days of vaccination whereas in naturally infected animals it persisted longer (Boh and Lascelles, 1973).

A centrifugation method to cut short the time of slow agglutination test has been described by Laschke (1974). A result in slow agglutination test could be obtained in five minutes by centrifusing the tubes at 2,000 rpm. The method compared well with standard agglutination test and complement fixation test. The agglutination test combined with gel diffusion could also be used to distinguish between Br. melitensis and Br.ovis infections. The sera samples positive by agglutination test were further tested by gel diffusion test using polysaccharide extracts of Br. melitensis and Br. ovis by Gelev (1974). It was found that all the 32 samples gave precipitation lines with Br.melitensis but only 8 samples gave precipitation lines with Br.ovis indicating that the precipitation lines with Br.ovis antigen were suggestive of Br.ovis infection. Working on agglutination, haemagglutination complement fixation, milk ring and gel diffusion tests, Panjarathinam and Gulrajani (1974) have recommended that

milk ring test can be used as a supportive test for agglutination in milch cows and gel diffusion in dry cows. While testing prevalence of brucellosis in mares of Haryana, Kulshrestha et al. (1975) found that reactor percentage in mares was 22.7% as against 1.8% in cows in organised farms. The high reactor percentage in mares was suspicious for nonspecificity and, therefore, the authors have recommended that the co-relation between standard agglutination and heat inactivation test be ascertained in experimentally infected animals before any conclusion could be drawn from naturally infected animals. In an attempt by Singh and Pathak (1975) to modify milk ring test for use in individual cows they evolved the serial dilution test which compared well with standard tube test, so far as the high titre serum samples were concerned, but teat variation in titre of MRT was detected and it was concluded that MRT could not be used as a routine test for individual animals in village conditions. The formation of button ABR deposit was regarded as a positive reaction in sheep and goats but a ring in cows and buffaloes were to be searched for.

The influence of storage temperature, repeated freezing and melting, mechanical stress and U.V. irradiation on the titre of agglutinating and complement fixing antibodies was studied by Kunter (1975) and his results showed considerable stability over a long period in titres of agglutinating and complement fixing antibodies in the infected animal. To differentiate between vaccinal and

infection titres in tube agglutination test the mercaptoethanol test has been described by Alton and Jones (1975). This test was carried out in presence of mercaptoethanol which inactivates macroimmunoglobulins and served as an indicator of microimmunoglobulins present in the serum. The tube agglutination test can easily be modified to become mercaptoethanol test, incubation and recording being done in the same way as tube agglutination test.

Argottee et al. (1977) tested 19 serum samples positive for Coombs test at monthly intervals for 5 months. Coombs, mercaptoethanol, rivanol, complement fixation and slow agglutination tests were used but the Coombs test was found superior to all other tests. In an attempt to find a suitable serological test for buffaloes, Soni (1978) tried standard tube, rapid plate, blocking antibody test and Castaneda's surface fixation test and milk ring test on haemolysed serum samples. Other tests included were spot test in citrated blood samples, ABR, whey plate and whey tube, but the results of various tests co-related well at higher titres with discrepancies at lower titres. For cattle, however, standard tube test and complement fixation tests were found suitable by Chapell et al. (1978). The Rose Bengal test have 11.1 % false positive reactions in goats and 6% in sheep as compared to slow agglutination and complement fixation tests in a study conducted by Papadopoulos et al. (1978). Khristoforov (1979 examined

7,416 blood samples from 34 sheep and 4 goats at weekly intervals over a period of 4 years and found the Coombs test to be the best for blocking antibodies. Muhammed et al. (1980) tried counterimmunoelectrophoresis for diagnosis of brucellosis in sheep and compared this test with Rose Bengal plate and standard agglutination tests and found that the counter immunoelectrophoresis test was the most sensitive of the three. Saeed and Salim (1980) found complement fixation test to be the best for cattle. Firuzi and Kita (1981) examined 599 serum samples from ewes that had history of abortion and found 399 samples positive for brucellosis and 10 for salmonellosis. Krolack et al. (1981) examined 994 blood samples from ewes, 134 from pigs and 176 from sheep showing weakly positive or doubtful agglutinins by antiglobulin and mercaptoethanol tests and in their opinion those negative to both tests were considered negative for cattle and pigs. An experimentally infected cow was studied intensively by Hock et al. (1981) by enzyme linked immunosorbent assay and other conventional methods. It was found that the test detected antibodies two weeks earlier and 3 weeks later than the conventional methods. The standard agglutination test was also conducted on microtitration plates by Herr et al. (1982) which saved time and labour.

CULTURE AND ISOLATION

Liver extract broth or agar is used for cultivation of these organisms. Other media include

Albimi agar and tryptose agar. Brucella organisms can also be cultivated in the yolk sac of fertilised or unfertilised eggs. The organisms are aerobic and only Br.abortus requires 10% CO_2 tension on primary isolation. They are slow growing and require about 15 to 20 days for primary isolation and one to two days subsequently. They can also grow in a temperature of 6-45°C. The colonies are small coneshaped on agar and liquid growth becomes turbid and mucilaginous. For primary isolation, selective media containing dyes and antibiotics are used. As usual, prolonged cultivation in artificial media causes loss of Vi antigen and weakening of virulence. The organisms do not produce exotoxin neither their carbohydrate fermentation is conspicuous. Some strains only produce hydrogen peroxide, break down urea and reduce nitrates to nitrites. As early as (1941) Ardrey has shown that tryptose agar or a complete peptone medium was satisfactory for isolation of Brucella. He also pointed out that bacteriostatic action of dyes was influenced by the pH of the media and peptone content. Mathur (1963) has pointed out that in view of the paucity of clinical symptoms in animals, it would be better to try isolation from the serologically positive animals.

Isolated organisms can be classified by phage. Phage Tb is specific for Brucella classification. For a long time standard method of differentiation of the three classical types was by dye inhibition(Huddleson,1943) and and reaction to monospecific sera (Stableforth,1961). An

attempt to phage type various strains of Brucella isolated from cattle, buffaloes, sheep, goats and pigs was made by Bhambani and Krishnamurty (1964) who included 9 phages and 37 Brucella strains in their study. Out of the 37 strains 24 were lysed by all phages whereas other strains showed irregular lysis and rough variants showed no lysis.

Sensitivity of Indian strain to chemotherapeutic agents was tested by Krishnamurty and Vershney (1964) by paper disc method using 31 agents in two dilutions. Thirty six strains were tested and all of them were sensitive to chlorotetracycline, tetracycline and oxytetracycline and kanamycin. Strains were in general resistant to salfonamides and penicillin.

Brucella biotypes are usually differentiated by dye inhibition, cross agglutination, phage lysis and metabolic activities. From Orissa Br. abortus biotype-I and Br.abortus-melitensis type were isolated by Pat and Panigrahi (1969). Earlier, Polding (1947) had isolated A/M (intermediate) strains of Brucella from cases of abortion in cattle of Orissa. Mathur (1967) isolated 37 Brucella strains from goats in Punjab out of which 10 were Br. abortus biotype-I. Twenty nine Brucella strains were isolated from milk of sheep out of which six were Br.abortus biotype-I.He, therefore, concluded that herds of sheep and goats could either infected with Br.abortus or Br.melitensis.

The addition of erythrytol to culture media as a growth stimulant is not necessary for Brucella strain-19.

Keppie (1967) found that erythrytol in the concentration of one micron mole per ml of culture media inhibited growth of strain 19. Other strains were stimulated by addition of erythrytol including the variant strain of strain-19.

Antiphage serum can be added to culture to neutralise the effect of any phage in sheep and goats in whom milk is a convenient source for isolation of the orgenisms. Samples positive to MRT are ideal for culturing. Mathur (1968) isolated 50 strains of Brucella from goats and 38 strains from sheep by milk culture. According to him, pooled milk from herds showing abortions were very likely to become positive on culture and 15 to 20% of the strains isolated from sheep and goat source were Br.abortus biotype-I and rest were Br.melitensis which included all the 3 biotypes. Biotype 2, however, was not isolated from sheep. Br.suis biotype 1 was isolated from the vagina of a virgin goat in Rhodesia by Manley (1968) who infected two goats isolated the organisms. Br.melitensis mainly infects sheep and goats and throughout the world, but curiously Indian sheep and goats show a higher incidence of infection with Br.abortus than any where else in the world (Mathur, 1968). Infection from sheep and goats neither pass down to cows and buffaloes nor the human beings are infected with Br.abortus in nature. After a study of 8 years in dairy farms, Mathur (1971) found that 52% of the infected cows excreted Brucella organisms in

their milk. Semen of all breeding bulls should also be cultured as there is possibility of isolating organisms from it. Isolation of Br.abortus biotype-I has also been reported from goats by Panjarathinam and Gulrajani (1974) who also isolated organisms from cows but only from placenta and stomach contents of foetus.

Jones and Early (1975) attempted isolation from vaccinated ewes. Seventy ewes were vaccinated with Br. melitensis Rev I vaccine and attempts for isolation failed. Only weak agglutination titres persisted in ewes as a residual effect of vaccination. Sen and Sharma (1975) have reported Br.abortus strains from man. In an study of 78 strains of Brucella isolated from different sources from India during 1965-70, they have encountered Br. abortus biotypes 1,2,3,4,6,9 and Br.melitensis biotypes 2,3 in India.Br.suis biotype 2 was isolated from a Government pig farm in Tamilnadu by Rajendra et al.(1980). This farm showed high percentage of serological reactors among pigs. Br.abortus strains have also been isolated from a Karnataka dairy farm by Ramachandra et al.(1981).

Pathogenesis:

Cattle, goats, sheep, pigs, horses, camel, deer, dogs, cats and rodents are susceptible to infection. The high concentration of Brucellae in the placenta of cattle is due to erythrytol. Abortion is the most constant symptom in animals. There may be loss of milk and flesh. The disease may produce arthritis, bursitis, orchitis and

epididymitis. In horses and camels, the disease is normally latent, emaciation and lassitude being the visible symptoms. The excretions and secretions of sick animals are the sources of infection (Pyatkin and Krivoshein, 1980).

Among laboratory animals, guineapig is the animal of choice in which the disease lasts for 3 months and the animal dies showing lesions in bones, joints, cartilages and eyes. During the course of the disease, emaciation, skin atrophy, fall of hair and orchitis develops (Pyatkin and Krivoshein, op.cit).The fundamental tissue reactions in guineapigs appear to be same in all strains. There may be considerable variation in the ability of the different strains to produce lesions. Foetal pneumonia may be a frequent condition in aborted calves. Hallman et al. (1950) studied pathology of udder and described degeneration and desquamation of parenchyma and cell exudate into the acini of glands. The primary lesion consisted of a variable degrees of endothelial cells and fibroblasts.

An exclusive study on the pathogenesis of Brucella species for the fowl was conducted by Huddleson and Enmel (1929). The results indicated that all the three species produced disease of chronic nature in birds which nearly ..... always had a fatal termination in experimental birds. For diagnosis, the agglutination test is reliable in flock but not for individual birds. The isolation of the organism from the infected bird is almost difficult to

accomplish. The lesions have been observed in liver, kidney and spleen and the disease also exists in naturally infected birds in whom the disease is not necessarily fatal but may cause drop in egg production. In India, the first report on the occurrence of brucellosis in fowl was in Orissa (Das et al. 1963).

The findings of Edjington and Donham (1939) probably gave a clue to the proper time of vaccination in brucellosis. They found that Brucella infected animals might or might not abort. Some animals aborted two to three times and then did not abort, the difference being attributed to the status of infection.

Studies on the leucocyte picture of the animals due to classical Brucella organisms, their endotoxin and strain 19 vaccine were conducted by Munger (1941 a, b) and Krishnamurty and Hajela (1962). In Br.melitensis infection, there occurred an initial leucopaenia with lymphocytosis and slight monocytosis. Liver endothelial cells were consistently found in the blood. Varying degree of basophilic granulation also occurred in neutrophils. When lethal endotoxin was injected to guineapigs in sublethal dose, 70% of the leucocytes were reduced from the blood. The uric acid content of urine was increased to 260%. The Brucella toxin fraction had also cytolytic effect on the neutrophils in the blood vessels. Strain 19 vaccine caused neutropaenia and leucopaenia after 2 or 3 days of vaccination and thereafter neutrophils increased and lymphocytes decreased. The ESR of blood increased and specific gravity decreased. In any

case, the haematological and biochemical changes are not diagnostic of Brucella infection in animals. Above all, there was positive response towards formation of specific agglutinins. The digestive juices, the tissue lysosome content and quality of dietary proteins have got their parts to play in Brucella infection because of the fact that infection in most cases occur through oral route. The normal sheep saliva has got antibacterial effect against Br.abortus strain-19 as reported by Lynov et al.(1967). The saliva could kill the bacteria after 20-24 hours of exposure. Rumen content, abdmasal content and juice killed bacteria in 10-30 minutes. However, pancreatic juice and bile were mildly bacteriostatic. Inoculation of strain 19 vaccine five times at six months interval or inoculation of virulent Br.melitensis organisms reduced the lysosome (L) activity in the blood,liver, spleen and brain (Grekova, 1967). The blood L activity returned to normal in five to thirteen days whereas the spleen and liver L activity was reduced and the brain L activity became completely nil. Antibody production in response to Brucella depended on dietary protein. Goreymisch et al. (1967) reported that poor quality protein such as gelatin reduced antibody production in rats and yeast improved the antibody production. Methionine not only improved antibody production but also provided a protection against toxic effects of living vaccine.

