A Quantitative Fluorometric Assay for the Measurement of Antibody to *Pasteurella haemolytica* in Cattle

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ABSTRACT

A rapid, simple fluorometric method is described for measuring antibody to *Pasteurella haemolytica* in sera of cattle. Various antigen preparations were compared for the test including live, formalin-killed and phenol-killed *P. haemolytica*. A preparation composed of formalin-killed organisms from a 22 hour culture gave consistent results and was used in the studies. The test was reproducible with percent coefficients of variation for fluorescent signal unit values on ten or more replicate samples ranging from 5.7 to 28.0. Sera from calves vaccinated by aerosol exposure to live *P. haemolytica* had up to a five-fold increase in antibody titer as measured by the fluorometric method test during a 21 day period. Fluorometric method titers were comparable to those obtained by the indirect bacterial agglutination test. There was no seroconversion to *P. haemolytica* in calves vaccinated by aerosol exposure of *P. multocida*. The major advantages of the fluorometric method test over conventional methods are that the assay does not require serial dilutions of serum samples and thus limits time and effort to determine antibody titers.

RÉSUMÉ

Cet article décrit une méthode fluorométrique, rapide et simple, destinée à mesurer les anticorps sériques contre *Pasteurella haemolytica*, chez les bovins. Les auteurs essayèrent les trois antigènes suivants: un premier, qui était vivant; un deuxième, inactivé avec de la formaline et un troisième, inactivé avec du phénol. Leur choix se porta finalement sur l'antigène préparé avec des cultures de *P. haemolytica*, âgées de 22 heures et inactivées avec de la formaline, parce qu'il donnait des résultats constants. Cette méthode se révéla reproductible et afficha des coefficients du pourcentage de variation relatifs aux valeurs unitaires du signal fluorescent qui varièrent de 5,7% à 28%, lorsqu'on l'utilisa avec des doubles de dix échantillons de sérum ou plus. Le sérum des veaux vaccinés au moyen d'aérosols de *P. haemolytica* vivantes afficha un titre d'anticorps jusqu'à cinq fois plus élevé, comme le démontra la méthode fluorométrique, sur une période expérimentale de 21 jours. Ces titres s'avérèrent comparables à ceux qu'on obtint par l'épreuve de l'agglutination indirecte des bactéries. Les veaux vaccinés contre *Pasteurella multocida*, au moyen d'aérosols, ne développèrent pas d'anticorps à l'endroit de *P. haemolytica*. Le principal avantage de la méthode fluorométrique, par rapport aux méthodes conventionnelles, réside dans le fait qu'elle ne requiert pas de dilutions en série des échantillons de sérum; cette particularité limite par conséquent le temps et l'effort requis pour déterminer les titres d'anticorps.

INTRODUCTION

Pasteurella haemolytica serotype 1 is the primary cause of a severe fibrinous pneumonia that occurs in shipping fever of cattle (8). Commensal infection of the upper respiratory tract with *P. haemolytica* is widespread in cattle populations as determined by isolation of organisms from nasal and tracheal swabs from normal animals (3, 11). The mechanism by which the organism becomes a lower respiratory pathogen and the role of the immune system in preventing pulmonary disease have been the subject of intense study for several years (2, 4, 7, 10, 13, 14).

Several serological methods have been used to study the immune response to *P. haemolytica* including indirect hemagglutination (IHA), whole cell agglutination, indirect bacterial agglutin-

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Journal article No. 4107 of the Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma.

Submitted February 19, 1982.

nation (IBA), and the bacteriocidal tests (4, 10, 11, 13, 15). The IHA test appears to be sensitive to *P. haemolytica* serotypes; but, in vaccination studies, changes in serum titers between pre- and postvaccination samples were minimal (4). Studies in healthy and sick feedlot cattle failed to demonstrate a correlation between IHA titers and shipping fever (10). Bacteriocidal activity toward *P. haemolytica* could not be consistently detected in sera from vaccinated, controlled, or challenged cattle (4). The whole cell agglutination and IBA tests have been shown to be most useful in comparing pre- and postvaccination titers (4, 13).

