Fluorescence Polarization Detection of Brucella abortus and Mycobacterium bovis specific antibodies Using Kits from Diachemix

Veterinary Diagnosis of Brucellosis and Tuberculosis

Paul Held Ph.D., Principle Scientist, BioTek Instruments, Inc., Winooski, VT
Michael E. Jolley Ph.D., Diachemix Corporation

Brucella abortus and Mycobacterium bovis are zoonotic pathogens that cause Brucellosis and Tuberculosis in cattle, respectively. Besides being a serious public health threat, these agents cause significant economic losses throughout the world. Here we use the fluorescence polarization capabilities of the Synergy™ 2 Multi-Mode Microplate Reader to run Brucella and Mycobacterium bovis antibody detection kits from Diachemix.

Introduction

Contagious diseases in livestock herds represent a serious public health threat as well as causing significant economic loss. Brucellosis infection in ruminants is highly contagious and causes abortion or premature calving of recently infected animals. Mycobacterium bovis (M. bovis) is a slow growing anaerobic bacterium that is the causative agent for tuberculosis in cattle. Both of these agents have the ability to infect a number of other animals including humans. The presence of antibodies against these pathogens in bovine serum is indicative of infection.

Fluorescence polarization (FP) is a fluorescence detection technique first described in 1926 by Perrin. It is based on the observation that fluorescent molecules, when excited by polarized light, will emit polarized light. In solution, the polarization of the emitted light is inversely proportional to the molecule's rotational speed, which is influenced by solution viscosity, absolute temperature, molecular volume and the gas constant. If one keeps viscosity and temperature constant, then the key variable for rotational speed differences is molecular volume or molecular weight.

Fluorescence polarization (FP) measurements are made through two different polarizing filters that are parallel and perpendicular to the plane of the polarized excitation source. Polarization values for any fluorophore complex are inversely related to the speed of molecular rotation of that complex.

Because the speed of rotation is also inversely related to the size of the molecule, polarization values will be high with large molecule complexes, and low with small molecules. The fluorescence intensity of the emitted light is monitored through both parallel and perpendicular filters.

The degree of transfer of emission intensity from parallel to perpendicular is proportional to the rotational speed of the fluorescent molecule. If a molecule is very large, there is very little movement during excitation and thus little transfer from the parallel signal to the perpendicular signal. However, if the fluorescent molecule is small, rotational speed is faster, resulting in a greater degree of transfer from the parallel to perpendicular signal.

Basis for Assays

Brucella-specific antibodies are detected using an O-polysaccharide (OPS) extract conjugated with fluorescein. Lipopolysaccharides are constituents of the bacterial cell surface and serve as antigenic epitopes for host antibodies. The OPS-fluorescein conjugate is relatively small in size and as such can rotate freely in solution. This results in a relatively small polarization value when measured. If antibodies specific to OPS are present, they will bind to OPS and effectively make the molecule complex much larger. This in turn increases the polarization value.

Mycobacterium bovis specific antibodies are detected in a very similar fashion. This test uses a fluorescein-labeled peptide that corresponds to the amino acid sequence of an epitopic region of the bacterium MPB70 protein. When epitope specific antibodies bind to the fluorescein-peptide conjugate, the polarization increases (Figure 1).

Key Words:

Brucellosis

Tuberculosis

Diagnostic

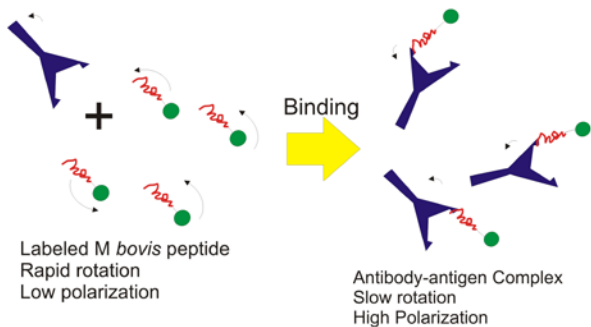Microplate

Fluorescence Polarization

Figure 1 illustration.

Figure 1. Illustration of the Antibody-Tracer binding of Mycobacterium Bovis Antibody Test.

Materials and Methods

Both Mycobacterium bovis FPA Antibody and Brucella FPA Antibody test kits were from Diachemix (Milwaukee, WI). Kit positive and negative controls were assayed as per the kit instructions. With the M. bovis antibody test kit, 100 µL of reaction buffer was added to all wells, then 100 µL of positive control or buffer (negative control) was added and mixed with care to avoid bubbles. The plate was incubated for 30 minutes at room temperature and 10 µL of conjugate was added to all wells and mixed thoroughly. After a 10-minute incubation, the fluorescence polarization was determined.