In india, a type of epididymitis occurring in an epidemic form akin to epididymitis due to Br.ovis

was investigated by Kulshrestha and Kalra (1978) and, to their astonishment, they isolated Br.abortus biotype 2 from such cases.

Incidence and epidemiology:

Hutchings et al. (1950) isolated Br. suis from 35 areas of carcase of an infected sow. The pathogen has also been isolated from 39 areas of an infected gilt. Out of the 68 serologically positive animals, 48 were culturally positive . Carcases held at 40°F had yielded Br.suis on culture and the organisms could usually be isolated 4 to 6 mont after exposure.Similarly, Br.abortus was recovered from the semen of a naturally infected bull in 80 consecutive collections. The maximum number of viable organisms per ml of semen was 49,500. This bull showed Brucella agglutination titre higher in the semen plasma than in the blood plasma (Mathei and Detray, 1950). Das et al (1962) found 4.2% reactors among 119 bulls tested in Orissa and Brucella agglutinins could be demonstrated in semen of bulls suffering from orchitis. Hutchings (1952) also reported that brucellosis was not transmitted from man to man and animals were the chief source for human beings. Kadyrov (1965) studied five farms harbouring large number of Brucella infected sheep for 3 years along with 2,877 cattle who were in contact with sheep and found that transmission of Brucella from sheep to cattle, horses and camels was very very rare.

Satyaprakash et al. (1967) studying the incidence of brucellosis in man and animals in Delhi

found 0.7% reactors in goats and 0.6% in sheep. Mathur (1968) found that 2% of the villages of Haryana had infected sheep and 15.75% of the strains isolated from sheep were Br.abortus. Sheep were normally found to be infected when they were reared along with infected goats. Zaki et al (1975) could not find any reactor among sheep in Karnataka. The number (1.) of reactors among sheep of villages around Karnal was 6.18%, Bihar 1.9%, U.P. 1.5%, Calcutta 4% and Punjab 0.47% (Sen, 1968).

How long the Brucellae can survive in soil, water and animals buildings? This was the subject of study by Daminavo (1967). The higher the humus content of the soil, the longer the survival of Brucella. When the humidity was 91%, the survival was 13 days in grey desert soil and 48 days in sandy loam. Pastures and infected pond waters should be rested for 3 months inorder to disinfect them of Brucella organisms. These organisms, mixed with faeces on internal and external walls of buildings, lived for one day in summer and 53 days in winter.

Chaterjee (1968) estimated the incidence of brucellosis among workers of military dairy farm at Kirkee and Pimpri and workers of butcheries at Kirkee and Poona to be 2%. In some tracts of Orissa, the incidence of brucellosis was as high as 50%, but significantly low among goats and sheep (Panda, 1968). Cross reactions were known to occur between Br.abortus, Vibrio cholerae, Salmonella pullorum and Proteus ox 19 . Animals vaccinated

against H.S, with P 52 vaccine might reveal . . a titre against Br.abortus upto 220 days.

Does whole blood spot test work in animals? Pathak (1968) found this test quite suitable at least for buffaloes. He also examined 110 goat sera samples for blocking antibodies but could not find further reactors. Blocking antibodies were also found absent in pig sera (Soni and Pathak, 1969). In Madhya pradesh, the incidence of brucellosis in cows and buffaloes was recorded to be 5.25% and 3.30% respectively by Kataria and Verma (1969). In Andhra Pradesh, the percentage of reactors among cows was 9 to 39% and in buffaloes 3 to 5% as reported by Nandgoankar and Narayan Rao (1971). Similarly, Kulashrestha et al. (1973) reported the reactor percentage in cows to be 2.3% and in buffaloes 13.10% during the period from 1966 to 1971 in Rajasthan. Zaki et al. (1975) have reported reactor percentage among sheep and goats in Karnataka as 0% and 1.1% respectively. Kumar et al. (1976) found 0.33% reactors among goats in Nellore District of Madras. Human brucellosis was found to be 1.86% among men and 3.21% among women of Madhya Pradesh by Soni (1976) who also found blocking antibodies to be equally distributed among both sexes.

Nag et al. (1977) reported incidence of brucellosis among cows to be 24.5% in West Bengal without finding any reactor from among the buffaloes tested. Do cross-breds show higher susceptibility to brucellosis? This

was the problem before Haldar et al. (1979) who studied the crosses like Jersey X Haryana, Holstein X Haryana and Brown Swiss X Haryana and found that crossbreds showed lower infection rate than the parent stock. Higher percentage of exotic blood upto 75% showed a tendency towards higher incidence. These findings have been confirmed by Bala and Sidhu (1982). The survey of brucellosis among animals of the farms of Nagaland revealed 8 to 50% reactors in three farms (Maiti et al. 1980)

Polydorou (1979) has detailed the procedure of examining sheep and goats in Cyprus. The flocks were first tested by allergic tests and then positive animals were confirmed serologically. The policy of slaughter had paid adequate dividends by reducing brucellosis to 0.06%. In France, survey of brucellosis in sheep was done by complement fixation test during 1979-80 and 1980-81 by Dolley (1982) who detected incidence of 2.7% in ewes and 14.2% in rams. Rahman et al (1983) reported seroprevalance of brucellosis among farm cows of Bangladesh and found higher percentage of reactors in farms than the rural cattle.

Immunity

The use of abortion vaccine in calves at six months has a special advantage over its use in more matured heifers. According to Buck (1938), the agglutinins that appear in the blood of calves disappear more readily and more regularly. Stahl and Hamann (1941) tried to ascertain the immunising effect of glucolipid antigen from Brucella cells. This antigen failed to protect 19 out of 21 guineapigs

which were vaccinated with this antigen. The total antigenicity of Br.abortus strain-19 vaccine could be preserved by storing at low temperature. However, Mitchel and Moore (1942) tried alkali-free vials for storing this vaccine, but the advantage of such vials did not appear spectacular.

Strain 19 vaccine is usually used once in the calfhood. Revaccination results in rapid rise of agglutination titre followed by relatively rapid decline. Abortion rate decreases and butter fat increases, but the long term effect of revaccination is difficult to assess (Burman, 1950). The multiplication of virulent organisms inside the monocytes of guineapigs has been taken as an index to study the effect of vaccine. Braun et al. (1962) found that virulent organisms multiplied most in the monocytes of normal guineapigs more inside the monocytes of guineapigs vaccinated with killed vaccine and least inside the monocytes of guineapigs vaccinated with live vaccine. Prenatal immunization of lamb to Brucella was tried by Richardson et al. (1968) who injected killed suspension of a Br.abortus into the heart of sheep foetuses. Results of agglutination of foetal blood showed that foetal lamb was immunologically competent and responded to antigens better than new born lambs. The relative unresponsiveness of the new-born lambs was thought to be due to interference by colostral antibody.

A new method of vaccination by conjunctival route has been advocated by Plomet and Fansterbank (1976)

who reported that under field condition, a booster vaccination by conjunctival route would provide more protection than standard vaccination without serious interference in routine diagnostic tests. Two vaccinations by conjunctival route would be simpler, economical and as effective as the standard vaccination and would have the advantage of vaccination at any age without risk of serological response.

The efficacy of using living vaccine also has been stressed by Plomet and Plomet (1978) who suggested that living bacteria containing at least 10^{10} organisms per dose should be used. Animals receiving doses of 10^{10} bacteria per dose developed high titre whereas those receiving lesser dose developed poor or no titres when seventy one calves were injected subcutaneously with 10^{4} to 10^{10} living bacteria and different organs of such experimental calves were cultured, bacteria could be isolated from calves receiving 10^{10} dose only.

Zoonotic significance

Brucellosis in human beings (Malta fever, undulent fever) is mostly an occupational disease and the natural reservoirs are domestic animals, mostly cattle goats and sheep.As long as reservoir of brucellosis persists in these animals, human brucellosis will occur (Thorn et al. 1977). In view of the fact that brucellosis in animals is endemic in certain parts of India, the occurrence of human brucellosis assumes special significance There have been several reports of human brucellosis in

different parts of this country ever since the first case was recorded by Pandalai and Raman (1941). Other important reports include those of Mathur (1959, 1960, 1967); Dhandhera et al. 1964; Grace and Sayers, 1967 and Sharma et al. 1974). Mathur carried out extensive studies on human brucellosis and his papers have thrown much light on the zoonotic significance of this disease. According to him, persons in the age group of 15 to 50 were found to be most susceptible and incidence was more in males (71%) than in females (29%). From human health point of view, goat is the most important human reservior. Brucellosis was more in families who kept goats and less in families who kept cows since 85% of the sheep and goat brucellosis in India is due to Br.melitensis. An important conclusion one arrives at from the above reports is that mostly small ruminants which predominently suffer from Br.melitensis infections are the chief sources of human infection in India but not cattle and buffaloes which suffer from Br.abortus infection.

Human cases of brucellosis have been reported from all states in India, except NEFA and Nagaland, have been recorded and all the three biotypes of Br. melitensis have been recorded. The incidence of human brucellosis appears to be higher in places where sheep and goats population is high such as Delhi, Calcutta, Orissa, Aurangabad and some parts of Andhra Pradesh. The incidence was more in shepherds who came in contact with sheep (Rao,1972). Mahakur and Panda (1972) reported isolation of 5 strains of Br.melitensis

from cases of human brucellosis in Orissa.

Control:

The control of brucellosis among domestic ruminants in India can conviniently be discussed separately for cattle, sheep and goats. The control programme in cattle has economic bearing on the livestock prospects of the country. In India where percentage of reactors is very high in some states, vaccination may be utilised as a ground work for control when storms of abortion are there in a farm. Vaccination of adult animals may be undertaken to abate clinical disease. Some breeds of cattle, particularly Jersey, react severely to strain-19 living vaccine. In such cases, dead strain 45/20 vaccine may be used for adult animals. However, after the abortion storm has subsided one may switch over to calf hood vaccination of all the calves born in the farm. There is no urgency to use dead vaccine in calves as strain-19 vaccine can conveniently be used in young subjects. There is no meaning in vaccinating bulls as vaccination does not reduce active infection. Once the disease has come under control by vaccination and quarantine methods, the test and segregation method may be adopted. The continuous testing and segregating may work towards eradication in lightly infected herds, provided the surrounding areas are also free and there is no possibility of the disease entering the farm. The eradication programme may be taken up on a country-wide scale as has been done in Scandinavian countries such as Norway, Sweden, Finland and Denmark, U.K., U.S.A., Australia and Newzealand have

reduced the incidence of the disease and are working towards eradication from their countries (Blood et al. 1979).

As regards the control of the disease in sheep and goats, it has special significance from the public health point of view. In India, vaccination programme is not undertaken for small ruminants because of the fact that economic loss due to brucellosis has not attracted so much attention. Nevertheless, since the major reservoir of brucellosis in man is these small ruminants, the eradication of brucellosis from sheep and goats will go a long way in minimising human brucellosis. Test and slaughter method can be adopted for these animals with provisign of adequate diagnostic and vaccination facilities where necessary (Uppal, 1979).

In India, Dhanda and Rajgopalan (1949) have recommended detailed procedures for control of brucellosis. They have suggested three methods. For herds with under 5% reactors test and elimination has been suggested. For herds with over 5% and less 15% reactors, test and segregation method has been suggested. Badly infected herds are to be controlled by vaccination. In herds showing high percentage of reactors all calves and all new entrants are to be vaccinated. The facility for efficient method of reporting and also the availability of efficient vaccines have been stressed by Gangules et al. (1967).

Mentioning some of the experiences in eradication of brucellosis, Greene (1951) stated that several sleeper cases of brucellosis can be discovered not by blood test but by testing milk and uterine samples. Reduction of abortion and mastitis in a herd is a clear indication of reduction in brucellosis.

Ghosh et al. (1968) have studied three vaccines for use in sheep. The efficacy of vaccines was assessed by bacteriological status of tissues and urine after artificial infection in vaccinated and control animals. The difference between the indices of infection between the vaccinated and control groups indicated that killed 53 H 38 vaccine conferred the highest degree of immunity followed by Rev 1 and strain-19 vaccine. Therefore, the value of using a killed vaccine in control of brucellosis in sheep was advised as practicable. At present there is no policy of vaccination in sheep in our country in view of limited work done so far.