We report the adaptation of a simple fluorometric method (FIAX)[1] for the detection and quantitation of antibody to *P. haemolytica* in cattle.

MATERIALS AND METHODS

BACTERIA

Bacterial cultures of *P. haemolytica* serotype 1 and *P. multocida* serotype 3, both originally isolated from feedlot steers, were used in these studies (7). The organisms were grown on brain heart infusion agar with 5% citrated bovine blood, 1% horse serum, and 1% filtered yeast hydrolysate at 37°C in candle jars. Cultures were harvested at six and 22 hours after incubation. Numbers of live organisms were determined by a modified plate count method and the concentrations are expressed as colony forming units (CFU) (7).

ANTIGEN PREPARATIONS

In the FIAX test, live, formalin-killed, and phenol-killed *P. haemolytica* preparations were used. All preparations were standardized such that a 1:10 dilution in phosphate buffered saline (PBS), (0.01M, pH 7.2) corresponded to an optical density reading of 0.36 at a 650 nm wavelength (equivalent to 10^9 CFU). Three live *P. haemolytica* preparations were evaluated in two trials. These consisted of organisms from 22 hour and six hour cultures suspended in PBS and organisms from six hour cultures with their capsules extracted by incubation in PBS at 41°C for 60 minutes as recently described (5). A different preparation was used in each trial. Preparations of *P. haemolytica* from similar cultures were suspended in 0.4% formalized PBS for 30 minutes. After two washes in PBS, the formalin-killed organisms were suspended at the desired concentration in 0.4% formalized PBS. Phenol-killed preparations were similarly prepared but in a 0.75% phenol-PBS solution containing 10% horse serum. Killed antigen preparations were stored at 4°C. The same stocks of formalin-killed organisms were used in each trial.

INDIRECT BACTERIAL AGGLUTINATION TEST

The IBA test was performed as previously described (13). A formalin-killed 22 hour *P. haemolytica* antigen preparation was used. Each serum sample was tested in serial twofold dilutions (in PBS) from 1:10 to 1:2560. The end point titer was defined as the reciprocal of the last serum dilution in which agglutination could be observed.

FIAX TEST

The test was performed using key shaped plastic STIQ samplers[2] that support a cellulose acetate-nitrate polymer-coated disk on both sides of the terminal portion. A 25 μL volume of antigen was evenly distributed on one side of each sampler and allowed to dry at room temperature before use. Samplers were used within four hours after sensitization.

All dilutions and wash steps were made in PBS (0.01M) with 0.15% (vol/vol) Tween 20, pH 7.4. Serum samples were assayed in duplicate or triplicate. Using an automated diluter,[3] sera were diluted to a working dilution of 1:76 (10 μL serum in 750 μL PBS-Tween 20 buffer) and 0.6 mL volume placed in 12 × 75 mm glass tubes. The assays were performed at room temperature. Tubes were placed in racks that maintained them at a 45 degree incline on a horizontal shaker. Samplers sensitized with antigen were placed in the tubes and incubated 30 minutes with constant agitation. The samplers were transferred to tubes containing 0.6 mL PBS-Tween 20 buffer and shaken for ten minutes. Samplers were subsequently placed in 0.5 mL of fluorescein isothiocyanate (FITC)-conjugated rabbit antibovine IgG (heavy chain and light chain specific)[4] that had been optimally diluted 1:200 with PBS-Tween 20 buffer. The optimal concentration had been determined in a standard titration experiment. Tubes were shaken for 20 minutes. Samplers were transferred and subjected to another ten minute wash in PBS-Tween 20 buffer.

The amount of fluorescence on the samplers was measured using a fluorometer.[5] The baseline of the fluorometer was adjusted to zero, then the fluorescence on both sides of each sampler was measured and recorded as the pregain values. The highest pregain sampler was reinserted, antigen side up, and the gain was set such that the fluorometric reading was 160 units. The fluorescence was measured on both the antigen and control sides of the samplers and recorded as fluorescent signal units (FSU). The specific fluorescence due to the reaction of the FITC conjugate with bound antibody was designated as ΔFSU and calculated by subtracting the control FSU value from the FSU value of the antigen side of the samples. A percent coefficient of variation was determined for several replicate serum samples.

