Chapter 21. ANIMAL SPECIES DETERMINATION, IMMUNOLOGICAL

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PART A

21.1 (Presumptive) Tube Ring Precipitin Test

21.11 Introduction

The accurate identification of animal meat species at a significant level of sensitivity in raw meat and poultry products is an important aspect of the Agency's ability to meet the legislative mandate providing for the assurance of a safe, wholesome, unadulterated and accurately labeled meat and poultry supply to consumers. Raw meat species identification can generally be accomplished by physicochemical procedures such as electrophoresis, isoelectric focusing (see Chapter 16) or high performance liquid chromatography and by immunological procedures such as immunoprecipitin (immunodiffusion) reactions (see Chapter 18) or enzyme-linked immunosorbent assay (ELISA), (see Chapter 17).

The immunological methods described in Parts A, B, and C of this chapter of the Microbiology Laboratory Guidebook concerning raw meat species identification have been selected, adapted and implemented for use in the FSIS Technical Support Laboratories because of their suitability as scientifically sound methods, defendable in a court of law when litigation arises from violative results and their practical working use in high volume, routine sample analysis in regulatory laboratories. The methods in Parts A and B are to be used only as presumptive screen tests and all positive, violative results are to be further subjected to a final confirmation by the procedure described in Part C.

The analytical screen test formerly used by the Technical Support Laboratories for determination of the species of animal tissue in raw meat and poultry products was the Ring Precipitin test. Although this immunoassay was successfully used for many years, it was subject to certain limitations or requirements. These consisted of such factors as unremarkable and variable sensitivity levels for species adulterants in different base meat tissues; the availability of significant quantities of expensive, specific anti-species sera; the exact titration of these antisera against standardized reference 30,000 total protein solutions in a specific timed reaction interval; preparation of crystal clear sample

extracts for test reactivity against the titered, specific antisera in the timed reaction interval; and the known observation that certain meat product ingredients such as spices or soy proteins may interfere with obtaining correct test results when certain samples containing such are analyzed by this standardized procedure. In short, this assay required several immunochemical reagents and much, exact standardization of reagents and test performance in order to insure reliable test results. Although this assay has been replaced for routine use by a commercial Immunostick ELISA screen test procedure described in Part B, the Ring Precipitin test procedure is presented below in detail to provide information as an alternative acceptable method if the need should arise.

21.12 Equipment and Materials

a. Culture tubes, glass, 6 x 50 mm, disposable.
b. Pipettes, Pasteur type, 9" (22.8 cm) and 5-3/4" (14.6 cm), disposable, sterile.
c. Pipettes, calibrated, assorted sizes, sterile.
d. Serum vials, rubber stoppered, 15 and 30 ml size, sterile.
e. Racks for holding 6 x 50 mm culture tubes.
f. Culture tubes, glass, 20 x 150 mm or larger.
g. Filter paper, Whatman #42, 11 cm diameter.
h. Millipore Millex® disposable membrane filter units, Luer-lock fitting, 0.45 or 0.22 µm porosity.
i. Syringes, disposable, assorted sizes.
j. Hypodermic needles, disposable, 20 and 22 gauge by 1" (2.5 cm) long; 19 gauge by 1-1/2" (3.8 cm) long.
k. Centrifuge, preferably refrigerated.
l. Centrifuge tubes, plastic, autoclavable, 50 ml capacity.
m. Spectrophotometer, Bausch & Lomb Spectronic 20.
n. Calworth Stomacher®, Model 80.
o. Whirl-Pak® polyethylene bags, 22.8 x 11.4 cm size.
p. Mechanical Shaker.
q. New Zealand albino rabbits, 2.3 kg.

Precaution: All non-disposable glassware must be thoroughly cleaned in detergent, followed by final distilled water rinse and heating in a drying oven for at least 2 h at 200°C to prevent foreign protein contamination.

