DETECTION OF *BRUCELLA ABORTUS* IN MAMMALIAN TISSUE USING BIOTINYLATED, WHOLE GENOMIC DNA AS A MOLECULAR PROBE

By

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CHAPTER I

INTRODUCTION

Goal of the Research

This study was to develop a detection method and system for Brucella abortus which causes infectious abortion in cattle as well as a zoonosis in man. The constraints of the research were that the techniques developed be capable of detecting the organism in a complex mixture of tissue and bacteria, of being performed in an ordinary animal disease diagnostic laboratory; not use any radioactive molecules requiring special handling or expertise, that it be relatively rapid, and give an unambiguous answer. Toward these goals, a DNA detection system was developed which utilizes biotinylated, B. abortus S19 DNA as a molecular probe. The bacteria were separated from mammalian tissue by selective filtration. DNA-DNA hybridization was utilized to detect the presence of the bacteria in the mixture.

Organism

Genus characteristics

The genus Brucella consists of six species recognized in the ninth edition of Bergey's Manual of Comparative Bacteriology. They are B. melentisis, B. abortus, B. suis, B. ovis, B. neotomae; and B. canis. The organisms stain negatively by Gram's method, showing coccoid

to coccobacillary cellular morphology. These are normally about 0.8-1.8 um in length and 0.4-0.6 um in diameter. They occur singly or in pairs and if freshly isolated from tissue they may appear in clumps or short chains. The method of staining may change the general appearance of the organism making them more or less coccoidal (Corbel and Brinley-Morgan 1984, Meyer and Shaw 1920).

Cell Wall

Brucellae, like many Gram negative bacteria, have a smooth colonial morphology. They appear complete and round on solid media. There appears to be a link between smoothness in colony morphology and virulence. Rough strains are generally less virulent than smooth strains and are characterized by an undulant colony morphology when viewed with oblique light. The difference between rough and smooth strains has been linked to the composition of the cell wall.

The cell wall of the genus is typically Gram negative, showing gracilocutian features such as outer membrane structure containing lipopolysaccharide and protein which is about 9 nm thick. The outer membrane is attached to the peptidoglycan by a Braun-like lipoprotein, which may be present in higher concentrations in smooth than in rough strains, accounting for up to 40% of the outer membrane proteins (Gomez-Miguel et al 1987). The peptidoglycan is typically a crosslinked muramic acid moeity, about 3-4 nm in thickness (Corbel and Brinley-Morgan 1984, Dubray 1976, Dubray and Plommet 1976). This cross-linking may also be greater in smooth strains than in rough strains (Corbel and Brinley-Morgan 1984, Moreno 1981). Beneath this is

a periplasmic space of 3-6 nm in smooth strains (Corbel and Brinley-Morgan 1984). This space may contain certain enzymes for cell wall synthesis and cell wall degradation but there is little evidence for this. Beneath this periplasmic space is the cytoplasmic membrane which consists of a typical tri-laminar layer, with the electron dense portions being 1-2 nm thick each with an electron dense portions of about 3 nm intervening. Granular aggregations are located in close proximity to the cytoplasmic membrane which are clusters of polyribosomes (Dubray 1976).

The cell wall accounts for about 21% of the Brucella dry weight in smooth strains and about 14% in non-smooth strains. This is composed of 37% protein, 14% carbohydrate, 18% lipid, 0.46% muramic acid and 0.1% 2-keto-3-deoxyoctulosonic acid (KDO). The ratios for non-smooth strains are 47% protein, 13% carbohydrate, 17% lipid, 0.4% muramic acid; and 0.1% KDO. Alpha-epsilon-diaminopimelic acid has been found in the peptidoglycan layer. This is a common amino acid analogue which is found in many bacterial peptidoglycan (Corbel and Brinley-Morgan 1984, Kreutzer 1977).

The other characteristics of the Brucella cell wall are significantly different from the typical Gram negatives of the family Enterobacteriaceae such as Escherichia coli. These differences allow for brucellae to be distinguished from the other gracilocutian by the fatty acid composition of the organisms. Brucella contains unique lipopolysaccharide-protein complexes which partition into both the phenol and aqueous phases upon phenol-water extraction. Another unique feature of the Brucella cell envelop is its lack of heptose which is an

integral part of the "model" E. coli envelope (Corbel and Brinley-Morgan 1984, Kreutzer and Robertson 1979).

Brucellae can, in general, mutate readily into rough colonial forms. When this occurs certain quantitative differences and also some qualititative differences become evident upon comparison of the two cell types (De Petris 1964). Kreutzer and Robertson studied smooth and rough strains of the Brucella abortus strain 45: 45/0 and 45/20. The 45/0 strain is the smooth, virulent strain while the 45/20 strain is the rough, avirulent strain. General properties of the smooth strain are: smooth, complete, round appearance, poor absorption of crystal violet, non-agglutination by saline, and acriflavin and susceptibility to Brucella phage. The rough strain, however, has a rough, undulant appearance when observed with oblique light, absorbs crystal violet more readily, is agglutinated by saline, and acriflavin and Brucella phage adsorbs poorly to the rough cell surface. Chemical analysis of the two strains shows a minor difference in composition with the 45/0 strain having a four-fold higher concentration of dideoxyaldoses in the cell wall, which is a component of the lipolysaccharide (LPS), than the 45/20 strain. However, when the two strains were subjected to trypsin digestion, the smooth strain resisted digestion and showed no decrease in optical density (O.D.) at 278 nm. The rough strain showed a drop in O.D. of 0.13 units. Upon digestion with pronase and papain the smooth 45/0 strain showed only a drop in absorbance of 0.10 and 0.03 O.D. units respectively, while the rough 45/20 strain showed a drop in O.D. units of 0.20 and 0.25 repectively for the two enzymes. Lysozyme decreased the absorbance of the smooth strain by 0.10 O.D. units while the rough

strain was nearly twice as susceptible with a drop of 0.20 O.D. units. When lysozyme and trypsin were used consecutively on the same sample, the 45/20 strain quickly disaggregated while the 45/0 strain did not disaggregate under the same conditions (Kreutzer and Robertson 1979).

Upon extraction with phenol, it was discovered that the 45/0 strain had a phenol soluble fraction of its LPS along with a water soluble fraction. The 45/20 strain had only a water soluble LPS fraction. This may be a major factor in virulence, since phenolic LPS fractions have been isolated from virulent B. abortus and B. meletensis.

The differences between the smooth (virulent) and rough (avirulent) strains suggest that these attributes contribute to virulence. It is possible that the chemical differences which cause smoothness aids the cell in survival within the macrophage. Researchers have not identified which of the attributes actually contributes to virulence but it appears that phenol soluble LPS is a major component of the smooth strain.

When the extracted phenol-phase LPS was injected into mice, the mice died. When the aqueous-phase LPS was injected, no lethal affects were noticed. However, when both phases were injected into mice at sub-lethal dosages, carbohydrate depletion was detected. It can be speculated that the phenol-phase LPS is an important contributor to Brucella pathogenicity, allowing the cell to evade the host defences such as proteases, lysozyme, and other lysozomal enzymes (Kreutzer et al 1978).

Metabolism

The species of the genus Brucella all have certain biochemical characteristics in common. They also have certain differences which allow for them to be distinguished. In general, the genus Brucella has an optimal growth temperature range of 36°C - 38°C with a total growth range of 20°C - 40°C. They are obligate aerobes, requiring oxygen as the final electron acceptor. Some strains also require increased CO_2 tension as well. If increased CO_2 is utilized, 5% is required. The requirement for CO_2 is considered to be a species identifying character (Corbel and Brinley-Morgan 1984).

The organisms of the genus do not ferment sugars to organic acids and gas but rather oxidize the sugars to pyruvate which is then utilized by an electron transport system to produce ATP (Rest and Robertson 1975). The following list of amino acids and carbohydrates are utilized by all or most of the strains of Brucella (Gerhardt 1958, Meyer and Cameron 1961a, Pickett and Nelson 1954).

TABLE 1

LIST OF AMINO ACIDS AND CARBOHYDRATES UTILIZED BY Brucella

AMINO ACIDS	CARBOHYDRATES
ALANINE	L-ARABINOSE
ASPARAGINE	GALACTOSE
GLUTAMATE	D-RIBOSE
L-ARGININE	D-GLUCOSE
DL-CITRULLINE	D-XYLOSE
DL-ORNITHINE	meso-ERYTHRITOL
L-LYSINE	L-MALATE
	LACTATE
	FRUCTOSE
	MALTOSE
	RHAMNOSE

Brucellae are capable of utilizing glucose as a carbon and energy source but not as efficiently as some other sugars. Brucellae are capable of very specific transport of glucose; experiments show that glucose analogues are not transported with the exception of 2-deoxy-glucose which is transported with the same efficiency as D-glucose (Rest and Robertson 1975). The mechanism of transport of glucose is not known exactly but it does not utilize a phosho-enol-pyruvate-phospho-transferase system as in E. coli but rather seems to be linked to the transport of galactose. This information was due to work on the organism of bovine abortion, B. abortus. It is noteworthy that other strains do not utilize galactose (B. melentisis, B. ovis, B.suis biovar 3; biovar 4). When grown on fructose, galactose, and erythritol, glucose transport is constituitive. When electron transport chain inhibitors are added to the culture, glucose transport is inhibited 100%. When membrane-bound ATPase inhibitor dicyclo-hexylcarbodiimide is added, glucose tranport is also inhibited which indicates that the transport of glucose is linked to electron transport, proton motive force and ATP production (Robertson and McCullough 1968).

Once inside the cell the glucose is not fermented to ethanol, volatile organic acids or CO_2. Experiments to determine the enzyme composition of the metabolic pathway for brucellae show that all the enzymes for the Embden-Meyerhof-Parnas pathway are present with the exception of the substrate level phosphorylation enzyme phospho-fructokinase. The enzyme fructose-diphospho-aldolase is present with weak activity. The Entner-Doudoroff pathway is also defective, having only partial activity. When the enzymes of the Entner-Doudoroff pathway

were analyzed it was found that there was no phospho-gluconate dehydratase activity. This caused the production of only one of the two possible pyruvates from the catabolism of each glucose.

These missing enzymes mean that another pathway must be the major energy acquiring pathway. It was determined that the major pathway for energy and carbon acquisition was the hexose-monophosphate pathway (HMP) which is coupled to the tricarboxylic acid cycle by pyruvate. The HMP utilizes both NADPH + H^+ and NADH + H^+ for reduction which is unique to microorganisms. This coupling of the HMP and tricarboxylic acid cycle for sole carbon and energy production is also unique in microorganisms. The HMP pathway has been found in other organisms such as Acetobacter and Pasteurella however these organisms also have either the Embden-Meyerhof-Parnas or Entner-Doudoroff pathways for energy production (Robertson and McCullough 1968).

Brucella are capable of growing on the polyol erythritol and indeed grows preferentially on this sugar. Studies have shown that, given the choice between glucose and erythritol, brucellae will utilize erythritol. Anderson and Smith showed that Brucella abortus growth was stimulated by addition of erythritol to glucose cultures. During growth on erythritol it was found, by using radioisotopes incorporated into the erythritol, that erythritol was utilized at a rate of one and one-half times the weight of the bacteria. The ratio of incorporation of the various carbons of the polyol was nearly constant among the different cellular fractions. The erythritol carbons were found in cell wall, and membrane fractions, amino acids, nucleic acids, and in excreted CO_2 (Anderson and Smith 1965).

When cells are grown in glucose and erythritol is added, the utilization of glucose is inhibited. Although glucose uptake is constituitive, glucose metabolism is limited in the cell; while the metabolism of erythritol is well developed (see below), therefore, the difference in utilization in the two substrates is due to the rate of metabolism of the substrate (Anderson and Smith 1965).

The catabolism of erythritol has been studied by Sperry and Robertson in cell free extracts of B. abortus. They determined that in order for erythritol to be utilized, ATP, NAD^+, Mg^{++}, inorganic orthophosphate, and reduced glutathione were required. Upon addition of meso-erythritol, the molecule was phosphorylated by an ATP-dependent kinase which formed D-erythritol 1-phosphate. This was oxidized by an NAD-dependent dehydrogenase (probably membrane-bound) to D-erythrulose 1-phosphate. D-erythrulose 1-phosphate was oxidized to 3-keto-L-erythrose 4-phosphate by another NAD-dependent dehydrogenase. This was in turn further oxidized to 3-keto-erythronate-4-phosphate by a membrane bound dehydrogenase in the electron transport chain with O_2 or NO_3 as electron acceptor. This keto acid was decarboxylated to dihydroxyacetonephosphate (DHAP) by a soluble decarboxylase. DHAP was converted to pyruvate by the final enzymes of glycolysis: erythritol -> D-erythritol-1-phosphate -> D-erythrulose-1-phosphate -> 3-keto-L-erythrose-4-phosphate -> 3-keto-L-erythronate-4-phosphate -> DHAP (Sperry and Robertson 1975).

The electron transport system of B. abortus was studied by Rest and Robertson. They determined the presence of membrane bound dehydrogenases, flavoproteins, coenzyme Q_{10} and cytochromes b, c, a + a_3

and the b class cytochrome with oxidase activity, o. All of the results were obtained with cell free extracts of membranes. They utilized spectral analysis, oxygen electrodes, and polarography to determine the presence of these compounds. They found that the electron transport chain was probably branched with cytochromes a + a_3 acting as terminal cytochrome before oxygen only in late-log phase organisms while cytochrome o was found in mid and late log phase organisms. The order of the cytochromes, flavoproteins and quinones has yet to be determined but the authors assume it to be similar to other bacteria. The cytochromes are present in relatively smaller concentrations in B. abortus than in other (non-brucellae) bacteria which may explain the relatively slow generation time for the organism (2.5-3.0 hr) (Rest and Robertson 1975).

The above information on the metabolism of B. abortus is thought to be indicative of the genus. The data points to an aerobic respiratory metabolism with the organism getting its energy for most systems including transport and anabolism from the coupling of electron transport and proton motive force to the production of energy rich ATP. Molecular oxygen or, anaerobically, nitrate, is the terminal electron acceptor with much of the substrate going to CO_2 (Rest and Robertson 1975).

The genus is a fastidious organism requiring complex molecules for growth as opposed to the "model" E. coli which is capable of growth in salts and glucose. However, defined media have been developed for Brucella by several investigators. These media require salts, a substrate, nitrogen source, and certain vitamins.

Listed below are two defined media in use today:

TABLE 2

DEFINED MEDIA USED TO GROW *Brucella*

Substituent	Rode *et al*	Gerhardt *et al*
NaCl	7.5g/l	7.5g/l
K_2HPO_4	1.0g/l	10.0g/l
Na-thiosulfate	0.1g/l	0.1g/l
Glucose	4.0g/l	-
Glycerol	-	30.0g/l
Lactic acid	-	5.0g/l
Mg^{++}	10.0mg/l	10.0mg/l
Fe^{++}	0.1mg/l	0.1mg/l
Thiamine	0.2mg/l	0.2mg/l
Nicotinic acid	0.2mg/l	0.2mg/l
Pantothenate	0.04mg/l	0.04mg/l
Biotin	0.0001mg/l	0.0001mg/l
DL-Asparagine	-	3000.0mg/l
Ammonium sulfate	1.35g/l	-
DL-Alanine	1200.0mg/l	-
L-Glutamate	3900.0mg/l	-
L-Cystine	100.0mg/l	-
DL-Methionine	940.0mg/l	-
L-Arginine	770.0mg/l	-
L-Lysine	770.0mg/l	-
Initial pH	7.5	6.8-7.0

The medium by Rode has been used as a minimal medium for several years and has the advantage of giving high yields but at the expense of simplicity. The Gerhardt medium is a recent development and is very simple but does not support rapid growth or high yields. The two media are also distinguished by the size of inocula required for initial growth. The most simple medium, Gerhardt's requires a large inoculum while the less simple medium of Rode requires only a small inoculum. The use of high quantities of amino acids is not necessary since the major reason for the amino acids is for a nitrogen source. Ammonium

salts are usually sufficient, however, a good supply of readily deaminated compounds enhances growth (Gerhardt 1958).

