Sudan J. Vet. Res. Vol 5 1983

SEROLOGICAL DIAGNOSIS OF BOVINE BRUCELLOSIS: CLASS AND SUBCLASS SPECIFIC ENZUME LINKED IMMUNOSORBENT ASSAY

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INTRODUCTION

Diagnosis in bovine brucellosis depends on isolation and identification of the causative agent or demonstration of specific antibody using serological tests. The difficulty in reaching positive identification of infected animals arises from the fact that there is a long incubation period which varies widely among individuals. The incubation period may be shorter only when infection has taken place late in pregnancy. There were animals which remain negative to all tests weeks after infection (Kerr 1983, Miller 1971, and Robertson 1971). Lapraik et al (1975) and Wilesmith (1978) found that a small number of heifers had become serologically positive during or just after their first pregnancy although they harbored the organisms since their birth. As an approach to overcome the problem posed by the incubation period a large number of serological tests have been developed for the diagnosis of bovine brucellosis and in attempts to increase their sensitivity without sacrificing specificity. Still these tests have a number of limitations. SAT gives negative results on sera from animals from which brucella can be isolated (Alton et al 1975a) and (Hess 1953). In SAT they were due to non-specific agglutinins. The CFT is probably the most accurate test in widespread use but prozoning occurs. Prozoning is due to abnormally high ratio of specific IgG2 to specific IgG1 and in some sera the high level of IgG2 block CFT (Plackett and Alton, 1975, Mc Naught et al 1977)/

The purpose of this study was to quantify and characterize the specific immunoglobulin produced in response to Brucella abortus in recently infected cows using different serological tests, with a view towards improving diagnostic efficiency.

MATERIALS AND METHODS

The standard serum tube agglutination test (SAT) (USDA) was performed as described by Alton et al (1975b) with the 1:200 dilution being the highest tested. Results were recorded as positive, incomplete (1) or negative.

The card test (buffered brucella antigen test) was performed as described by Alton et al (1975b). Weak reactions on the card test were considered positive.

The rivanol test (USDA) was done according to the supplemental test procedures for the diagnosis of brucellosis, NADL Diagnostic Reagents, Manual65E. USDA, NADL, Diagnostic Services, Diagnostic Reagents, Ames, Lowa. The maximum serum dilution used was 1:200.

The complement fixation test (CFT) was a microtiter adaptation of the cold fixation method described by Jones et al (1963). The standard B. abortus tube agglutination antigen was diluted 1:500. 1+ fixation (75% haemolysis) at 1:40 dilution of test serum or higher was considered positive.

The indirect haemolysis test (IHLT) was performed according to Jones et al (1980). Lysis or 75% or more of the cells at a serum dilution of 1:10 or higher was considered positive.

The radial immunodiffusion test (RID) was perfomed as described by Diaz et al. (1979) using a concentration of 200 ug/ml of polysaccharide B (poly B). The results were recorded every hour for the first 8 hours and left over night and read through the next day's working hours. The readings were based on a positive or negative visual evaluation. Positive and negative controls were usually added each time the test was done.

Enzyme linked immunosorbent assay (ELISA) was performed as described by Lamb et al (1979). The antigens used were B. abortus smooth lipopolysaccharide (S-LPS) solution at 1 ug/ ml in 0.06 M carbonate buffer (pH 9.6) and brucella rough lipopolysaccharide (R-LPS) solution at the same concentration but in a barbital acetate buffer (pH 4.6).

Rabbit anti-bovine IgM, IgG, IgG1 and IgG2 were purchased from Miles, Elkhart, Indiana. The conjugates were prepared with these antisera according to the method of Nakane and Kawaoi (1974), modified to eliminiate the final gel filtration step for separation of bound and unbound enzyme, as suggested by Saunders et al (1977). All the conjugate dilutions were made in phosphate buffered saline (pH 7.2) - 0.05% Tween 20. Working substrate was made up fresh and consisted of 1ml of 2% O-phenylenediamine (Eastman Kodak Co.) in methanol, 99ml of distilled water and 0.1ml of 3% H2 O2. The optical density (OD) was measured at 490nm.