21.121 Reagents

a. **Normal Saline (0.85% sodium chloride solution):**

Dissolve 8.5 g NaCl in 1000 ml distilled water.

b. **2X Saline (1.7% sodium chloride solution):**

Dissolve 17 g NaCl in 1000 ml distilled water. Add merthiolate to a final concentration of 1:10,000.

c. **2X Saline Containing 10% Normal Rabbit Serum:**

Add 10 ml of normal rabbit serum to 90 ml of 2X saline (above) and mix thoroughly.

d. **Merthiolated Saline:**

To normal saline add sufficient powdered merthiolate to produce a final concentration of 1:10,000.

e. **Normal Sera:**

Obtain authentic normal horse, beef, pork, sheep, chicken, and turkey sera from a reputable commercial source or by directly bleeding the appropriate animal.

f. **10% Solution of Aluminum Potassium Sulfate in Distilled Water**

g. **Specific Antisera to Animal Species:**

Obtain anti-horse, beef, pork, sheep, chicken, and turkey sera following rabbit immunizations.

h. **Biuret Solution**

21.13 Preparation of Proom's Alum Precipitated (PAP) Antigens for Rabbit Immunizations

The preparation of alum precipitated antigens from the normal serum of various animal species is as follows by the method of Proom (Proom, 1943).

a. Obtain 25 ml of authentic normal serum of the particular species required and thaw completely from the preserved frozen state.

b. To this 25 ml of normal serum add 80 ml of sterile distilled water and 90 ml of 10% aluminum potassium sulfate solution and mix thoroughly.

c. Using a pH meter, adjust the pH of the resulting solution to 6.35 very carefully with 5 N NaOH.

d. Pour the adjusted solution into centrifuge tubes, centrifuge in the cold at 3,000 RPM for 20 minutes and discard the supernatant fluid.

e. To the packed precipitate add 100 ml of merthiolated saline, thoroughly resuspend the precipitate and pour into a large plastic bottle.

f. Place this bottle and solution on a mechanical shaker and shake vigorously at room temperature for 25 minutes.

g. Pour the solution back into centrifuge tubes and centrifuge as described in Step (d). (Or centrifuge in large bottles.)

h. Repeat steps (e thru g) for a total of 4 times.

i. After the final centrifugation and liquid discard, add merthiolated saline to the fluffy white precipitate for a final volume of 100 ml and thoroughly resuspend.

j. Place 25 ml aliquots of this alum precipitated antigen into sterile serum vials and label the appropriate species represented.

k. Store this antigen in the refrigerator until needed for rabbit immunization. **DO NOT FREEZE**.

l. Prepare alum precipitated antigens, as outlined above, for each species of animal to which specific antiserum is required.

21.14 Antiserum Production

Prepare specific antisera against each species required by the following method:

a. Obtain a healthy 2.3 kg New Zealand albino rabbit and using a syringe fitted with a 20 gauge, 2.5 cm long needle obtain 5 ml of blood from the medial artery of the ear.

b. Separate the serum and test this preimmune serum against the prepared test antigens by the tube ring precipitin test to assure that the rabbit is free of existing antibodies.

c. Using a syringe fitted with a 22 gauge, 2.5 cm long needle inject 0.5 ml of thoroughly mixed, previously prepared alum precipitated antigen of the desired species, intramuscularly into each hind leg of the rabbit (1.0 ml total) as the primary injection.

d. On Day 21 post primary injection, inject 0.5 ml antigen into each leg, as the initial booster.

e. On Day 28 post primary injection, trial bleed the rabbit from the medial artery of the ear, obtain the serum and perform a titration to determine the relative antibody content as described under Section 21.16, Antiserum Titration and Specificity Tests. If the immune serum has a titre of 1:10 or greater, proceed to obtain a large bleeding from the rabbit.

f. If the serum titer of the above trial bleeding is considerably lower than 1:10, proceed to give a second booster injection of antigen as in step (d) on Day 36 post primary injection.

g. After 14 days from this second booster injection of antigen obtain a large bleeding from the rabbit.

h. Large bleedings may be obtained by using a large syringe fitted with a 20 gauge, 2.5 cm needle and bleeding carefully through the medial artery of the ear or by placing the rabbit ventral side up in an appropriate restraining device and performing intracardiac bleeding with a 100 ml disposable syringe fitted with a 19 gauge,

3.8 cm long needle. If the rabbit is to be kept for subsequent bleeding or reimmunizations, DO NOT bleed for more than 35 ml at any one time. Remove the needle and gently aspirate the blood from the syringe into a sterile container.