The requirement for Na^+ ions is debatable since experiments have shown that K^+ ions may be substituted. The sulfur component may be in the form of inorganic or organic sulfur although there is an order of preferrence with cystine being the most desirable and sulfite and thiosulfate being the least. Thiosulfate and cystine are utilized by the two media. The other two inorganic ions, Mg^{++}, and Fe^{++} are utilized by both and are required for brucellae growth. The other major inorganic ions do not seem to be stimulatory for growth. Mn^{++} and Ca^{++} may be provided with some small enhancement but the minor ions do not seem to be helpful. This may be due to contamination of other components with these elements (Gerhardt 1958).

The use of glucose as a carbon and energy source has been shown to be lacking. The alternative use of glycerol and lactate may also not be optimal. In fact, the presence of glycerol may be non-nutritionally beneficial in that it may be contaminated with other factors necessary for growth. D-glutamate has been substituted as sole C-E source and appears to give better yields (Gerhardt 1958).

The vitamins listed are not all required for growth. Thiamine is required for growth of all strains of Brucella, niacin is essential for growth of strains of B. suis B. melitensis, while biotin is essential only for B. abortus. Pantothenate, biotin and niacin are stimulatory for all strains. Other factors are stimulatory for growth rate but not necessarily for yield. These include ribose nucleic acids, or adenine, guanine, cytosine by themselves (Gerhardt 1958).

The genus Brucella oxidizes several carbohydrates, and amino acids including those listed in Table 1. The rate of oxidation is often the only method of determining the identity of a field isolate. Meyer and Cameron performed experiments to determine if the difference between the species is qualitative or quantitative (Meyer and Cameron 1961a-1961c).

Genetics

The genetics of the genus Brucella have been neglected until recently. Variation among the brucellae is limited and will be discussed shortly. The nucleic acid of the genus has not been studied in depth until recently, largely due to the problems of growth of the organism, limited techniques, and to the emphasis upon detection, and prevention rather than taxonomy. Marmur and Doty applied their technique of thermal denaturation to determine the guanine + cytosine (G+C) content of several bacteria including Brucella. They determined that Brucella have a percent G+C of 55% (Marmur and Doty 1962). Later studies by Schildkraut, Marmur and Doty on B. abortus using buoyant density in CsCl confirmed that indeed, the percent G+C of B. abortus is 55% - 56% (Schildkraut et al 1962).

Hoyer and McCullough performed competition experiments between unlabeled and radio-labeled nucleic acid from the species B. abortus, B. melitensis, B. suis, B. neotomae, and B. ovis. against the immobilized, labeled nucleic acids of each of the species. The results they obtained indicated that there was equal and reciprocol homologies between the different species of the genus with the exception of B. ovis

which showed a difference of 4-5 % in competition. These reactions were not very stringent (0.2 M Na^+ concentration which is rather high for good dissociation of dissimilar strands), but they show that the genus is highly related between species (Hoyer and McCullough 1968a).

In a companion work, Hoyer and McCullough determined that the difference between B. ovis and the rest of the genus is due to a deletion rather than unique sequences and that this deletion is the reason for the lack of homology. This was determined by hybridization competition two different ways. DNA from B. ovis was labeled with ^{32}P and was used to probe, along with unlabeled B. suis DNA, DNA from B. suis immobilized on a nitrocellulose filter. The amount of unbound, labeled DNA was determined by scintillation spectrometry. To determine whether the results were from a deletion or an insertion, B. ovis DNA was immoblized on a nitrocellulose filter and probed with ^{32}P labeled B. suis DNA and the amount of unbound, labeled DNA was also determined by scintillation spectrometry. The reassociation products of the mixed species hybrids was determined by temperature of disassociation and concluded that the hybrids formed were completely homologous. Enrichment experiments were also performed where the labeled DNA of B. suis was successively passed over fixed B. suis DNA in the presence of large quantities of B. ovis DNA. The competion between the B. ovis and the B. suis left only the non-homologous portions in solution. The reciprocal experiment with labeled B. ovis DNA did not reveal any unbound B. ovis sequences. The results of these experiments showed that a 6% deletion existed in B. ovis which did not exist in the other Brucella species (Hoyer and McCullough 1968b).

The determination that B. ovis had a slightly reduced homology to the rest of the genus raised questions about the relatedness of the species to the genus. Meyer performed experiments to determine the place of B. ovis in the genus. She created cell wall defective mutants by the addition of physiological amounts of progesterone into the growth medium of the bacteria. Some of the resultant mutants, when reverted back to cell wall-intact forms were altered physiologically as determined by biochemical tests. A B. meletensis strain, upon reversion had lost the ability to oxidize erythritol, and glucose, and had gained the ability to oxidize adonitol, D-asparagine, and D-glutamate which it did not have previously. When B. abortus type 2 strain 3074 was subjected to growth in progesterone at physiological concentrations a filterable (cell wall-defective) mutant was isolated. This mutant had metabolic characteristics nearly identical to B. ovis including the loss of ability to utilize glucose and erythritol. This evidence, when added to the evidence of Hoyer and McCullough seems to suggest that B. ovis species arises out of a defective revertant from one of the other Brucella species (Meyer 1976).

Meyer and Cameron and Meyer performed exhaustive research on the metabolism of brucellae. They determined that at the metabolism level, the difference between species is minute and variable. The major difference between species seems to be in the host of choice, with the species nomenclature coming from the host rather than from other characteristics (Meyer and Cameron 1961, Meyer 1961).

Molecular corroboration of the Meyer and Meyer and Cameron findings comes from recent studies by Verger et al and De Ley et al. De Ley et

al has studied the taxonomic position of Brucella using the method of De Ley where ribosomal RNA is hybridized to genomic DNA which attached to a nitrocellulose filter. The filter with the hybrid on it is then subjected to heat to denature the hybrids. The hybrids which have the highest T_m have the most homology. De Ley has determined the taxonomic position of several genera including Brucella. Using this technique, De Ley places Brucella in a taxonomic class by itself. It is most closely related, by this method, to the rRNA Super-family IV which contains the fast growing Agrobacterium-Rhizobium but is most closely related to Alcaligenes sp. and Achromobacter sp. (CDC group Vd which a group of Pseudomonas-like organisms which have not been classified into a particular family). These homologies suggest that Brucella diverged from the other gram negative organisms long ago and thus should have much less in common with other bacteria than some other genera might show (De Ley et al 1970, De Ley et al 1987).

Verger et al examined DNA from 51 strains representing all of the genus, species and biovars by stringent hybridization and subsequent digestion with S1 nuclease which cleaves single stranded, non-hybridized DNA. They hybridized the DNA from each strain with each other strain and digested with S1 nuclease at the T_m (the point where 50% of the hybrid becomes S1 nuclease sensitive), and determined the homology. They determined that there was at least 87% homology between all species and biovars of Brucella with a mean homology of 96 $\pm$ 5%. They proposed that the genus be limited to a single species, Brucella meletensis with biovar designation limited to a vernacular name (e.g. Brucella meletensis biovar Abortus 1) (Verger et al 1985). It would appear that

there is some utility in this proposed nomenclature since the difference between species at the genetic level is minute.

The other aspects of classical genetics such as transformation, conjugation, and transduction have not been demonstrated in the brucellae. No plasmids have been found, nor any evidence of plasmid bourne genetics such as antibiotic resistance. There are a large number of phages which attack brucellae but they do not appear to be lysogenic or have imprecise excision (Corbel and Brinley-Morgan 1984).

Disease

History

The diseases caused by the genus Brucella affect many mammals. The animals most often affected are cattle, sheep, goats, swine, and dogs; with man, elk and bison also being affected. The disease has been known by several names including undulant fever, Mediterranean fever, intermittent fever and brucellosis. Hippocrates may have been the first to describe the disease in Epidemics where he described, (circa 450 B.C.) a disease in man similar to undulant fever. Many years passed before another reference to the disease was recorded. In the late 1700's an affliction which caused abortion in cows and pigs was described in Germany and an affliction which caused abortion in cows and mares in France. In 1804, two French scientists reported that farmers in Flandin were

> ...so convinced that these abortions were contagious that they always say that, in order to avoid recurrences, the calves must be

> carefully wrapped up and taken out by the window or a hole in the wall, but not the door, and especial care must be taken that no cow follows it or goes by the path which it has taken. (Dalrymple-Champneys 1960 pg 1)

In 1807, a book titled The Complete Farmer also described an ailment of cattle which caused abortion and which seemed to be contagious. In 1861 Marston described a disease which afflicted the British soldiers on Malta, including himself. He named this Mediteranean or intermittent fever.

> By this is meant a fever characterized by the following symptoms and course: a preliminary stage of subacute dyspepsia, anorexia, nausea, headache, feeling of weakness, lassitude, and inaptitude for exertion, mental or physical; chills, muscular pains, and lastly, a fever having a long course, 3 to 5 or 10 weeks, marked by irregular exacerbations and remissions, great derangement of the assimilative organs tenderness in the epigastric region, and splenic enlargement. It is prone to relapses, has a protracted convalescence, and is frequently marked by rheumatism. (Huddleson 1939 pg 51)

The organism responsible for this human ailment was isolated by David Bruce in 1887 from the spleen of patients which had succumbed to the disease, Mediteranean fever (Huddleson 1939, Bruce 1887). He named the organism Micrococcus melitensis. In 1897 Wright and Semple demonstrated that the organism identified by Bruce was agglutinated by serum from persons affected by undulant or Mediteranean fever. In 1904 Bruce was appointed to head a Commission for Mediteranean Fever on Malta to find the source of undulant fever. A researcher named Zammit, working for the commission, checked the serum of milch goats (the major supply of milk for the island) to determine if they would be a good experimental model. He found a high titer against the organism of Bruce. He tried to grow the organism from the milk of these goats and found that the milch goats of Malta were the primary source of the

disease on the island (Huddleson 1939, Dalrymple-Champneys 1961).

Zammit also developed the first detection method for detecting Brucella. He found that the milk of the infected goats contained specific agglutinins which could be detected and give a reliable indication of Brucella infection. B. abortus was first isolated by Bang and Stribolt working in Denmark in 1897 from the aborted fetuses of cattle. The organism was grown on artificial media; and subsequently reintroduced into a pregnant cow and abortion was induced. This work was confirmed in England in 1909 and it was thus determined that the organism, named Bacillus abortus was the causitive agent of infectious abortion in cattle. In 1911, in simultaneous experiments Schroeder and Cotton, and Smith and Fabyan inoculated guinea pigs with the milk of presumably healthy cows. Both groups found that the guinea pigs had lesions on their spleen and liver which, when cultured, produced the Bang bacillus. In 1913, Zwick and Krage, in Germany, isolated the bacillus from cows artificially infected with Bang's bacillus (Huddleson 1939, Dalrymple-Champneys 1961).

In 1918, Alice Evans determined that there was a close relationship between Bacillus abortus and Micrococcus melitensis. She determined that serum which agglutinated one would also agglutinate the other. Biochemical tests which she ran were identical, within the experimental error of her techniques. She thus postulated that, although they were organisms of different hosts both could be responsible for undulant fever in man, one from goat's milk and the other from cow's milk. In support of this theory, Keefer and Duncan showed in 1924 that the organism responsible for bovine infectious abortion could cause an

undulant fever-like disease in man (Dalrymple-Champneys 1961).

Evans, in 1918, suggested therefore, that the two organisms be grouped together under the name of Brucella in honor of the original isolator of the organism, David Bruce. In 1920, Meyer and Shaw also performed a comparison of B. abortus and B. melitensis and determined that the genus nomenclature of Brucella should hold and that the two types should be listed as related generically (Meyer and Shaw 1920).

Since the genus was established, other species have been added, all of which cause infectious abortion in the natural host, with the possible exception of B. neotomae which is found in desert wood rats and is of very low infectivity for any mammal. There is presently some discussion about whether the genus should be considered mono-specific since there is very little difference between species except for host (Corbel and Brinley-Morgan 1984, Verger et al 1985).

Pathogenicity

Brucellosis is a true zoonosis. It generally affects sexually mature animals, causing abortion. It may affect sexually mature male animals and induce epidymitis and has been linked, along with Actinomyces bovis, with fistula of the withers in horses. The disease is readily transmitted to man through ingestion of infected material, invasion of open sores, mucous membranes and conjunctiva; and injection of the live attenuated vaccine by veterinarians. There is also some evidence that the disease may be acquired through the application of large quantities of the causitive organism to the unbroken skin. There has never been any documentation of the disease being transfered from

human to human by casual contact or by aerosol of the breath (Gillepsie 1981).

The disease is usually manifested as a series of fever spikes with general malaise and perhaps nausea. Marston described the disease in humans very eloquently (see above). The epidemiology of the disease is thus: The organism generally enters the body, orally it is thought, and enters the bloodstream along with nutrients. Once inside the bloodstream, the organism is met by circulating polymorphonuclear leukocytes (PMN) which are partially capable of neutralizing the organisms. The PMN phagocytizes the bacterium and starts the process of killing. The phagosome fuses with the lysozome and degranulation is initiated. However, before degranulation has proceded very far it is inhibitied. Experiments show that the smooth strains of the organism are more resistant to killing than rough strains. The mechanism of the resistance is not clearly understood. All brucellae which are virulent, be they smooth or rough, inhibit degranulation. The smooth strains of the brucellae seem to be somewhat more resistent to the initial degranulation (Canning et al 1985). Riley and Robertson performed experiments on intracellular survival of the brucellae, specifically B. abortus 45/0 and 45/20. They determined that the relative ability to survive this initial attack of the PMN was to survive the myeloperoxidase-H_2O_2-halide (MPO) system within the granules (Riley and Robertson 1984b). Canning et al discovered two low molecular weight proteins which conveyed resistance to the MPO system. These proteins inhibited the ability of the PMN to iodinate proteins which is an essential part of the MPO system (Canning et al 1985).

Once the organism has successfully invaded and colonized the PMN, the organisms are delivered to the lymph nodes and other lymphatic tissue where they destroy the PMN after multiplying and invade the surrounding macrophages which are even more susceptible hosts. The major factor in brucellae survival in macrophages is slow growth. The organism must be benign until sufficient numbers have been accumulated in the cell to lyse the cell and spread the infection. It has been shown that smooth intermediate strains which grow too fast in the macrophage are cleared from the system faster than the smooth strains which grow slower. This cycle of intracellular growth and extracellular release is the cause of the major symptoms of undulant fever and the reason it was mistaken for malaria in the early years of epidemiology (Smith and Fitzgeorge 1963, Fitzgeorge and Smith 1966, Riley and Robertson 1984a).

The organism is however, more notorious for its ability to produce abortion. The reason for this abililty is its predelection for erythritol which is produced in high quantities in the pregnant bovine uterus. The organism then migrates from the lymph nodes and other lymphatic tissue to the uterus where it overgrows the uterus, infecting the fetus, allantoic fluids, umbilicus, placenta, and cotyledons (Alexander et al 1981). This overgrowth deprives the fetus of nutrients and oxygen. The fetus is usually aborted; if full term, the fetus is often dead, or severly underweight (Gillespie 1981).

Detection Methods

Bacteriological

There are several methods utilized in the detection of Brucella infection of mammals. The World Health Organization has provided a complete manual of Brucella laboratory methods (Alton et al 1975)). Veterinary textbooks also list several methods for the detection of brucellosis in a herd (Gillespie 1981). In general, since there are several causes of abortion, two major types of detection methods are indicated at present. These are bacteriological methods which entail the direct culture of the organism from infected individuals and immunological methods which identify circulating antibody against the organism.

Isolation and identification of the organism from suspect individuals should be made from several sources in the suspect animal. If the reason for the suspicion is an abortion, samples should be taken from the aborted fetal stomach contents, lung tissue, and intestines. The placenta should be sampled, as well as the uterine exudate (Gillespie 1981)). If the milk is suspected, it too should be sampled. The adult animal should have the vagina swabbed and the blood may also be cultured.