Immunoelectrophoresis (IEP) and immunodiffusion (ID) were used to test the rabbit anti-bovine IgM, IgG1, and IgG2 against whole bovine serum with veronal buffer (pH 8.6) in IEP while in ID the buffer was a borate buffer (pH 8.3).

The sera were from animals in a large, recently infected herd. The entire herd was bled in a regular fashion. The herd belonged to a large family dairy operation of 1544 cows when first tested. There was no history of vaccination

against brucellosis. The first reactor was found on a market cattle test performed. The serum sample was traced back to the herd of origin, the whole herd was tested and 26 cows were found to be reactors using the card and the milk ring tests. The results were confirmed in all these cases using the complement fixation test. The authorities decided to slaughter those reactors and retest the whole herd every month to see if they could eliminate infection. Four to six weeks later the numbers of reactors detected were 62 and 55, respectively. The authorities decided after that to retest every 15 days which led to detection of more reactors. The total number of reactors disclosed in a five month period was 437. These results were based on card, SAT, CFT, and serum plate agglutination test. Sera were available from animals that became reactors after being negative on the card and other standard tests or having titers below the diagnostic level on those tests. These sequential serum samples were used in class and subclass specific ELISA to evaluate and characterize the immunoglobulins produced in such sera in response to B. abortus. from milk or tissue samples from this herd was confirmed.

RESULTS

The strategy of this investigation was to look for a more sensitive indicator of infection in recently infected animals. The need was demonstrated by the fact that in this study 36 animals identified as card and SAT or CFT and SAT 2 4 weeks earlier. The highest SAT titer detected on them was incomplete at 1:100 and the highest CFT titers was 1+ at 1:20. Results using SAT, CFT, IHLT 1, IHLT-2 and RID are summarized in Table 1. Sixteen of these 105 sera were negative on the card test and properly identified as reactors by the CFT or SAT. The CFT was negative with 11 sera which had titers below + at 1:50 on the SAT but none of them was completely negative on SAT. In all 105 sera tested, IHLT-2 was more sensitive than IHLT-1 permitting the identifcation of 18 more sera as positive than IHLT 1. On these 105 early reactors the RID test identified only 18 as reactors. The specificity of the rabbit antibovine IgM, IgG1, and IgG2 used to prepare conjugates for the ELISA was tested by IEP and ID. Both rabbit anti-bovine IgM and IgG showed characteristic lines by both techniques while IgG1 and IgG2 showed characteristic lines by IEP and no line was observed on ID. The selection of appropriate dilutions of sera and conjugates was based on preliminary tests using several dilutions of sera and conjugates. Class and subclass specific ELISA was done on all 36 sera when they were done on antigen and serum controls. There was substantial specific binding to both S-LPS and R-LPS for all classes and subclasses tested with sera from the infected animals and those below the reactor levels on

the conventional tests. The ranges and mean OD's with both antigens are shown in Tables 2 and 3 for comparison with the other group tested. In this ELISA the optical density is proportional to the amount of conjugate bound to the antigen-antibody complexes and proportional to amount of anti-lipopolysaccharide of that specific isotype. Competion between immunoglobulin classes and subclasses for antigen binding sites on S-LPS was observed with some sera with high IgM binding ie using concentrated serum sample (1:50 dilution) showed low OD values for IgG, IgG1 and IgG2 but when more diluted sera were tested (1:250 dilution), there was an increase in the OD with the IgG, IgG1 and IgG2 conjugates. This may have been due to more efficient specific binding by IgG which blocks the binding of the other immunoglobulin classes at high concentrations.

DISCUSSION

A number of serological tests are applied now in the diagnosis of bovine brucellosis. The ability of the test to avoid false positive and false negative reactions is one step towards high sensitivity and specificity which are needed in a good test. Unfortunatly all serological tests applied in negative brucellosis are susecptible to false positive and false neative reactions.