-xox-

C H A P T E R - III

MATERIALS AND METHODS

C H A P T E R - III

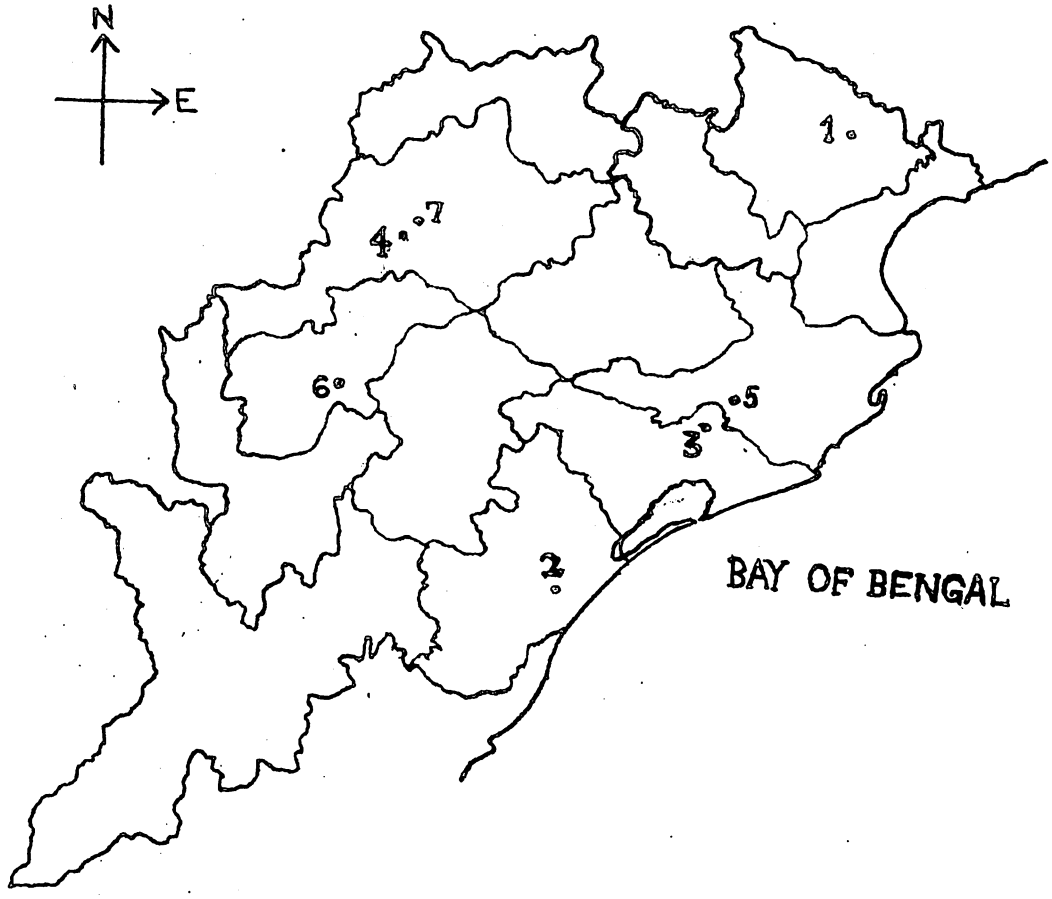
MATERIALS AND METHODS

The role of different serological tests in the detection of Brucella infection with special reference to domestic animals, has already been reviewed in the previous chapter. Compared to other available tests, the blocking antibody and Coombs antiglobulin tests have been proved to be far more specific and highly sensitive in serodiagnostic procedures employed in the study of brucellosis.

Collection of serum samples:

It was decided to collect serum samples from four agro-climatic zones of the state including the two Government organised sheep breeding farms. For this, the following places were selected:

Bhubaneswar and Cuttack of the eastern zone, Sambalpur, Deogan and Chiplima of western zone, Berhampur of southern zone and Baripada of northern zone. Out of these, blood samples were collected from the slaughter houses located at Bhubaneswar, Cuttack, Sambalpur, Berhampur and Baripada, whereas Deogan and Chiplima represented Government sheep breeding farms. The number of samples collected at different places have been mentioned below against each place of collection.



1. BARIPADA
2. BERHAMPUR
3. BHUBANESWAR
4. CHIPLIMA
5. CUTTACK
6. DEOGAN
7. SAMBALPUR

Map of Orissa Showing the places of collection of sheep serum samples from four zones

Locality	Source	Nos. of sample
Baripada	Slaughter house	63
Berhampur	-do-	72
Bhubaneswar	-do-	146
Chiplima	Sheep breeding farm	79
Cuttack	Slaughter house	112
Deogan	Sheep breeding farm	48
Sambalpur	Slaughter house	26
	Total	546

Except in the two organised farms viz. Deogan and Chiplima where cross breds of local and Corridale as well as Madras red and Corridale are being maintained, the rest of the blood samples were collected in slaughter houses which received sheep from the respective nearby areas belonging to the local nondescript breed. In all these places, including the farms, blood samples were obtained from only adult animals varying between 2 to 5 years in age. The sex ratio of animals slaughtered in the abbattoirs was roughly 50 : 50 as per the data available from the slaughter house authorities. In the two farms, however, the blood samples obtained were mostly from the ewes and 10 rams maintained for breeding purpose. As may be seen from the figures shown above a total of 546 blood samples were collected for this investigation. It was not possible to obtain samples of almost the same number of animals at each place because of variable number of goats and sheep slaughtered at different slaughter houses. At the two farms,

however, random samples had to be collected out of nearly 200 adult sheep maintained at Deogan and approximately 400 at Chiplima. The blood samples in slaughter houses were collected individually into sterilised screw capped vials (5 to 10 ml) from the jugular vein at the time of slaughter, mostly in the early hours of the morning. Blood from farm sheep, on the other hand, was collected into similar vials after drawing 5 to 10 ml by a sterilised syringe and needle also from the jugular vein. The syringe were rinsed 5 times with sterilised normal saline before bleeding the next animal. The blood samples, so obtained, were stored inside a refrigerator ($\pm$ 4°C) and transported in thermoflask with ice to the laboratory within 48 hours after collection. Sera samples were separated by centrifuging the vials at 3,000 rpm for 30 minutes and any sample showing haemolysis was discarded. The sera were transferred to small tubes with rubber corks and stored inside a deep freeze at -20°C without adding any preservative. They were taken out and thawed in small lots of six for a days work.

Pattern of designing the tests:

It was decided to test all the sera samples both for complete and incomplete antibodies against Brucella. The plate agglutination test was performed as described by Dhanda and Rajgopalan (1949). The tube agglutination test with 5 per cent saline without carbolic acid was used for testing bivalent agglutinating antibodies. For testing

incomplete antibodies, two methods viz. blocking antibody and Coombs antiglobulin tests were attempted. Necessary modifications were done to suit antiglobulin test for sheep. To start with, every sample was put to standard plate and tube agglutination tests. The results of the positive tubes were noted carefully. Those samples showing negative or doubtful reaction were subjected to blocking antibody and Coombs antiglobulin tests. Each serum sample was put to tube test in two sets to facilitate testing of one set for blocking antibody and the other set was utilised for antisheep globulin test. The standard antigens used in these tests were obtained from the Indian Veterinary Research Institute(IVRI), Izatnagar and Institute of Veterinary Preventive Medicine, Ranipet.

Preparation of hyperimmune serum:

The method used by Panda and Pat (1967) was followed. Three adult rabbits of robust health were selected for this purpose. Fresh Brucella tube antigen was used for immunization. On the 1st day, 1 ml of tube antigen was injected to each rabbit intravenously with aseptic precautions. The injection of the same quantity was repeated on the 3rd day and thereafter, weekly injections of two ml each were administered to each rabbit for four weeks. After the last injection, 10 days time was allowed to elapse. Exploratory bleeding of the rabbits was done on the eleventh day to know the anti-Brucella titre of the serum obtained from the blood collected by cutting an ear vein by means of a sharp blade. For testing, serum samples

were subjected to Brucella tube agglutination test as outlined by Sen (1983). All the three rabbits showed equal anti-Brucella titre of 1280 International Units (I.U.) per ml. Since this titre was considered satisfactory, all the immunized rabbits were bled next day, first from the heart and then by cutting the throat. The blood samples were stored in sterile tubes and sera separated aseptically and the hyperimmune serum was stored in the deep freeze in aliquots of four ml in sterile screw-capped containers. No preservative was added.

Preparation of sheep antiglobulin

The whole serum technique of Alton et al. (1972) was followed. Blood from seven sheep were collected separately in seven sterile test tubes and serum were separated and pooled together. They were tested for presence of Brucella agglutinins by tube test and found to have no observable titre even at 1 : 10 dilution level. The pooled serum sample was stored in the deep freeze in four ml aliquots for use as antigen. Three healthy adult stout rabbits were selected for production of antiglobulin. On the 1st day 0.75 ml of undiluted whole serum was injected to each rabbit intraperitoneally. The intraperitoneal injection was followed within 24 hours by injection of 0.5 ml of serum diluted with 0.5 ml of sterile saline intravenously. For the next four days all the three rabbits were injected 1 ml of whole serum intravenously through the ear vein. The rabbits were bled 10 days after the last

injection. Bleeding was done first through heart and then completed by cutting the throat. Blood was collected in separate sterile tubes and serum separated. Pooled serum was stored in deep freeze at - 20°C in four ml aliquots in screw capped vials.

Titration of antiglobulin by precipitation:

Test:

The Coombs reagent prepared against pooled sheep whole serum was titrated by two methods: first on the lines of Wisniowski and Remniukowa (1964) and next according to the method of Malik (1967). The former method was originally used by the authors for titration of bovine antiglobulin by tube precipitation method and the latter for the demonstration of gel diffusion technique.

In the present experiment, however, antisheep globulin was titrated in place of antibovine globulin all other techniques of the test remaining the same for the tube precipitation. Equal quantities (0.2 ml) of antiglobulin was layered with the sheep serum in a precipitation tube with the help of a Pasteur pipette. The tubes were kept at room temperature and the development of precipitation ring was observed at intervals of 15 minutes for one hour.

Titration by gel diffusion test:

Coombs reagent was also titrated in agar gel following the diffusion technique of Ouchterlony (1949). Agar gel was prepared with one per cent Difco agar and 0.85 per cent chemically pure sodium chloride. Merthiolate was

added to make the final concentration 1 : 10,000 for preventing bacterial contamination. The pH was adjusted to 7.4 by Elico pH meter. The molten gel was then poured in sterile petridishes to a theikness of four mm and seven wells were made by a corkborer after setting of the gel. A central well was surrounded by six wells, the distance between any two wells being 4 mm. The bottom of the wells was sealed with the same agar.

Coombs reagent was filled to the central well. The surrounding wells were filled with saline, whole sheep serum and sera is doubling dilutions from 1 : 2 to 1 : 16. The last well was filled with saline to serve as negative control. The gel plates, after filling, were kept at refrigerator at 4°C to prevent drying.

The plates were observed daily till precipitation lines developed and maximum upto 7 days.

Titration by known Coombs positive serum

The methdd of Alton et al.(1975) was followed in toto for the titration of Coombs reagent. A Coombs positive ovine serum negative to ordinary agglutination test but positive to Coombs titre at 1 : 80 was used.

An ordinary tube agglutination test was carried out on the known Coombs test positive serum. This was done in 5 rows of identical doubling dilution with 6 tubes in each row.

After incubating for 24 hours, the tubes were examined to note complete agglutination. None of the tubes showed agglutination of any degree. Hence all the tubes were

used for block titration of the Coombs reagent.

Before adding Coombs reagent, the tubes were centrifuged, the supernatent fluid discarded and deposit resuspended with 5 per cent normal saline which was used in the tests throughout. The process of centrifuging and resuspending was repeated for three times. After the 3rd centrifugation, the deposit was resuspended in the Coombs reagent diluted with 5 per cent saline. A different dilution for each horizontal row viz. 1 : 50; 1 : 100; 1 : 200; 1: 400 and 1 : 800 was used.

The tubes were further incubated for a period of 24 hours at 37°C and results recorded in the same way as in the ordinary agglutination test. The dilution of the Coombs reagent to be used in the diagnostic test was the most dilute solution to show maximum sensitivity in the titration. However, in practice a slightly less dilution i.e. 1 : 100 was used.

Principle of blocking antibody and Coombs antiglobulin test

Sera from some infected animals may contain specific antibodies that combine with antigen but are not capable of causing agglutination. Some of these antibodies by occupying the combining sites on the antigen prevent agglutinating to cause agglutination. The Coombs reagent brings about agglutination in presence of the so called incomplete antibodies. The Coombs test when applied to sheep sera can be called antisheep globulin test (ASG).

Ordinary agglutination test was used as a starting point for blocking antibody and Coombs test. The tubes that did not show agglutination were retested for incomplete antibodies with hyperimmune serum and Coombs reagent for adherent antibodies. The blocking antibody technique was on the lines of Panda and Pat (1969) and the Coombs test technique used in the present study was on the lines of Coombs et al. (1945) as modified by Alton et al (Op.cit).

Plate agglutination test:

This was performed and results interpreted as outlined by Dhanda and Rajgopalan (Op.cit).

Tube agglutination test:

Tube agglutination test was performed as a starting point for antisheep globulin test as per the technique (Sen, op.cit) with 5 per cent saline.

Antigen - Heat killed carbolised suspension of Brucella abortus, strain 99 standardised to give 50 per cent agglutination at 1 : 500 dilution of international standard anti-Br.abortus serum was used. This antigen was centrifuged at 7500 rpm in a IEC centrifuge for 20 minutes and supernatent was discarded. The volume was again made up and resuspended with 5 per cent sterile saline and the process was repeated twice. The object of twice washing the suspended cells in the antigen was to get rid of the phenol which according to Alton et al. (Op.cit) might some times interfere with

the final results of the Coombs test..

Serum - Unpreserved sero-samples without heat inactivation were used in the test in lots of six as described earlier.

