The Use of Fluorescence Polarization Assays for the Detection of Infectious Diseases

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Abstract: Fluorescence Polarization Assays (FPAs) have been shown to have great utility in the detection of infectious diseases. Examples are presented of the use of O-polysaccharides (OPSs) for the detection of antibodies in serum, whole milk and whole blood to gram negative organisms (*Brucella* spp., *Salmonella* spp.). The use of proteins and peptides are also described for the detection of *Mycobacterium bovis* and Equine Infectious Anemia Virus. Fluorescence Polarization Inhibition Assays (FPIAs) are discussed for the specific and sensitive detection and quantitation of *Salmonella* spp. cells from culture. An example of the detection of enterohemorrhagic *E. coli* (EHECS) by Strand Displacement Amplification (SDA), coupled with FP, down to the single cell level, within thirty minutes, is described.

INTRODUCTION

Fluorescence polarization (FP) measures the sizes of fluorescent molecules by measuring their rates of rotation in solution. The bigger the molecule is, the slower it rotates, because of frictional drag. Therefore FP has found great utility in the detection and measurement of the binding of small fluorescent-labeled molecules, such as epitopes, antigens and hormones, to large molecules, such as antibodies and receptors.

The key to FP's success is the equation P=(V-H)/(V+H). V and H are the vertical and horizontal intensities, respectively, of the emitted light when a fluorescent molecule (fluorophore) is exposed to a vertically polarized beam of light. The importance of this equation is that, since V and H are intensities which, when divided out of the equation, P becomes a dimensionless entity. The FP of a fluorophore is therefore independent of its intensity and concentration. FP assays are therefore resistant to cloudy suspensions, such as milk, lipemic sera, egg yolks and grain extracts. They are also unaffected by colored solutions, such as hemolyzed sera and whole blood. We have recently reviewed the principles and applications of FP [1].

FPAs have been in use in the human clinical laboratory for over two decades [2-5]. We have recently described the use of FP assays, using a fluorescein-labeled OPS from *Brucella abortus,* for the detection of antibodies to *Brucella* spp. (a gram negative bacterium) in bovids, bison, cervids and swine [6-21]. We have shown that this approach, in principle, can be applied to all gram negative bacteria, which possess OPSs, by extending it to the detection of antibodies to *Salmonella* spp. in chicken sera and egg yolks [23-26]. We have recently converted these FPAs to FP inhibition assays (FPIAs) for the detection of *Salmonella* spp. cells in culture broths [27-28] with a sensitivity of 10^4 to 10^5 colony forming units (CFUs), which was equivalent to a commercially available lateral flow immunogold assay. We have extended this assay to the detection and grouping of *Salmonella* spp.from colonies [28].

Proteins may be used as "tracers". We have reported the detection of antibodies to *Mycobacterium bovis* in bovine sera by the use of the protein MPB70 labeled with fluorescein [31-32]. We have subsequently identified peptide epitopes from MPB70 which we have used to develop FPAs for the detection of antibodies to *M. bovis* with enhanced performance over that of the whole protein assay (unpublished data, which will be described here). We have also shown that peptides may be used for the detection of antibodies to viruses. An example is that of Equine Infectious Anemia Virus [39]. Finally, FP, coupled with some form of DNA amplification, has been used for the sensitive detection of enterohemorrhagic *E. coli* [41-45,49], *Mycobacterium tuberculosis* [47-48] and *Chlamydia trachomatis* [46].

All of these above-mentioned assays can be, and some have been, employed "in the field". The instrument used is the Sentry-FP™ (Diachemix LLC). This is powered and controlled by a lap top computer and can operate for a limited period of time (approximately one hour) without external power or can employ either mains or a twelve volt supply (such as a cigarette lighter in a car or truck). In addition, FPAs have been adapted to the 96-well format. The Polarion™ and Ultra™ FP microplate readers (both from Tecan) have been approved by the USDA for brucellosis testing. These instruments are capable of throughputs of 1000 samples per hour.

This review will discuss these applications and, in addition, will present some relevant unpublished data.

GRAM NEGATIVE BACTERIA

All gram negative bacteria possess a lipopolysacharride (LPS; endotoxin), which is a major constituent of their cell walls. The LPS of a "smooth" organism is composed of three parts: lipid A, which is common to most gram negative bacteria, the core polysaccharide, which is shared by members of a particular genus of bacterium, and the O-polysaccharide, which is serotype specific. The LPS of a

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"rough" organism lacks the OPS. The LPS can easily be purified from gram negative bacteria by a number of methods, the most common of which is phenol/water extraction [50].

The FPA for the Detection of *Brucella* spp. in Animal Body Fluids

Brucellosis is a zoonotic disease which has great economic importance to live stock industries. The three major "smooth" organisms are *B. abortus* (bovids and cervids), *B. melitensis* (sheep and goats) and *B. suis* (pigs). The OPSs from these organisms are immunologically cross-reactive. The major "rough" organisms are *B. canis* (canids) and *B. ovis* (goats and sheep). Brucellosis in animals causes miscarriage, infertility and a general failure to thrive. In humans it causes malaria-like symptoms ("undulant fever"; "the bangs"), which requires an extensive treatment with a combination of powerful antibiotics (for example doxycycline and streptomycin). Humans can contract brucellosis by direct blood-to-blood contact with infected animals, through contact with aborted materials and discharges, consumption of infected unpasteurized milk and cheese, and by aerosols. It is an occupational hazard of meat processors, veterinarians and laboratory workers. In addition, persons that consume unpasteurized milk products are at great risk for contracting Brucellosis [8].