i. The serum is obtained by allowing the blood to clot at room temperature for 2-4 h, ring the clot from the walls of the container and place in the refrigerator overnight. Decant the serum, centrifuge at 3,000 RPM to remove all RBC's and filter sterilize through a 0.22 µm Millex® membrane filter unit directly into a sterile, rubber stoppered serum vial. Merthiolate may be added to a final concentration of 1:10,000 as a preservative, but only as a last resort in lieu of strict aseptic handling of the serum at all times. More information relative to Steps (h) and (i) may be found in "Methods in Immunology", 1977.

j. Label the vial as to the specific anti-species serum represented and keep refrigerated until further use in the tube ring precipitin test. DO NOT FREEZE.

21.15 Preparation of Normal Serum Antigens for Controls in The Ring Precipitin Test

Antigens to be used for controls and antisera titering in ring precipitin tests are prepared from authentic normal sera obtained from various animal species. Maintain these normal sera in a frozen sterile condition prior to dilution and use.

Since the protein content of sera varies from animal to animal within a species, as well as among species, it is necessary to determine and adjust the amount of antigen used for controls. This is done on the basis of the total protein (TP) content of each normal sera.

The TP content of each sera is determined by the biuret method (Section 21.19). Prepare a 1:500 working dilution of TP using the following formula:

(5 x % TP) - 1 = D_v 500. In which % TP = % TP in serum, and D_v 500 = Volume of normal saline to be added to one volume of serum to attain a 1:500 dilution.

Examples:

Serum A = 7% TP	Serum B = 6.5% TP
(5 x 7) - 1 = 34	(5 x 6.5) - 1 = 31.5
1 ml Serum A + 34 ml	1 ml Serum B + 31.5 ml
Normal Saline = 1:500 TP	Normal Saline = 1:500 TP

From this 1:500 working dilution of TP prepare the following TP dilutions in normal saline: 1:1,500 TP; 1:3,000 TP; and 1:30,000 TP. The 30,000 TP serum antigen will serve as the homologous test antigen, while the 3,000 TP and 1,500 TP serum antigens will serve as heterologous test antigens in the procedures that follow.

Filter these diluted serum antigens through a Millex® filter (0.45 µm) into sterile vials or screw cap tubes. Store these diluted antigens at 4-6°C. DO NOT FREEZE. Discard after 8 weeks, or if cloudy or precipitated.

21.16 Antiserum Titration and Specificity Tests

Since the specific antibody content varies within different lots of a particular prepared anti-species serum, it is necessary to quantitate and standardize this antibody level for use in routine sample analysis by the ring precipitin test. It is also necessary to verify the specificity of the reactivity of an anti-species serum towards its homologous antigen at this time.

a. Using 2X saline containing 10% normal rabbit serum, prepare the following dilution series of the anti-species serum to be titered: undilute; 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:9, 1:10, 1:15, 1:20, and higher if deemed necessary. (Large volumes are not necessary.)

b. Test each of the above dilutions against 30,000 TP homologous serum antigen and 1,500 TP heterologous sera antigens previously prepared using the described Ring Precipitin Test. NOTE EXCEPTIONS: To test anti-bovine and anti-ovine sera with their respective heterologous sheep and beef antigens, use 3,000 TP instead of 1,500 TP. Make the same exception for anti-turkey and anti-chicken sera.

c. Choose as the working dilution of antiserum for subsequent use in routine ring precipitin testing on unknowns the highest dilution of antiserum that gives a positive test with the 30,000 TP homologous antigen

within 6 minutes, and fails to give a positive test with the 1,500 TP heterologous antigen (Note Above Exceptions) within 10 minutes. This establishes the antiserum titer and confirms specificity.

An additional test on specificity may be performed by the agar gel immunodiffusion test using undiluted antiserum and saline extracts of tissues from authentic heterologous and homologous animal species.

d. Prepare a 5-6 ml volume of working dilution of each anti-species sera required in 2X saline containing 10% normal rabbit serum and filter sterilize through Millex® filters (0.22 µm) into sterile 15 ml screw cap vials. Refrigerate at 4-6°C until needed. DO NOT FREEZE. Reconfirm the titer and specificity of the working dilution of antisera against appropriate TP antigens each week and discard the sera upon loss of titer or specificity, or development of autoprecipitation or microbial contamination.