[1]Fluorescent Immunoassay System, International Diagnostic Technology, Santa Clara, California.
[2]Surface Technique for Immunoquantitation, International Diagnostic Technology, Santa Clara, California.
[3]IDT Automatic Pipettor, International Diagnostic Technology, Santa Clara, California.
[4]Cappel Laboratories, Inc., Cockranville, Pennsylvania.
[5]FIAX 100, International Diagnostic Technology, Santa Clara, California.

DETERMINATION OF FIAX TITER

Four calibrator sera (#398, #393, #389, and #384) were chosen from calf experiment A (described below) based on ΔFSU values ranging from five to 135. End point titrations were performed in triplicate on sera sequentially diluted twofold in PBS from an initial dilution of 1:5. For each serum dilution, a working dilution of 1:76 was made with the automated pipettor and the FIAX test performed as described above. Formalin-killed 22 hour *P. haemolytica* was used as antigen. The average ΔFSU value was determined for each serum at each dilution. The end point titer was the reciprocal of the last dilution that had an average ΔFSU value higher than the minimal ΔFSU value observed as background fluorescence on the antigen-sensitized side of the samplers.

ANALYSIS OF DATA

Calibrator sera were placed in tubes containing 0.1% sodium azide, aliquoted in 0.1 mL volumes and stored at -70°C until used. Once sera were thawed, they were maintained at 4°C for two weeks after which time remaining sera were discarded. The ΔFSU values for the four calibrator serum samples at the working dilution of 1:76 were determined each time a FIAX test was performed. A line of best fit was determined by least-squares linear regression analysis of the predetermined FIAX end point titers and the ΔFSU values of the calibrator sera (1). Titers for unknown samples were determined by extraprolation of ΔFSU values from the best fit line. Regression analysis was used to determine the correlation between titer values obtained by the FIAX and IBA tests.

VACCINATION EXPERIMENTS

Sera were obtained from Hereford, Angus, and Hereford-Angus cross-bred male and female calves, six to eight months old, used in vaccination experiments (A, B and C). All calves were obtained from a closed herd that had exhibited a low incidence of respiratory disease.

In calf experiment A, prevaccination serum samples were obtained from 30 calves. On day 0, ten calves were sham vaccinated by a 15 minute exposure to an aerosol of PBS as described previously (2). Another ten calves were vaccinated by a 15 minute exposure to an aerosol containing a suspension of *P. haemolytica* in PBS (approximately 10^9 CFU) from a six hour culture and the last ten calves were vaccinated by a 15 minute exposure to an aerosol containing *P. haemolytica* (10^9 CFU) from a 22 hour culture. The above procedures were repeated on the same animals on day 7. Two weeks after the second vaccination (day 21) serum samples were obtained from all the calves. Calf #398 (22 hour culture vaccinate) was vaccinated with live *P. haemolytica* by the above procedure two additional times (days 35 and 49) to produce a hyperimmune serum.

Calf experiments B and C were similar to calf experiment A and consisted of two 15 minute aerosol vaccinations at one week intervals. In calf experiment B, two calves were vaccinated with PBS and six calves were vaccinated with a 22 hour culture of *P. haemolytica*. Serum samples were obtained on days 0, 14 and 21 of the experiment. Calf experiment C consisted of eight calves vaccinated with PBS and six calves vaccinated with a 22 hour culture of *P. multocida*. Serum samples were obtained on days 0, 7 and 21 of the experiment.