The World Health Organization gives instruction for the isolation of the organism from these sources. Briefly, milk is cultured by centrifuging the milk at 6000-7000 X g for 15 minutes and sediment and cream either mixed or separated are spread onto the surface of agar plates with a small cotton swab or with an inoculating loop. Vaginal

swabs are taken with sterile cotton swabs attached to a long probe which can reach deep into the vagina. The uninoculated end of the swab is flamed and the swab placed into a sealable sterile tube for transportation. The swab is then rubbed over the surface of the agar plate. Blood culture is performed by asceptically removing 10 ml of blood from suspected animal into sodium citrate and transferring directly to a Casteñeda blood-culture bottle containing culture medium. The bottles are incubated in 5% CO_2. Fetal membranes are cultured by washing three times with sterile saline and then wiping the membrane over the surface of the agar plate. Stomach contents of the aborted fetus are spread over the surface of the agar plate in the standard colony isolation technique (quadrant method). Tissues are flamed with alcohol, cut to reveal inner surface and this inner surface is spread over the surface of the agar plate. For more thorough search and quantitative isolatio of the organism, the tissue is cleaned of fat, flamed with ethanol, placed in buffer (normal saline), homogenized either in a blender or Ten Broeck homogenizer (or Dounce homogenizer), the homogenate diluted and samples spread over agar plates (Alton et al 1975). A time consuming but highly reliable method for the isolation of Brucella from infected tissue is to solublize the sample by homogenization and injection of the homogenate intraperitoneally, subcutaneously or intramuscularly into two guinea pigs. The guinea pigs may die within a few days of inoculation. The surviving animals are sacrificed at 3 weeks and 6 weeks and the tissue examined for lesions. The lymph glands draining the inoculation site are cultured and the blood examined for circulating antibody (see below) (Alton et al 1975)

The media used for isolation and identification of Brucella are as follows. Initial isolation of colonies is accomplished on basal medium such as tryptose agar, or Albimi agar to which serum and selected antibiotics are added: 100 mg cycloheximide to inhibit fungi; 20,000 U bacitracin to inhibit Gram positive organisms; 6000 U polymyxin B against Gram negative bacteria; and ethyl violet dye at 1:800,000. In addition to these inhibitors, vancomycin, nalidixic acid and nystantin may be added to further select for Brucella over other organisms (Gillespie 1981, Alton et al 1975)).

Once suspected Brucella colonies have come up on agar they are restreaked for purity on selective medium (see above). The resulting colonies are examined for white, convex colonies as seen from above. From below, with light source, they appear clear, and honey colored. When stained by Gram's method they are Gram negative, short coccoid rods.

The organisms should be cultured in 5% CO_2. No growth in CO_2 does not indicate the absence of Brucella however, positive growth is a good indicator of B. abortus. Lactose fermentors on MacKonkey's agar are eliminated as are those which produce haemolysis on blood plates. Hydrogen sulfide is usually produced by B. abortus. The growth in the presence of the dyes thionin and fuchsin is indicative. Brucella abortus can oxidize L-alanine, L-aparagine, L-arginine, L-lysine, L-arabinose, D-galactose, D-ribose, D-glucose, and i-erythritol. If all these are positive, phage susceptibility is the final strain determination factor (Alton et al 1975).

All of these tests show the presence of the etiological agent in

the suspected animal. They are time consuming, expensitive and require some microbiological expertise. Other, faster methods of identifying the presence of Brucella which are indirect yet powerful are the immunological methods.

Immunological

There are two types of immunological detection systems. Detection of antibody (Ab) and detection of antigen (Ag). Both are utilized in the identification of Brucella infection. The fluorescence antibody method (FAb) of scrutinizing tissue utilizes an antibody against Brucella which has a fluorescien or rhodamiene moeity attached. The tissue is flooded with the antibody, washed to remove unbound antibody and observed under a fluorescent microscope. If the antigen is present (Brucella) the antibody attached to it will fluoresce. This technique is utilized along with the bacteriological methods to aid in identification. It has the disadvantage of cross-reactivity. Ab are usually very specific, however they can also react with molecules of similar shape and structure. For this reason, the direct FAb method is usually followed by bacteriological methods for verification. (CRC Brucella book in print).

Another Ag detection technique which is very powerful is the enzyme-linked immunosorbent assay (ELISA). Perera et al have developed a technique by which sub-picogram quantities of Brucella Ag can be detected. Anti-Brucella Ab is attached to nylon beads which is exposed to Ag (killed Brucella cells). The bead-Ab-Ag complex is then sandwiched with another anti-Brucella Ab which is linked to a horse

radish peroxidase (HRP) enzyme. The HRP substrate is added and the presence of the Ag is determined indirectly by the production of color (Perera et al 1983).

The majority of immunological techniques detect Ab by the use of an agglutination reaction. Basically, Ag (whole, killed Brucella cells) are stained with a dye and mixed with the serum of a suspected animal. If Ab against Brucella are present they will agglutinate the Ag which will be detected by the clumping of the stained Ag. Several variations of this have been developed. The slide agglutination test is used along with the bacteriological methods in which the suspect is the organism isolated and the serum is known to have Brucella Ab. The card test is a field test in which the serum of a suspected animal is mixed with Ag in the field without prior dilution. The tube agglutination tests utilize dilutions to determine not only the presence of Ab but also the titer of the Ab to determine the magnitude of the response and hopefully the recentness or severity of the disease. The milk ring test is used to test milk samples for contamination. Killed B. abortus, stained with hemotoxylin is mixed with milk and incubated at 37°C for 60 minutes. The test utilizes the fact that agglutinated organisms rise to the surface with fat globules resulting in a white lower milk layer with a blue frothy top (Alton et al 1975, Gillespie 1981).

The other major test for Ab against Brucella is the complement fixation test. In this test, suspected sera is mixed with Ag and a known dilution of complement. The anti-Brucella Ab will combine with the Ag and will fix complement. The mixture is incubated at 37°C for 30 minutes and is then mixed with sheep red blood cells (RBC) sensitized

with anti-sheep Ab (hemolysin). If there is enough anti-Brucella Ab in the serum to fix the dilution of compement, no hemolysis will occur with the RBCs. The titer of Ab can be detected in this way (Alton et al 1975).

The disadvantage of the immunological techniques where Ab is detected is the problem of vaccinated versus infected animals. Both types have Ab against Brucella. The family of immunoglobulin (Ig)which is prevailent during infection is a function of time. In a recent, acute infection as well as in vaccination the major type of Ig is IgM while in chronic infections the major type is usually IgG. The mercaptoethanol test can distinguish between the two types of Ig by inactivating the IgM types and allowing only the IgG to participate in agglutination. As far as these two types of Ig are related to vaccination and infection, the mercaptoethanol test can distinguish between vaccination and infection. (Barrett 1978)

DNA Detection

Recently there has been a surge in the state of the art of detection methods. A technique has been developed which is both very sensitive, direct, and relatively rapid. This technique is the use of DNA as the target and probe to detect organisms.

Renaturation

DNA hybridization utilizes the affinity that the positive sense strand of DNA has for the negative sense strand. Due to Watson and Crick base pairing, each strand will preferentially anneal to it's

complementary strand in an appropriate solution (Watson and Crick 1953).

Marmur and Doty showed that DNA from different species of bacteria had different bouyant densities in CsCl. They related this to G+C content. They also determined that differing G+C contents allowed for differentiation between the DNA from different species on the basis of heat denaturation (Marmur and Doty 1962).

Marmur and Doty measured the change in the extinction coefficient of DNA as it denatured as heat was applied. They termed the point at which DNA was 50% denatured to be T_m or the melting temperature. They examined this phenomenon with the goal of determining the %G+C in different bacterial species (Marmur and Doty 1962).

During their studies, Marmur and Doty noted that the temperature of denaturation increased as the cation concentration increased. This was seen from KCl concentrations of between 0.01 M to 1.0 M. This stabilization is due to the effect of the cations on the outer phosphate ions of the DNA strand. As the concentration of cation increases, the better the stablization of the phosphate. Friefelder reports in Molecular Biology that the increase continues to around 4.0 M NaCl concentration at which point the stabilizing effect of the cations is overcome and salting out begins (Marmur and Doty 1962, Freifelder 1987).

Marmur and Doty tested the DNA from several genera of bacteria as well as some bacteriophage, plants and animals. Their findings were that related organisms had similar T_ms and similar G+C contents (Marmur and Doty 1962).

Several studies have built upon the work of Marmur and Doty, (De ley et al 1970, Britton and Kohn 1968). It has become more effective

and easier to measure renaturation rather than denaturation. Renaturation can be used to determine the complexity of a genome. It can be used to measure relatedness of species. And it can be used for the detection of a species.

Basically, renaturation utilizes the fact that DNA is hydrogen bonded in a specific way. During DNA replication, one strand of the DNA acts as a template for the construction of an identical, anti-sense, inverted mirror image of the template. The new strand is perfectly complimentary to the template strand. They are bound by hydrogen bonds which upon heating, break, upon reheating, they re-anneal thus creating a double stranded molecule which is identical to the original. When this occurs in a solution where there are several identical molecules of DNA, the exact strands do not necessarily recombine, they rather make a hybrid of DNA. This is termed hybridization (Britton and Kohn 1968).

Several methods can be used to determine renaturation rates. The hyperchromic shift in absorbance at 260 nm wavelength as the DNA renatures is easy to detect but the rate is often difficult to determine. Labeling the molecule with a radioactive moeity such as ^{32}P or ^{3}H allows for better quantitation. To quantify renaturation using a label such as this, the DNA is placed in solution, denatured, allowed to renature, and, as the molecules renature, they are passed over hydroxyapatite which is a clay which binds double stranded molecules at low salt concentration and elutes them at high salt concentrations. By binding the double stranded molecules at different times and subsequently counting the radioactivity in the bound, double stranded DNA, the rate of renaturation can be detrmined. The reaction is a

second order reaction. Using a formula:

$$\text{rate} = dC/dt = -KC_aC_b$$

where C_a and C_b are the concentrations (in nucleotides) which should be equal, t is time in seconds and K is a second order rate constant. The formula simplifies to:

$$C = C_o/(1+KC_ot)$$

or, since it is often more convenient to refer to the fraction of reactants remaining:

$$C/C_o = 1/(1+KC_ot)$$

and

$$C/C_o = 1/(1+KC_ot_{1/2}) = 1/2$$

This is a measure of the renaturation rate of any DNA molecule. Half Cot's, as they are called, can then be compared to determine the genome complexity of an unkown species. The higher the Cot, the lower the renaturation rate since Cot measures the concentration of nucleotides in the solution with respect to time. Renaturation is a function of collision. This is where concentration comes into play. The rate of collision is high with a high concentration, thus more collisions occur and the Cot is high. Once two molecules collide, if their bases match they will reanneal quickly. If the bases do not match, they will not reanneal and will continue to collide until a match occurs. If the DNA in the solution is very homogenous, i.e. if there is only one strand of adenine and one strand of thymine, the renaturation will occur very rapidly since the reanealing will be very quick (Ham and Voemett et al 1980).

The relatedness of two species can also be determined in a similar

way by determining the amount of renaturation between a labeled species and an unlabeled species. Measuring the amount of double stranded DNA as renaturation occurs will determine the amount of relatedness. If the labeled fraction is kept low, then the renaturation with itself will be reduced considerably and may be discounted. The rate of hybridization as well as the specificity is controlled by controlling the stringency of the reaction. Since DNA base pairs specifically and is a long chain of nucleotides, hybridization can occur rapidly between similar strands. Once the two strands collide, the hybridization is a matter of "zipping" up the two strands as the base pairs re-hydrogen bond. If the two strands are identical, Tm will be at Tm for the species. If there are some dissimilarities, the Tm will drop (at the specified ionic concentration). When the temperature of hybridization is above the Tm for non-identical strands, they will not form a stable hybrid. This is termed stringency (Meinkoth and Wahl 1984).

The stringency of a hybridization reaction is related to the ionic concentration and to the reaction temperature. As was noted earlier, increasing the ionic strength lowers stringency while increasing temperature increases stringency (De Ley *et al* 1969).

Detection

DNA:DNA hybridization utilizing the renaturation theories of Marmur and Doty, and De Ley can be used to detect the presence of a bacterial species in a complex mixture (Marmur and Doty 1962, De Ley et al 1970, Moreley et al 1980, Horn et al 198-); and detect the presence of a specific gene in a chromosome for analyzing heritable diseases such as

sickle cell anemia (Conner et al 1983). They can also be used to map specific sequences in plants to determine evolutionary divergence (Rayburn et al 1985).

For these applications, it has become easier to perform the renaturation with one of the reactants attached to a solid support. Southern developed a method whereby DNA could be transfered from an agarose gel to a nitrocellulose membrane in a high salt solution (Southern 1975). DNA attaches to nitrocellulose in high salt concentrations (Nagamine et al 1980), and can be immobilized indefinitely by heating the DNA/ nitrocellulose to 80°C. If the transfer is performed after denaturating the DNA in NaOH, the DNA will attach as a single-stranded molecule capable of undergoing hybridization.

Once the nitrocellulose has been baked to permanently attach the DNA, it can be place in a solution containing the probe DNA (labeled with a radioactive label or biotin). Since nitrocellulose is rather heat labile, the temperature of hybridization (Tm) is reduced by the addition of formamide. After hybridization is complete, the nitrocellulose is washed to remove any non-hybridized probe. The stringency of the was can be altered to increase specificity of the probe. With a wash at low ionic strength, and at a temperature approaching Tm, the match between probe and target must be near 100% (Meinkoth and Wahl 1984).

Clinical geneticists can detect a single base-pair difference using Southern blot hybridization, stringent washes and ^{32}P labeled oligonucleotide probes. Using this technique, diseases such as sickle

cell anemia (Conner et al 1983) and beta-thalassemia (Pirastu et al 1983) can be detected. The DNA is extracted from cells, cut with restriction endonucleases, separated with agarose gel electrophoresis or polyacrylamide gel electrophoresis, and transfered to nitrocellulose by Southern's method. The probe is applied, hybridized, and very stringently washed to remove hybrids that are not 100% base paired. If one base out of the total probe length is not hydrogen bonded the washes will denature the strand. In this way, the single base change can be detected (Conner et al 1983).

Two variations upon the Southern blot have been developed. They are the colony blot and the dot blot. The colony blot entails placing or growing a colony af bacteria on a nitrocellulose membrane, lysing the cells and hybridizing with a probe specific for a sequence suspected of being the bacteria. This method is useful for screening cloned libraries (Maniatis t al 198-). It is also useful for detecting bacteria in complex mixtures (Grunstein and Hogness 1975, Moseley 1980).

A probe is made of a plasmid which carries a disease causing gene. Bacteria suspected of causing the disease are colony blotted onto nitrocellulose, lysed, baked to attach the DNA to the nitrocellulose, hybridized, and washed. If the label is detected, the presence of the disease causing gene is indicated. This has bee used to detect enterotoxigenic E. coli directly from stool samples from infected patients (Moseley et al 1980).

The dot blot is the third method of applying DNA to a solid support for hybridization. It is essentially the same as the original Southern

technique except the DNA is spotted directly onto the nitrocellulose and not transfered from agarose or polyacrylamide gels. The size of the DNA applied is rather critical. If the DNA is less than 200-300 base pairs in length, it will not bind to the nitrocellulose but will rather pass through during initial application (Meinkoth and Wahl 1984, Berent et al 1985).

The dot blot procedure consists of dissolving purified DNA in a low salt solution (or water). The solution is heated to denature the DNA. After the DNA is denatured, salt is added to stablize the denatured DNA and make the DNA attach to the nitrocellulose. While still warm, the DNA-salt solution is applied to the nitrocellulose, usually in an apparatus which reduces the surface area exposed to the DNA solution. Vacuum is slowly applied to draw the solution through the membrane and the DNA onto the membrane. After application the membrane is washed in a medium slat buffer to remove any un-attached DNA and then baked at 80°C (Meinkoth and Wahl 1984, Brandsma and Miller 1980).