21.17 Sample Extraction

a. Fresh Tissue

Weigh 25 g of fresh tissue, using the inner portion of the piece if possible. Dice the tissue and place into an appropriate receptacle (polyethylene bag or beaker) and add 100 ml normal saline. Allow to stand for 1-1/2 to 2 h at room temperature. Filter 5-6 ml of the extract through three-fold filter paper (Whatman #42) into 20 x 150 mm tubes. The filtrate must be crystal clear, but may be colored from straw to dark red. If the filtrate is not crystal clear, subject it to centrifugation and/or filtration through a Millex® syringe filter unit (0.45 or 0.22 µm pore size). Run the test as soon as possible, before the filtrate becomes cloudy.

b. Partially Cooked or Cured Tissue

When a tissue has been heated above 165-175°F, the proteins become insoluble and cannot be extracted. Frequently, however, an interior section may not have reached the denaturing temperature and will release enough soluble proteins for a test. The same applies to cured products. For cooked, uncured tissues, extract as

for fresh tissue and let stand in the refrigerator at least 18 h, then test aliquots at intervals for 5 days. If no reaction occurs after 5 days' extraction, report sample as not giving an antigenic response. If possible, perform the ELISA cooked meat species procedure (see Chapter 17) to identify and differentiate these non-reactive samples. Use the same procedure for cured tissue, but extract with distilled water instead of saline.

c. Chopped, Ground or Emulsified Tissue

Proceed as for fresh tissue.

d. Alternative Extraction Method

Place 12.5 g of tissue and 50 ml normal saline in a 22.8 X 11.4 cm Whirl-Pak® polyethylene bag. (Do not deviate from above amounts.) Place the bag and contents in a Calworth Stomacher®, model 80, and stomach for the following times found to be optimum for the various types of sample products listed (Table 1):

Table 1. Stomaching Time for Samples

Sample Types	Stomaching Time/seconds
Raw ground meats, emulsions and sausage formulations	0 (manually knead bag and contents)
Raw muscular tissue, diced	5-10 (maximum)
Cooked and cured samples, hard processed meats (salami, bologna, frankfurters, etc)	15-30 (maximum)

After stomaching, allow the bag and contents to sit at room temperature for 15-20 minutes. Proceed to prepare a crystal clear filtrate of this extract in the usual manner outlined for fresh tissue extraction.

21.18 Ring Precipitin Tube Test

In an appropriately marked rack, place one 6 X 50 mm tube for each species for which the sample is to be tested (e.g., horse, beef, pork, sheep, chicken, turkey). Place in each tube about 0.2 ml of the working dilution of respective anti-species serum using individual, sterile Pasteur pipettes. Fill another Pasteur pipette with the unknown tissue extract to be tested. Tilt the tube at a 45^{o} angle and slide the pipette down the side of the tube just above the antiserum. Then allow the extract of the unknown to flow gently over the surface of the antiserum, while withdrawing the pipette, keeping it ahead of the advancing interface. Do not allow the pipette to touch the antiserum, or to disturb the interface. Clean the surface of the tube with moist toweling, then wipe it dry. After 3 to 5 minutes, and again up to 10 minutes, read the tube by indirect light against a black background.

A cloudy white ring at the interface is a positive test. Also test heterologous TP dilutions, and read up to 10 minutes as a test of acceptability of antisera. If the heterologous TP dilution for one species gives a positive test against the serum of another species within 10 minutes, check for possible contamination of the antiserum. (Note: Quality Control Section, 21.110)

Retest the antiserum for specificity and retest the sample, extracting at least two times. If more than one piece of tissue was used, then retest each piece separately using, if possible, the innermost portions of the pieces. If the sample is ground or chopped, retest another extraction of the sample; repeat two times if the reaction indicates possible violation. Record the reaction times.

21.19 Total Protein by Biuret Method

21.191 Biuret Solution

In a one liter volumetric flask place 1.5 g cupric sulfate, and 6.0 g fine crystals of potassium sodium tartrate. Add sufficient distilled water to dissolve. Add slowly with agitation of the flask, 300 ml 2.5 N sodium hydroxide and mix. Add 1 g potassium iodide and shake until dissolved. Dilute to one liter total volume. Discard when black or reddish precipitate forms.