A negative control serum was obtained from umbilical cord blood of a germfree calf.[6]

RESULTS

COMPARISON OF ANTIGEN PREPARATIONS

The results from the *P. haemolytica* antigen preparations are compared in Table I. In trials 1 and 2, the highest ΔFSU values were found when serum #1 was reacted with the live six hour organisms without capsules. Nonspecific con-

TABLE I. Comparison of Specific Fluorescent Signal Units (ΔFSU) Values for Various *Pasteurella haemolytica* Antigen Preparations in the FIAX Test

Antigen preparation	Serum #[a] 1	2	3	GF[c]	Conjugate[d]
Trial 1					
22 h — live	102.5[b]	68.5	43.5	24.5	26.0
— formalin	95.0	36.5	17.5	7.0	5.5
6 h — live	105.5	36.0	21.5	13.0	18.5
— live/extracted[e]	131.5	74.5	25.5	8.5	8.5
Trial 2					
22 h — live	87.5	62.0	52.5	ND	37.0
— formalin	102.0	43.5	19.0	ND	12.0
6 h — live	86.0	45.0	20.0	ND	15.0
— live/extracted	127.5	49.0	26.0	ND	7.5
— formalin	87.5	49.0	15.0	ND	3.0
— formalin/extracted	96.0	78.0	25.0	ND	10.0
— phenol	76.5	46.5	30.5	ND	16.0
— phenol/extracted	73.5	42.0	34.5	ND	13.5
Trial 3					
22 h — formalin	116.0	40.0	11.0	ND	5.5
6 h — formalin	62.0	17.0	1.0	ND	0
— formalin/extracted	102.0	36.5	13.0	ND	3.5

[a]Sera #1 and 2 obtained from calves 14 days after two aerosol exposures to *P. haemolytica*. Serum #3 obtained from a calf seven days after two aerosol exposures to *P. haemolytica*
[b]Average ΔFSU value of two replicates
[c]GF = Germ free calf serum
[d]Test performed with FITC-conjugated rabbit antibovine immunoglobulin serum without a primary serum
[e]Capsule extracted in PBS at 41°C for 60 minutes
ND = Not done

[6]Provided by Dr. M. Frey, University of Nebraska, Lincoln, Nebraska.

jugate binding was greatest in live 22 hour and six hour *P. haemolytica* preparations as demonstrated by ΔFSU values of 26.0 and 18.5, respectively, when a primary incubation step with serum was eliminated. In contrast, lower nonspecific ΔFSU values of 5.5 and 12.0 were observed when formalin-killed 22 hour preparations were reacted with conjugate alone. The ΔFSU values for germfree calf serum were similar to those when the conjugate was used alone in trial 1. Additional testing of germfree calf serum was not done.

Formalin-killed 22 hour *P. haemolytica* preparations gave relatively consistent results with each serum tested in all three trials. Use of the formalin-killed 22 hour and six hour extracted preparations resulted in high ΔFSU values for serum #1 (95.0 and 116.0 and 96.0 to 102.0 respectively) and low nonspecific ΔFSU values (5.5 to 12.0 and 3.5 to 10.0 respectively). The ΔFSU values for sera #2 and #3 varied between trials 2 and 3 by nearly twofold when formalin-killed six hour extracted cells were used as antigen. A marked variation was seen in the ΔFSU values for all three sera when the formalin-killed six hour whole cell preparations was used in trials 2 and 3. Phenol-killed whole cell and extracted preparations had low ΔFSU values (73.5 and 76.5) for serum #1 and relatively high nonspecific ΔFSU values (13.5 and 16.0). For the remaining experiments the formalin-killed 22 hour *P. haemolytica* preparation was used as antigen in the FIAX assay.

The effect of the concentration of formalin-killed 22 hour *P. haemolytica* on ΔFSU values was determined (Table II). For each serum tested, the antigen preparation equivalent to 10^9 CFU had the highest ΔFSU values.

TABLE II. Titration of *Pasteurella haemolytica* Antigen in the FIAX Test[a]

Serum #	ΔFSU[b] 1:1[c]	1:10	1:20
1	120	84.5	69.5
7	126	88.0	68.0
8	98.5	69.5	50.0

[a]22 h formalinized culture
[b]Average ΔFSU value of two replicates
[c]Equivalent to 10^9 colony forming units

REPLICATE SAMPLES

Ten to thirteen replicates of six sera were tested in the FIAX test (Table III). The coefficient of variations for ΔFSU values ranged from 5.7% to 28.0%.