We have developed a FPA for the detection of antibodies to *B. abortus* [6-9]. The LPS from *B. abortus* was first isolated [22]. It was then hydrolyzed by 1% acetic acid and the OPS isolated by passage through a polymixin B-agarose column. The OPS was labeled with fluorescein isothyocyanate isomer I (FITC) under alkaline conditions and purified by Sepharose A25 chromatography to yield the "tracer" [6].

In the original assay, 10µl of serum was added to 1ml of buffer (PBSA-LDS; 0.01 M sodium phosphate buffer, pH 7.5, containing 0.9% sodium chloride, 0.1% sodium azide and 0.05% lithium dodecyl sulfate). The sensitivity of the assay could be increased by adding more serum. A serum fluorescence blank measurement was performed and 10µl of stock tracer added to give a tracer intensity of approximately 1.5 nM fluorescein equivalents. Samples with a FP value equal to, or greater than 10 mP (the mean of the negative controls plus 5 standard deviations), were considered positive.

The assay was evaluated against a variety of commonly used assays (the buffered plate antigen test, the rose bengal plate test, the complement fixation test, the rivanol agglutination test, competitive ELISA, indirect ELISA and culture) in bovine sera [6,10-12,19-20], porcine sera [13-14], bison sera [15], cervid sera [16], bovine whole blood [8,17,19], and bovine milk [8,18,19,21]. In all these studies the FPA was equal, and many times superior to the commonly used assays in terms of sensitivity and specificity, often achieving 99+% for both.

An example of the field use of the brucellosis FPA (for *B. melitensis* in goats) is shown in Figures **1** and **2**. Sera from a Rev 1-vaccinated goat herd in Obregon in the State of Senora, Mexico, were screened for antibodies against *B. melitensis.*Three populations of animals were observed. The first were clearly negative (mean approximately 80 mP). The second was weakly positive (mean approximately 92 mP). These were assumed to be residual vaccine titers. However, one animal was strongly positive (148 mP). Milk was taken from the animals with the positive reactions and tested by the FPA. The milk from the previously strongly positive animal was even more positive than its serum (222 mP). The milk from the weakly serum-positive animals were negative, indicating that the weak serum positivity was

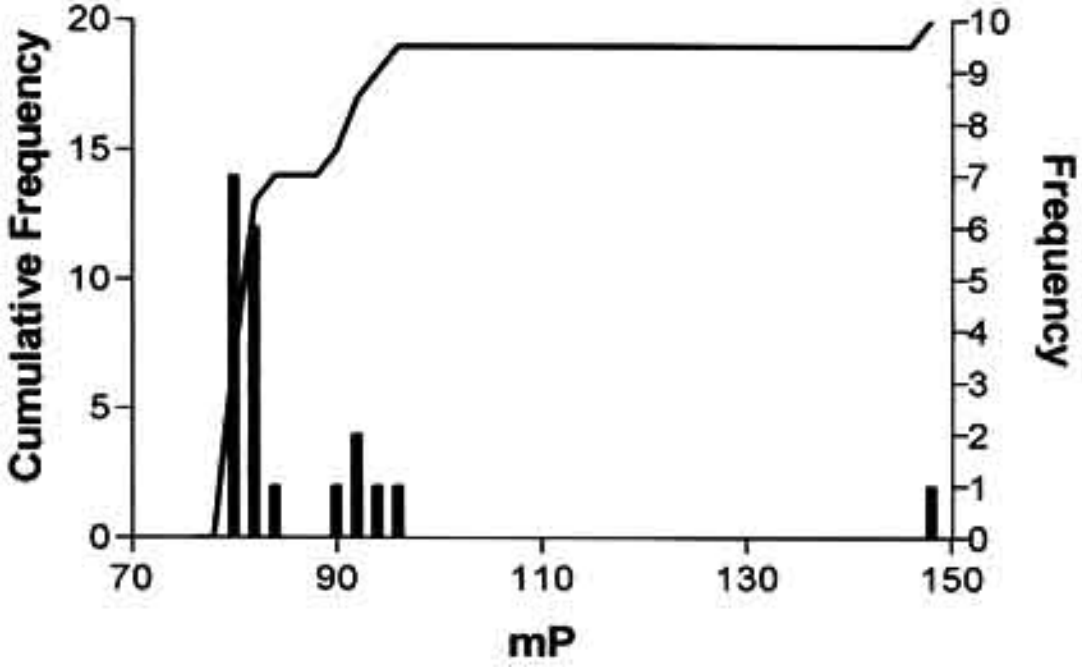

Fig. (1). Screening of serum, by FPA, from a goat herd, for antibodies to *B. melitensis*