The test proper - Five uniform-sized round bottomed tubes were taken for each sample and different reagents were added as per the protocol given below. One set of control was kept for all the tests performed in a day. All the tubes were left in the incubator for 18-24 hours before the results were read.

Protocol for standard tube agglutination test:

Tube	Test serum					Standard antigen control				
	1	2	3	4	5	1	2	3	4	5
5% saline in ml	0.8	0.5	0.5	0.5	0.5	1.0	1.25	1.50	1.75	2.0
Test serum in ml.	0.2 mixed and transferred									
		0.5	0.5	0.5	0.5	0.5	discarded			
Modified antigen in ml.	0.5	0.5	0.5	0.5	0.5	1.0	0.75	0.50	0.25	Nil
Final serum dilution	1:10	1:20	1:40	1:80	1:160	0%	25%	50%	75%	100%

Results were noted by considering the opacity or clearance of the supernatant fluids of the tube and not by sediment of the agglutinated mass. The tubes in the test series were compared with tubes of the control series and degree of agglutination was judged by opacity as follows :

0% agglutination compared to control tube No. 1

25% -do- No. 2

50% -do- No. 3

75% -do- No. 4

100% -do- No. 5

The serum dilution showing 50% agglutination was taken as the titre of the serum. To express the result in I.U. double the reciprocal of the serum titre was taken as the number of I.U. per ml of the serum. For example, when the serum titre was 1:40 antibody content of the serum was expressed as 80 I.U.. Since sheep sera were tested, a titre of 1:20 equivalent to 40 I.U. was considered positive and 20 I.U. was considered doubtful (Anonymous, 1964). Samples showing no visible agglutination even in the first tube were declared negative.

Blocking antibody test:

The blocking antibody test was conducted as per the technique described by Panda and Pat (Op.cit.). With the serum samples, the standard tube agglutination test was conducted as usual. The results of the positive tubes were noted and to the negative tubes one drop of Brucella hyperimmune serum having a titre of 640 I.U. was added and gently shaken and further incubated for 18-24 hours. Negative tubes were expected to show complete agglutination in all the tubes including the positive serum control tubes whereas those tubes showing no agglutination or less than 50% agglutination were taken as positive for the blocking

antibody test.

Anti-sheep globulin test:

The negative tubes of the standard agglutination test were labelled individually and centrifuged at 2,500 rpm for 20 minutes and the supernatant was poured off. The deposit was resuspended to the original volume with 5% saline. A clean Pasteur pipette was used for resuspending, starting from the tube that had the highest serum dilution and working towards the tube that had the lowest. A separate Pasteur pipette was used for each set of tubes. The washing process was repeated three times and after the third (final) centrifugation , the deposit was resuspended in the Coombs reagent which was prepared by diluting anti-sheep globulin serum to 1: 100 with 5 per cent saline. The tubes were reincubated for another 18-24 hours after which they were read as in the ordinary agglutination test. The dilution of the last tube showing 50% or more of agglutination was considered as the end titre irrespective of the result of tube agglutinnation test.

Using the technique described above a Coombs titre of 1:10 and above was taken to indicate that the animal tested had suffered from specific stimulus following infection as has been specified in respect of sheep and goats (Stableforth and Galloway, 1959).

C H A P T E R - IV

R E S U L T

C H A P T E R - IV

RESULT

Preparation of Brucella antiserum:

On bleeding the three rabbits, which were injected with the Br.abortus plain antigen , the sera samples, when tested separately, revealed uniform titre of 1280 I.U. per ml. As such, these sera were pooled and used throughout the studies as hyperimmune serum. Since the above titre was adequate for use in all the serological tests conducted in this investigation, it was not considered necessary to inject more doses of the antigen to raise the titre still higher.

Preparation and standardisation of antisheep globulin:

The antisheep globulin obtained after immunization of three rabbits with sheep serum was also pooled and on titration exhibited clear precipitation rings starting from 1:10 upto 1:100 in ten fold dilutions. There was no observable precipitation at 1:1,000 or 1: 10,000 dilution. As such,the titre of the antiglobulin was taken as 1:100 and this was adequate according to Wisniowski and Romniukowa (1964).

Titration of the antiglobulin by agar gel diffusion method resulted in the development of clear precipitation lines against whole sheep serum and faint but decernible lines in the other dilutions of sheep serum used viz. 1:2, 1:4 and 1:8. It was observed that the precipitation lines against the whole serum was the

lst to develop with three simultaneous distinct precipitation lines by the 3rd day, whereas only one line was visible against diluted sera by the fifth day and by this time the three separate precipitation lines with undiluted serum merged to form a thick line and formed a common precipitation line with other three dilutions as shown in the figure I & II. There was no precipitation line against the negative saline control. These results of the gel test lent additional support to the potency of the antiglobulin as titrated by the tube method (vide supra).

Titration of antiglobulin by Coombs positive serum:

Titration of the antiglobulin by Coombs positive serum showed that the highest dilution at which maximum titre was exhibited ended at 1:80 and as such was accepted as the end titre as shown in table. 1.

TABLE -I

Titration of Coombs reagent

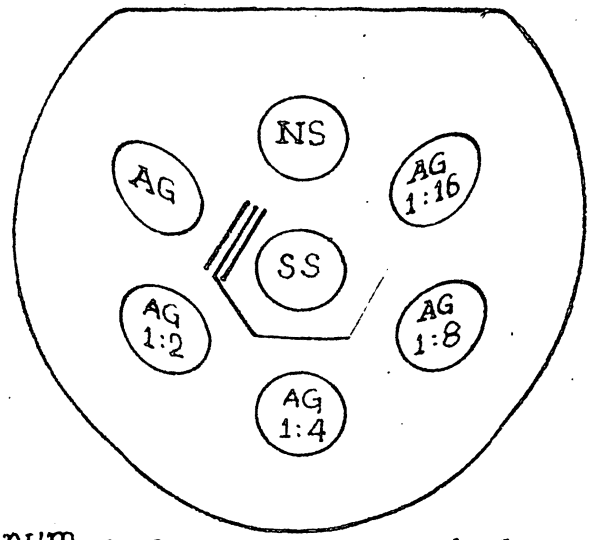
Coombs reagent	Serum dilution					
	1:10	1:20	1:40	1:80	1:160	1:320
1:50	++++	++++	++++	++++	-	-
1:100	++++	++++	++++	++++	-	-
1:200	++++	++++	++++	++++	-	-
1:400	++++	++++	++++	-	-	-
1:800	++++	++++	-	-	-	-

Plate agglutination test:

The results of the plate agglutination test performed with 546 sera sample have been summarized in table 2.

FIGURE-1

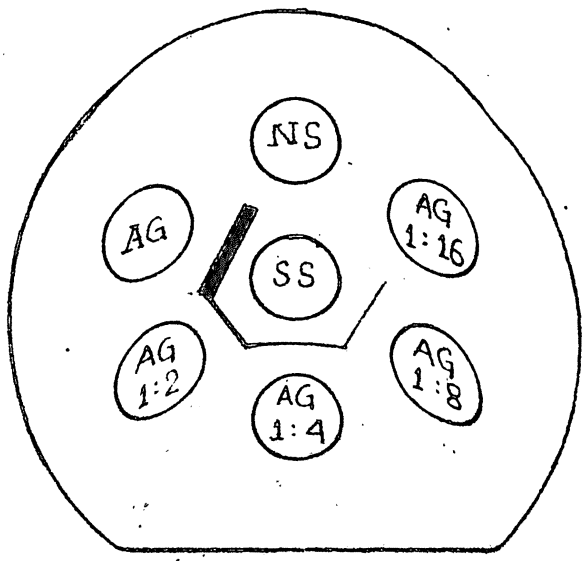
Schematic drawing of the precipitation lines on 3rd day



SS = Sheep serum ; NS = Normal Saline ;
AG = Antiglobulin ;
AG 1:2 - AG 1:16 = Antiglobulin diluted 1:2 - 1:16

FIGURE-2

Schematic drawing of the precipitation lines on 5th day



SS = Sheep serum ; NS = Normal saline ;
AG = Antiglobulin ;
AG 1:2 - AG 1:16 = Antiglobulin diluted 1:2 - 1:16

TABLE - 2

Results of plate agglutination test:

Place	No. of samples tested	Positive	Doubtful	Negative
Baripada	63	0	1	62
Berhampur	72	3	1	68
Bhubaneswar	146	1	1	144
Cuttack	112	2	1	109
Chiplima	79	0	4	75
Deogan	48	0	0	48
Sambalpur	26	0	2	24
Total	546	6	10	530
Reactor		1.1%	1.8%	97.1%

It may be seen from the above table that out of 546 sheep sera tested, there were six positive reactors in the plate test, while 10 animals were found doubtful. It may further be seen that the plate agglutination test detected positive reactors in only 3 places viz. Berhampur , Cuttack and Bhubaneswar but not in any other of the four places from where sera samples were collected.

Tube agglutination test:

In this test, the six sera samples of sheep, which were found clearly positive to the plate agglutination test, were not subjected to any other serological test(s) and the rest 540 samples including the doubtful reactors were subjected to the tube agglutination test, details of which may be seen in table 3.

TABLE - 3

Results of tube agglutination test:

Place	No. of sample tested	No. Positive	No. Doubtful	No. Negative
Baripada	63	0	1	62
Berhampur	69	1	3	65
Bhubaneswar	145	1	0	144
Cuttack	110	1	2	107
Chiplima	79	0	4	75
Deogan	48	0	0	48
Sambalpur	26	2	0	24
Total	540	5	10	525
Reactor		0.9%	1.8%	97.3%

Perusal of the above table reveals that out of 540 samples which included 10 doubtful reactors in the plate test, 5 samples revealed 40 I.U. titre and above and were, as such , declared positive. An additional 10 samples showed doubtful reaction i.e. a titre 20 I.U. As may also be seen from the table, the positive reactors were detected in four out of seven areas as against three in the plate test.

The results of the tube agglutination test interestingly revealed that out of 10 doubtful reactors in the plate test, five were positive in the tube test and the other five reacted doubtfully. It was further found that 530 animals, which were declared negative by the plate

test, had no detectable titre to be declared as doubtful/positive on the basis of results of tube agglutination test details of which have been provided in table 4.

TABLE 4

Analysis of results in the plate and the agglutination test:

No. of animals found doubtful in plate test	Results of tube test		
	No. positive	No. doubtful	No negative
10	5	5	-

It may thus be observed that out of 10 doubtful reactors in the plate test, five revealed 40 I.U. or more titre in the tube test and were taken as positive. The other samples revealed 20 I.U. i.e. a doubtful reaction.

When 530 negative samples in the plate test (vide table 2) were subjected to the tube test, it was found that five samples revealed 20 I.U. titre and these were considered doubtful.

Blocking antibody test:

Five hundred and twenty five samples found negative in the tube agglutination test (vide table 3) were subjected to blocking antibody test and the results have been analysed in table 5.

TABLE 5

Results of blocking antibody test

Places	No. of samples tested	No. Positive	No. Negative
Baripada	62	0	62
Berhampur	65	3	62
Bhubaneswar	144	1	143
Cuttack	107	2	105
Chiplima	75	0	75
Deogan	48	0	48
Sambalpur	24	0	24
Total	525	6	519
Reactor rate		1.1%	98.9%

It may be observed from the above table that six sera samples had blocking antibodies against Brucella sp. thus revealing six additional animals positive to infection which were missed in the tube agglutination test. Further, it may be noted that out of six samples, three had significant titre of 1:20, whereas the other three had comparatively low titre of 1:10.

Anti-sheep globulin test

This test was conducted by subjecting such of the samples which had reacted negatively to tube agglutination as well as blocking antibody tests numbering 519 in all. For the purpose of comparision, the six samples

which had revealed blocking antibodies (vide table 5) were also included in the Coombs test separately and the results have been summarized in table 6.

TABLE 6

Results of anti-sheep globulin (Coombs) test:

Place	No of sample tested	No. Positive	No. Negative
Baripada	62	0	62
Berhampur	62	0	62
Bhubaneswar	143	6	137
Cuttack	105	3	102
Chiplima	75	0	75
Deogan	48	0	48
Sambalpur	24	0	24
Total	519	9	510
Reactor rate		1.7%	98.3%

It was observed that out of 519 samples tested nine (1.7%) were positive to the Coombs test excluding six animals which had been found positive to the blocking antibody test. Hence nine more animals were detected as positive in the Coombs antiglobulin test.

Zone wise prevalence of brucellosis among sheep in Orissa

As has been indicated earlier, for purposes of this investigation the state of Orissa was divided into

four agroclimatic zones. The prevalence of Brucella reactors in respect of each zone, as revealed by the four serological tests employed in this investigation, have been shown in table 7.

TABLE 7

Name of zone	Name of the test	No. of samples examined	No. positive	No. Doubtful	No. Negative
Eastern	Plate agg. test	258	3	2	253
	Tube agg. test	255	2	2	251
	Blocking antibody test	251	3	0	248
	Antiglobulin test	248	9	0	239
Northern	Plate agg. test	63	0	1	62
	Tube agg. test	63	0	1	62
	Blocking antibody test	62	0	0	62
	Antiglobulin test	62	0	0	62
Southern	Plate agg. test	72	3	1	68
	Tube agg. test	69	1	3	65
	Blocking antibody test	65	3	0	62
	Antiglobulin test	62	3	0	59
Western	Plate agg. test	153	0	6	147
	Tube agg. test	153	2	4	147
	Blocking antibody test	147	0	0	147
	Antiglobulin test	147	0	0	147

Table eight summarises the results of this investigation in its totality taking into account the prevalence of positive seroreactors in different zones of the state.