Hybridization is performed the same as for Southern blot hybridization. The probe is labeled and denatured. The nitrocellulose is submerged in formamide and salt for higher stringency and lower temperature. The probe mixture is incubated at an appropriate stringency. After incubating, the nitrocellulose is removed, washed and the label developed.

There are two other important variations on the hybridization technique. They are both in situ techniques. One is known as colony blotting and entails growing bacteria on a nitrocellulose membrane or somehow getting bacteria directly onto nitrocellulose. Once the

bacteria are on the nitrocellulose, they are lysed with NaOH, which causes the DNA to leak onto the nitrocellulose in a denatured state. Once the bacteria have been lysed, proteinases are added to digest away the non-nuclear material. The digested proteins, the proteinases and the other non-nuclear materials are then washed away by soaking the filter in chloroform. The resultant filter then has effectively only DNA and RNA available to be attached by baking. Rinsing the filter with several washes of a salt solution removes most of the RNA leaving only the single stranded DNA loosely attached to it. The filter is then baked, and hybridization is carried out in the usual manner. This method allows for screening of cloning libraries and clinical samples which have been isolated previously on solid media by the use of specific probes (Grunstein and Hogness 1975).

The second in situ method is a true in situ method. Tissue samples are sectioned as for histological study. Proteinase and detergent are added to open the cell membrane and allow access to the interior of the cells by a probe specific for a suspected DNA sequence inside the cell. If a radioactive label is used on the probe, photographic emulsions are added post-hybridization which develop, by autoradiography and give black dots where the target sequences are (see below). If a non-radioactive label is used, the standard alkaline phospatase-streptavidin system can be used. In this case, blue dots appear in the cell where the target sequences are (see below).

Labels

Several molecules have been proposed and used for labeling DNA for

detection of hybridization. The most common is a radio-label such as ^{32}P which can be added to a nucleotide and applied to DNA by nick translation. This label is very powerful in that high sensitivities can be achieved. Radioactive phosphorus emits gamma rays which have very high energy. These are capable of exposing film at a high rate and are therefore rather quick in giving an answer to the question of hybridization. They are also rather dangerous due to their high energy radiation and therefore require special handling (Pollice and Yang, 1985). Also, the half-life of such high energy isotopes as ^{32}P prohibit their storage for long periods. Lower energy labels are also used but they can take much longer to develop and although they have longer storage lives due to a longer half-life, the emissions are capable of cleaving the bonds between the nucleotides of a strand and therefore shortening the length of the probe.

Radioactive probes also require special handling, equipment, expertise, containment, and disposal. The disposal problem alone is becoming a problem in the environment. For these reasons, an alternative label has been devised for DNA hybridizations. This label is vitamin H or biotin which is used by nearly all cells in the transportation of carbonate molecules in the cell (Gottschalk 1986). Biotin has been chosen due to its small size, it causes very little steric hindrance for hybridization, and for another molecule which can be used to detect the presence of biotin: avidin.

Avidin, which is produced in egg white, or streptavidin which is produced by the bacterium Streptomyces avidinii has a very high affinity for biotin, with a dissociation constant on the order of 10^{-15}, and a

high specificity for the molecule as well. This allows for the attachment of an enzyme to a streptavidin molecule which can be used to produce a reaction which can be detected.

The reaction briefly is, biotin is attached via a linker molecule to a nucleotide which is then placed into a probe by nick translation or another method to be described below. The probe is hybridized in the usual manner except that a lower concentration of formamide is used. After washes of required stringency, streptavidin is added which binds to the biotin of the probe. The streptavidin may be bound to an enzyme or the enzyme may be bound to another biotin and added after the streptavidin is bound to the probe. Once the enzyme is linked to the probe through the streptavidin, the substrate analog for the enzyme is added along with a chromogen which will turn color when acted upon by the changed analog (Pollice and Yang 1985). Two common enzymes are horse radish peroxidase (Pollice and Yang 1985), and alkaline phosphatase (Leary et al 1983).

Using biotin as a label, as little as 32 fg of DNA can be detected on a nitrocellulose filter (Chan et al 1985). A single gene in a single chromosome can be detected in a chromosome squash (Lawrence et al 1988). This rivals the sensitivity and specificity of ^{32}P labels without the hazard and expense of radio-labels.

The use of nick translation to label nucleic acids for probes limited the use of probes to only double-stranded molecules, which are the only types of molecules that DNA pol I can act upon. Forster et al developed a method whereby a molecule of biotin attached to a linker could be attached to almost any molecule whether it be single-stranded

DNA, RNA, double-stranded DNA or protein. This method utilizes an aryl azide moeity on the end of the linker which is photo-reactable. When the molecule is irradiated with ultra-violet light, the aryl azide becomes a highly reactive aryl nitrene which is capable of reacting with the aromatic base moeity of the nucleotide or any other aromatic rings on molecules. When the aryl azide-linker-biotin molecule is placed in solution with the nucleic acid and irradiated, the biotin is attached to the nucleotide strand. This attachment occurs about every 40 bases and does not hinder the ability of the molecule to hybridize. In this way, single-stranded nucleic acids such as RNA or M13 may be labeled (Forster et al 1985).

The utility of labeling single-stranded molecules lies in the fact that hybridization is a second order reaction. The double-stranded probe must compete with itself for unlabeled strands. There exists the possibility that some of the signal is due to probe hybridizing with probe and not detecting sequences in the unlabeled DNA. Single-stranded probes can be used to alleviate this potential problem. Also by using an unlabeled single-stranded probe made from M13 (a single-stranded phage which has been engineered for molecular biology from an E. coli phage [Messing, 1977]) and then following with a labeled double-stranded M13 which does not contain foreign sequences, a sandwich can be created which increases the signal by forming ladders of biotin labeled DNA above the amount which would normally be present in a hybridization (Meinkoth and Wahl 1984).

Diagnostic Detection Methods

DNA hybridization techniques have been applied to the detection of a variety of etiological agents. Both human and veterinary medicine has been enhanced by the use of these techniques in the detection of a variety of organisms. Viruses, bacteria and eukaryotes have all been detected using radioactive labeled probes and recently, advances have been made which utilize non-radioactive labeled probes.

In 1980, Brandsma and Miller used ^{32}P labeled probes to detect Epstein-Barr viral sequences from inside lymphoid cell lines derived from patients afflicted with Burkitt's lymphoma. It is a DNA virus of which the DNA intercollates into the genome of infected cells and goes latent.

The study used DNA which was extracted from the cell lines and spotted onto nitrocellulose. The probe was total genome, Epstein-Barr viral sequences labeled by nick translation. The specific activity was not reported, rather the total amount of radioactivity used in the hybridization was given as $1.4 \times 10^5 - 7.2 \times 10^6$ cpm. The hybridiation was detected using autoradiography at 20 hr exposure. The DNA from 10^5 lymphoid cells was used. They were able to detect 5 - 50 pg of viral DNA which is about 3×10^4 to 30×10^4 genomes in the sample which related to a level of detection of between 0.06 and 0.6 genomes per cell (Brandsma and Miller 1980).

In 1985, McFarlane et al used ^{32}P labeled pseudorabies virus (PRV) DNA fragments to detect latent PRV in swine tissue. They tested several Bam H1 fragments which had been cloned into E. coli and labeled by nick

translation to a specific activity of 10^8 cpm/ug. To test the probes they isolated the DNA from the swine tissue by phenol extraction; cut it with Bam H1; and separated it by agarose electrophoresis. A Southern blot was performed prior to hybridization. They found three fragments which gave good sensitivity and specificity. The filters were used to expose photographic film for up to three weeks at -70° C. These probes were able to detect 10^{-11} g of PRV DNA in the presence of 15 ug of swine tissue DNA which amounts to a detection level of 1 PRV genome per 40 swine cells. This level is about 10 fg which is extraordinary (McFarlane et al 1985).

Linne also use a 1.8 kb ^{32}P labeled PRV fragment to detect PRV in infected tissue but by dot blot hybridization rather than Southern blot hybridization. He labeled this probe to a specific activity of 1 X 10^7 cpm/ug. He extracted the DNA from approximately 3 mm^3 of infected organ tissue; and spotted the DNA onto nitrocellulose. He used autoradiography to detect hybridization. With this radio-labeled probe he was able to detect 20 - 50 pg of viral DNA in 6 ug of host DNA (Linne 1987).

Bacteria have also been detected using radioactive labeled probes. In 1980, Moseley et al were successful in detecting the presence of enterotoxigenic E. coli by colony hybridization using ^{32}P labeled fragments of the heat-stable (ST) and heat-labile (LT) toxins. The LT probe was a 0.5 megadalton fragment of the LT toxin gene labeled by nick translation to a specific activity of 2.5 X 10^7 cpm/ug. The ST probe was a 157 bp fragment of the ST toxin gene also labeled by nick translation to a specific activity of 5 X 10^7. The hybridizations were

detected by autoradiography. All of the LT producing cells were detected by the LT probe while 71% of the ST producing cells were detected by the ST probe. The quantity of DNA detected was not reported (Moseley et al 1980).

DNA probe hybridization was used by Totten et al to detect the presence of Neisseria gonorrhoeae in men with urethritis. They placed exudate swabbed from the urethra of men directly onto nitrocellulose. The DNA was extracted similar to a colony blot by deproteination and extraction with chloroform. Hybridization was performed with a 2.6 megadalton cryptic plasmid from N. gonorrhoeae, labeled with ^{32}P by nick translation to a specific activity of 10^8 cpm/ug. The hybridization was detected by autoradiography. They were able to detect 89% of the culture positive specimens and 0% of the culture negatives. The reason for the less-than-100% success with the positives was due to a lack of the cryptic plasmid in the remaining 11% (Totten et al 1983).

Kuritza et al were able to detect 10^7 Bacteroides cells per ml of a pure culture isolated from stool samples. They required 10% of a sample to be Bacteroides when mixed with other species. The probe they used was a cloned fragment labeled by nick translation with ^{32}P to a specific activity of 10^7 cpm/ug. They used autoradiography to detect the hybridization (Kuritza et al 1986).

Eukarotic organisms have also been detected using DNA hybridization with radio-labeled probes. Both Plasmodium falciparum and Trypansoma cruzi have been detected with the general technique. T. cruzi was detected by Sanchez et al in 1984 by the use of ^{32}P whole kinetoplast DNA from T. cruzi and autoradiography. They used a modified colony blot

where whole parasites were spotted onto nitrocellulose; lysed with NaOH to release and denature the DNA and hybridized with the kDNA probe. They also used smaller fragments of kDNA as probes which allowed them to determine the geographic origins of particular strains (Sanchez et al 1984).

P. falciparum was detected down to a level of 0.001% parasitemia in 50 ul of blood using a P. falciparum DNA fragment which was specific for P. falciparum without cross-reacting with other Plasmodium species. The probe was a fragment of 1.7 kb in length which was labeled by nick translation to a specific activity of 3 X 10^7 cpm/ug. The samples were analyzed by lysing 50 ul of blood with salt solution; and extracting the DNA from the parasites with phenol. The purified DNA was then analysed using dot blot hybridization and subsequent detection with autoradiography (Franzen et al 1984).

In recent years, the detection of many organisms which are important to veterinary science has been facilitated by DNA hybridization. The American College of Veterinary Microbiologists held a workshop in nucleic acid probes in 1988. Proceedings of the meeting included the detection of several viruses, bacteria and a rickettsia with the technique. Listeria monocytogenes, a Gram positive bacterium which causes food poisoning was detected by colony blot and Southern blot using a ^{32}P labeled synthetic oligomer (Wesley et al 1988). A conserved plasmid was used as a probe to detect Moraxella bovis. The plasmid was shown to be species specific for M. bovis and did not react with cromosomal DNA at all. The probe was labeled with ^{32}P (Rosenbusch et al 1988). Campylobacter hyointestinalis, which inhabits the

intestines of swine and causes disease there, was detected using a ^{32}P labeled from C. hyointestinalis which did not hybridize with other Campylobacter species including C. fetus, C. jejuni, C. coli, and C. mucosalis. The probe was capable of detecting 10^3 organisms from pure culture in a dot blot. They also used the probe to detect the organism in fecal samples by first filtering through a 0.8 um filter. This reduced the background but also reduced the sensitivity ten fold (Gebhart et al 1988).

Another group of organisms which were subjected to DNA hybridization detection with success are the Rickettsia. Goff et al used dot blot hybridization to detect Anaplasma marginale in infected blood from cattle as well as to detect the organisms in the arthropod vector Amblyoma americana and study the development of the rickettsia in the tick (Goff et al 1988). The probe was a 2 kb A. marginale fragment, which coded for a surface protein of the organism, labeled with ^{32}P by nick translation. They were able to detect between 500 and 1000 infected erythrocytes which correlates to the detection of a parasitemia of 2.5×10^{-5} (Goff et al 1988).

The challenge has been to develop non-radioactive labeled probes which are sensitive yet which do not have the problems and hazards associated with radioactive labels (see above). One reason for the late entry of non-radioactive labels is the lack of sensitivity. With radioactive probes, a low signal can be enhanced by simply increasing the exposure time for autoradiography. If this is not feasible due to background, scintillation spectrometry can be used to detect hybridization. However, with non-radioactive labels, an enzyme must be

used which acts to produce color. Enzymes have limited activity and indeed, the activity of most enzymes *in vitro* is on the order of a few minutes to a few hours. This means that, with low target DNA quantity, not much enzyme will be available to produce color. Recently, however, non-radioactive probes have been employed in the detection of several organisms.

Biotin was used in the detection of human cytomegalovirus (HCMV) in both *in situ* and in dot blots. Mifflin *et al* compared ^{32}P, ^{35}S and biotin as labels for the detection of HCMV in a dot blot. They determined that biotin, coupled with alkaline phosphatase, allowed detection of HCMV sequences of purified DNA from tissue infected with HCMV at quantities as low as 5 pg and in only 1 - 3 hours. The ^{32}P label allowed for detection of 1 pg in 4 hours by autoradiography. The ^{35}S labeled probe could also detect 1 pg of HCMV DNA but required 96 hours by scintillation spectrometry (Mifflin *et al* 1987).

Keh *et al* were able to detect the presence of HCMV from tissue samples collected from patients with AIDS using biotinylated probes and *in situ* hybridization. The hybridization was detected using streptavidin-alkaline phosphatase conjugates (Keh *et al* 1988).

Human papilloma virus (HPV), a DNA virus responsible for warts, has been detected *in situ* using biotinylated probes from a commercial source. Walboomers *et al* and Syrjanen *et al* were able to detect HPV in frozen sections which contained 20 HPV genomes per cell using biotin labeled probes followed by streptavidin-alkaline phosphatase conjugates.

Dot blots have also been used to detect Hepatitis B virus. The

viruses were isolated from serum of infected patients; the DNA extracted with phenol and spotted onto nitrocellulose. Biotinylated probes were used for hybridization. Hepatitis B DNA was detected by this method at quantities as low as 1 pg (Saldanha et al 1987).

Biotinylated probes have also been used to detect the presence of enterotoxigenic E. coli. Olive et al used unlabeled, single stranded M13 cloned fragments of the LT toxin gene. The probes were detected by the sandwich technique using biotin-labeled RF M13mp18 as a second probe. The labeled M13 produced crosslinking which increased the signal. They were able to detect 10 pg of LT DNA using this method. They also used polymerase chain reaction to amplify the LT signal prior to probing. With the increased amplification, they could detect as little as 1 fg of initial LT DNA (Olive et al 1988).

Other bacteria which have been detected using biotinylated DNA are the Mollicutes. These are bacteria which naturally lack cell walls. Saglie et al detected Mycoplasma pneumoniae in situ from patients with chronic inflammatory periodonal disease. They used the technique to determine the intracellular dispersal of the organisms (Saglie et al 1988).