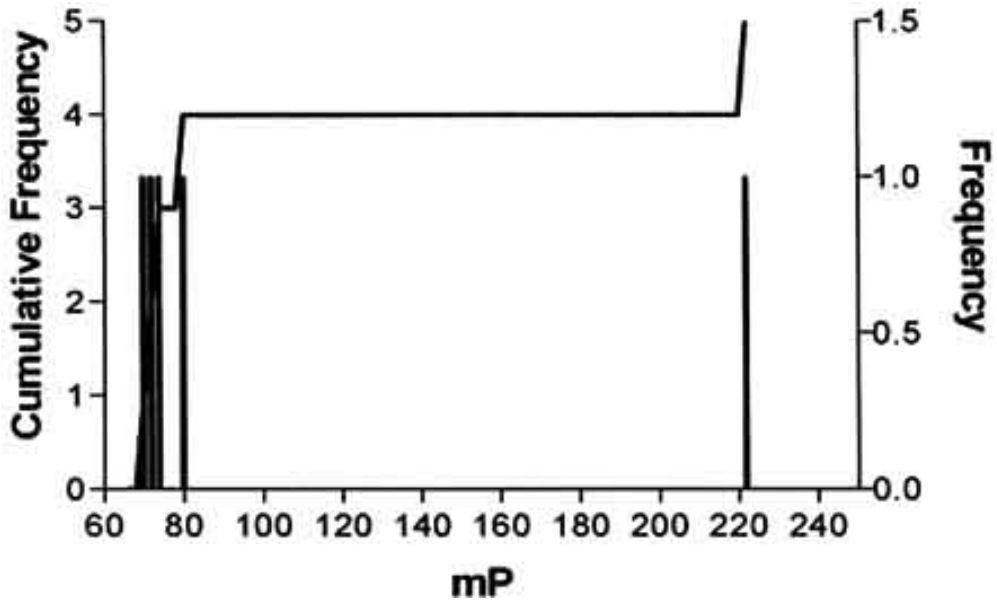

Fig. (2). Screening of milk, by FPA, from a goat herd, for antibodies to *B. melitensis.*

indeed due to residual vaccine titers. *B. melitensis* was isolated from the strongly positive animal's milk and the goat was subsequently destroyed.

FPAs for the Detection of *Salmonella* spp.

The *Salmonellae* are a genus of gram negative organisms, which are major causative agents of food poisoning. There are over 2000 species, belonging to 64 groups, based on their O-antigens. Ninety eight percent of human isolates belong to five groups (A, B, C, D1 and E). The two most important of the *Salmonellae* are *S. enteritidis* (SE; Group D1, O:1,9,12) and *S. typhimurium* (ST; Group B, O:1,4,5,12). SE is usually found in eggs and ST in meat (poultry, beef and pork) and unpasteurized raw milk [24,25,27].

We have developed FPAs for the detection of antibodies to SE and ST in chicken sera and egg yolks [23-25]. The LPSs from SE and ST were purchased from Sigma Chemical Co., (St. Louis MO), the OPSs prepared, and labeled with FITC [6, 23]. Using experimentally infected birds the SE and ST FPAs performed well, exceeding a flagellin-based ELISA in terms of sensitivity and specificity. There was about a 30% crossreactivity between the two FPAs. Since these latter studies were performed with experimentally infected chickens, under laboratory conditions, it was thought necessary to validate the assays on field samples. Therefore a number of panels, consisting of an unvaccinated "negative" flock, a vaccinated "negative" flock and a vaccinated "infected" flock were tested by the FPAs (Groups D1 and B). For the purpose of this review we will not discuss their Group B status (the manuscript discussing this is in preparation and will be submitted elsewhere). Some panels also comprised SE challenged birds. These panels were also assayed by currently employed agglutination-based assays [the plate test, the pullorum tube test (PT), and the microagglutination test (MAT)]. Birds were also cultured for the presence of *Salmonella* spp. The following is part of a recent presentation [26].

Study 1

This study was performed with the collaboration of the Ohio Department of Agriculture, Columbus, Ohio, under the auspices of Dr. B. Byrum.

Normally in FPAs one uses a cutoff of 10mP delta (the sample gives a reading 10mP, or more, higher than the mean of the negative controls). This corresponds to 5 standard deviations above that of the negative control. However, since this study involved vaccinated birds, it is more useful to divide the data into populations of birds. "Population A" (centered at approximately 0mP delta) refers to those birds which are clearly negative. "Population B" (centered at approximately 10mP delta) refers to those birds which are either weakly positive or suspects. "Population C" (20-40mP delta) refers to those birds which are clearly moderately positive. "Population D" (greater than 40mP delta) refers to strong positives and greater (150mP delta is not uncommon in an infected flock). It must be pointed out that the Group D1 and Group B FPAs are not totally specific. In fact, there is an approximately 30% cross-reaction between the two. Therefore both assays must be performed in order to determine the (probable) status of the bird in question, and therefore the flock status.

The histograms of the delta mPs for an unvaccinated "negative" flock, a vaccinated "negative" flock and a vaccinated "infected" flock are shown in Figures 3-5. Figure **3** shows the distribution of delta mPs for the Group D1 FPA in an unvaccinated "negative" flock. The plate test was negative on these samples. It can be seen that the vast majority of samples (approximately 80%) fell into population A. Of cause for concern is the presence of a small population falling into population B, and especially the