Brucella antibody determinations were made using a similar procedure. Briefly, 20 µL aliquots of samples and controls were placed in the wells of a microplate along with 180 µL of reagent buffer. After incubating for 5-60 minutes, 10 µL of conjugate was added to all the wells and the plate incubated for 2-60 minutes at room temperature and the fluorescence polarization determined.

The polarization of samples was determined using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT), equipped with the fluorescence polarization (FP) and time-resolved fluorescence (TRF) optical modules. Parallel and perpendicular polarized fluorescence values were obtained using a 485/20-excitation filter and a 528/20-emission filter with a 510 nm cut off dichroic mirror. In accordance with Diachemix' setup recommendations; the reader's xenon flash lamp (TRF module) was used. The PMT sensitivity was set such that raw parallel measurements were approximately 30,000 RFUs. The reader was controlled and polarization values automatically calculated using Gen5™ Data Analysis Software with an instrument G factor of 0.85.

The Synergy 2 reader function is controlled using Gen5 Data Analysis software (BioTek Instruments). Besides controlling the reader, the software is capable of performing polarization calculations, assay validation, reporting assay results, and generating a color-coded report that easily identifies positive, negative and equivocal sample results (Figure 2).

Figure 2 Gen5 Sample Results Report.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	SPL5	SPL13	SPL21	SPL29	SPL37	SPL45					
	1	26	153	195	37	198	197					
	---	POS	POS	POS	POS	POS	POS					
B	NC	SPL6	SPL14	SPL22	SPL30	SPL38	SPL46					
	2	199	146	133	46	1	198					
	---	POS	POS	POS	POS	---	POS					
C	NC	SPL7	SPL15	SPL23	SPL31	SPL39	SPL47					
	0	156	140	155	227	227	216					
	---	POS	POS	POS	POS	POS	POS					
D	PC	SPL8	SPL16	SPL24	SPL32	SPL40	SPL48					
	193	202	175	172	191	46	158					
	POS	POS	POS	POS	POS	POS	POS					
E	SPL1	SPL9	SPL17	SPL25	SPL33	SPL41	SPL49					
	6	231	196	152	12	268	203					
	---	POS	POS	POS	suspect	POS	POS					
F	SPL2	SPL10	SPL18	SPL26	SPL34	SPL42	SPL50					
	181	189	201	214	238	217	211					
	POS	POS	POS	POS	POS	POS	POS					
G	SPL3	SPL11	SPL19	SPL27	SPL35	SPL43	SPL51					
	18	206	187	185	211	155	202					
	suspect	POS	POS	POS	POS	POS	POS					
H	SPL4	SPL12	SPL20	SPL28	SPL36	SPL44	SPL52					
	160	4	135	97	205	161	3					
	POS	---	POS	POS	POS	POS	---					

Figure 2. Gen5 Sample Results Report.

Results

The polarization values of negative and positive controls, as well as the OPS-fluorescein tracer only for the Brucella test kits were determined and compared. As demonstrated in Figure 3, the positive control had a mean value of 222.8 mP, while the Tracer and negative control had mean values of 86.1 mP and 89.2 mp respectively. A positive sample, such as the positive control has a significantly higher polarization value than the negative control or the fluorescent tracer only samples. A sample that has a polarization value of 20 mP greater than the negative control is considered a positive sample.
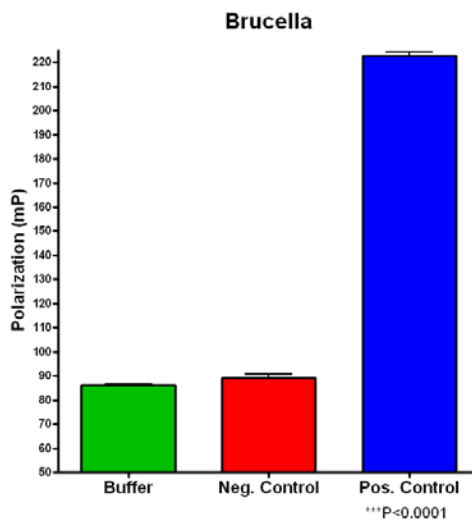
Figure 3 Brucella bar chart.

Figure 3. Comparison of Brucella Positive and Negative Control Polarization Values.

When the polarization values of the tracer and positive control of a Mycobacterium bovis assay kit are compared similar results are observed (Figure 4). In these experiments, the positive control had a mean polarization value of 137.8 mP, while the tracer only was 72.4 mP. With this test kit, samples with a polarization value that is 15 mP greater than the negative control are considered to be positive.

The accuracy and specificity of the Brucella test with bovine serum samples is demonstrated in Table 1. When a series of samples from the National Veterinary Service Laboratories (NVSL) enriched for Brucella positive samples is compared to random bovine serum samples a marked difference in the number of positive samples identified is observed. The general bovine population has not been infected with Brucella, which is reflected in the low number (2) of positive samples identified out of 920 tested samples. However, when true positive samples are present, as is the case with the test group from the NVSL, they are identified accordingly.