21.192 Method

a. Place 9.5 ml 0.85% NaCl in a test tube. Add 0.5 ml of sample. Rinse out pipette by drawing in and expelling some of the mixture.

b. Into one of 2 test tubes place 2 ml of the diluted sample, above; in the other, 2 ml 0.85% NaCl solution (blank).

c. Add 8 ml biuret reagent (above) to each tube, and mix.

d. Set 100% transmission with "blank" at wavelength 540 nm.

e. Immediately after adding biuret reagent read transmission of sample and obtain concentration from the following (Table 2).

Table 2. Percent protein, as determined by percent transmission of Biuret reaction in Bausch and Lomb Spectronic 20. (Note: Quality Control Section).

% TR* (540 nm)	0	1	2	3	4	5	6	7	8	9
	Percent Protein									
0										
10										
20										
30								13.8	13.4	13.0
40	12.7	12.4	12.0	11.7	11.4	11.1	10.8	10.5	10.2	9.9
50	9.6	9.3	9.0	8.8	8.6	8.3	8.0	7.8	7.6	7.3
60	7.1	6.9	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2
70	5.0	4.8	4.6	4.4	4.2	4.0	3.8	3.7	3.5	3.3
80	3.1	2.9	2.8	2.6	2.4	2.3	2.1	2.0	1.8	1.6
90	1.5	1.4	1.2	1.0						

* TR (Transmission)

Example: % transmission = 47. Concentration of protein = 10.5%

21.110 Quality Control Procedures

In order to assure the integrity and reproducibility of the procedures previously outlined, special attention should be given to the following considerations cited for each section listed below:

a. **Normal serum of authentic species: It is absolutely essential that the species authenticity of the normal sera be initially established since these sera serve as the starting material for anti-species sera production and standardized test antigens. This can be accomplished by directly bleeding the live animal species required and preparing the serum from the blood. If a commercial source of normal serum of a particular species is used, it should be verified in a known, correctly functioning, serological test system.**

b. **Total Protein Determinations and TP Dilutions: Care and attention should be given to the correct test performance, data interpretation and calculations to arrive at the total protein content of each normal sera. Caution must also be exercised in the mechanical preparation of the correct TP dilutions of heterologous and homologous sera antigens. Improperly prepared or calculated values for the above will lead to erroneous anti-species sera titration or specificity data. This in turn might render the antisera dilution finally chosen for use, totally ineffective for reacting with an adulterant tissue in an unknown sample.**

c. **Antiserum Titration and Specificity Checks: The most important component of the ring precipitin test system which ultimately is responsible for the successful detection of an adulterant tissue is the standardized anti-species serum. It cannot be stressed too strongly that periodic checks on the performance characteristics of these diluted antisera must be made with the 1,500 TP and 30,000 TP normal serum antigens to assure that the antisera are reacting in the expected manner. Previously titered antisera can on occasion, with age, produce a change in the titration endpoint. Appropriate adjustments in the working dilutions of these antisera would therefore need to be made in order to compensate for this fact.**

d. Biuret Protein Determination Table: It should be noted that the convenient table provided for the determination of protein by the Biuret reaction is valid only if the exact test procedure is followed and the percent transmission values are obtained using a Bausch and Lomb Spectronic 20 spectrophotometer with the standard, round, tube shaped cuvettes. If a different protein determination test or spectrophotometer is to be employed, then a new standard table must first be prepared with the use of known protein standards.

e. Sample Extracts and Anti-species Sera Working Dilutions: Reagents must be crystal clear following Millex® filtrations just prior to performing the ring precipitin test. Any degree of cloudiness will make it more difficult to visualize any reacting immunoprecipitin line at the interface.

f. Overlaying the Working Dilution of Each Respective Anti-species Serum with the Sample Extracts: Overlayering must be done in a careful, gentle manner so as to not create a mixture of the two reagents at the interface. A mixture at the interface will tend to create a broad, diffuse immunoprecipitin band and cause difficulty in visualizing a positive reaction within the specified time period, rather than the usually expected sharp band.