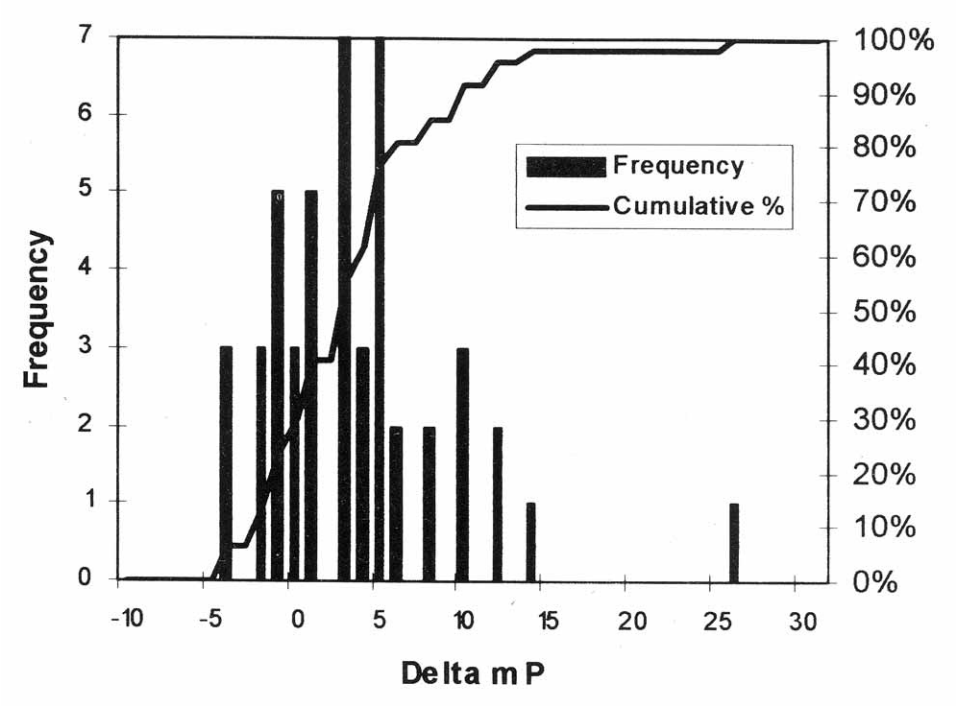

Fig. (3). The Group D1 *Salmonella* delta mPs for a "negative" unvaccinated flock, by FPA.

single sample which fell into population C. This latter sample was repeatedly positive by the Group D1 FPA but fell into population A (negative) in the Group B FPA. Furthermore, one sample fell into population B in the Group B assay. This sample was in population A in the Group D1 FPA. These observations imply that this flock, although called "negative" by the plate assay was, in fact, in the early stages of seroconversion for both Groups D1 and B.

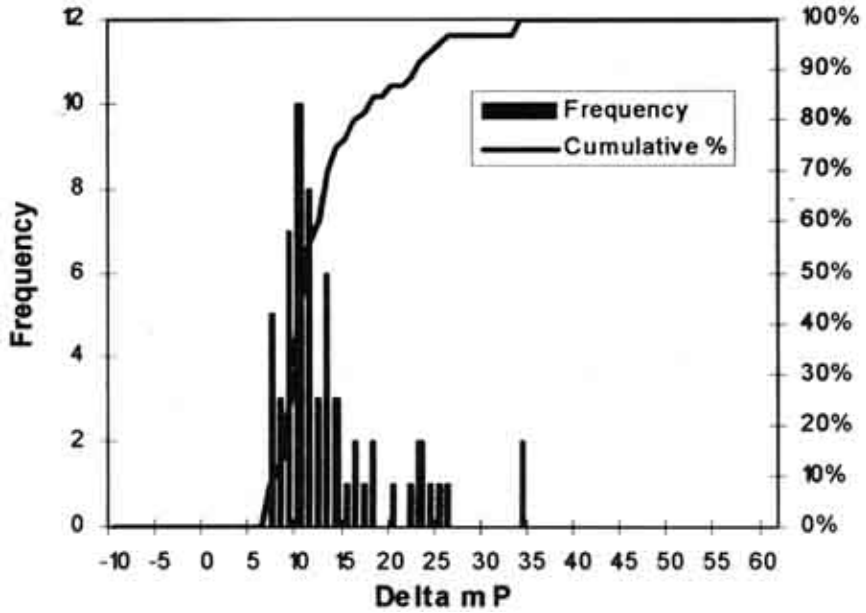

Fig. (4). The Group D1 *Salmonella* delta mPs, for a vaccinated flock, by FPA.

Figure **4** shows the distribution of delta mPs for the Group D1 FPA in a vaccinated "negative" flock. The difference in the Group D1 distribution between this and the unvaccinated flock is remarkable. The vast majority of samples now fall into population B (approximately 80%); population A is virtually absent. This would indicate that vaccination has had a significant impact on the Group D1 status of this flock. The presence of members of population C is consistent with this observation. One sample (population B) was positive by the plate test, but negative by the tube test.

Figure **5** shows the distribution of delta mPs for the Group D1 FPA in a vaccinated "infected" flock. The remarkable observation here is that, even though heavily exposed to Group D1, approximately 30% of samples fall into population A. Of the 57 samples represented here, 19 were positive by the plate test. Of these, 16 were also positive by FPA, two of which were MAT positive, with 4 MAT suspects. The three plate test positive-FPA negatives, clearly falling into population A, indicate that these were plate test false positives (these samples were grossly hemolyzed – gross hemolysis is known to interfere with agglutination tests but does not interfere with FPAs). In addition, 5 population C samples were negative by the plate test as were a further 5 population B samples. This is indicative of the superior sensitivity of the FPA.

Study 2

This is part of an ongoing vaccine study at The Georgia Poultry Laboratory, Athens, Georgia, conducted by Dr. W. D. Waltman. Birds were unvaccinated or vaccinated and challenged or not challenged with SE.

Table **1** summarizes some preliminary findings. For the purposes of the study described here, vaccine status is irrelevant, since we wanted to compare the ability of the FPA and the PT test to identify infected birds. Therefore we only analyzed the data from birds from which Group D1 was isolated. Table **1** shows that of 39 birds in whom D1 was cultured, 29 showed exposure to D1 by FPA; only 10 did so by PT. There were no samples positive by PT and negative by FPA, again indicating the superior sensitivity of the FPA.