Plackett et al (1976) showed that the IHLT-1 had a disadvantage of being unable to detect recent infection. In this study, IHLT-1 was found to be entirely negative on 14 sera, which were identified as reactors by other methods. The results in Table 1 show IHLT-2 was more sensitive than IHLY-1 and this agrees with previous findings (Jones et al 1980). The CFT became negative earlier in calfhood vaccinated animals than the IHLT which made the CFT a better to differentiate between vaccinated and infected animals.

The use of class and subclass specific ELISA on sera from infected animals with titers below the reactor level and negative controls (Tables 2 and 3), gave a clear indication of a rise in all classes and subclasses of specific anti B. abortus antibodies in both the infected group of sera and those which showed a titer below the reactor level according to the conventional tests. ELISA showed high binding for both S-LPS and R-LPS with the infected group of sera and those which showed titer below the reactor level on conventional tests and made it possible to identify the immunoglobulin raised against B. abortus in the latter group of sera approximately 2-4 weeks before all other conventional tests. The greater sensitivity of ELISA was demonstrated also by the studies of Carlesson et al (1976), who found ELISA to be 10 to 100 fold more sensitive than SAT, as well as Saunders et al (1977) who found it more sensitive than the other tests.

There are major differences on ELISA done at different laboratories due to differences in the nature of

the antigen used and the isotype tested in those studies. The nature of the anti-immunoglobulins, their dilutions and the specificity of the different conjugates also vary among described procedures. Byrd et al (1979) found ELISA to be less sensitive than SAT on sera from infected animals and also Ruppanner et al (1980) using USDA tube agglutination antigen, found a CFT able to detect antibodies to Brucella abortus at earlier stage than SAT and ELISA. These two reports contradict the results obtained (using the same antigen with differences in the sera and the concentrations of antigen and conjugates) by other researchers (Heck et al 1980, Stemshorn 1980 and Bullock and Walls 1977) who found ELISA to be more sensitive than the other test now in use to diagnose bovine brucellosis.

Competition or steric blocking among different isotypes tested was reported by Lamb et al (1979) and was confirmed by this study. Tedder and Hoffmann (1981) found the binding of specific bovine antibodies of the different immunoglobulin classes and subclasses to B. abortus antigens, non-linear and they suggested that the non-linearity due to the competition between antibodies for antigen sites. The competion was also observed by Bruins et al (1978) on a work done on mutants of Salmonella minnesota and also by Vos et al (1979) when they used ELISA to quantitate IgM and IgG antibodies to LPS. The use of both S-LPS and R-LPS in this study showed that the smooth LPS binds more antibodies of all isotypes in these sera than R-LPS, however there was a substantial amount of specific binding of all isotypes to the R-LPS. This is best explained on the basis of shared antigenic determinants between the S-LPS and the R-LPS. An ELISA that measures the response to more than one class or subclass of immunogolbulin as in this study gives a more complete understanding of the ELISA assay for bovine immunglobulin response to B. abortus than the use of a single dilution or class of reagent as was done in other experiments.

SUMMARY

In the management of bovine brucellosis eradication programs, a herd history of recently introduced and rapidly spreading infection is often associated with epizootiological and immunological problems. The problems arise because infected cows, with titers below the reactor levels with standard diagnostic tests performed at frequent intervals, are usually left in the herd, and constitute a continuous reservoir. Later on they become reactors, but will already have served as a source of further transmission. A battery of standard diagnostic tests (serum tube agglutination test (SAT) rivanol test (RIV), radial immunodiffusion test (RID), indirect haemolysis test (RID), indirect haemolysis test (IHLT), complement fixation test (CFT), and the card test and class and subclass specific enzyme-linked

immunosorbent assay (ELISA) were used on sera from such a herd. Conjugates of rabbit anti bovine IgG, IgG1, IgG2 and IgM were used in the ELISA. Class and subclass specific ELISA was found to permit identification of such sera as positive appoximately 2-4 weeks earlier than the conventional tests.