A thorough search of the literature has revealed several instances where DNA hybridization has been used to detect the presence of etiological agents and organisms which are of interest to science. Most of these instances have involved the use of radioactive labels. Very few attempts have been made to detect organisms from inside tissue using biotinylated probes. A major obstacle to the detection by dot blots and other procedures which utilize purified DNA is the problem noted above

in the study by Kuritza et al. This is the reduction in signal which accompanies the presence of contaminating host DNA.

The purpose of the present study was to determine if the presence of Brucella abortus organisms could be detected in complex mixtures of bacteria and mammalian tissue by using DNA probe technology. Further constraints were to keep the system as simple as possible and to use non-radioactive labels. No limit was set for detection level initially since the study was a pilot study. Once experimentation began, the goal was simply to detect as low a number of bacteria per gram of tissue as possible.

The study was begun prior to the publishing of the above cited literature, therefore much of the information about dot blot hybridization was unknown. A series of experiments was performed to determine the detection level of B. abortus DNA purified alone, in a mixture with alien DNA, and from cells mixed with tissue. Several concepts were considered as to how to overcome the problem of contaminating mammalian DNA. It was finally decided that the simplest method was to remove the contaminating DNA by simple filtration.

CHAPTER II

MATERIALS AND METHODS

Extraction of Brucella abortus DNA

DNA was extracted from Brucella abortus S19 by the method of Hoyer and McCullough (Hoyer and McCullough 1968a). The organisms were grown on tryptic soy broth medium with ultra-pure agarose for substrate. Sixteen plates at a time were grown for the extraction. The cells were harvested by adding a small amount of buffer to each plate (Buffer A: 0.03 M Tris-HCl pH 8.2, 0.1 M NaCl, 0.05 M EDTA), and suspending the cells in the buffer by scraping them off of the agarose with a sterile oese or plastic loop.

The extraction consisted of several washes of the harvested cells in Buffer A. The cells were then resuspended in 10 mls of Buffer A and incubated in 100 ug/ml lysozyme (stock: 10 mg/ml in ddsH_2O) for several hours at 37°C. Proteinase K was then added to a concentration of 100 ug/ml (stock: 20 mg/ml in ddsH_2O) along with 100 ug/ml of RNase A (stock: 10 mg/ml in ddsH_2O, heated to 100°C for 15 min to remove DNase activity). SDS was added to a final concentration of 0.1 % by the addition of a 10% solution. The cells were allowed to incubate in the enzyme solution overnight at 37°C.

After the enzyme incubation, the lysed cells were extracted in capped Oak Ridge tubes with a phenol-chloroform-isoamyl alcohol mixture (25:24:1) two times with agitation by inversion of the tubes followed by

low speed centrifugation to separate the phases.

Phenol-chloroform was made according to the Molecular Cloning Manual (Maniatis et al 1980). Phenol was equilibrated with 1.0 M Tris-HCl pH 8.0 to a pH of about 7.5. 8-hydroxyquinoline was added to a final concentration of 0.01% as a deoxicant. An equal volume of 25:1 chloroform-isoamyl alcohol was then added to the phenol. The mixture was then stored at 4°C until use.

After the phenol-chloroform-isoamyl alcohol extraction, the aqueous phase was extracted two times with chloroform-isoamyl alcohol to remove residual phenol. After the chloroform extraction, the DNA was precipitated from the aqueous phase by the addition of 2 volumes of 100% ethanol (ETOH). The phenol-chloroform was then extracted with new Buffer A to remove any residual DNA trapped in the phenol phase.

The precipitated DNA was stored at -20°C stably until needed. When needed, the DNA was pelleted with high speed centrifugation in a Sorvall RC-2B centrifuge with either an SS-34 rotor or HB-4 swinging bucket rotor. Centrifugation was at 12,000 x g for 30 min to 1 hr at 4°C. Occasionally, an Eppendorff model 5415 micro-centrifuge was used to pellet small amounts of DNA. The centrifugation was carried out at a setting of 14 at room temperature.

The DNA was resuspended in either a buffer which contained 0.1 M NaCl (NET: 0.1 M NaCl, 0.01 M Tris-HCl pH8.0, 0.001 M EDTA) or double distilled, sterile water (ddsH_2O). All water used in these studies was glass purified, filtered through a carbon filter, heat distilled and autoclaved. The concentration of the DNA was then determined by the absorbance of light at 260 nm wavelength. The purity of the preparation

was also determined by comparing the absorbance at 260 nm and at 280 nm. If the 260:280 ratio was between 1.8 and 2.0 the DNA was considered clean. If the ratio was above 2.0, the DNA was considered contaminated with RNA and required further RNase A digestion. If the ratio was below 1.8, the DNA was considered contaminated with protein and required further Proteinase K digestion. (Maniatis et al 1980).

Once the DNA was determined to be cleaned, it was stored at 4°C to be used within one week. If longer storage was required, a small drop of chloroform was added to discourage microbial growth. If storage of longer than 2 weeks was anticipated, the DNA was re-precipated with ETOH and stored in 70 % ETOH at -20C.

Attachment of DNA to Nitrocellulose: Dot Blot

Nitrocellulose (BA85 Schleicher and Schuell, Keene, NH) was prepared prior to application of DNA by wetting it in 5 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na citrate pH 7) at room temperature. The nitrocellulose was cut to fit a dot blot manifold (Bethesda Research Laboratories [BRL] Bethesda, MD. or Schleicher and Schuell), and floated on the surface of the buffer. Once the nitrocellulose was wetted, about 5 min, it was placed in the dot blot manifold and the apparatus clamped down (Leary et al 1983).

B. abortus DNA with 260:280 ratio of between 1.8 and 2.0 was used in a dot blot. The DNA was first cleaned of endogenous biotin by spun column (Maniatis et al, 1980). Briefly, a 1 ml syringe is plugged with silanized glass wool, and filled with Sephadex G-50 swollen in ddsH_2O or buffer. The syringe with G-50 is centrifuged at approximately 1400 X g

for 4 min at room temperature or cooler. More G-50 is added and centrifuged until the volume of packed G-50 is 0.9 mls. Buffer or ddsH_2O is added until dot blot consisted of diluting the DNA to an appropriate concentration in 100 ul of ddsH_2O. The diluted DNA was then denatured at a temperature of 105°C for 5 min in a glycerol bath. The unusually high temperature used for denaturation was determined empirically. Temperatures of less than 100°C were ineffective at denaturing B. abortus DNA.

Upon denaturation, the DNA was placed at 65°C. One volume of 20 x SSC, which had been prewarmed, to 65°C was added to the denatured DNA. The DNA solution which was then at 10 x SSC was then applied in full volume to a well in the dot blot manifold. The solution was allowed to set upon the nitrocellulose for 5 min at which time vacuum was applied to pull the solution through the nitrocellulose. The well was rinsed 3 times with 5 x SSC by the application of 400 ul volumes with full vacuum. The nitrocellulose was then removed from the manifold and placed in a vacuum container, vacuum applied, and the container sealed to prevent air from entering. The container was then placed in an 80°C oven for 2 hrs. The baked nitrocellulose could be stored at 4°C for months prior to use (Leary et al 1983).

Hybridization

Probe

The probe used in this study was a whole genome, biotinylated, B. abortus S19 DNA probe. The probe was prepared initially be nick

translation and subsequently by the method of Forster, a photochemical method (Maniatis et al 1980, Forster et al 1985).

A commercial nick translation system was used (BRL). The reaction was carried out at 14°C to avoid snap-back DNA (Maniatis et al 1980). The reaction consisted of mixing 45 ul of solution A4 (0.2 mM each of dATP, dGTP, dCTP, 500 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 100 mM 2-mercaptethanol, 100 ug/ml bovine serum albumin [BSA]) with 22.5 ul of biotin-11C-dUTP (0.4 mM biotin labeled dUTP linked by an 11 carbon linker in the same buffer as A2). Ten micrograms of B. abortus DNA was added in 30.5 ul along with 352 ul of $ddsH_2O$. Finally, 45 ul of a solution C (0.4 U/ul DNA pol I, 40 pg/ul DNase I, 50 mM Tris-HCl pH 7.2, 5 mM Mg-acetate, 1 mM 2-mercaptoethanol, 50 % glycerol, 100 ug/ml BSA) was added, mixed thoroughly but gently, and incubated for 60 min. After incubation, 45 ul of 300 mM EDTA was added to stop the reaction.

A timed reaction was initially performed to determine the optimal incubation time. This was followed by substituting ^3H-dCTP for the biotin-dUTP and using liquid scintillation spectrometry to determine incorporation of the nucleotide. A sample of the reaction was removed, precipitated in 5% trichloroacetic acid (which precipitates DNA polymers but not nucleotides), and filtered through a glass filter which bound the precipitated polymers but not the unincorporated nucleotides. The filter was dried and counted in a scintillation counter. The amount of counts per filter found as the reaction progressed was compared for each filter and the point at which the counts plateaued was taken to be the optimal incubation period (Maniatis et al 1980).

The biotin-labeled probe was purified by gel chromatography. A

column of Sephadex G-100 was prepared and equilibrated with 1 x SSC. The void volume and elution volume were determined by following blue dextran and phenol red as they passed through the column. The DNA was applied and fractions collected at the blue dextran volume. The cleaned probe was stored in aliquots at -20°C.

The majority of the probes used for the study were produced by using the method of Forster et al. DNA was suspended in ddsH_2O at 1 ug/ml and 1 volume of the photoreactive reagent (photobiotin acetate Vector) was added. The solution was placed on ice and exposed to ultra-violet light from a sun lamp for 10 min at 10 cm below the light source. Buffer was added to bring the volume to 100 ul (0.01 M Tris-HCl pH9.0). The unincorporated photobiotin was removed by extraction with 1 volume n-butanol. The DNA was then precipitated by the addition of NaCl to final concentration of 0.1 M and 2 volumes of 100 % ETOH (Forster et al 1985).

The DNA was recovered from the ETOH by high speed centrifugation and resuspended in ddsH_2O. The labeling was determined by spotting some of the probe onto nitrocellulose and detecting it.

Hybridization

The DNA extracted from B. abortus and other organisms and attached to nitrocellulose was detected by DNA:DNA hybridization. The nitrocellulose with the DNA attached was placed in a heat sealable plastice bag. Prehybridization solution (50% formamide, 5 X SSC, 5 X Denhardt's reagent [1 X Denhardt's = 0.1 % polyvinyl pyrrolidone, 0.1 % BSA, 0.1% Ficoll 400], 0.025 M Na phosphate buffer pH 7.0) was warmed to

42°C, and 4 mls are added to the bag. Calf thymus DNA was denatured at 100°C and then cooled to 65°C. One ml of the prehybridization solution was warmed to 65°C and mixed with the calf thymus DNA. This mixture was then added to the prehybridization solution in the bag at 100-500 ug/ml. All air was purged from the bag and the bag was heat-sealed. The bag was then incubated at 42°C in a water bath for 2-4 hrs.

After the prehybridization, the bag was opened and the prehybridization solution removed. Calf thymus DNA (100 ug/ml final hybridization concentration) was mixed with 200 ng - 5 ug of probe DNA and denatured at 105°C in a glycerol bath. Hybridization solution (45 % formamide, 5 X SSC, 1 X Denhardt's, 0.02 M Na phosphate buffer pH 7.0) was warmed to 42° C and 1 ml placed at 65°C. The denatured DNA was added to the 65°C solution and mixed. One - four mls of the warmed hybridization solution was added to the nitrocellulose in the bag along with the DNA-hybridization solution. The air was purged from the bag. The bag was then sealed, and incubated at 42°C for 18-48 hrs. It was very important that the temperature of the incubation be constant at 42°C.

Once the incubation was complete, the hybridiation buffer was removed and saved it as it may be used again. The nitrocellulose was removed from the bag and place in a large receptacle such as a 1000 ml beaker. The nitrocellulose was washed 2 times in 2 X SSC, 0.1 % SDS, at room temperature for 3 min each, 2 times in 0.2 X SSC, 0.1 % SDS at room temperature for 3 min each, and 2 times in 0.16 X SSC, 0.1 % SDS at 60°C for 15 min each. The nitrocellulose was rinsed in 2 X SSC and then placed in blocking buffer (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 0.002 M

$MgCl_2$, 0.1 % Triton X-100, 3 % non-fat dry milk).

The nitrocellulose was incubated in the blocking buffer for 10 min at 42°C. The nitrocellulose was removed, placed in a vacuum container, and then baked at 80°C for 10-20 min. The blocked filters could be stored indefinitely

Detection

For detection, the Bethesda Research Laboratories DNA Detection System (BRL) was utilized. Test strips were made using the biotinylated Lambda DNA provided with the system. Test strips were made by diluting the Lambda DNA in the included dilution buffer, and spotting directly onto nitrocellulose and baking. Both test strips and experimental nitrocellulose were re-hydrated in blocking buffer at 42°C for 10 min. Streptavidin (stock provided in the system at a concentration of 1 mg/ml in 50 mM Tris-HCl pH 7.5, 0.2 mg/ml Na azide) was made 2 ug/ml in 10 mls of Buffer I (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 0.002 M $MgCl_2$, 0.1 % Triton X-100) by the additioof 20 ulThe streptavidin solution was added to the nitrocellulose filters and incubated with agitation for 10 min at room temperature.

Three washes of Buffer I of 100 mls each for 3 min removed excess streptavidin. This was followed by incubation with 10 mls poly-alkaline phosphatase-biotin conjugate (stock provided in the system as concatemers of alkaline phosphatase at a concentration of 1 mg/ml) with agitation for 10 min at room temperature. Two washes in 100 mls of Buffer I, 3 min each at room temperature were followed by 2 washes in Buffer III (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$).

Development was carried out in 7.5 mls Buffer III which contained 33 ul of nitro blue tetrazolium and 25 ul 5-bromo-4-chloro-3-indolyl-phosphate (nitro blue tetrazolium is 75 mg/ml in 70 % dimethylformamide, 5-bromo-4-chloro-3-indolyl-phosphate is 50 mg/ml in 70 % dimethyl-formamide). The nitrocellulose was placed in heat sealable plastic bags along with the development solution, sealed, and incubated at room temperature in subdued light for 15 min - 4 hours. After incubation, the filters were removed from the solution, rinsed in NET buffer, and dried between two paper towels. They were stored in plastic bags in the dark.

Filtration

Tritium-labeling of *Serratia marcescens*

Serratia marcescens ATCC was labeled in situ with ^{3}H-thymidine. The organisms were grown in 10 ml LB broth to turbidity (Maniatis et al 1980). M9 salts (0.04 M Na_2PO_4, 0.02 M KH_2PO_4, 1 mM NaCl, 0.2 M NH_4Cl) with 2 mg/ml casamino acids was made and 1 ml of the growing S. marcescens was added to 250 mls and incubated at 37°C until early log phase (Maniatis et al 1980). One uCi/ml of ^{3}H-thymidine with a specific activity of 69 Ci/mM (ICN Irvine, CA) was added and the organisms incubated for 4 hrs at 37°C. The organisms were then centrifuged at 3000 X g avg. for 20 min at 4°C, the pellet resuspended in NET buffer, and another centrifugation was performed. The pellet was resuspended in 30 mls NET bufer and dispensed into microcentrifuge tubes (1 ml/tube) and frozen at -20°C.

The ^{3}H-label was detected by precipitating the cells in 5 % trichloroacetic acid on ice. The precipitate was pelleted by low speed centrifugation at 3000 X g, 4°C. The pellet was resuspended in 1 ml tissue solublizer (Research Products International [RPI], Mt. Prospect, Illinois) which is a toluene-NaOH solution. The mixture was incubated at 65°C until the pellet dissolved. The dissolved tissue and solubilizer were added to 18 mls of neutralizer cocktail (RPI) and the activity determined by liquid scintillation spectrometry using the tritium window at 33 % efficiency.