Table 1. Comparison of Plate Test (PT) and Group D1 *Salmonella* FPA with Group D1 *Salmonella* Culture Positives, all Ages, Non Vaccinates and Vaccinates, SE Challenged and Unchallenged (N=39)

	PT –ve	PT +ve
FPA –ve	10	0
FPA +ve	19	10

FPIA (Antigen) Protocol for the Detection of Salmonella cells

A FPA can easily be converted to a FPIA by incorporating a defined quantity of antiserum into the dilution buffer. We have described FPAs for the detection of *Salmonella* spp. cells in culture broths [27], which were

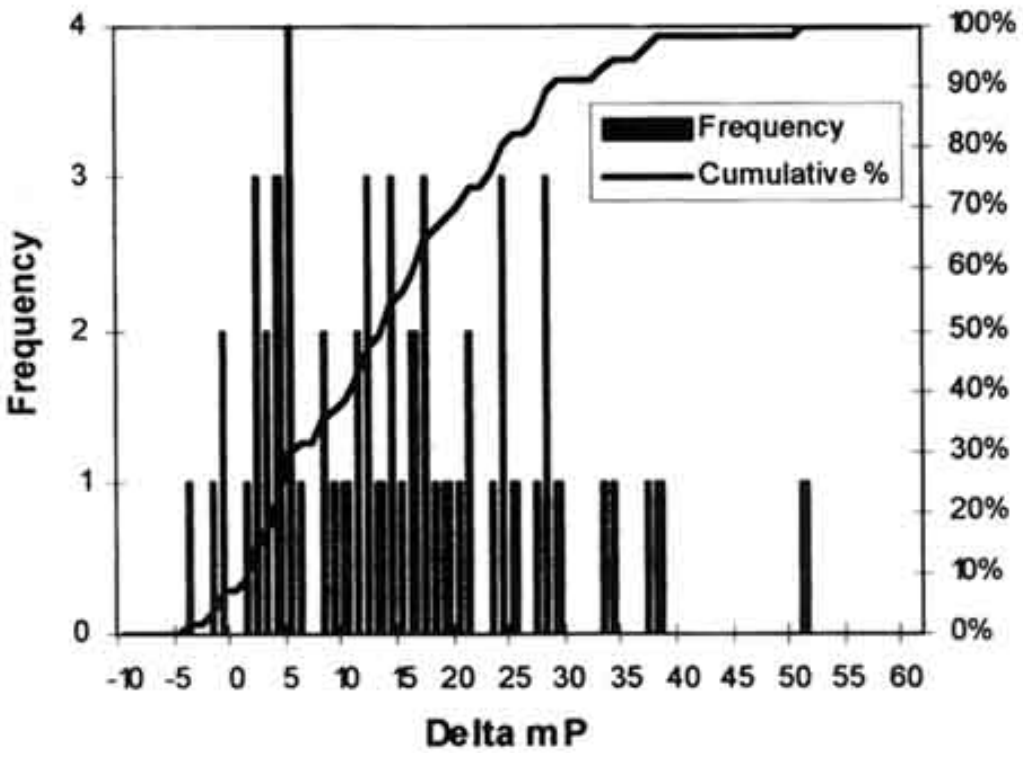

Fig. (5). The Group D1 *Salmonella* delta mPs, for a vaccinated, infected, flock by FPA.

shown to be equivalent, or sometimes superior, to a commercially available lateral flow immunogold assay [28]. We have modified these assays to detect cells from colonies on culture plates [26].

A colony was picked from a culture plate and added to 1ml of water. The mixture was then boiled for five minutes. 100µl of the suspension was added to 1ml of pre-diluted antiserum and vortexed. The background fluorescence was determined and 10µl of the appropriate tracer (approximately 100nM) was added and vortexed. After two minutes (or longer) incubation, the blank-subtracted fluorescence polarization of the tracer was measured.

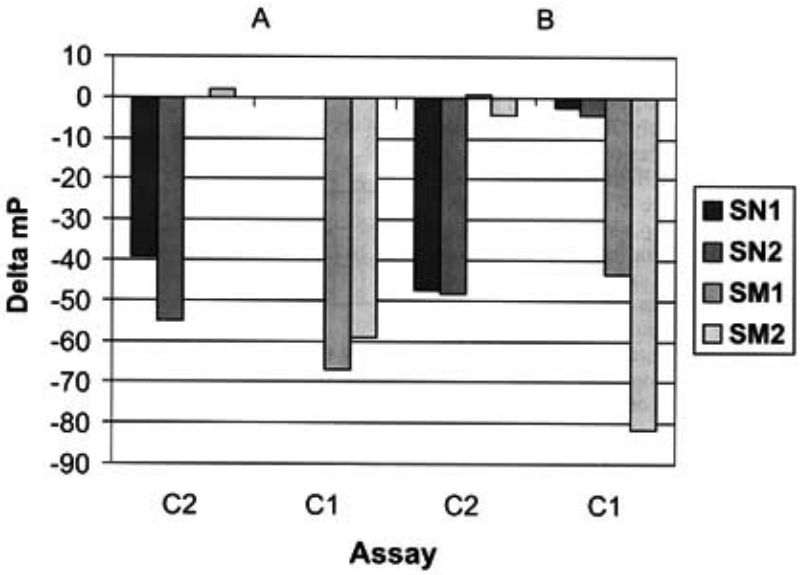

Fig. (6). FPIAs of colonies of *Salmonella* spp. picked from A) Onoz Agar and B) Brilliant Green Agar