Critical examination of the table which show that the percentage of reactors is highest in the southern zone (9.7%) and lowest in the northern zone (0%).The eastern zone, where reactor percentage is 6.6%, occupies the second place and the western zone where reactor percentage is 1.2% ranks the third position. The reactor percentage of the state as a whole, on the basis of this investigation, works out to 4.7% taking into account results of all the serological tests.

Further, some important conclusions emerge regarding the relative efficacy of the four serodiagnostic tests deployed in the detection of brucellosis in sheep. With the plate agglutination test alone only six (1.1%) out of 546 sheep were found positive, whereas this number increased to 11 (2%) when the tube agglutination test was performed. The reactor rate increased further to 17 (3.1%) with the inclusion of the blocking antibody test and finally when Coombs test was deployed 26 (4.7%) reactors were detected.

Table 8. Consolidated figures showing prevalance of positive Brucella reactors by different tests in different zones of Orissa

Zone	Plate agg. test				Tube agg. test				Blocking antibody test				Coombs test				Total no. reactor	Reactor percentage
	No. of sample tested	Positive	Doubtful	Neg	No. of sample tested	Positive	Doubtful	Neg.	No. of sample tested	Positive	Doubtful	Neg	No. of sample tested	Positive	Doubtful	Neg.		
Eastern	258	3	2	253	255	2	2	251	251	3	0	248	248	9	0	239	17	6.6
Northern	63	0	1	62	63	0	1	62	62	0	0	62	62	0	0	62	0	0
Southern	72	3	1	68	69	1	3	65	65	3	0	62	62	0	0	62	7	9.7
Western	153	0	6	147	153	2	4	147	147	0	0	147	147	0	0	147	2	1.2
Total	546	6	10	530	540	5	10	525	525	6	0	519	519	9	0	510	26	4.7
Reactor rate		1.1%	1.8%	97.1%		0.9%	1.8%	97.3%		1.1%	0%	98.8%		1.7%	0%	98.3%	4.7%	

C H A P T E R - V

DISCUSSION

C H A P T E R - V

DISCUSSION

Research work on brucellosis among animals in Orissa, which is known to be endemic for brucellosis, was chiefly confined to cattle both under farm and village conditions. Polding as early as 1947 had confirmed beyond doubt that not only was brucellosis an important disease among village and farm cattle but he had also isolated, from infected cattle, several Brucella strains which on the basis of laboratory studies could not be classified either as Brucella abortus or Brucella melitensis and were regarded by him as intermediate (A/M) strains. A somewhat systematic work, on a wider scale, was once again initiated in Orissa, mostly confined to cattle from 1961 onwards, the results of which drew attention of veterinarians both inside and outside the state. In a series of papers, the results of large-scale survey of bovine brucellosis in the state of Orissa were published (Panda and Das, 1965; Das et al. 1961; Pat and Panda, 1968; Pat and Panigrahi, 1968 and Panda, 1968). In addition to cattle, some work was initiated on the incidence of Brucella reactors among goats and sheep (Das et al. 1961) but the work was confined to Bhubaneswar and its surrounding areas. In view of the high incidence of this disease among cattle, the question that invariably arose was as to what could be the position in respect of sheep and goats, as under village conditions the latter two species of animals lived in close proximity to infected herds of cattle. No doubt,

cattle mostly suffer from Br.abortus while sheep and goats from Br. melitensis; but, there are several reports both in India and abroad of sheep and goats suffering from Br.abortus under natural conditions (Bruce, 1936; Huddleson, 1943; Karston, 1950; Young, 1953 and Mathur, 1967).

Studies on brucellosis among goats in Orissa have already revealed 5.19% positive reactors basing upon standard plate and tube agglutination tests (Das et al. Op.cit.). However, the work of Panda and Pat (Op.cit) revealed a higher rate i.e. 12-13% reactors when, in addition to the above two classical tests, the blocking antibody test was used. The presence of blocking antibodies in goats automatically arises the question whether such antibodies do or donot occur in sheep. This aspect, i.e. the occurrence of incomplete antibodies, which mask agglutination by ordinary methods, has not been probed among sheep. In view of the economic importance and public health significance of this disease in sheep, it was considered worthwhile to start investigations among sheep from where it was left by the earlier workers.

While planning the study, it was visualised on the basis of earlier work in goats by Panda and Pat (Op.cit.) that, in addition to the standard plate and tube agglutination tests, the inclusion of blocking antibody test was a must. It was further contemplated to deploy at least another serological test which would possibly detect

all the reactors in any given population of sheep infected with Brucella organisms other than Brucella ovis. Perusal of available literature showed that the Coombs antiglobulin test was by far the most sensitive one in identifying most if not all infected animals as compared to other available serological tests (Hajdu , 1963; Lepennec and Goyson, 1965; Argotte et al. 1977 and Khristoforov, 1979).

It was the authors' aim to collect sera samples from the four agroclimatic zones of Orissa and subject these samples simultaneously to all the four serological tests viz., plate, tube agglutination, blocking antibody and Coombs antiglobulin tests so that a picture might emerge regarding the occurrence of brucellosis in sheep and efficacy of the agglutination and blocking antibody tests as compared to the Coombs antiglobulin test.

The overall picture which emerged out of these investigation is rather interesting in as much as only 26 sheep out of 546 tested had detectable titres to Br.abortus antigen, thus revealing a rather low incidence of 4.7% reactors among sheep of this state. This situation is in agreement with the reactor rates of 6.6%, 2.2% and 4.2% in Uttar Pradesh, West Bengal and Punjab (Misra, 1967; Sen and Joshi, 1968 and Randhwa and Dhillon, 1979). In some other states, however, much higher rate of prevalence of brucellosis in sheep has been reported. The highest rate of 58% was encountered in Haryana by Kalra and Kulshreatha (1978) among aborted ewes followed by 20% in Kasmir (Rao, 1952

and Maharastra (Despande, 1960). It must be mentioned in this connection that Polding (1947) had also come across stray Brucella reactors among sheep in Orissa, while the situation was rather alarming in cattle of some of the Government farms and villages. He had further reported that his attempts to isolate Brucella organisms from cases of ovine abortion were not rewarding which further indicated either no infection or rare cases of the disease in so far as Orissa sheep were concerned.

Zonewise, the highest prevalence of ovine brucellosis in Orissa was detected in the southern zone (9.7%) followed by the eastern zone (6.6%), western zone (1.2%) and northern zone (0%). It was significant that none of the sera samples obtained from the two Government sheep farms, both located in the western zone, as well as those of the northern zone, had no clear-cut positive titre in any of the four serological tests. Even though the situation is very fortuitous, yet more samples are required to be tested before these farms are declared free from brucellosis. However, only four sera samples of sheep of Chiplima had doubtful titre of 20 I.U. in the tube test, but failed to exhibit any reaction in the Coombs test. The significance of such results will be discussed separately.

It is now relevant to discuss in some detail as to why the prevalence of brucellosis in sheep is even lower than that in goats and cattle (4.7% in sheep, 13% in goats and 30% in cattle) [illegible] inspite of the fact that cattle, sheep and goats, specially under village conditions, are

stalled and reared together. The observation of Mathur (1967) and others, based on isolation of species of Brucella from cattle, sheep, goats and human beings, has shown that nearly 85% cases of brucellosis in sheep are caused by Br. melitensis and the rest 15% by Br. abortus. Since Br. abortus infection among cattle is rampant in Orissa, it is likely that inspite of coming in contact with infected cattle, only a very few sheep actually suffer from Br. abortus infection. Obviously, sheep of Orissa, and perhaps else where, have natural resistance against Br. abortus infection and this, in all probability, has kept the infection rate at a very low level. However, under certain conditions the organisms might overtake the body resistance and the disease assumes an epidemic form as has been encountered by Rao (Op. cit.) in Kasmir and Kalra and Kulshreetha (Op. cit.) in Haryana. One can postulate breed resistance in sheep in areas where the prevalence rate is rather low and if it is proved true, the nondescript native sheep of Orissa are perhaps more resistant to Brucella infection than the sheep of Kasmir and Haryana. Another probable cause of such low rate of prevalence is perhaps due to the fact that the disease is less protracted in sheep than cattle and goats and the infection gets cleared off within a few weeks thereby reducing chances of transmission to a very great extent.

In view of the fact that a total number of 546 sheep sera were screened, a statistical analysis of the zonewise reactor rate may not be rewarding. However, sheep of the southern zone certainly have a higher reactor rate

as compared to other three zone. Incidentally, the percentage of Brucella reactors among cattle in south Orissa is highest in the state (Panda and Das, op.cit.) and this could be explained as one of the reasons of slightly higher incidence of this disease in sheep. However, until isolation and identification of Brucella organisms are made, all the factors associated with the epidemiology of this disease cannot be fully explained. Similarly, further work is needed to find out the relative susceptibility of ewes and rams under natural conditions as in this study, almost equal number of male and female animals were screened and it was not possible, due to practical difficulties, to label male and female sera separately under slaughter house conditions. Interestingly, none of the 10 rams tested in the two Government farms had detectable titres in any of the tests.

For a long time, the standard plate and tube agglutination tests have been extensively used in detecting cases of brucellosis not only among cattle including buffaloes but also in sheep and goats (Dhanda and Rajgopalan, 1949 and Alton et al. 1975). So far as sheep and goats are concerned, a titre of 40 I.U. is considered diagnostic as against 80 - 100 I.U. for cattle (Report, 1964). Obviously, this is due to a comparatively weaker antibody response in sheep and goats to Brucella infection. The plate agglutination test, in particular, suffers from serious limitations on account of weak agglutination reaction

with a thick suspension of the organism which makes interpretation of results quite difficult as compared to positive cattle sera. It is, therefore, not surprising that only 1.1% sheep sera could be detected as positive in this investigation by the plate test as against 4.7% reactors detected when additional tests were deployed. It was the experience of the author that the titre of the positive sera samples never exceeded beyond 80 I.U. and, as such, the dependability of the plate agglutination test in the correct appraisal of infection status among sheep is highly questionable. With the availability of Br.abortus tube antigen, standardised according to FAO/WHO stipulation, it is now possible to ascertain the titre of sera samples in International Units at 50% agglutination level. Several workers including Alton et al.(Op.cit.) have recommended 5% saline solution for sheep and this has been followed throughout this investigation while performing the tube agglutination test. This test was able to detect more positive reactors which had not shown any detectable agglutination in the plate test. In view of the difficulties encountered while using the plate tests, the detection of a few more reactors by the tube test is not surprising and could also be attributed to the use of hypertonic saline as diluent in the tube agglutination test. As is so often the case with investigations on brucellosis in animals and man, 10 sheep sera revealed a titre of 20 I.U. and were classified as doubtful reactors. However, these samples were found negative to the blocking antibody as well as

Coombs antiglobulin tests. Obviously, these animals had normal agglutinins against Br.abortus antigen but were free from incomplete antibodies. In all probability, such animals were in the early stage of infection but further follow up was not possible.

The appearance of blocking antibodies in the course of Brucella infection has been posing serious difficulties in detecting infection in animals and also in man. Available literature confirm beyond doubt that blocking antibodies are more frequently encountered in chronic human cases and more so in sheep and goats (Stableforth and Galloway, 1959; Report, 1964 and Spink, 1977).

Zinnemann et al. (1959) developed a method of detecting the presence of blocking antibodies in sera samples negative to the tube agglutination test and this has been widely followed in medical as well as veterinary fields. Earlier work by Panda and Pat (Op.cit.) confirmed the presence of blocking antibodies against Br.abortus antigen in goats of Orissa but the position with regard to sheep was not investigated. The present investigation has revealed that, as in goats, the sheep also had blocking antibodies against Br.abortus antigen to the extent of 1.1% out of 525 sera samples tested , all of which were negative to the tube agglutination test. Since the sera of only adult animals were screened , it is not unusual to assume that most, if not all, animals having blocking antibodies were sufferers of this infection. This assumption is further

strengthened by the fact that following infection, normal agglutinins appear first and blocking antibodies at somewhat later stage and persists for a protracted period extending over several years (Hajdu, 1970 and Knyazeve et al.1974). By comparing the percentage of sheep found positive to the blocking antibody test with that of goats (Panda and Pat, Op.cit.) it appears that the occurrence of these antibodies in sheep (1.1%) is distinctly lower than goats. This disparity could be explained either due to a relatively lower prevalence of the disease in sheep in this area as compared to caprine brucellosis or difference in the immunological responses to infection in these two different host species. According to Stableforth and Galloway (1959) blocking antibodies are heat labile and are inactivated at 56°C for 15 minutes. Now that the presence of blocking antibodies in sheep has been confirmed, prior inactivation of serum samples would perhaps solve the problem of missing the infected sheep possessing blocking antibodies. Among the sera samples which were positive to blocking antibody test, it was observed that inhibition of agglutination was observed upto the 3rd tube i.e. at serum dilutions of 1:10 to 1 : 40. Since inhibition of agglutination even in the 1st tube was taken as positive for blocking antibodies it was not considered necessary for practical purposes to classify the titres of blocking antibodies.