Sample quantities were normalized to the same volume as the homogenization by dividing by the percent of total homogenization volume. The prefilter normalization equation was: percentage of homogenate value divided by 0.9. The five micron percentage value was divided by 0.8.

General filtration

The mammals used in this investigation were female BALB/C mice (Charles River Laboratories, Wilmington, MA). There were a total of 17 mice used. The rats used were males or females Sprague-Dawley CD strain (Sprague-Dawley, Charles River Laboratories, Wilmington, MA). All animals were over 6 weeks old and were fed on Purina Lab Chow, ad libitum. For most of the studies, liver was removed from a rat, sectioned into 1 g portions, and frozen until use.

The filtration apparatus consisted of a vacuum source, a nylon mesh (nylon hosiery, pore size 125 um - 500 um, generic, Walmart), a prefilter (Millipore, Bedford, MA) in a Swinnex/syringe holder

(Millipore), a five um pore size filter (Gelman Ann, Arbor, MI) attached to a 10 ml syringe. The tissue was cut into small pieces and placed in a 15 ml Dounce homogenizer, B pestle along with 9 or 10 ml of NETTS buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 8.0, 0.001 M EDTA and 0.25 M sucrose, nucleic acid grade, 0.1% Triton X-100). Control bacteria were added at this point, if necessary. Control bacteria were 1 ml aliquots of 10 fold serial dilutions of B. abortus S19 beginning with 1 x 10^9 bacteria/ml (see below). The tissue was then homogenized with 10-20 strokes. The homogenate was filtered through the nylon mesh, the prefilter (with vacuum pressure), and pushed through the 5 um filter with a syringe. The filtrate was collected, centrifuged at 3000 x g for 20 min at room temperature and the pellet resuspended in NET buffer for DNA extraction.

The removal of host DNA by the filtration process was examined by using 1 g rat liver and removing 1 ml aliquots at each step. These samples were extracted by the method of Hoyer and McCullough (Hoyer and McCullough 1968a).

Briefly, the aliquots were subjected to 100 ug/ml RNase A for 1 hr at 37°C, 100 ug/ml Proteinase K, 0.1% SDS (sodium dodecyl sulfate) overnight at 37°C and extracted with phenol/chloroform/isoamyl alcohol (25:24:1)and chloroform/isoamyl alcohol (24:1). The aqueous phase was precipitated with 2 volumes of 100% ethanol. The DNA was then resuspended in 1 ml of NET buffer and the concentration of DNA determined by absorbance at 260 nm in a Beckman DU spectrophotometer with a Gilford (Oberlin, OH) upgrade.

Mouse Challenge Experiment

The sample mice were injected with 0.1 ml of a bacterial suspension in phosphate buffered saline (PBS) containing either 7.1 X 10^9 or 9.0 X 10^{11} B. abortus strain 2308S per ml as determined by spectrophotometric readings on a Bausch and Lomb Spectronic 20 (Bausch and Lomb, Rochester,N.Y.) at 420 nm (Alton et al 1975) and later corroborated by plate count. The control mice were injected with 0.1 ml of PBS only (Montaraz et al 1986).

The surviving mice were sacrificed 6 days post infection, by cervical dislocation and the spleens and livers removed from all mice. The organs were observed for disease, frozen in liquid nitrogen, sealed in microcentrifuge tubes and stored at -70°C until processed. All manipulations were done in a laminar flow hood or within sealed containers.

The spleens or livers were processed together using the above filtration method with some modification. The organs were removed from the -70°C storage and weighed on an OHaus (Florham N.J.) model 1600 pan balance. One g or less of each organ was placed in 10 ml of NETS buffer. Five control tissues received Brucella abortus S19 which was diluted ten fold serially from 10^{10} in 1 ml and the 10^9 through 10^5 organisms/ml dilutions were used. The organ was homogenized with 10 strokes of a Dounce homogenizer, B pestle. One ml of the homogenate was removed for a plate count. Nucleic acid grade Triton X-100 (Pierce Chem. Co., Rockford, IL) was added to the remaining homogenate to a concentration of 0.1% and the tissue was homogenized with another 10

strokes. The homogenate was filtered by the above method.

Cell counts of the organisms isolated from the mice were made by making 10 fold serial dilutions of samples in 1 ml and spotting 10 ul of each dilution onto blood agar plates. Seven dilutions were spotted onto one plate and triplicate plates were made. The organisms were also subjected to bacteriological methods to determine the genus and species. These identifications were performed by the Oklahoma Disease Animal Diagnostic Laboratory (Stillwater, OK). The tests were: colony observation, Gram stain, motility, indole utilization, citrate utilization, urea utilization, Vogues-Proskauer, and growth in 5% CO_2.

Cross Reactivity Tests

The bacterial cultures used in this investigation consisted of enterics, common contaminants of bovine culture samples, and bovine pathogens from the genital tract as well as other types. Also included in the cross-reactivity tests were fungi which could be present in samples. Table 3 lists the organisms which were tested.

TABLE 3

LIST OF ORGANISMS TESTED FOR HOMOLOGY WITH *BRUCELLA* DNA

Species	Strain	Source
Bacteria		
Brucella abortus	S19	Colorado Serum Co.
Brucella abortus	2308S	USDA, Ames, Iowa

TABLE 3 (Cont.)

Species	Strain	Source
Bacteria		
Escherichia coli	HB101	John Donelson, Univ. of Iowa (H.W. Boyer, and D. Roulland-Dussoix, J. Mol. Biol. 41:459)
Klebsiella pneumonia	ATCC 13882	American Type Culture Collection
Proteus mirabilis	ATCC 25933	American Type Culture Collection
Salmonella typhimurium	ATCC 29630	American Type Culture Collection
Serratia marcescens	ATCC 13880	American Type Culture Collection
Yersinia enterocolitica	ATCC 23715	American Type Culture Collection
Fungi		
Absidia sp.	OSU strain M2	H.S. Vishniac, Oklahoma State Univ.
Filobasidiella neoformans var. neoformans	NIH 12	H.S. Vishniac, Oklahoma State Univ.
Mucor racemosus	OSU strain M3	H.S. Vishniac, Oklahoma State Univ.
Rhizopus nigricans	OSU M1	H.S. Vishniac, Oklahoma State Univ.

The bacterial DNA was extracted by the method of Hoyer and McCullough with modifications (Hoyer and McCullough, 1968a). Briefly, cells were grown on an appropriate medium for an appropriate time at the Oklahoma Animal Disease Diagnostic Laboratory. Cells were harvested the

from agar plates with 5 mls of Buffer A. The cell harvest was divided into 2 Oakridge tubes, 5 ml per tube. The Oakridge tubes were centrifuged in the SS-34 rotor at 5000 rpm for 10 minutes at room temperature. The supernatant was then decanted into a 1000 ml beaker marked "contaminated waste" which contained disinfectant. The pellet was resuspended by finger vortex or pipet tituration in 5 mls of Buffer A and centrifuged at 5000 rpm in the SS-34 rotor for 10 minutes at room temperature. The supernatant from this spin was decanted into the "contaminated waste" beaker. The pellets were resuspended in 10 mls Buffer A and combined into 1 tube. Lysozyme was added to a final concentration of 100 ug/ml (100 ul of a 10 mg/ml stock solution) and incubated at 37°C all day (several hours).

Proteinase K was added to a final concentration of 200 ug/ml (100 ul of a 20 mg/ml stock solution). RNase was added to final concentration of 100 ug/ml (100 ul of 10 mg/ml stock solution heated to 100°C for 15 minutes). Finally, SDS was added to a final concentration of 5 mg/ml, (100 ul of a 10 % [w/v] solution). The tube was incubated overnight at 37°C.

The DNA was extracted by phenol extraction followed by chloroform extraction as described above. The DNA was precipitated by the addition of 2 volumes of 100% ethanol. The DNA from the bacteria was stored at -20°C until ready for use.

The DNA was extracted from the fungi by one of two methods. The yeasts were extracted by the method of Bahareen and Vishniac using a Braun homogenizer (Baharaeen et al 1983). Briefly, The cells are grown PYG broth at 37° C until turbid. (PYG: 1.0 % peptone, 0.5 % yeast

extract, 1.0 % glucose). The cells were then centrifuged out of the broth at 5000 rpm for 20 minutes and subsequently washed in 10 mls NET buffer. The cells were recentrifuged at 5000 rpm for 20 minutes. The pellet was rinsed gently 2 times with distilled water, air dried and weighed.

The dried pellet was resuspended in 40 mls of a sucrose buffer (20 mM Tris pH 7.8, 10mM EDTA, 15% sucrose) and placed in a steriled ampule provided with the Braun homogenizer. An equal weight of sterile 0.45 micrometer diameter glass beads was added to the pellet in the sterile ampule. The ampule was then placed in the Braun homogenizer and strapped down tightly. The machine was run at 4000 rpm for 2 min with CO_2 injections of 5 seconds every 15 secs. (The CO_2 cools the ampule, freezing the cells in the buffer and allowing the glass beads to better break the cell walls).

After homogenization the homogenate was transfered to a sterile 600 ml polypropylene beaker and the beads were allowed to settle out. The supernatant, containing the broken fragments of cells, was decanted into a sterile Oak Ridge tube. The remaining glass beads were washed with 10 mls of detergent solution (20 mM Tris-HCl pH 7.8, 10 mM EDTA, 1.0 % SDS) to remove any residual fragments of the yeast cells. The detergent solution (minus the glass beads) was then added to the homogenate mixture in the container. The broken cells were then subjected to RNase, Proteinase K and SDS to purify the DNA. The DNA was then extracted with phenol:chloroform in the usual manner and the DNA precipitated with 2 volumes of ETOH.

Another method of breaking the fungi was employed for the

filamentous fungi. These organisms were grown in broth as were the yeasts. The organisms formed a pellicle on the surface of the broth which was removed and centrifuged twice with NET buffer washes to remove residual medium. The organism was placed in a mortar and liquid nitrogen was added to the pestle to freeze the organism. The organism was then ground with a pestle into a fine powder. As the temperature in the mortar rose, additional liquid nitrogen was added to keep the organism hard enough to grind. Once the organism was reduced to powder, NET buffer was added and the mixture subjected to RNase, Proteinase K and SDS to purify the DNA. The DNA was then extracted with phenol in the usual manner and the DNA precipitated with 2 volumes 100 % ETOH.

CHAPTER III

RESULTS

Detection of Brucella abortus DNA on Nitrocellulose

In the initial experiment, to determine if Brucella DNA could be attached to nitrocellulose and detected by hybridization with a biotin labeled probe, 1 ug of purified B. abortus DNA was spotted onto nitrocellulose along with 1 ug of calf thymus DNA as a control. The filter was hybridized for 48 hours in hybridization solution containing 200 ng of whole genomic B. abortus DNA labeled with biotin. Figure 1 is a dot blot hybridization filter in which 1 ug of B. abortus DNA was spotted onto nitrocellulose in triplicate (column Ba) while 1 ug of calf thumus DNA was spotted, in triplicate (column CT).

The next experiment was perfomed to determine the possible limits of the detection system. B. abortus DNA was spotted onto nitrocellulose in dilutions from 1 ug to 0.001 ug. with 1 ug of calf thymus DNA as a control. Three identical blottings were carried out. The hybridization conditions were the same as in the initial experiment. Figure 2 shows the gradual lessening of detection signal, from a dark spot in column 1.0 Ba to practically no darkening in 0.001 Ba while the control column, 1.0 CT shows no darkening.

In order to have a viable detection system, it was crucial to be able to detect B. abortus DNA in a mixture of DNA. Figure 3A is a filter upon which 1 ug of B. abortus DNA, mixed with from 1 ug to 100 ug

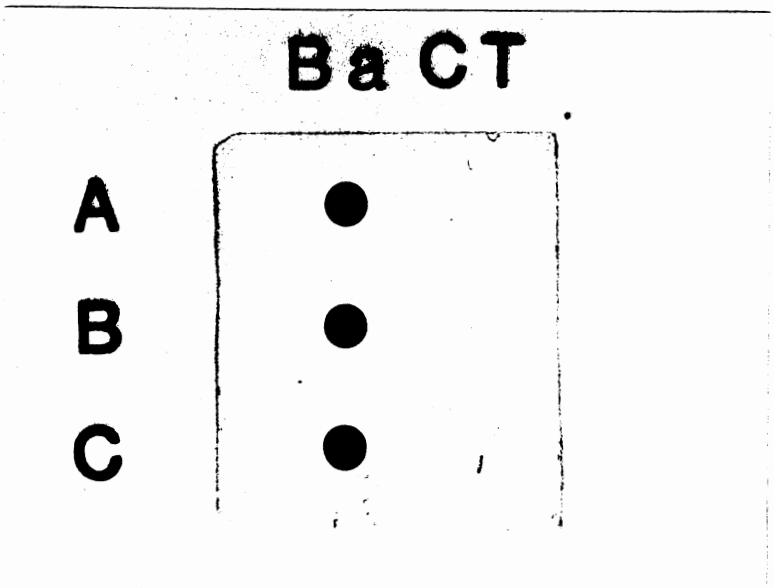

Figure 1. Brucella abortus Dot Blot. Ba. 1 ug B. abortus S19 purified DNA spotted onto nitrocellulose. CT. 1 ug calf thymus DNA spotted onto nitrocellulose. Probe was biotin labeled whole genomic B. abortus S19 DNA.

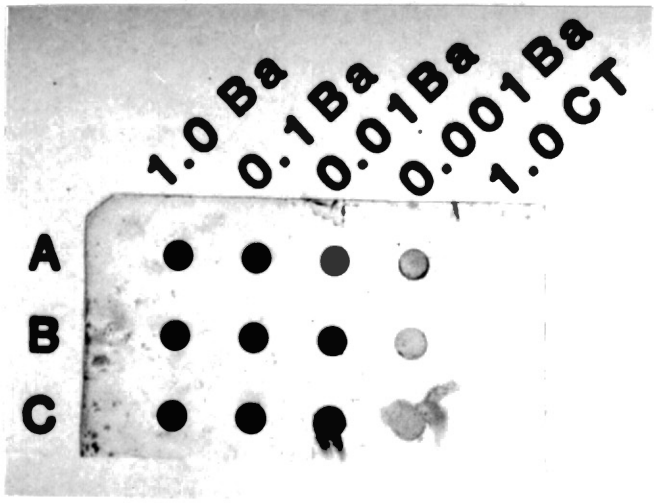

Figure 2. Brucella abortus Dilution. 1.0 Ba. 1.0 ug B. abortus DNA spotted onto nitrocellulose. 0.1 Ba 0.1 ug B. abortus DNA spotted onto nitrocellulose. 0.01 Ba. 0.01 ug B. abortus DNA spotted onto nitrocellulose. 0.001 Ba. 0.001 B. abortus DNA spotted onto nitrocellulose. b1.0 CT 1.0 ug calf thymus DNA spotted onto nitrocellulose. A,B,C are exact replicas.

of calf thymus DNA, was spotted, hybridized as above and detected. This figure shows that in a mixture of B. abortus DNA and calf thymus DNA, the B. abortus signal gradually falls to the level of the blank control between 50 and 100 ug of foreign DNA to 1 ug of target. Figure 3B is of a filter upon which 1 ug of B. abortus DNA mixed with 1, 10, 20, 40, 60, and 80 ug of calf thymus DNA was blotted and detected to determine more precisely the limit of detection in a mixture of DNAs.

Probe Concentration Test

A hybridization kinetics reaction was performed using various concentrations of probe in the hybridization mixture to increase the rate of hybridization, which would in turn decrease the time necessary to detect the organism in tissue. For this experiment, B. abortus DNA and DNA from rat liver was extracted and purified and spotted, along with calf thymus DNA onto nitrocellulose. In Figure 4 the DNA was spotted in the following quantities: 1.0 ug of B. abortus DNA; 1, 5, 10, 20, 40, 60, and 80 ug of rat liver DNA; and 10, 20, 40, 60, 80 and 80 ug of calf thymus DNA. Three identical filters were made: A, B; C. The filters were subjected to hybridization for four hours at the normal conditions with the exception of probe concentration. The probe concentrations used were: A, 1 ug/ml; B, 2.5 ug/ml and C, 5 ug/ml. As can be seen, there was no apparent non-specific hybridization at any of the probe concentrations. The amount of hybridization in each reaction was similar, therefore, all subsequent hybridizations were carried out in 1 ug/ml probe concentration.