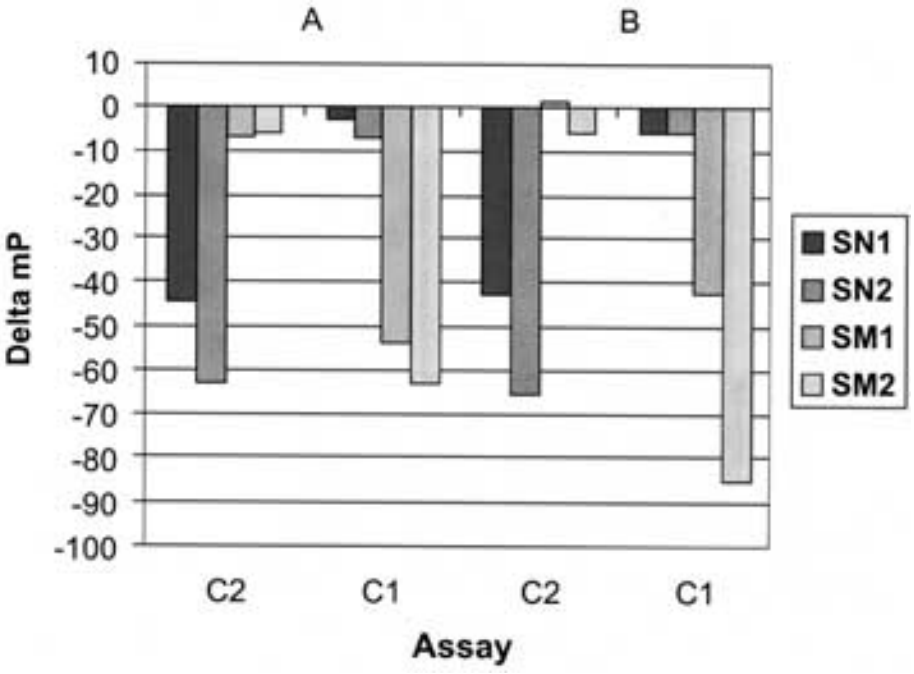

Fig. (7). FPIAs of colonies of *Salmonella* spp. picked from A) XLT4 Agar and B) SS Agar.

Figures **6** and **7** show that the FPIAs are very useful in grouping *Salmonella* spp. by colony picking. SN1 and SN2 are two isolates of *S. newport* (Group C2; one from a cow, the other from a horse; O: 6,8). SM1 and SM2 are two isolates of *S. montevideo* (Group C1; both from chickens; O: 6,7). Two colonies of each, from each plate, were picked. Since we are now dealing with inhibition assays, a delta mP of minus 10 or less is considered positive. A number of agars were tried, since there was a concern that the various dyes added to the agars could interfere (for example, be fluorescent). No interference was observed. As can be seen, there was no doubt as to which group the colonies belonged.

FPAs using Proteins and Peptides

The principles of FP dictate that the tracers employed should be as small as possible in order to get the greatest span out of an assay [1-2]. In practice, proteins up to 50 kDa can be used, depending upon the molecular weight of the receptor. When detecting immunoglobulin G (160 kDa) the practical limit for the tracer size is approximately 20 kDa. Wherever possible, efforts should be taken to identify the major epitopic and nonepitopic regions of the antigen and excluding as much of the non-essential regions as possible. These principles are exemplified by the development of FPAs for the detection of antibodies to *Mycobacterium bovis* and Equine Infectious Anemia.

Mycobacterium Bovis

M. bovis causes tuberculosis in a variety of farm and wild animals and also in humans. Together with *M. tuberculosis, M. africanum,* and *M. microti, M. bovis* is a member of the taxonomically closely related *M. tuberculosis* complex [29]. The protein antigen MPB70 is secreted from *M. bovis* cells following cleavage of a 30 amino acid signal peptide which directs the active transport of the protein across the cytoplasmic membrane [30]. MPB70, whose function is unknown, may comprise between 10% to 23% of the protein excreted by various *M. bovis* strains [31]. The molecular mass of MPB70 was estimated to be between 18 and 23 kDa by sodium docecyl sulfate-polyacrylamide gel electrophoresis, 15 kDa by sedimentation equilibrium analysis and 16 kDa as deduced from the gene-derived amino acid sequence [31]. This indicates that MPB70 can exist with various degrees of post-translational modification.

We have developed a FPA for the detection of antibodies to *M. bovis* using fluorescein-labeled MPB70 [31]. On initial evaluation with a small panel (n=28) of culture positive bovine sera and a large number of presumed negative sera (n=5666) the results looked very promising [32]. The sensitivity was 92.9% and the specificity was 98.3%. However, in further evaluations with samples from animals at various stages of infection, the sensitivity dropped to approximately 50%, which was no better than existing ELISAs. Because of this observation, and the problems in the manufacture of the tracer, it was decided to convert the assay to one based on peptides. To do this, we had to resort to epitope scanning.

Epitope Scanning by FP

A protein has two types of epitopes (the constituents of antigens), which activate T cells. This, in turn leads to B cell activation, which causes the humoral immune response to be generated (the production of soluble antibodies), and T helper cell activation, which causes the cellular immune response to be generated (the production of a variety of cells which kill the invading organism). The latter pathway is usually activated before the former, but is of comparatively limited longevity. Epitopes are either linear (sequential) or conformational (two or more parts of the protein come together to form the epitope). Epitope scanning [33,34] will only work with the former.

Epitope scanning gives one an alternative to the molecular biological approach for finding epitopes, which involves cloning, sequencing, restriction enzyme digests and expression – a very time consuming and low resolution process. If the amino acid sequence of a protein is known, then it is amenable to epitope scanning. A number of overlapping peptides are synthesized and tested for activity in some sort of assay (usually ELISA or in vitro lymphocyte activation, depending on which type of epitope one is looking for). We have developed an FP-based alternative to the use of ELISA, whereby fluorescein is attached to the peptide during its synthesis. The product is then released from the solid phase and screened for activity by FP.

MPB70 was first scanned by Radford *et al.* in 1990 [35]. Amino acid 1 in Figure **8** is number 15 in this sequence. For convenience, we will use the numbering of amino acids in Figure **8**. Radford *et al.*, using octapeptides (8-mers), overlapping by one amino acid, found that mouse monoclonal antibodies reacted to peptides 1 to 13 and cows to peptides 18 to 22. Wiker *et al.* [36], using 20-mers, overlapping every 10, confirmed Radford *et al.* with regard to mouse monoclonal antibodies. They also showed that rabbits responded similarly to cows in Radford *et al.* but cows themselves showed a very broad response, centering in the "Radford region". Lightbody *et al.* [37], using 20-mers overlapping every 10, and Pollock *et al.* [38], with 16-mers,overlapping every 5, looking for T-cell epitopes by in vitro lymphocyte activation, found epitopes which were completely unrelated to those of Radford *et al.* [35] and Wiker *et al.* [36].