The antiglobulin test, originally invented by Coombs et al. (1945) , was deployed to detect incomplete Rh antibodies occasionally present in human serum. In course of time, this test, popularly known as Coombs antiglobulin test, was extended for the detection of incomplete antibodies against several microbial antigens and has been frequently used as a reliable tool for the diagnosis of brucellosis particularly in human beings, cattle, sheep and goats. According to Hajdu (Op.cit.), Lepennec (Op. cit.) and Argotte (Op.cit), this test is almost foolproof and the results are as dependable as actual isolation of the causative agent (s).

Qualitative titration of the antiglobulin was done by tube precipitation method and a visible precipitation indicated that enough antiglobulin had been formed in the rabbits. Gel diffusion test was also found to be an adjunct to the above method. However, the maximum dilution of the antiglobulin which showed satisfactory reaction with known Coombs positive serum, appeared to be the only reliable method for quantitative titration of antiglobulin Accordingly, Coombs test was designed by using 1 :100 dilution of the antiglobulin and gel diffusion test was of no avail in this regard.

The results obtained in the course of this investigation have amply confirmed that the Coombs test in sheep could detect 1.7% more reactors over and above the blocking antibody test. In so far as the incomplete antibodies against the Brucella species are concerned, there

appears to be no difference between those which mask agglutination in blocking antibody test and the Coombs test. However, by detecting atleast some reactors found negative in the blocking antibody test, the antiglobulin test justifiably has proved itself as the most sensitive test in the field of diagnostic immunology out of the currently available techniques except, perhaps, the fluorescent antibody technique (FAT). Thus, leaving aside the plate agglutination test, the tube agglutination test could detect 1.8% reactors, the blocking antibody test another 1½1% and the Coombs test additional 1.7% reactors and thereby the total reactor rate among sheep of Orissa works out to 4.7%. Not withstanding the close antigenic relationship between Br.abortus and Br.melitensis the advisability of using Br.abortus antigens for detecting reactors among sheep, as has been followed in this investigation, is a distinct shortcoming in the face of overwhelming evidence that Br.melitensis is much more frequently isolated than Br.abortus from cases of ovine brucellosis in this country and elsewhere. Indeed, Joshi (1979) has observed that Br.melitensis antigens are to be preferred over Br.abortus antigens in serodiagnostic tests in sheep and goats. Unfortunately, Br. melitensis antigen of approved standard is not available in India but it may be worthwhile to produce this antigen for use in sheep and goats, so that comparative studies could be taken up to

evaluate the relative efficacy of Br.melitensis and Br.abortus antigens in the serological diagnosis of brucellosis in these two species of animals.

-XOX-

C H A P T E R - VI

SUMMARY

C H A P T E R - VI

SUMMARY

A small-scale survey was undertaken in Orissa to investigate into the prevalence of Brucella reactors among sheep. For this purpose the state was divided into four agroclimatic zones viz., eastern, northern, southern and western zones and two organised Government sheep breeding farms were also included in this study. A total of 546 sera samples, 258 from eastern zone, 63 from northern zone, 72 from southern zone and 153 from western zone, including Deogan and Chiplima farms, both located in the western zone, were screened for the presence of Brucella antibodies. Four serological tests viz, plate agglutination , tube agglutination, blocking antibody and Coombs antiglobulin tests were deployed in this investigation. The preparation of the antiglobulin and the test procedures followed were on the lines of FAO/WHO specifications. Only Brucella abortus antigen was used throughout this investigation. At first, all the sera samples were subjected to the plate agglutination test and those found negative were tested by tube agglutination method taking 40 International Units (I.U.) as positive and 20 I.U. as doubtful. Agglutination titres below 20 I.U. were declared negative. Each of the serum sample which did not reveal significant titre in the tube test was examined by the blocking antibody test. Lastly, the remaining samples, which were negative to the three previously mentioned tests, were screened for the presence of incomplete

antibodies by the Coombs anti-sheep globulin test. The anti-sheep globulin, raised in rabbits, revealed tube precipitation titre of 1 : 100 and distinct lines developed in the gel diffusion test upto 1: 8 dilution. For the Coombs test, the antiglobulin was standardized against a known Coombs positive serum and a 1 : 100 dilution was found suitable for use in the test proper.

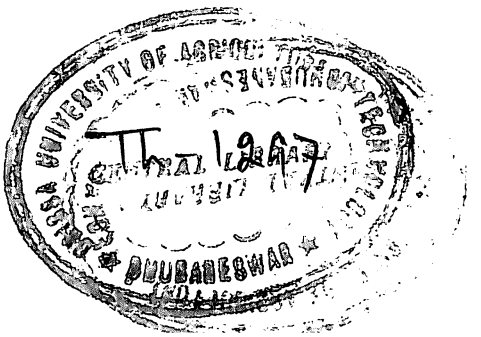
On the basis of all the four serodiagnostic tests performed, 3 out of 258 (1.2%) animals of the eastern zone were found positive to the plate test, 2 out of 255 (0.8%) to the tube test, 3 out of 251 (1.2%) to the blocking antibody test and 9 out of 248 (3.6%) to the Coombs test. In the northern zone, where 63 animals were tested, there were no positive reactors to any of the four tests. However, in the southern zone, 3 out of 72 (4.2%) animals were positive to the plate test, one out of 69 (1.4%) to the tube test, 3 out of 65 (4.1%) to the blocking antibody test and 3 out of 62 (4.8%) to the Coombs test. As regards the western zone, none of the 153 (0%) animals were positive to the plate test, 2 out of 153 (1.3%) to the tube test, none out of 147 (0%) to the blocking antibody and Coombs tests. Both the Government farms were free from Brucella reactors although four samples of Chiplima revealed doubtful reactors but were confirmed as non-reactors on the basis on blocking antibody and Coombs tests. The overall Brucella reactor rate, taking all the four zones and Government farms into consideration, were found to be 4.7%. Zonewise, the reactor percentage was highest in the southern zone (9.7%), followed

by eastern zone (6.6%), western zone (1.2%) and northern zone (0%). The final picture that emerged has revealed that the reactor rate among sheep was comparatively less than in goats as reported by earlier workers.

Among the tests used in this investigation, the plate agglutination test was found to be of very little value as a diagnostic tool. The tube agglutination test missed 15 (2.8%) reactors which were detected by the blocking antibody and Coombs tests. Between the blocking antibody and Coombs tests, the latter proved superior by detecting 9 (1.7%) more reactors on the basis of results obtained. The Coombs test was obviously most sensitive and hence may be recommended as the test of choice in detecting, perhaps, all the infected animals in a flock.

The limitations of using Br.abortus antigen in this investigation as well as problems arising out of brucellosis in sheep including the public health significance have been discussed and the importance of using Br.melitensis antigens in future investigations has been stressed.

***



BIBLIOGRAPHY

B I B L I O G R A P H Y

Alton,G.G.; Jones,L.M. and Pietz, D.E.(1975). Laboratory Techniques in Brucellosis. WHO, Geneva (2nd Ed.)

Anansides and Misoulis (1931). Cited by Marsh,H. (1960).

Ardrey, W.B. (1941). A study of factors influencing the isolation, cultivation and differentiation of the species of Brucella, Technical Bull. No. 177, Michigan State College, U.S.A.

Argotte,E.; Herrera,F.; Cardosa, M. and Cebarrubias, A. (1977). A behaviour of different serological tests in the diagnosis of brucellosis in cattle. Revista cubana de ciencias veterinarias,8: 31-36. (Abst.Vet.Bull. 48: 5261)

Bala, A.K. and Sindu,M.S. (1982). Studies on disease resistance vis-a-vis susceptibility in farm animals, genetic group difference for the incidence of Brucellosis in cattle. Indian J. Anim.Hlth. 21: 61-64.

Bang, B. (1896). Cited by Merchant,I.A. and Packer,R.A.(1966)

Boh, K.J. and Lascelles, A.K. (1973). The use of the antiglobulin test in the diagnosis of Bovine brucellosis. Res.Vet.Sci. 14: 239-244.

Bhambani, B.D. and Krishnamurty, D. (1964). Studies on phase typing of the strains of Brucella isolated from human and animal sources in India. Indian J.Vet.Sci. and A.H. 34: 177-188.

Blood,D.C. and Henderson, J.A. and Redostits,O.M. (1979). Veterinary Medicine, 5th Ed. Bailliere and Tindoll, London

Braun, W.; Kessel, E.W.P. and Pomales Leborn,A. (1962). Failure of vaccination with killed Brucella to modify bacterium interaction. Proc. Soc. Exp. Biol. N.Y. 109: 875-878

Bruce, E.A.(1930). Cited by Marsh, H. (1960).

Bruce, D. (1937). Cited by Merchant,I.A. and Packer,R.A.(1966)

Buchmeiser, R.(1961). Serological diagnosis of Bovine brucellos by agglutination complement fixation and Coombs test. Rindertubrek U.Brucellose, 10: 143-150. (Abst. Vet.Bull. 32: 2023).

Buck, J.M. (1936). Vaccination of calves and yearlings against Bang's disease. Tech.Bull. No. 658 U.S.states Deptt. of Agril, Washington,D.C.

Buddle, M.B. and Boyes, B.W. (1953). Cited by Wilson,G.S. and Miles,A.A(1975).

Cedro,U.C.F.; Cisalo,H.O. and Cocchione, R.A. (1959). The Coomb test for brucellosis in animals. Rev. Aust. Ganard. 15: 43-46.

Chapell, R.J.; MacNaught, D.J.; Bourke,J.A. and Allan,G.S. (1978). Comparison on the results of some serological tests for Bovine brucellosis. J. of Hyg. 80: 365-371.

Charmichael, I.E. (1967). Cited by Alton, G.C.; Jones, L.M. and Pietz, D.E.(1975).

Chaterjee, A.N.(1966). An experimental study of brucellosis among the workers of the Military Dairy Farms at Kirkee and Pimpri and workers ofbutcheris at Kirkee and Poona. Proc. National Seminar on Zoonosis. New Delhi.

Coombs,R.R.A.; Mourant,A.E. and Rice, R.R. (1945). Detection of weak and incomplete Rh. antibodies. A new test. Lancent II:15-16.

Crawford,R.P. and Hedalgo, E.J. (1977). Bovine brucellosis. An International Symposium, Texas Univ. Press.

Deminova,I.F. (1967). Survival of brucella in soil, water and animal buildings. Veterinariya, Moscow, 8:103-105. (Trans. Vet. Bull. 38: 1239)

Das,J.; Panda, S.N. and Dutta,N.K. (1961). Incidence of Brucella reactor among goats and sheep in Orissa. Indian Vet.J. 38: 547-550.

Das,J.; Panda, S.N.; Dutta,N.K. and Acharya,B.N. (1963). An investigation into incidence of Brucellosis in fowls. Orissa Vet.J. 1:5-9.

Das,L.N.;Panda, S.N. and Biswal, G. (1962). Some observations on the incidence of Brucella reactors among bulls. Indian Vet.J. 39: 30-33.

Darlymple Champenys (1960). Brucella infection and undulent fever in man. Oxford Univ. Press,London.

Dennis, S.M. (1972). Infectious ovine abortion in Australia. Vet.Bull. 42: 415-419.

Despande, K.Y.(1968). Brucellosis in man and animals. Proc. National Seminar on Zoonoses, New Delhi

Dhanda, M.R. and Rajgopalan, V.R. (1949). Brucellosis and its control in India. Indian Vet.J. 26:2-16.

Dhandhera,M.K.; Bhagawant,R.B. ; Sainani, G.S.; Deshmukh, P.Y.; Patel, S.D. and Gangrani,J.(1964). Outbreak of brucellosis in a family. Indian J.Med.Sci. 18: 145-149.

Diaz, R.; Jomes,L.M.; Leong, D. and Wilson,J.B. (1967). Difference between Brucella antigens involved in indirect HA test with normal and tanned red blood cells. J.Bact.94: 409-505.

_____(1968). Surface antigens of smooth Brucella. J.Bact. 96: 893-901.

Dolezal, M.; Lytynski, I. and Wisniewski, J. (1959). Use of human antiglobulin serum in the Coombs test in Bovine brucellosis.Med. Vet, Warszawa. 15:334 -336. (Abst. Vet. Bull. 30: 971)

Dolly, P.; Geral, M.F. and Polerin,J.L. (1982). Infectious epididymis of rams.Br.ovis infection. (Abst.Vet.Bull. 52: 5959).

Dubois, C. (1910). Cited by Marsh,H. (1960).

Ebadi,A.(1971). Comparsion of various serological tests on the milk of sheep in relation to isolation of Br.melitensis. Brit.Vet.J. 127: 105-112.

Edington,B.H. and Donham, C.R. (1939). Infection and reinfection experiences in Bang's disease J.Agri. Res. 59: 609-616.

Enchev,S.; Ivanov,I. and Khristoforov, L.(1971). The surface fixation test in ovine brucellosis. Veterinarno Medit-Sinski Nauki 8:9-16. (Abst. Vet. Bull. 42: 1740).