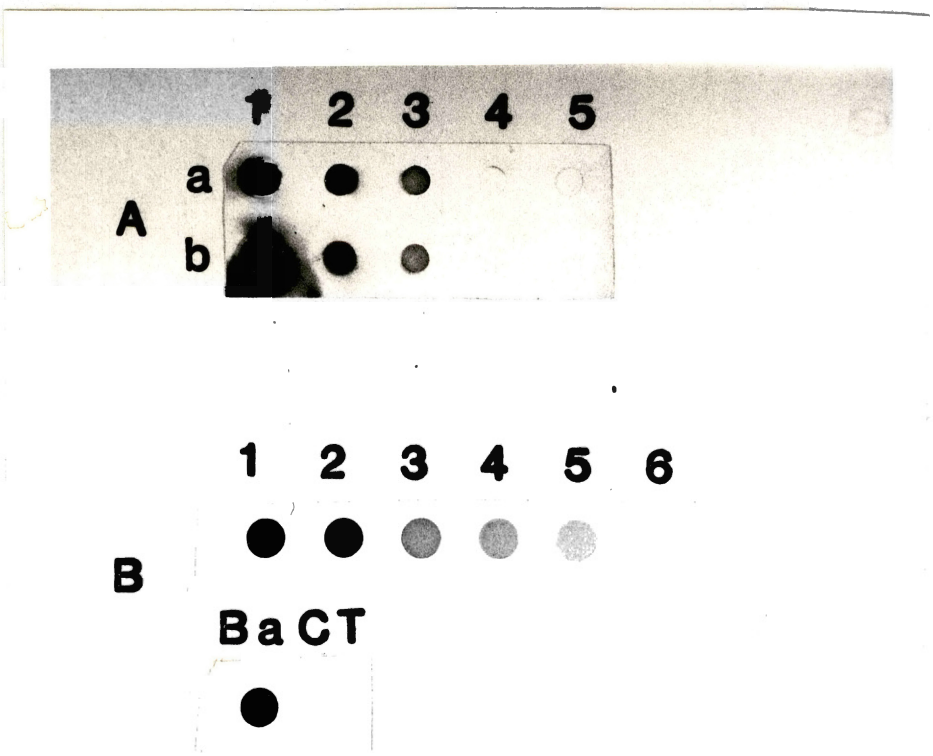

Figure 3. Dilution of Brucella abortus S19 DNA in calf thymus DNA.
A. 1.0 ug B. abortus DNA mixed with various quantities of calf thymus DNA. 1. 1.0 ug calf thymus DNA. 2. 10.0 ug calf thymus DNA. 3. 50 ug calf thymus DNA. 4. 100 ug calf thymus DNA. 5. Control with 100 ug calf thymus DNA alone.
B. 1.0u B. abortus DNA mixed with various quantities of calf thymus DNA. 1. 1.0 ug calf thymus DNA. 2. 10.0 ug calf thymus DNA. 3. 20.0 ug calf thymus DNA 4. 40.0ug claf thumus DNA. 5. 60.0 ug claf thymus DNA. 6. 80.0 ug calf thymus DNA. 7. Control with 80.0 ug calf thymus DNA alone.

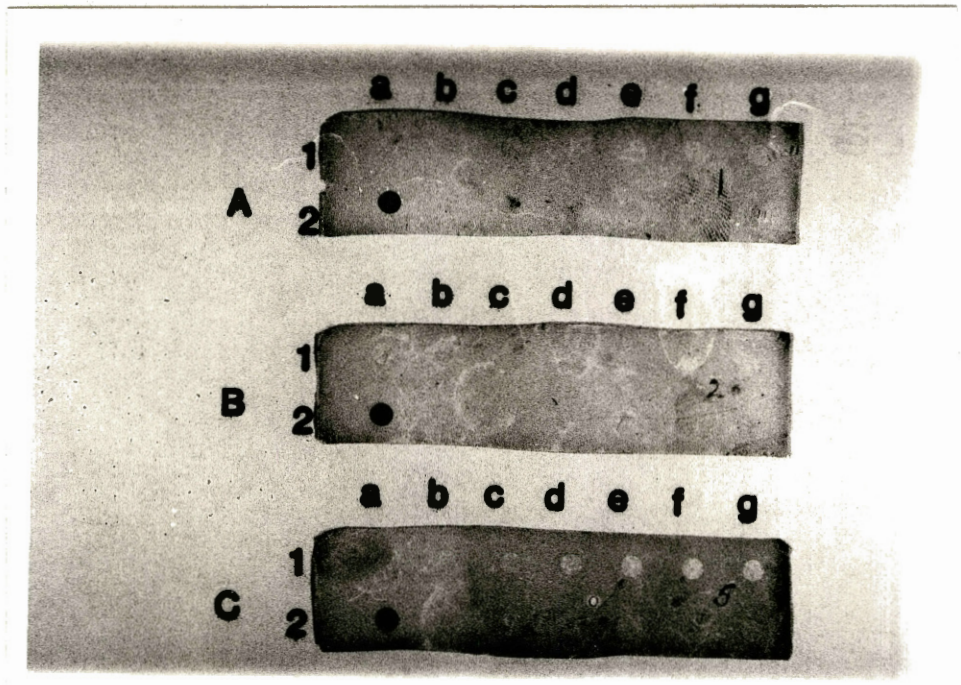

Figure 4. Hybridization Kinetics. A. Hybridization with 1 ug/ml probe concentration. Row 1 is various concentration of rat liver DNA. 1a: 1.0 ug. 1b: 5 ug. 1c: 10 ug. 1d: 20 ug. 1e 40 ug. 1f: 60 ug. 1g: 80 ug.
Row 2 is calf thymus with a B. abortus control. 2a: 1 ug B. abortus. 2b-2g is calf thymus at various concentration. 2b: 10 ug. 2c: 20 ug. 2d: 40 ug. 2e: 60 ug. 2f: 80 ug. 2g: 80 ug.
B. Exact replica of A except probe concentration was 2.5 ug/ml.
C. Exact replica of A except probe concentration was 5.0 ug/ml.

^{3}H-*Serratia marcescens* filtration

Previous experiments with mixed DNA populations showed that there was a limit to detection of target DNA diluted in contaminating DNA. To reduce this effect it was evident that some method be employed to remove the contaminating DNA. Toward this end, a physical filtration technique was developed to remove the mammalian nuclei which contains most of the contaminating DNA, while leaving the *B. abortus* cells intact and available for DNA extraction.

To verify the success of the filtration procedure thus devised, *S. marcescens* labeled with ^{3}H-thymidine was used to follow the reduction of bacteria during filtration. ^{3}H-thymidine was used as an *in situ* marker fue to the high incorporation of this nucleotide into the genome of growing cells. The genomic incorporation was found to be >90% of the total radioactivity of the cell. *S. marcescens* was used as a model because it was similar to *B. abortus* in size, cellular morphology, and Gram reaction.

The labeled bacteria were mixed with mammalian (1 g rat liver) tissue and subjected to the filtration technique. The amount of radioactivity in the homogenate was taken to be 100% of the radioactivity present. This was determined by removing 1 ml of the homogenate, precipitating the large molecules with 1 volume of 10% TCA, and dissolving the precipitate in NaOH. The radioactivity of the precipitate was measured by liquid scintillation spectrometry after neutralization. The subsequent steps in the procedure were analyzed in like manner and the sample radioactivity normalized to the entire

radioactivity in the homogenate. There was an average of 8344 CPM/ml recovered from the homogenate in 10 ml total; an average of 3119 CPM/ml recovered after prefiltration; and an average of 2978 CPM/ml recovered from the 5 micron filtrate. The homogenate was considered 100% and the normalized quantities of prefiltrate and 5 micron filtrate were compared to this standard. Forty-two percent of total radioactivity contained in the homogenate was retained by the pre-filter while 44% was retained by the 5 micron filter. Therefore, 58% of the S. marcescens applied was passed through the pre-filter and this amount was also passed through the 5 micron filter.

The percent reduction of mammalian DNA was determined by removing 1 ml samples from each stage of the filtration procedure, extracting the DNA with phenol and determining the absorbance of the extracted sample at 260 nm. There was an average of 511 ug/ml of DNA in 1 ml of the homogenate; an average of 243 ug/ml DNA in the prefiltrate; and an average of 110 ug/ml DNA in the 5 micron filtrate. Again, homogenization samples were considered 100% and the subsequent quantities were normalized and the percentage of this total was determined. For the prefiltrate, 47% of the DNA was lost, while 73% was lost in the 5 micron filtrate. Figure 5 shows that 55% of the bacterial DNA was lost, and mammalian DNA was reduced by 73%. This filtration procedure was used for all subsequent experiments.

Filtration Experiments

Bacterial counts were made of each infected sample after homogenization to determine the number of bacteria/g of tissue, allowing

for correlation with the dot blot sensitivity. Table 4 shows the colony forming units/g of tissue.

TABLE 4

COLONY FORMING UNITS OF Brucella abortus ISOLATED FROM INFECTED TISSUE

SPLEEN			
MOUSE CODE	WEIGHT g	*B. abortus* cfu/g[a,b]	cfu/spleen
2B1	0.7	$4.4 \times 10^8 \pm 1.7 \times 10^7$	3.0×10^8
2B2	0.7	$1.6 \times 10^8 \pm 6.9 \times 10^6$	1.1×10^8
2B3	0.6	ND	ND
2B4	0.6	$5.0 \times 10^7 \pm 1.3 \times 10^6$	3.0×10^7
2B5	0.5	$4.0 \times 10^8 \pm 3.6 \times 10^6$	2.0×10^8
6B1	0.1	$3.3 \times 10^9 \pm 1.4 \times 10^8$	3.3×10^8
LIVER			
MOUSE CODE	WEIGHT g	*B. abortus* cfu/g[a,b]	cfu/liver
2BL1	1.5	$1.5 \times 10^8 \pm 6.1 \times 10^6$	2.2×10^8
2BL2	1.6	$9.4 \times 10^7 \pm 2.0 \times 10^6$	1.5×10^8
2BL3	1.5	$6.0 \times 10^7 \pm 2.7 \times 10^4$	9.0×10^7
2BL4	1.7	$1.8 \times 10^8 \pm 4.7 \times 10^6$	3.0×10^8
2BL5	1.2	$1.7 \times 10^{13} \pm 4.7 \times 10^{11}$	2.0×10^{13}
6BL1	1.0	$7.0 \times 10^{11} \pm 2.4 \times 10^{10}$	7.0×10^{11}
6BL2	0.7	ND	ND

[a] Average of triplicate counts.

[b] Standard error of the mean.

ND: Not Done

Mouse infection: Dot blots of Spleen Samples

In this experiment, the DNA from infected spleens, which had been homogenized, filtered, centrifuged, and ethanolprecipitated was suspended in 1 ml double distilled H_2O to determine the ability of the test to detect bacteria in infected tissue. Spun column chromotagraphy using Sephadex G-50 was performed on 100 ul of this solution. The chromatographed DNA solution was dot blotted onto nitrocellulose, hybridized, and detected (Figure 5). Positive controls were purified B. abortus S19 DNA diluted 10 fold serially from 0.1 ng to 100 ng. These indicated that the hybridization was successful and showed the sensitivity of the probe to be below 10 ng but above 1 ng of target DNA (Figure 2). Negative controls included a commercial extract of calf thymus DNA, which would indicate non-specific hybridization and give a qualitative background measure; and mouse spleen DNA from animals injected with PBS only. Five of the uninfected spleen tissues were mixed with 10^9, 10^8, 10^7, 10^6, or 10^5 B. abortus S19 organisms respectively. These showed the lower limit of detection, and also indicated that the filtration technique was successful. The two non-infected mouse spleen tissues were included to give a maximum background.

Mouse infection: Dot blots of Liver Samples

The same controls were used for the livers as above and the samples were handled in the same manner with two exceptions: the PBS control mouse tissue used was liver (Figure 6); and an extra control was used

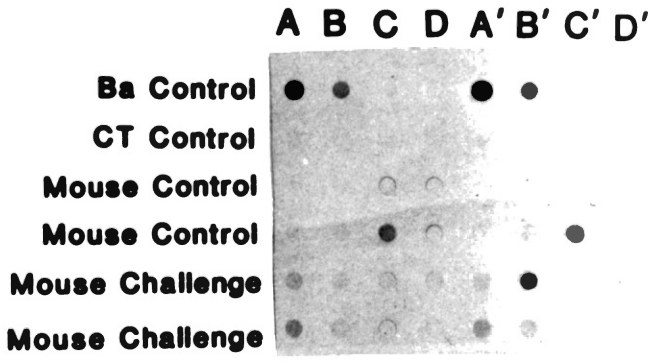

Figure 5. Detection of Brucella abortus 2308S in artificially infected mouse spleen samples using B. abortus S19 DNA as a probe.
Ba. Control: Purified B. abortus S19 DNA spotted onto the nitrocellulose. A. 100 ng, B. 10 ng, C. 1 ng, D. 0.1 ng.
CT control: claf thymus DNA spotted onto the nitrocellulose. A. 1 ug, B. 5 ug, C. 10 ug, D. 20 ug.
Mouse Control (upper): 1 g control mouse tissue was mixed with . abortus S19, DNA extracted, chromatographed with G-50 to remove small contaminating molecules and spotted onto the nitrcellulose. A. Mouse only, no B. abortus, B. Mouse only, no B. abortus, C. 10^5 B. abortus, D. 10^6 B. abortus.
Mouse Control (lower): A. 10^7 B. abortus, B. 10^8 B. abortus, C. 10^9 B. abortus, D. blank.
Mouse challenge (upper): Mouse tissue from infected animals, DNA extracted, passed over G-50 Sephadex, and spotted onto the nitrocellulose, one tissue per dot. A-D. Low dose tissue (7.1 X 10^9). A. 2B1, B. 2B2, C. 2B3, D. 2B4.
Mouse Challenge (lower): A. Low dose (7.1 X 10+9) 2B5, B. High dose (9.0 X 10^{11}) 6B1, C-D. blank.
A'-D'. Duplicate hybridization.