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1 NPTGPASVQG MSQDPVAVAA SNNPELTTLT AALSGQLNPQ
VNLVDTLNSG QYTVFAPTNA 6061 AFSKLPASTI DELKTNSSLL
TSILTYHVVA GQTSPANVVG TRQTLQGASV 110
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Fig. (8). The sequence of the fragment of MPB70 which was epitope-scanned by FP.

MPB70 Epitope Scanning

Using the Multipin™ peptide synthesis kit and a PinAid™ from Chiron Technologies (Mimotope, Inc.), trace quantities of 96 peptides were synthesized on derivatized polypropylene gears coupled with a cleavable diketopiperazine (DKP) linker (1-2 μmole/gear) in a block.

A complete block consists of one unit containing 96 pins. Each of these pins contained one detachable gear. Synthesized peptides were covalently attached to these gears. After complete synthesis, these peptides were screened using FP.

A computer program keeps track of weights, volumes and dispensing of various amino acids during peptide synthesis. A schedule for the synthesis of peptides on a

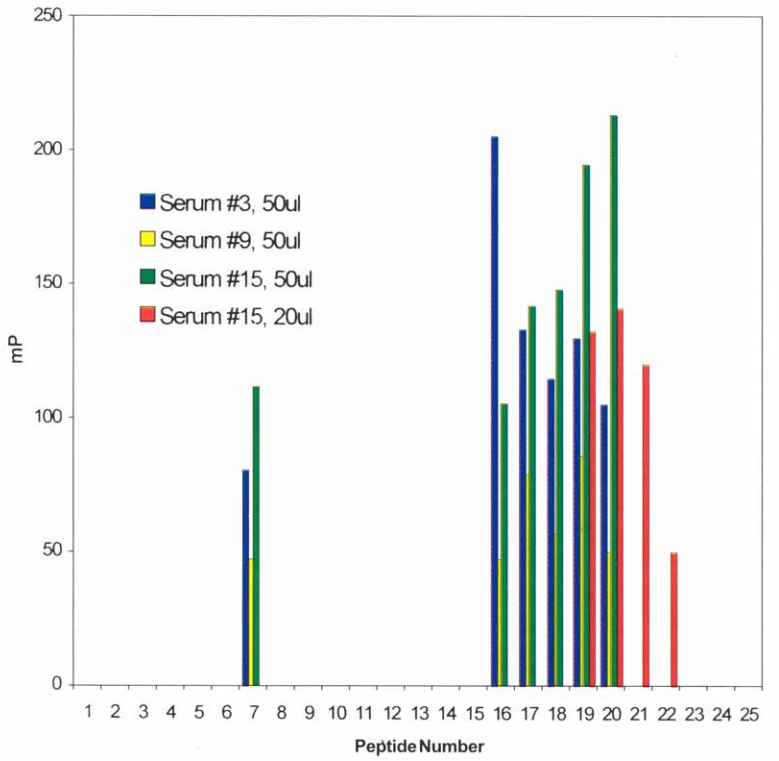

Fig. (9). Epitope Scan of purified MPB70 peptides, by FP, with three Antisera.

PinAid™ was generated. These gears were Fmoc deprotected before use in peptide synthesis. The Fmoc and DIC/HOBT chemistry was used for peptide synthesis by utilizing the amino acids with side chains protected with groups other than Fmoc.

Using 150µl of 30mM amino acid solutions in each well, 96 peptides of 15 amino acid each were synthesized with a spacing of one amino acid. Each coupling cycle was carried out overnight. After the last cycle, gears were washed, and Fmoc deprotected.

The last coupling was done with 6-carboxyfluorescein using standard coupling of DIC/HOBT. After overnight reaction, the entire block was washed with DMF and methanol, side chains deprotected using TFA/Anisole (19/1), and peptides cleaved with a 40% solution of CH_3CN in PBS (pH 7.4). These peptides were diluted appropriately and tested with M. *Bovis* positive sera.

Amino acid number 1 is number 45 in the full MPB70 sequence. In a preliminary screen against a single positive serum we identified peptide 16 as the most reactive peptide, confirming the observations of Radford *et al.* [35] and Wiker *et al.* [36]. Since the preliminary peptides obtained from the gears are usually less than 30% pure, all peptides through this region were purified by HPLC and screened with three different positive sera. The results are shown in Figure **9,** which is a compilation of the data from two experiments. Because of the shortage of serum number 15, the determination of the end of the epitope was performed with 20ul of serum. It must be noted that the FPs of the various peptides were between 40 and 45 mP in buffer. Interestingly, in negative sera they tended to have a slightly lower FP, for reasons which we do not understand. However, this does show that the elevation in FP is not due to nonspecific binding. As can be seen the sera reacted somewhat differently with the individual peptides, confirming the observations of Wiker *et al.* [36]. Peptide 7 is a 20-mer (peptide 555), the beginning of which corresponds to the reactivity of mouse monoclonal antibodies (Wiker *et al.* [36]). As can be seen this epitope extends to peptide 34 (20 plus 14). Subsequent screening identified a minority of bovine sera which reacted preferentially to peptide 555. Therefore a peptide was made, starting at position 7, and ending at position 34. This peptide is currently is in clinical trials and is showing very promising results, having superior performance over MPB70 itself in terms of sensitivity and specificity.