FIAX TITER DETERMINATION

Fluorometric method titers were determined for four sera (Table IV). Serial dilution of sera resulted in a parallel reduction in ΔFSU values. The minimal ΔFSU values were consistently around four (3.7 ± 0.6 to 4.0 ± 0) and a ΔFSU value of five (standard deviations of ± 0 or ± 1) was considered to be the last ΔFSU value to represent a positive reaction. Fluorometric method titers of 640, 160, 40 and < 5 were designated for sera #398, 393, 389, and 384, respectively. For the purpose of regression analysis, a titer of < 5 was designated as 1. In this experiment, a correlation coefficient (r) of 0.999 ($p < 0.001$) as calculated when ΔFSU values of the undiluted sera were regressed against the end point titers.

CALF VACCINATION EXPERIMENTS

Serum samples from ten calves

TABLE III. Mean Specific Fluorescent Signal Units (ΔFSU) Values and Percent Coefficient of Variation for Selected Serum Samples

Serum #	Replicates	Mean ΔFSU Value ± SD	% Coeff. Variation
398	11	131.9 ± 7.6	5.7
393	13	73.8 ± 7.2	9.7
389	11	35.7 ± 5.0	13.6
367	10	75.7 ± 8.2	11.0
382	10	55.1 ± 9.2	16.7
378	12	13.6 ± 3.8	28.0

SD = Standard deviation

TABLE IV. Determination of FIAX End Point Titers of Calibrator Serum Samples

Serum #	Inverse Serum Dilution 0	5	10	20	40	80	160	320	640	1280	2560	5120	Endpoint FIAX Titer
398	128[a] ± 8	86.7 ± 4	61.7 ± 4	35 ± 2	25 ± 2	16.3 ± 2	10 ± 1	7 ± 1	*5*[b] ± 1	3.7 ± 0.6	4 ± 0	4 ± 0	640
393	71.3 ± 4	25.3 ± 3	15.3 ± 2	10 ± 1	6.6 ± 2	6 ± 1	5 ± 0	4 ± 0	3.7 ± 0.6	4 ± 0	ND	ND	160
389	30.7 ± 4	8.3 ± 0.6	6.7 ± 0.6	5.3 ± 0.6	*5* ± 1	4 ± 0	4 ± 0	4 ± 0	3.7 ± 0.6	4 ± 0	ND	ND	40
384	*5* ± 0	4 ± 0	4 ± 0	4 ± 0	3.7 ± 0.6	4 ± 0	4 ± 0	ND	ND	ND	ND	ND	<5

[a]Mean ΔFSU value ± SD of three replicates
[b]Italicized are the ΔFSU values that correspond to the end point titer
ND = Not done

TABLE V. Comparison of Mean FIAX and Mean Indirect Bacterial Agglutination (IBA) Titers for Calves Vaccinated by Aerosol Exposure to Live *Pasteurella haemolytica*

Aerosol Vaccine[b]	# Animals	Geometric Mean Titers ± SD[a]			
		Before vaccination		After vaccination[c]	
		FIAX	IBA	FIAX	IBA
PBS	10	8.1 ± 3.0	ND	5.0 ± 3.9	ND
6 hr culture	10	6.3 ± 1.8	3.6 ± 3.3	114.3 ± 4.9	65.3 ± 5.4
22 hr culture	10	10.0 ± 6.1	23.8 ± 2.1	83.4 ± 2.9	68.6 ± 2.3

[a]SD = Standard deviation
[b]Two 15 minute aerosol exposures to PBS or 10^9 live organisms at one week intervals
[c]Sera obtained two weeks after last vaccination
ND = Not done

that were vaccinated with PBS (calf experiment A) had a geometric mean prevaccination FIAX titer of 8.0 ± 3.0 (range 0.4 to 26.5) at the start of the experiment (Table V). Serum samples obtained two weeks after the seconds PBS vaccination had a geometric mean FIAX titer of 5.0 ± 3.9 (range 0.02 to 44.3). Only one of these calves (#389) had a rise in titer during this period (18.7 to 44.3).