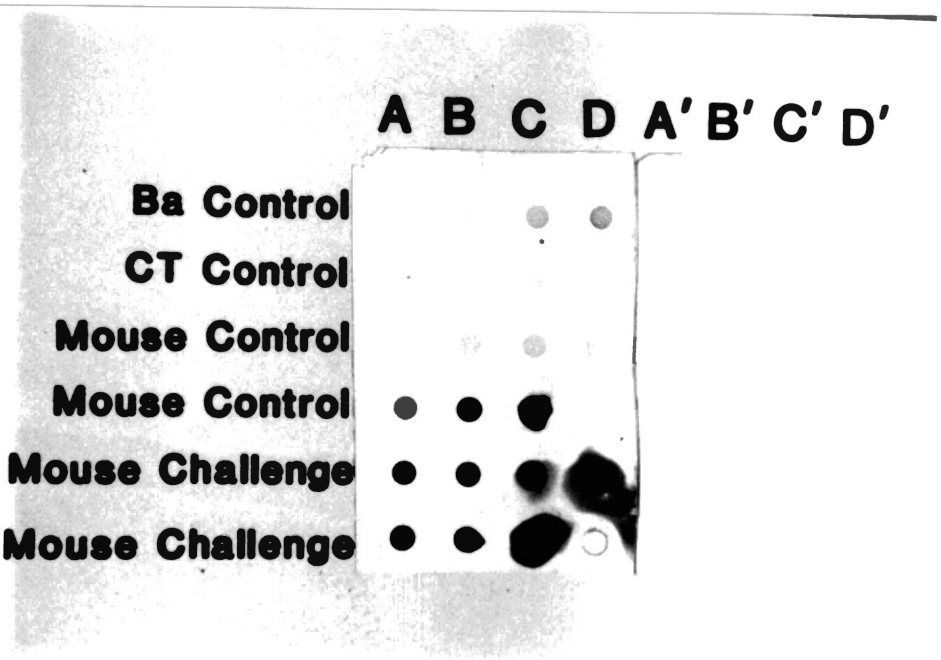

Figure 6. Detection of Brucella abortus 2308S in artificially infected mouse liver samples using B. abortus S19 DNA as a probe.
Ba. Control: Purified B. abortus S19 DNA spotted onto the nitrocellulose. A. 100 ng, B. 10 ng, C. 1 ng, D. 0.1 ng.
CT control: claf thymus DNA spotted onto the nitrocellulose. A. 1 ug, B. 5 ug, C. 10 ug, D. 20 ug.
Mouse Control (upper): 1 g control mouse tissue was mixed with . abortus S19, DNA extracted, chromatographed with G-50 to remove small contaminating molecules and spotted onto the nitrcellulose. A. Mouse only, no B. abortus, B. Mouse only, no B. abortus, C. 10^5 B. abortus, D. 10^6 B. abortus.
Mouse Control (lower): A. 10^7 B. abortus, B. 10^8 B. abortus, C. 10^9 B. abortus, D. blank.
Mouse challenge (upper): Mouse tissue from infected animals, DNA extracted, passed over G-50 Sephadex, and spotted onto the nitrocellulose, one tissue per dot. A-D. Low dose tissue (7.1 X 10^9). A. 2BL1, B. 2BL2, C. 2BL3, D. 2BL4.
Mouse Challenge (lower): A. Low dose (7.1 X 10+9) 2BL5, B. High dose (9.0 X 10^{11}) 6BL1, C. High dose 6BL2, D. blank.
A'-D'. Duplicate hybridization without probe.

which showed the amount of non-label binding of the detection streptavidin after hybridization (Figure 6 A'- D'). This was a replica dot blot which was subjected to hybridization conditions without probe.

Cross-reactivity

Five enteric bacteria, Y. enterocolitica and five fungi, were tested by dot blot for cross-reactivity to the whole genomic B. abortus S19 probe to determine the specificity of the probe. These organisms were chosen for the pilot test because they were easy to work with in the laboratory and Y. enterocolitica has some antigenic cross-reactivity with Brucella (Corbel et al 1975). The positive controls were purified B. abortus S19 DNA diluted 10 fold serially from 1 ng to 100 ng and dot blotted, hybridized and detected to show the hybridization success and the efficiency of the probe. The negative control of calf thymus DNA showed the maximum background for non-specific hybridization. The negative control was dot blotted at the same concentrations as the samples (Figure 7). Only Y. enterocolitica DNA showed any significant cross-reactivity at 1 ug of applied DNA. S. marcescens DNA showed a slight positive reaction at 10 ug concentration.

The fungi also showed no cross-reactivity as can be seen from Figure 8. The reactions were carried out similarly to the enteric filter. All of the detections showed no sign of hybridization with the exception of Candida albicans. However, C. albicans also showed a positive detection in the negative control filter which was treated exactly as the test filter except no probe was added. This control filter showed similar detection for hybridized and non-hybridized C.

albicans DNA. The DNA from the suspect C. albicans was digested several times with proteinase, and extracted several times with phenol/chloroform prior to this hybridization to eliminate contaminating proteins which might react with the detection system. This does not suggest that there is no cross-reactivity between C. albicans and B. abortus but rather that none was detectable in this experiment due to high non-specific detection.

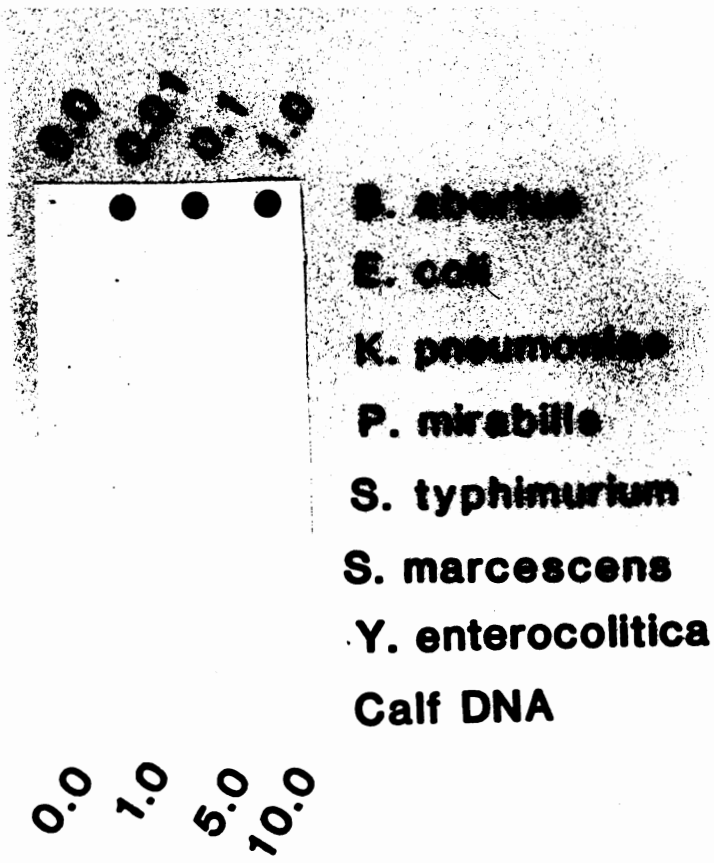

Figure 7. Cross-reactivity between Brucella abortus probe and five enteric species, Y. enterocolitica and calf thymus DNA. The upper scall (0-100 ng) shows increasing concentration of purified B. abortus DNA as a positive control. The lower scale (0-10 ug) shows increasing concentration of the purified test DNA from each organism listed at the right.

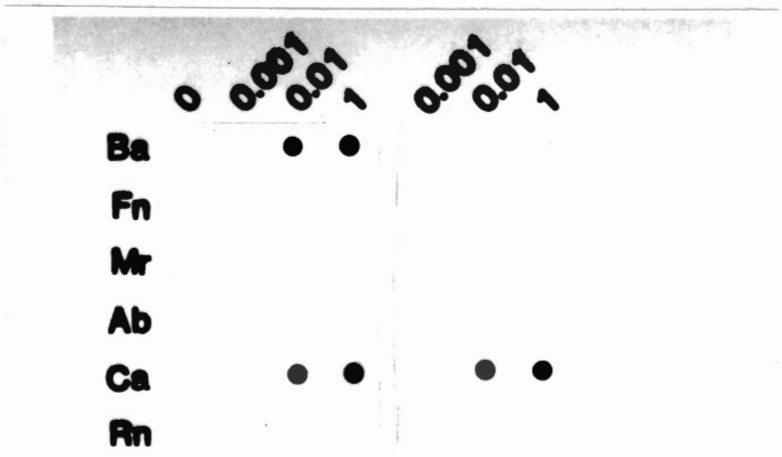

Figure 8. Cross-reactivity between Brucella abortus probe and five fungi. Filter A. Ba. Ten fold serial dilution of B. abortus DNA spotted onto nitrocellulose, from 1 ug down to 0.001 ug with a 0 ug negative control.
The fungi were spotted in quantities of 0.0 ug, 1.0 ug, 5.0 ug, and 10.0 ug. Fn = Filobasidiella neoformans, Mr = Mucor racemis, Ab = Absidia sp., Ca = Candida albicans, Rn = Rhizopus nigricans.
Filter B. Negative control with hybridization conditions and no probe to detect non-specific detection.

CHAPTER IV

DISCUSSION

The purpose of this study was to determine if the detection of B. abortus using biotin labeled DNA as a molecular probe was feasible. I have shown that, by using special filtration techniques, B. abortus can be preferrentially detected in infected tissue using a biotinylated, B. abortus DNA probe.

In order for a detection test to be feasible, two important criteria must be met: sensitivity and specificity. The results indicate that it is possible to get relative enrichment of bacterial DNA over contaminating mammalian DNA in a complex mixture by differential filtration. The filtration experiment shows that a reduction of up to 73% in mammalian DNA was achieved while a reduction of only 56% of bacterial DNA was observed. In relative terms, this means that for a complex mixture containing equal numbers of mammalian cells and bacterial cells, which would have DNA equivalents of about 1000 ug:1 ug respectively, the filtration would reduce the mammalian DNA to 26 ug and the bacterial DNA to ~440 ng. It has been shown that there can be in excess of 10^{13} B. abortus cells per gram of infected, aborted fetal tissue (Alexander et al 1981). This is about 1 cell of B. abortus cell for every mammalian cell in the infected tissue.

In practical terms, 1 g of mammalian tissue contains 5 X 10^3 ug of DNA. I determined this by the extraction of DNA from rat liver using

standard proteinase/SDS digestion followed by phenol extraction. The quantity of DNA was determined by spectral absorption at 260 nm. If this tissue were infected with 10^5 B. abortus/g which contains 100 ng of DNA, and was subsequently subjected to the filtration technique specified here, the resulting relative amounts of DNA would be 1300 ug of mammalian DNA, and 44 ng of B. abortus DNA. This amount of DNA, when resuspended in 1 ml of buffer would give 130 ug of DNA in a 100 ul dot blot sample. Twenty-five ug of this DNA would bind to the nitrocellulose, giving a final amount of bacterial DNA to be detected of 9 ng. The limit of our probe sensitivity was 1 ng. Therefore, this technique is 9 times more sensitive than necessary to detect 10^5 organisms per gram of tissue.

I have developed a system which allows for a reduction of the amount of contaminating mammalian DNA without a similar, concomitant decrease in the target bacterial DNA. The relative decrease is such that 10^5 B. abortus organisms/g can be detected in infected tissue. This system consists of homogenization of infected tissue in the presence of sucrose and Triton X-100 to disrupt the cells but leave the nucleus intact. The homogenate is then filtered through a prefilter and a five micron pore size filter to remove the intact mammalian nuclei while allowing the B. abortus cells to pass through. The DNA is then extracted from the filtrate, denatured and spotted onto nitrocellulose. The immobilized DNA is then probed (hybridized) with whole genomic B. abortus DNA which has been labeled with biotin. The biotin is then detected by an enzyme linked assay using avidin and alkaline phosphatase. The sensitivity in this system is such that B. abortus can

be detected in infected tissues at levels of bacteria reported to be present in aborted fetuses (Alexander, et al 1981).

The experiments show that 10^5 B. abortus organisms can be detected when mixed with 1 g of mammalian tissue (Figure 5). It has also been shown, through the mouse infection experiment that 5.0×10^7 B. abortus organisms/g of infected spleen and 9.4×10^7 B. abortus organisms/g of infected liver can be detected. These results can be related to the detection of B. abortus in aborted fetuses since the organs were infected rather than simply mixed with the bacteria. This level of detection is also in line with results on other bacteria: Kuritza et al were able to detect 10^6 Bacteroides in human stool samples using ^{32}P-labeled DNA probes (Kuritza et al 1985). I have not been able to find any studies published in the major literature in which bacteria has been detected from within infected tissue by the use of biotin labeled DNA.

Another aspect of sensitivity is signal to noise ratio. For detection of DNA using biotin, signal to noise ratio is the amount of signal (color development) due to streptavidin attaching either to endogenous biotin, or non-specifically to a non-DNA contaminant. To determine the signal to noise ratio, a control experiment was run. This control had all conditions equivalent to the experimental dot blot except no probe was added during the hybridization. The control is shown in Figure 6 A'-D'. Little color development is evident due to the passing of the samples over G-50 Sephadex to remove contaminating biotin prior to application to the nitrocellulose.

When the samples were not passed over G-50, high backgrounds were

observed. The results of negative controls such as extracted mouse DNA and calf DNA show no color development after hybridization with the probe; while purified B. abortus DNA and extracted B. abortus DNA both give excellent color development, an easily seen blue dot results that makes distinction of positives unambiguous (Figures 5, 6). The assay is reliable but as can be seen in the duplicate hybridization in Figure 5, some variation in color development is possible on occasion. The difference is in degree only and did not affect the interpretation of the results as the positives are still darker than negative controls.

The second important criterion is specificity. Using a whole genomic probe can cause problems with cross-reactivity. Cross-reactivity between the DNA of different species can occur due to the presence of genes with similar function; unrelated genes which have converged on a sequence, or even the retention of extraneous sequences which once were similar or were different and, due to mutation, acquired similar sequences. One method of circumventing these problems is to use a more specific set of B. abortus sequences; this would, however, reduce the sensitivity. De Ley has shown that the evolutionary neighborhood of the genus Brucella is sparsely populated, with certain Agrobacteria and CDC group V bacteria being the closest (De Ley et al1987). These evolutionarily close bacteria are not commonly found in cattle.

The cross-reactivity of the probe was tested by hybridization with DNA from common enteric contaminants as well as Y. enterocolitica DNA. Y. enterocolitica has been shown to have some antigenic cross-reactivity with B. abortus (Corbel et al 1975). The results indicate that the enteric organisms have practically no sequences in common with

B. abortus while the Y. enterocolitica has 0.1-1.0% cross-hybridization. Given this cross-hybridization, it would be possible to discount the significance of Y. enterocolitica contamination since the organism is not a normal inhabitant of the bovine reproductive tract; and any contamination with this organism would have to be of several orders of magnitude greater than a B. abortus infection to be detectable. Therefore, there is little difficulty in using a whole genomic probe for diagnostic purposes. In order to completely negate the possible effects of Y. enterocolitica contamination, however, hybridization subtraction chromatography could be performed to remove the 0.1-1.0% of the sequences which are in common (Vitek et al 1981).

Other considerations concerning the feasibility of using this detection test are warranted. Any new test needs to be faster, more accurate, less expensive, easier, more versatile, more reliable than the currently employed bacteriological or immunological techniques; or any combination of the above to be practical in a clinical setting. This test presently is faster than the current bacteriological methods which can take up to 2 weeks and many man hours to obtain an answer. The organism can be detected in infected tissue in two days with increased probe concentration. This test is more accurate, in a clinical setting analyzing aborted tissue, than the immunological methods used since it detects the presence of the organism directly, rather than the presence of circulating antibody. The versatility is evident, in that, by using the filtration system, we can increase any infecting bacterial DNA over mammalian DNA and thus screen for several different organism from a sample rather than just one. This would possibly be less expensive than

bacteriological methods since a single test would be used rather than the myriad biochemical tests now employed for bacteriological differentiation.

Summary

In summary, I have developed a technique which was capable of detecting 10^8 B. abortus organisms in artificially infected mouse tissue. This technique utilizes physical separation of bacteria from the mammalian DNA, immobilization of the extracted DNA on nitrocellulose, hybridization of the immobilized DNA with biotin labeled B. abortus DNA and detection with an enzyme-linked assay. assay

The reason for the research was to develop a detection method which can be applied in a clinical setting. For the technique to be useful, subsequent experiments need to be applied to field samples.

More research needs to be performed to improve the reliability of these preliminary results. The detection level is dependent upon several factors including the amount of biotin labeling of the probe DNA. Two probes of the same DNA, prepared at different times may give different sensitivities due to a difference in biotin labeling. This problem can be circumvented by careful screening of the probes prior to use.

Future research should focus on the possible elimination of the need for physical filtration. The present method, although adequate for the present study, may prove rather cumbersome and time consuming when applied to field samples due to the scarring of fetal tissue during the infection. This scarring could lead to an increase in connective

tissue which could clog the filters and prevent proper filtering and thus proper separation of materials. Hybridization subtraction chromatography could be used using calf thymus DNA attached to cellulose to remove the mammalian DNA without the need for filtration.

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VITA

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