_____(1971). The ring test on blood serum in the diagnosis of brucellosis in sheep. Veterinarno Medit-Sinski Nauki 8:3-8. (Abst.Vet.Bull. 42: 1739)

Enchev,S. and Khristoforov,L. (1971). Serological tests for ovine brucellosis on milk whey. Veterinaro Medit-Sinski Nauki 8:25-31. (Abst. Vet. Bull. 42: 2324)

Firuzi,S. and Kitu,J.(1981). Causes of abortion in Khorasan province zzand. Polskie Archewum. Veterynaryjne. 21: 89-94 (Abst.Vet.Bull. 51: 7621)

Foster,J.W. and Ribi, E. (1962). Immunological role of Br.abortus cell walls. J.Bact. 84: 158-168.

Ganguleo, P.C.; Sharma,G.L. and Sen,G.P. (1967). ICAR Technical Bull. No. 7, ICAR, New Delhi

Golev,I.(1974). Differential diagnosis of Brucella infections in sheep reacting in the agglutination test Vet. Med. Nauki. 11:71-81. (Abst.Vet.Bull. 44:5864)

Gerymisch, J.; Hock, A. and Strey, J. (1967). Effect of dietary protein quality of DLmethionine on antibody formation against brucella strain 19 in young albino rates. Arch.Exp.Vet.Med. 21: 881-893. (Abst. Vet. Bull. 38: 3435).

Ghosh, S.S.; Sen, G.P. and Singh, G. (1968). The use of 3 vaccines against Br.melitensis in sheep. J. Comp. Path. 78: 387-392.

Giannakoulas,D. and Xenos,G. (1971). Comparative study of serum ring test and serum agglutination test in the diagnosis of brucellosis in sheep. In Brucellae. Proceeding 4th symposium on Bacterial. Univ. Athens,Greece (1971): 205-213. Vet. Microbiol. Lab. Aristotalean Univ. Thessaloniki. (Abst. Vet. Bull. 42: 5108)

Glenenur,H.; Hall, W.H. and Zimmerman, H.H. (1959). Blocking antibodies in rabbits infected with Br. melitensis. Proc.Soc. Exp. Biol. N.Y. 101: 422-425.

Grace,K. and Meyers, N. (1967). Report on 20 bacteriologically diagnosed cases of brucellosis encountered in routine laboratory service over a period of 10 years. Indian J.Med. Sci. 21: 89-96.

Greene, H.F. (1951) Experience in eradicating brucellosis. Proc. Book Am. Vet.Med. Ass. 48-50.

Gruteva,M.A.(1967). Factors affecting natural resistance to brucellosis.I. Effect of immunization and infection on the lysosome content of the body organs. Zh.Microbiol.Epidem. Immunobiol 44: 42-44. (Abst.Vet.Bull. 38: 614)

Gwcund ,S. (1964). Electron microscopy of brucella at various stages of development. Berl.Munch. 1 tierarztl. wscher 77: 46-52 (Abst. Vet.Bull. 34: 2022)

Gulrajani,T.S.; Mishra, R.P. and Verma, J.C. (1968). Application of conglutinating complement adsorption test for detecting antibodies from natural cases of Bovine brucellosis. Indian Vet. J. 45: 18-22.

Hajdu,S. (1963). Antiglobulin test for diagnosis of Bovine brucellosis. Bull. Off Inst. Epizoot. 60: 433-445.

_____(1970). A study of heterogeneeity of Brucella antibodies-III. Contribution to the formation of heavy and light antibodies of IgG type in cattle. Vet.Cas. 13: 189-195. State Vet. Inst. Zvolin, Czechoslovakia (Abst.Vet.Bull. 41: 3810).

Haldar, S.K.; Choudhary,B.K.; Dutta,B. and Sen,G.P. (1979). Bovine brucellosis in relation to cross breeding in organised farm. Indian Vet.J. 56:76

Hallman, E.T.; Sholl, L.B. and Deloa,A.L. (1928). Observations on the Pathology of Bacterium abortus infections. Tech. Bull No. 93 Michigan state college,U.S.A.

Hand Book (1979). Abstract statistical of Orissa. Bureau of Statistics, Orissa

Heck, P.C.; Williams,J.D.; Zink,D.L.; Gilmore,W.C. and Adams,L.G.(1961). Serologic profile of a cow experimentally infested with Br.abortus. Brit. Vet.J. 117: 520-526.

Herr,S.; Burgge,L.A.F. and Guiney,M.C.M. (1962). The value of the microtitre serum agglutination test as a second screening test in Bovine brucellosis. Onderstepoot J.Vet.Res. 49: 23-28

Hines, W.D.; Freeman, B.A. and Pearson, G.P. (1964). Productio and characterisation of brucella sporoblasts. J.Bact. 87: 438-445

Hoyer, B.H. and McCullough, N.B. (1968). Homologies of deoxy-ribonucleic acids from Br.ovis canine abortion organisms and other Brucella species. J.Bact. 96: 1783-1790

Huddleson,I.F. and Munel,M.W.(1929). The pathogeneeity of the species of genus Brucella for the fowl. Tech.Bull No. 103 Michigan state College, U.S.A.

Huddleson, I.F.(1941). The presence of capsule on the Brucella cells. Tech.Bull.No. 177 11-14 Michigan State College, U.S.A.

_____(1943). Brucellosis in man and animals. Revised Edison,Commonwealth Fund,N.Y.

_____(1943). Cited by Marsh, H.(1960).

Hutchings, L.M.; Bunnel, D.E.; Dunham, C.R. and Bay, W.W. (1950). The viability of Brucella suis in swine carcases. Proc. Book Am.Vet. Med. Ass. PP 119-124

Hutchings. L.M. (1952). Swine Brucellosis. Its importance to human health. Proc. Book.Amer.Vet. Med. Ass PP 389-390

Jentiah, K.D. (1964). Fluorescent Coombs test to detect incoplete brucella antibodies. Zbl.Bakt i(orig) 192: 262-264(Abst Vet. Bull. 35: 1645)

Jones, L.M. and Marly, J. (1975). Serological and bacteriological studies of ewes vaccinated with Br.melitensis strain. Strain Ref.1 during isolation. Annales de Recherches Veterinaires, 6: 67-71. (Abst. Vet.Bull. 46: 512)

Joshi, T.P.(1979). Lab. procedure for diagnosis of brucellosis Proc. staff course Edip. prophylaxis and control of rabies brucellosis and tuberculosis in animals. I.V.R.I., Izatnagar

Kadyrov, O.M.(1965). Spread of Brucella melitensis to animals other than sheep in Turkmenia. Ser. biol. Nauk. No. 1 93-96. (Abst. Vet. Bull. 36: 1694)

Kalra, D.S. and Kulshrestha, T.C. (1978). Review of Vet.Path. Res. 1970-75. Hissar Agri. Univ. Hissar.

Karston (1950). Cited by Stableforth,A.W. and Galloway,I.A.(195
Kataria, B... and Verma,G.P. (1969). Bovine brucellosis in Madhya Pradesh a serological survey. Indian Vet.J.46: 13-18

Keppie,J.; Witt,K. and Smith,H. (1957). The effect of erythritol on the growth of S-19 and other attenuated strains of Br.abortus. Aus. Vet.Sci. 8: 294-296

Kessel, R.W.I.; Lepitris,D. and Karlsund, J. (1963). Electron microscopy of intra and extra cellular Br.abortus.Fed. Proc. 22: 618 (Abst.Vet.Bull. 32: 1381)

Koppie,J., Witt,K. and Smith,H.(1967). The effect of erythritol on the growth of S-19 and other attenuated strains of Br.abortus.Res.Vet. Sci. 8: 294-296

Kerr, (1955). Cited by Stableforth,A.W. and Galloway,I.A. (1959).

Khristoforov,L. and Peskov,Y.(1969). Studies on 'L' forms of Brucella. Vet.Med. Nauki. 6: 25-32.(Abst. Vet. Bull. 39 :3223).

Khristoforov,L. (1979). Comparision of ways of detecting blocking antibodies in sheep with Br.melitensis Vet.Med. Nauki. 16: 9-15.(Abst. Vet.Bull. 49: 4300).

Kiss, J. (1970). Value of antiglobulin test in brucellosis. Eradication in one year field study. Acta.Med. Hung. 20: 365-371.

Knyazeva, E.N.; Chernyaeva, M.I. and Branovskoya,Y.A. (1974). J.Hyg. Epidem. Microbiol and Immunol 18: 106-110.

Kondaurov,B.I. and Chekishev, V.M. (1969). Sequence of appearance of brucella agglutinins in cattle following vaccination or infection. J.Hyg.Epidem. Microbiol and Immunol.13 :23-25.

Krishnamurty,D. and Hajela, S.K.(1961). Haematological changes in adult bovine and buffalo heifers following vaccination with Br.abortus strain-19 vaccine. Indian J.Vet. Sci. & A.H.. 52: 125-138

Krishnamurty,D. and Verahony, P.R. (1964). Microbial sensitivity of strains of Brucella isolated from human and animal sources in India. Indian J.Vet. Sci. 34: 65-74

Krolack, M.; Chylinski, C.; Stryzak,A.; Nowosielski,F. and Marcicieski, W. (1961). Studies on the occurrence of nonspecific Brucella agglutinins in cattle, swine and sheep. Model procedure in diagnostic examinations. Medycyna Weterynaryjna 17: 71-75

Kulshreatha, R.C.; Kalra, D.S. and Arora, R.C.(1973). Prevalence of brucellosis in cattle and buffaloes of Haryana State. Indian Vet.J. 50: 228-230.

Kulshrostha,R.C.; Arora, R.C. and Kalra,D.S.(1975). Brucellosis in camels and horses. Indian J. Ani.Sci. 45: 673-675.

Kulshrestha,R.C. and Ramachandran,K. (1976). A study of complement fixation,agglutination and haemagglutination tests on bovine brucellosis. Indian Vet.J. 47: 103-107

Kulshrestha,R.C. and Kalra,D.S.(1976). A study on sheep brucellosis with particular reference to infectxious/epidedymitis/outbreak in rams. Indian Vet.J. 55:s357-362.

Kumar,K.; Quadar, S.A. and Arunachalam,T.N. (1976). Brucellosis in goats. Indian Vet.J. 51: 493-494

_____ (1976). Cited by Uppal,P.K.(1979).

Kunter, E.(1975). Storage temperature and time and other environmental factors, their effects on the titres of agglutination and complement fixing Brucella antibodies in cattle blood samples. Monatshafte fur Veterinarmedizin 30: 949-955 (Abst. Vet. Bull. 46: 4910)

Leschke, G.(1974). Centrifugation to accelerate the slow agglutination test in Brucella diagnosis. Monatshafte fur Veteriardedcrin 29:R36-139. (Abst. Vet.Bull. 44: 3563)

Lazareve, P.S.; Smolin, N.I. and Suvorova,G.V. (1969). Rapid test for bovine brucellosis. Agglutination test on filter paper. Veterinariya, Moscow No.11 113-114 (Abst. Vet. Bull. 40: 2726)

Leong,D.; Diaz, R. and Wilson, J.B. (1968). Identification of the toxic components of Br.abortus endotoxin and its labelling by radio active chromate. J.Bact. 95: 612-617.

LePennec,J. and Geyson,M. (1965). A study of bovine Brucella antibodies using Coombs test with warm serum and the complement fixation test. Bull.Soc. Vet.Pract. Fr. 49: 299-307 (Abst. Vet. Bull. 36: 1666)

LePennec, J.(1967). Serology of ovine brucellosis. Vaccinatio and infection antibodies. Serum negative to wrights agglutinatin test but positive to Cf test. Bull Soc. Vet. Pract. Fr. 51:94-112 (Abst. Vet.Bull. 37: 3071)

Lewkowicz,H. (1972). The antiglobulin test in the diagnosis of bovine brucellosis. Medycyna Weterynaryjna 28: 536-539.

Lynov, V.E. and Trutnev,E.I. (1967). Role of digestive juices in the nonspecific defence of body against infection. Uchen. Zap.Kazan. Vet.Inst. 98:114-120. (Abst.Vet.Bull 38: 34-35)

Maiti, C.R.; Ghosh,S.S.; Roy,D.J.; Sen,G.P. and Sharma,R.H. (1980). Survey of bovine brucellosis in cattle breeding farms of Nagaland. Indian J.Anim. Hlth. 19: 83-87

Mahakur, R.C. and Panda, J.K. (1972). Cited by Mishra, H.N. Proc. staff course Rabies Brucellosis and Tuberculosis, IVRI, Izatnagar.

Malik, B.S.(1967). Laboratory Manual for bacteriology and immunology. Brit. Book. Dept., Lucknow.

Manley.P.H. (1966). Br. suis (biotypes) isolated from a goat in Rhodesia. Rhod.J.Agric.Res.6: 55-57

Manthei, C.A. and Detray, D.E. (1950). Brucella infection in bulls and the spread ofbrucellosis in cattle by A.I. Proc. Book Am.Vet.Med.Ass. pp 177-184

Marsh, H.(1960). Sheep diseases. 2nd Ed. Williams and Wilkins, Baltimore.

Mathur, T.N. (1955). An outbreak of brucellosis in a family. Indian J.Med. Sci. 9:1-6

_____(1959). Cited by Khera,S.S. (1968). Animal Brucellosis, Leptospirosis and other bacterial infections of zoonotic importance. National Seminar on zoonoses, New Delhi.