Serum samples from ten calves that were aerosol vaccinated with six hour cultures of *P. haemolytica* had a geometric mean prevaccination FIAX titer of 6.3 ± 1.8 (range 2.1 to 71.9) and IBA titer of 3.6 ± 3.3 (range < 10 to 10). Two weeks after the second vaccination, serum samples from these calves had a geometric mean FIAX titer of 114.3 ± 4.9 (range 34.2 to 264.2) and mean IBA titer of 65.3 ± 5.4 (range 40 to 320). During the experiment, each calf experienced a rise in antibody titer of at least fourfold by the IBA and fivefold by the FIAX tests.

Vaccination with 22 hour *P. haemolytica* cultures resulted in seroconversions similar to those observed after vaccination with six hour cultures. At the start of the experiment, these calves had a mean FIAX titer of 10.1 ± 6.1 (range 0.0 to 85.5) and mean IBA titer of 23.8 ± 2.1 (range ten to 80). Two weeks after the second vaccination, calves had a mean FIAX titer of 83.4 ± 2.9 (range 11.6 to 314.9) and mean IBA titer of 68.6 ± 2.3 (range 20 to 320). During the experiment each calf had a rise in antibody titer of at least twofold but mostly fourfold or greater as measured by both IBA and FIAX. There was a high degree of correlation between titer values determined by the two techniques ($r = 0.5616$, $n = 40$, $p < 0.001$).

Calves vaccinated with *P. haemolytica*, in calf experiment B, had an average 4.5 fold increase in FIAX antibody titers to *P. haemolytica* over the 21 day observation period (Fig. 1). Phosphate buffered saline vaccinated calves had an average twofold decrease in titer. In calf experiment C, a rise in FIAX antibody titer to *P. haemolytica* was not observed in calves vaccinated with either PBS or *P. multocida*.

DISCUSSION

The FIAX system has been used to demonstrate antibody to a number of infectious agents (6, 9, 12). The results of the present studies indicate that the FIAX system can be successfully adapted for detecting antibodies to *P. haemolytica* in the sera of calves that have been experimentally vaccinated