Equine Infectious Anemia Virus (EIAV)

EIAV infection is confined to equids (horses, donkeys, mules, etc) and the possession of an infected animal can have an economically, and emotionally, devastating impact on a horse owner. EIAV is a blood-born member of the lentivirus family of retroviruses and is related genetically to human immunodeficiency viruses. It is not a human pathogen, but is of great interest to researchers studying retroviral-induced diseases. It is transmitted by the horse fly over a relatively short distance (200 yards), because of the unstable nature of the virus. Therefore EIAV is spread by close contact with an infected horse, and is thought to be a life-long, incurable, disease. Therefore the owner of an infected animal is faced with two choices – euthanasia, or the quarantine of the horse for its natural life in a strictly

controlled environment, at least 200 yards from any other horse [39].

The control of EIAV, over the past 20 years, has been based primarily on the identification and elimination of seropositive horses. This is accomplished by a standardized agar gel immunodiffusion assay (AGID), performed in centralized reference laboratories. Because of a high false positive rate, any AGID positive must be confirmed by a Western blot assay (which itself is not perfect). If positive by both assays, before condemning a million-dollar racehorse to death, the presence of EIAV RNA must be confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). There is a great need to reliably identify seropositive horses by a rapid field test. We believe that FP fulfills this need.

We have reported the development of a FPA for the detection of antibodies to EIAV [39]. The assay was based on a fluorescein-labeled peptide from the immunodominant region of the EIAV transmembrane protein gp45. This peptide was tested for its reactivity with 151 AGID-positive horse sera and 106 AGID-negative sera in a study conducted with the University of Pittsburgh School of Medicine. The FPA sensitivity was 89.4% and the specificity 100%. By the use of experimentally infected animals the FPA could detect antibodies produced early in infection (</=3 weeks post-infection). The 16 AGID-positive, FPA-negatives were retested by AGID, a peptide ELISA and Western blot. 6 were found to be true negatives, raising the FPA sensitivity to 93%.

In a subsequent study at the University of Kentucky at Lexington on a panel of 211 AGID negative and 109 positive samples, the FPA scored a sensitivity of 94.5% and a specificity of 100% [40]. The results of the previous study were confirmed. Interestingly, all of the FPA "false negatives" were from a feral herd on the Outer Banks of North Carolina. These horses were subsisting solely on salt-grass and other forages – a diet generally considered unfit for adequate nutrition. Eight horses, quarantined in North Carolina, were selected for study of the genetic material of EIAV in a sensitive RT-PCR assay (<50 copies of RNA in 2 ml of plasma). Only one of the eight had detectable RNA. Interestingly, this sample was positive by FPA. Three of the seven horses, negative in the RT-PCR assay, were also negative in the FPA. These observations raise the possibilities that either poor nutrition can interfere with conventional assays or that, in some cases, EIAV is not an incurable life-long disease. These issues have yet to be resolved.

Another study was performed at the Canadian Food Inspection Laboratory, Charlottetown, PEI. Based on AGID reactivity, 285 positive and 315 negative samples were gathered from various Canadian provinces. All AGID positives had been euthanised, but trace-back was possible for these animals. Of the negative samples, all were negative by the FPA (100% specificity). 14 of the positive samples were negative in the FPA (95.1% sensitivity). Interestingly, 7 of these samples originated from one owner, who also had 11 other positive animals (by both AGID and FPA). This raises the possibility that these animals were "early seroconverters".

The Detection of Infectious Agents by DNA-based FPAs

Although still in their infancy, when compared to immunoassay-based FPAs, DNA-based FPAs show great promise. For example Tsuruoka *et al.* were able to detect 40 fmol of DNA from methicillin resistant *Staphylococcus aureus*, by a DNA hybridization-based FPA, in 10 minutes [41]. Ye *et al.* used a fluorescein labeled primer in a FP-based PCR assay for a 256 bp fragment of the stx2 gene in *Escherichia coli* O:157H7 [42]. Ohiso *et al.*, using a hybridization-based FPA and PCR for the verotoxin type 2 gene, were able to detect 10^3 colony forming units (CFUs) within 5 minutes after PCR [43]. Hybridization-based FP-PCR was also used by Kido *et al.* to detect two types of Shiga toxin (verotoxin) in enterohemorrhagic *Echerichia coli* (EHEC), using the genes stx1 and stx2 as targets, in about 15 minutes [44]. Tsuruoka *et al.* used hybridization-based FP and assymmetric PCRs for the detection of verotoxins types 1 and 2 in EHECs and also the presence of hepatitis C (HCV) RNA by NASBA within 1 minute [45].

An alternative amplification method to PCR and NASBA is strand displacement amplification (SDA). SDA is a very rapid isothermal procedure and was used, in combination with FP, by Spears *et al.* to detect less than 5 elementary bodies (EBs) of *Chlamydia trachomatis* in 30 minutes [46]. Using the same technique, the same group detected less than 10 *M. tuberculosis* genomes in 20 minutes [47-48]. Similarly, using the hemolysin gene from EHECs (EHEC-hly A) we showed a sensitivity of between 1 and 4 CFUs for *E. coli* O:157:H7 and other EHEC serotypes within 30 minutes [49].

CONCLUSION

Ten years ago, most experts in fluorescence polarization would have said that it would be impossible to detect infectious diseases by FP. We have shown that FPAs and FPIAs are excellent alternatives to assays currently in use, and often exceed them in performance. FP-based assays are rapid, sensitive and easy to perform, requiring minimal operator training. The reagents are very stable and therefore are suitable for use in the field. They can be performed beside an animal, one sample at a time, in extremes of weather conditions, and also in the laboratory, at a rate of 1000 samples per hour. It will be very interesting to see what FP is doing in 2013.

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