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Table 1. Results of serological tests with sera from 105 recently infected cattle.

Card		SAT		CFT		IHLT-1a			IHLT-2b			RID	
+	-	> +1:100	negative	> 1+1:40	negative	> 3+1:16	< 3+1:16	negative	> 3+1:16	< 3+1:16	negative	+	
89	16	93	12	94	11	57	34	14	75	26	4	18	87

a = only guinea pig complement
b = a mixture of equal parts of guinea pig and rabbit complements

Table 2. Immunoglobulin class and subclass specific ELISA with Rough and Smooth Brucella LPS and 1:50 dilution of sera from infected, supspicious and negative cattle.

Animal Groups	Antigens	Conjugates IgM 1:600	IgG 1:750	IgG1 1:600	IgG1 1:400	IgG2 1:250	IgG2 1:100
		(a)			(b)		(b)
Infected	S	.462(.382-.517)	.643(.589-.684)	.302(.245-.379)	.325(.305-.345)	.216(.125-.258)	.212(.204-.219)
(N=18)	R	.386(.296-.401)	.513(.459-.555)	.205(.135-.276)	.256(.206-0299)	.165(0119-.205)	.162(.147-.178)
Below					(c)		(c)
Reactor	S	.406(.341-.496)	.413(.203-.526)	.221(.106-.307)	.293(.207-.391)	.135(.065-.183)	.167(.103-.218)
Level							
(N=36)	R	.307(.240-.360)	.329(.134-.390)	.200(.095-.270)	.221(.126-.275)	.099(.062-.147)	.135(.081-.178)
					(d)		(d)
Negative	S	.139(.091-.148)	.055(.041-.065)	.041(.029-.057)	.049(.031-.060)	.036(.028-.051)	.039(.031-.051)
(N=42)	R	.092(.081-.139)	.045(.038-.060)	.038(.027-.050)	.040(.029-.058)	.031(.023-.041)	.032(.027-.044)

N= numbers tested, R= Rough-LPS, S= Smooth LPS
(a) Numbers are mean and (range) of OD at 490 nm
(b) N=6
(c) N=20
(d) N=16

Table 3. Immunoglobulin class and subclass specific ELISA with Rough and Smooth Brucella LPS and 1:250 dilution of sera from infected, supspicious and negative cattle.

Animal Groups	Antigens	Conjugates IgM 1:600	IgG 1:750	IgG1 1:600	IgG1 1:400	IgG2 1:250	IgG2 1:100
		(a)			(b)		(b)
Infected	S	.345(.282-.413)	.563(.432-.628)	.299(.145-.303)	.306(.298-.313)	.146(.111-.197)	.201(.148-.253)
(N=18)	R	.241(.184-.269)	.371(.279-.432)	.200(.121-.232)	.234(.224-.244)	.114(.224-.244)	.148(.113-.184)
Below					(c)		(c)
Reactor	S	.287(.192-.396)	.289(.217-.387)	.149(.080-.192)	.237(.163-.348)	.089(.063-.123)	.131(.088-.182)
Level							
(N=36)	R	.227(.112-.246)	.204(.134-.291)	.199(.061-.129)	.169(.111-.226)	.071(.060-.112)	.102(.065-.158)
					(d)		(d)
Negative	S	.112(.088-.142)	.062(.038-.084)	.041(.028-.051)	.040(.029-.051)	.031(.021-.049)	.033(.024-.039)
(N=42)	R	.089(.087-.131)	.050(.031-.062)	.03_(.029-.041)	.037(.028-.046)	.029(.020-.039)	.030(.020-.041)

N= numbers tested, R= Rough-LPS, S= Smooth LPS
(a) Numbers are mean and (range) of OD at 490 nm
(b) N=6
(c) N=20
(d) N=16