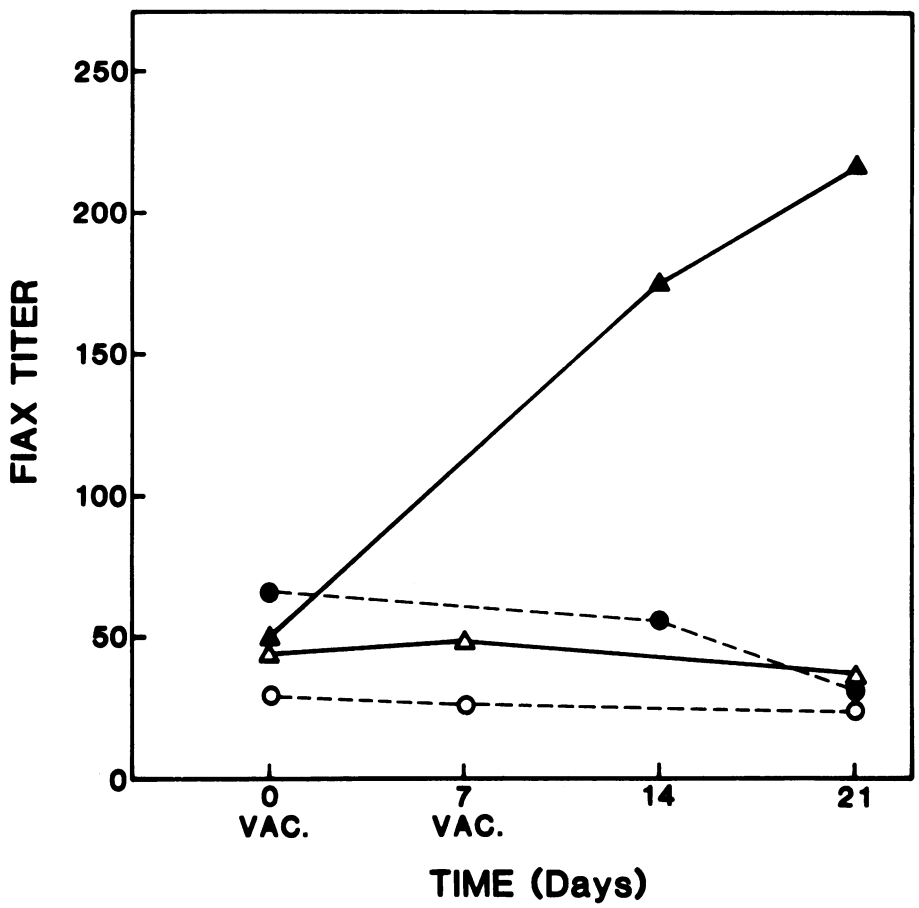

Fig. 1. Comparison of mean FIAX titers in calf experiments B and C.
Experiment B
▲——▲ ***P. haemolytica*** **vaccinated (six calves)**
●-------● **PBS vaccinated (two calves)**
Experiment C
△——△ ***P. multocida*** **vaccinated (six calves)**
○-------○ **PBS vaccinated (eight calves)**

by aerosol exposure to live *P. haemolytica* organisms.

Six hour organisms without capsules and 22 hour organisms were the most efficient antigens in the FIAX system. This finding correlates with the observation that in a direct agglutination assay using hyperimmune chicken sera six hour *P. haemolytica* without capsules and 22 hour organisms agglutinated more readily than six hour whole organisms (5). The results of our FIAX study support the concept that the presence of a capsule on six hour *P. haemolytica* may decrease antibody binding.

We determined that the formalin-killed *P. haemolytica* preparation from 22 hour cultures gave consistent results and was advantageous because of low nonspecific binding of the conjugate. The lower nonspecific binding of conjugate to the formalin-killed 22 hour preparation compared to the live 22 hour preparation is most likely due to the washing steps involved in preparing formalin-killed antigen. This may remove bacterial debris resulting in a "cleaner" antigenic preparation. The 22 hour formalin-killed preparation was also advantageous because 18 to 22 hour formalin-killed *P. haemolytica* preparations have been used most frequently in the IBA assay (4, 13). We were, therefore, able to compare the antibody titers in both the FIAX and IBA tests, and results of calf experiment A demonstrated that the FIAX test was of similar sensitivity to IBA. Regression analysis indicated that the titers determined by the two assays were comparable.

Repeatibility in the FIAX test was acceptable in that replicate samples had percent coefficients of variation of 28.0 or less. In our laboratory, variations in IBA titers from one test to another on the same serum samples can often be twofold and occasionally fourfold while variations in FIAX titers have consistently been less than twofold for individual samples. This difference in reproducibility in the IBA test may only reflect our limited experience with that test and may not be a valid concern for those laboratories that have extensive experience with the assay.

The FIAX test for *P. haemolytica* appears to be fairly specific in that calves vaccinated with *P. multocida* serotype 3 did not develop antibody that cross reacted with *P. haemolytica* serotype 1. Additional tests should be performed to determine the specificity of the FIAX test using other *P. haemolytica* and *P. multocida* serotypes.

There are several advantages to the FIAX test over other techniques for detecting antibody to *P. haemolytica*. The assay requires only small quantities of serum (20 μL for each serum performed in duplicate). The assay also does not require serial dilutions of serum samples. This limits the time per sample to determine an antibody titer. In our laboratory, 150 to 200 serum samples in duplicate can be tested easily in one working day, thereby making the FIAX test ideal for serological surveys or experiments involving multiple samplings of large numbers of cattle. Another potential advantage is that various types of antigen preparations can be used in the assay. The antibody response, therefore, could be detected to such things as *P. haemolytica* capsular antigens or cytotoxic factors. Finally, the FIAX test could be used to determine antibody titers associated with different classes of antibody by use of FITC-conjugated antisera that is immunoglobulin class specific.

In conclusion, the FIAX test for detection of antibody to *P. haemolytica* is sensitive, simple to perform, specific, and reproducible. The cost per test is not prohibitive. We estimate that in our laboratory the test costs about $2.00 per serum sample. The major disadvantage to the FIAX test is a high initial equipment cost.

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