_____(1960). The presence of Brucella organisms in the milk supply of Karnal town and surrounding villages and their pathogenecity for human beings and animals. Indian Vet.J. 40: 205-211.

_____(1960). Brucellosis stimulating a respiratory illness. A report of 11 cases. Indian J. Med.Sci. 14:1-5

_____(1967). Isolation of Br.abortus from goats and sheep in Punjab. Indian J.Vet.Sci. 37:277

_____(1967). An outbreak of brucellosis in an urban family. Indian J.Med.Sci. 21: 314-317.

_____(1968). Brucellosis in goats and sheep in Haryana. Indian J.Med.Sci. 15: 91-102

_____(1968). Brucellosis in Haryana. Proc. National Seminar on zoonosis, New Delhi.

Mathur,P.N.(1971). Brucellosis and farm management. Indian Vet. J. 46: 219-228

Merchant,I.A. and Packer,R.A.(1966). Veterinary Bacteriology and Virology. 6th Ed. Iowa State University Press, Ames, Iowa.

Misra, H.N. (1967). Studies of brucellosis in man and animals. M.V.Sc. Thesis Agra University

Mitchell, C.A. and Moore,E.(1942). Viability of Br.abortus strain 19 in vaccine. Cand.J.Comp.Med.2: 292-295.

Moller, J.A. and Wright,A.E. (1940). Benefits of eradicating Bang disease. Misc. Pub. No. 381 U.S. deppt. of Agriculture, Washington DC

Mollin,F.E.(1939). Bang's disease. American National livestock Assoc. Colorado

Muhammed, S.I.; Mohamadi, H. and Sadiram,H. (1980). A comparison of counter immunoelectrophoresis with the rose bengal and serum tube agglutination test in the diagnosis of brucellosis in sheep. Vety. Microbiol. 5: 223-228.

Munger, M. (1941). A study of the effect of toxic reaction from Brucella cells as the leucocytic picture of normal guineapigs. Tech. Bull 177:45-54.

_____(1941). A study of the leucocytic picture of brucellosis. Tech. Bull. 35-44

Murty, D.H. and Kamath,K.A. (1963). A rapid whole blood agglutination test for survey of brucellosis in rural areas. Indian Vet. J. 40: 742-752.

Nag, N.C.; Kanjilal,B.C. and Ray, J.P. (1977). Brucellosis in cows and buffaloes in West Bengal. Indian J.Anim. Hlth. 16: 89-90.

Naimanov, I.L.(1962). Incidence of brucellosis in sheep of different ages. Veterinariya, Moscow. 1: 48-50. (Abst. Vet.Bull. 32: 2545)

Nandgoonkar, D. and Narayan Rao,P.C. (1971). A survey of incidence of bovine brucellosis in the Integrated Milk project area and in some of the dairy farms of Andhra Pradesh. Indian Vet.J. 48: 12

Nilakantan, P.R. and Pande,P.G. (1948). Investigation of contagious abortion in goats with special reference to isolation of Br.abortus(Bang) from goats milk. Indian J.Vet.Sci. 18:61-67.

O'Reilly,L.J. and Cunningham,B. (1971). An assesment of the brucellosis card test.Vet.Rec. 88: 590-594

Orlov,E.S. and Karyanov, A.N. (1976). Stability of the ovine infectious epididymis agent Trudy Veseoyuznoge Institute experimentalanoi. Veterinarii. 44: 55-59 . (Abst. Vet. Bull. 45: 1519)

Ouchterlony,O.(1949). Cited by Merchant,I.A. and Packer,R.A. (1966).

Palczuk,N.V.; Braun,V.;Pleasoa, O.J. and Corepugueroa, M.E. (1961). Heat revealed antigen in DNA rich preparation from Br.abortus. Proc. Soc.Exp. Biol. 111: 166-171.

Panda, S.N. and Das,L.N. (1965). Survey of the incidence of bovine brucellosis in the state of Orissa. Indian J.Anim.Hlth. 4: 1-7.

Panda, S.N. and Pat,K.B. (1967). Significance of blocking antibodies in brucellosis in goats.Indian J. Anim. Hlth. 6: 267

Panda,S.N. (1968). Studies on animal brucellosis in Orissa. Proc. National Seminar of Zoonoses.

Pandali, K.G. and Mamun,I.R. (1941). Cited by Misra,H.N. (1979)

Panjarathinam, C. and Gairsani,I.S. (1974). Studies on brucellosis. II isolation of Brucella organisms in milk of cow, sheep and goats. Indian J.Anim.Hlth. 13:29

Papadopoulos, O. and Keptopoulos,G. (1976). Application of rose bengal plate test in the diagnosis of brucellosis. Acta Microbiologica Hellenica 21: 29-35.(Abst.Vet.Bull. 49: 5076)

Pat, K.B. and Panda, S.N. (1966). Fibre study of serum samples with tetrazolium stained (ABR) antigen in the diagnosis of brucellosis. Indian J. Anim.Hlth.5: 97-100.

Pat,K.B. and Panigrahi,B. (1966). Isolation and typing of Brucella strains from cattle of Orissa. Indian J.Anim.Hlth. 5: 17-21.

Pathak,R.C. (1966). Cited by Uppal,P.K. (1979). Proc. staff course. Rabies, brucellosis and tuberculosis in livestock, I.V.R.I., Izatnagar

_____(1968). Brucellosis. Proc. National Seminar on zoonosis, New Delhi.

_____(1968). Incidence of brucellosis in domestic animals in Uttar Pradesh with a note on preliminary survey of brucellosis in Mathura district. National Seminar on zoonosis

_____(1965) Comparative studies on the standard and supplement serological tests for the diagnosis of bovine brucellosis on Indian condition. National Seminar on Zoonosis.

Plommet, M. and Fensterbank,R. (1976). Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route.III. Annals of Vet. Res. 7: 9-23

Plommet,M. and Plommet, A.M. (1976). Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route. II. Annals of Vet.Res. 7: 1-8

Polding,J.B.(1943). Brucellosis in India. Indian J.Vet. Sc. and A.H. 13: 27-34.

_____(1947 a). Brucellosis in India. Indian J.Vet.Sc. and A.H. 17: 147-155

_____(1947 b). Indian species of brucella. Indian J.Vet.Sc.and A.H. 17: 157-163

_____(1949). Research into contagious abortion of cattle,sheep and goats. Indian J.Vet.Sc.and A.H. 19: 113-151

Polegdoru, K.(1979). Brucellosis in sheep and goats in Cyprus. Comp. Immunol. Microbiol. and Infectious diseases. 2: 99-106. (Abst. Vet. Bull. 50:1627)

Pyatkin, K.D. and Krivoshein,Y.S. (1980). Microbiology.2nd Ed. Mir Publishers Moscow.

Rajendra,M.P.; Kumar, K. and Narayan Rao, C.V. (1980). Studies on chemicopathology of ovine brucellosis Indian Vet.J. 27: 102-104

Ramachandran,K. (1964). Chemical constituents of Brucella species. Indian J.Microbiol. 4: 41-44.

Ramachandran,K.N.I.; Rabindranath,J.; Jayanath,D.M. and Subbaestri,C.N. (1961). A report on brucellosis in a dairy farm. Indian Vet.J. 38: 741-742.

Randhawa, A.S. and Dhillon,S.S. (1979). Seroprevalence of brucellosis in human and animals of Punjab Indian J.Pub.Hlth. 16:15-21.

Rao, S.R. (1963-72). A decade of Science in India. Progress of Vet. Science. Indian Science Congress. Calcutta.

Rao, K.R.A. (1952). Cited by Uppal,P.K.(1979). Proc.staff course, Rabies,Tuberculosis and brucellosis, Izatnagar

Reiman, H.H.; Chaudhary,A.I.A.P.; Reiman,A. and Farida Haque (1963). Seroprevalence of human and animal brucellosis in Bangladesh Indian Vet.J.60: 165-166.

Report No. 289 (1964). World health organisation Tech. Series No. Geneva.

Richardson,H.; Beck, G.G. and Clark,D.T. (1968). Prenatal immunization of lamb to Brucella. Dissociation of the immune competence and reactivity J. Immunol. 101: 1361-1366.

Ronsechino,F. and Redaelli,G. (1959). The Coombs test for bovine brucellosis. Arch.Vet.. Ital. 10: 319-332.(Abst.Vet. Bull. 30: 1707)

Saeed,A.E.A. and Salem,A.A. (1970). Evaluation of diagnostic procedure in bovine brucellosis. J.Egyptian Vet.Med. Ass. 40: 43-50. (Abst. Vet.Bull.52: 7526)

Satyprakash, Verma, B.M.L. and Ghose,J.N. (1967). Incidence of brucella in man and animals in Delhi. Proc. National Seminar on Zoonosis.

Schuster, E.(1961). Significance of brucellosis by rapid Coombs test. Succn. tierarzti ausenr. 60: 440-444. (Abst. Vet. Bull. 32: 667)

Sen,G.P. and Joshi, P.P. (1966). Cited by Khera,S.S. (1968). National Seminar on Zoonoses, Delhi

Sen,G.P.(1969). Prevalence of brucella infection and its control in man and animals in India. Proc. National Seminar on Zoonosis, New Delhi.

Sen,G.P. and Sharma,G.L. (1975). Speciation of 78 Indian strains of brucella. A epidemiological study Indian J.Ani.Sc.45 : 337-342.

Sen,G.P. (1983). Personal communication.

Sharma,A.D.; Patel, S.P. and Talib, V.H. (1974). Cited by Misra,K.D. (1979) Proc. staff course. Epid.prophylaxis and control of rabies tuberculosis and brucellosis in livestock, IVRI, Izatnagar

Singh,B.P. and Pathak,R.C. (1978). Value of whey antiglobulin (Coombs) test in the diagnosis of chronic brucellosis in cattle and buffaloes. Agra Univ. J.Res. 19: 77-81.

_____(1979). Evaluation of milk ring titre in the diagnosis of brucellosis in bovine. Indian Vet. J. 52: 46.

Sivakumar, D.S. and Patnak, P.R. (1969). Search for specific Brucella agglutinins in goats milk. IndianVet. J. 46: 275-276.

Soni, T.L. and Patnak, P.R.(1969). Serological investigation into bovine brucellosis. Indian Vet.J. 45: 191-195

Soni, T.L. (1976). Serological evidence of human brucellosis in Madhya Pradesh. Indian Vet.J. 53: 841-847.

_____(1976). Suitability of different serological tests for diagnosis of brucellosis in buffaloes. Indian J.Anim. Sci. 46: 571-601.

Spink,W.W.(1956). The nature of brucellosis. University of Minnesota Press, Minneapolis.

Stableforth,A.W. and Galloway,I.A. (1959). Infectious diseases of animals. Diseases due to bacteria Vol. I Butterworth Scientific Publication,London.

Stahl, W.H.(1941). Separation and study of the lipid fraction of Br.abortus. Tech.Bull.No. 177:29-34

Stahl,W.H.; and Hunnum,W.H. (1941). The immunising value of the glucolipid antigen from Brucella cells. Tech. Bull No. 177: 17-19.

Stoenner, H.G.(1951). Cited by Maruh,H.(1960) Sheep diseases. 2nd ed. Williams and Wilkins Co. Baltimore.

Stoenner, H.G. and Lackman,D.B. (1957). Cited by Allison,G.S. and Hiltn, A.A. (1960). Principles of Bacteriology immunity. 5th ed. Edward Arnold Publishers Ltd. London

Sukla,P.N.(1976). Biological and immunological properties of th different fractions of brucellin. Indian J.Anim.Sci. 44: 630-634.

Tanwani,S.K. and Pathak,P.H.(1971). Studies on ABR testing. Indian Vet.J. 48: 229-233.

Thorn, G.C.; Adams, D.R.; Braunwald,E.; Isselbacher,J.K. and Petersdorf,R.G.(1977). Harrisson's principles of internal medicine. 6th Ed. McGraw Hill, Kogakusa,Tokyo

Traum,J.(1914).Cited by Merchant and Packer,R.A. (1968).

Uppal, P.K. (1979). Ovine and caprine brucellosis. Proc. staff course Epidemiology, prophylaxis and control of rabies, tuberculosis and brucellosis, IVRI,Izatnagar.

Vysotaskii, V.V.; Kardina,G.S. and Ostrovskaya,M.N. (1967). Ultrastructure of brucella II. Ultrastructure of R.cells. Zh.mikrobiol Epidem. Immunobiol 44: 19-22

Wilson,G.S. and Miles,A.A(1975). Topley and Wilsons principles of bacteriology and Immunity 6th Ed. Edward Arnold,London

Wisniowski, J.; Romaniuk,K. and Drozdzynska,M. (1964). The antibiogram reaction in the diagnosis of bovine brucellosis. Med.Wet. 20: 327-332. (Abst. Vet. Bull. 35: 2861)

Young,S.(1953). Cited by Stableforth,A.M. and Galloway,I.A. (1959).

Zaki, S.; Krishnamurty, B.S.; Rao,S.A. and Manjish,B.D. (1975). Serological incidence of brucellosis in sheep and goats in Karnataka. Current Research News 11: 195-197

Zimmerman,M.H.; Glenchur,H. and Hall,W.W. (1959). The nature of blocking antibodies in human brucellosi J.Immunol.83: 208-212.