Determination	NVSL serum bank	Random samples
Negative	4	918
Suspect	2	----
Positive	38	2
Total	44	920

Table 1. Analysis Statistics from Brucella Testing.

Discussion

These data indicate that the fluorescence polarization kits provided by Diachemix in conjunction with the Synergy™ 2 Multi-Mode Microplate Reader provide an excellent platform for performing these veterinary tests. The assays are quick and easy to run, while the Synergy 2 Multi-Mode Reader automatically performs the fluorescence polarization measurements. While only two different test kits were demonstrated, Diachemix offers a range of FP-based kits that can be quantitated using the Synergy 2 reader.
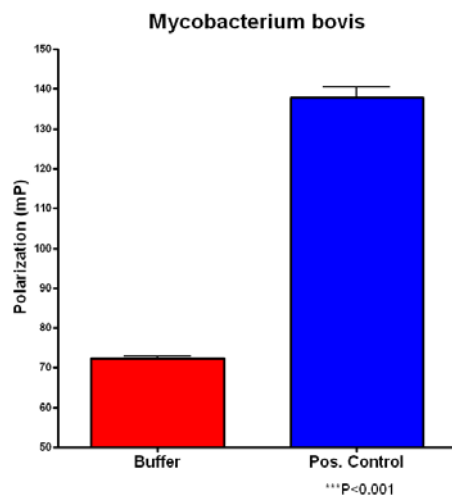

Figure 4. Comparison of Mycobacterium bovis Positive and Negative Control Polarization Values.

Fluorescence polarization technology provides very rapid answers with minimal effort. The homogeneous assay technology does not require any washing steps, which not only speeds up the assay process, but also eliminates the need for additional instrumentation. The assays are easy to run, require few reagent additions, and yet provide accurate quantitative results with a sensitivity and specificity equivalent to or greater than other existing methods.

The polarization value of the tracer is approximately 75-80 mP, for either assay conjugate, whereas the estimated value for unconjugated fluorescein is approximately 20 mP. This difference reflects the size difference between the molecule fluorescein and the addition of the antigenic epitope OPS or peptide for Brucella or M. bovis respectively. More importantly is the effective range of the assay, which is reflected in the difference in polarization values of the unbound tracer molecule and when the tracer is completely bound. This difference is related to the relative change rather than the absolute change in size of the complex upon binding. In other words, if the tracer by itself were a large molecule, binding would change the polarization value by a small degree. Likewise, if the tracer binds a small molecule little changes in polarization will be observed. The Brucella and M. bovis antibody detection test kits have been designed such that change in polarization upon binding is significant. The large effective range provides superior sensitivity when only small numbers of antibodies are present.

The Brucella kit has been approved by several different organizations for the testing of animals. The United States Department of Agriculture (USDA) has approved its use for the testing of cattle, bison and swine sera and has listed it as an official test for both screening and confirmation. The World Organization for Animal Health (OIE) has approved the test for bovine and as an alternative test for small ruminants. In addition, it is pending approval by the European Union (EFSA) for the testing of bovine and small ruminant series. The Mycobacterium bovis assay kit is currently under review in Canada, United States, South Africa, Greece, and other countries around the world.

References

[1] Perrin, F. (1926) Polarization de la Lumiere de Fluo-rescence. Vie Moyenne de Molecules dans L'etat Excite. J. Phys Radium 7:390.

[2] Nasir MS, Jolley ME (1999). Fluorescence polarization: an analytical tool for immunoassay and drug discovery. Comb. Chem. High Throughput Screen. 2(4), 177-90. Review.

[3] Jolley ME, Nasir MS, (2003) The use of fluorescence polarization assays for the detection of infectious diseases Comb. Chem. High Throughput Screen 6(3):235-44.

[4] Nielsen K, Lin M, Gall D, Jolley M (2000) Fluorescence polarization immunoassay: detection of antibody to Brucella abortus Methods, Sep;22(1):71-6.

[5] Lin M, Sugden EA, Jolley ME, Stilwell K. (1996) Modification of the Mycobacterium bovis extracellular protein MPB70 with fluorescein for rapid detection of specific serum antibodies by fluorescence polarization. Clin Diagn Lab Immunol. Jul;3(4):438-43.

[6] Jolley ME, Nasir MS, Suruballi OP, Romanowska A, Renteria TB, De la Mora A, Lim A, Bolin SR, Michel AL, Kostovic M, Corrigan EC (2007) Fluorescence polarization assay for the detection of antibodies to Mycobacterium bovis in bovine sera. Vet. Microbiol.120:113-121.