21.111 Selected References

Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Methods in Immunology: A Laboratory Text for Instruction and Research, p. 7-38. 3rd Edition. W. A. Benjamin Inc. Reading, MA.

Kenny, F. 1985. A practical species testing programme, p. 155-159. *In* R. L. S. Patterson (ed.), Biochemical Identification of Meat Species. Elsevier Science Publishing Co., Inc. New York, NY.

Proom, H. 1943. The preparation of precipitating sera for the identification of animal species. J. Path. Bact. 55:419-426.

PART B

21.2 (Presumptive) Commercial ELISA Immunostick Screen Test Kit.

21.21 Introduction

Modern developments in immunoassay technology have made available alternative procedures which have the advantage of eliminating or greatly reducing the limitations previously cited for the Ring Precipitin test. One such procedure is the Enzyme-Linked Immunosorbent Assay (ELISA) method, which is now available in a commercialized kit form capable of rapid, specific species identification of raw meat and poultry tissue products, inclusive of all current species of interest to our National Testing Program. The original ELISA raw species test kit was developed and manufactured as a solid phase microwell plate system. The system was subsequently modified slightly by incorporation of NUNC dip-stick paddles (immunosticks) as the solid phase and the use of pre-dispensed, standardized reagents in color coded tubes. It is currently marketed and distributed in the U.S. in a complete (25 test) kit form and is referred to as a commercial ELISA Immunostick Raw Meat Species Screening Test Kit.

This raw meat species screen test is a double antibody "sandwich" ELISA procedure with antibody specificity directed against the various species albumins which are contained in meat tissues. Specific antibody sensitized immunosticks are allowed to capture homologous species albumin from sample tissue extracts, then reacted with the second peroxidase labeled antibody of the same specificity, followed by a final reaction step in ABTS/H_2O_2 chromogen/substrate solution. A short incubation period and a brief tap water rinse is performed between each of the first two steps. A positive reaction, indicating the presence of the test species tissue in the sample, is evidenced by a distinct green color formation in the last reagent tube. Each species kit contains all necessary reagents, controls and accessories to perform the test in an extremely easy fashion with the production of very accurate results.

The Immunology Section of BCB, MD at Beltsville conducted an evaluation of the ELISA Immunostick Screen test kits for all available species. They were found to be very specific, reliable, easy to use and capable of detecting an adulterant tissue at the 1% sensitivity level. It is with the above considerations in mind and the aim of technical improvement over the previous screen test procedure that these commercial Immunostick Screen Tests were

implemented in all FSIS, Technical Support Laboratories for raw meat species determinations. The Immunostick Screen Test is now used in place of the standard Ring Precipitin test procedure. All positive Screen Test results which represent sample violations are to be confirmed in the usual manner by the standard agar-gel immunodiffusion procedure described in Part C.

A commercial ELISA Immunostick Screen Test employed for presumptive identification of species composition of raw meat and poultry tissues should meet or exceed the following performance characteristics:

Sensitivity - produce positive reactions down to the 1% level (W/W) of adulterant or contaminant tissue in a base meat tissue mixture such that a 0% False Negative Rate is observed.

Specificity - produces no positive cross reactions with any heterologous species tissues such that a 0% False Positive Rate is observed.

21.22 Reagents and Equipment

a. Commercial ELISA Immunostick Raw Meat Species Screen Test Kits. Color codes for individual species kits are as follows (Table 1):

Table 1. Color Codes for Commercial ELISA Immunostick Screen Test Kits.

Color Code	Species	Color Code	Species
Red	Beef	Orange	Horse
Yellow	Pork	Lilac	Rabbit
Blue	Poultry*	Grey	Kangaroo
Pink	Chicken*	Brown	Turkey*
Green	Sheep	Various	Mixed

* The ELISA immunostick Poultry screen test does not differentiate between chicken or turkey. If it should become necessary to do

so, this can be accomplished by performing the traditional agar-gel immunodiffusion procedure (Part C), or by using the ELISA immunostick chicken or turkey screen tests. Since these latter two screen tests have less than the required sensitivity, their use should be limited to whole meat or poultry tissues or a mixed meat/poultry emulsion where the poultry component is known to constitute over 5% of the final meat block.

Each individual species kit contains the following items:

i. Twenty-five - color coded, white plastic immunosticks sensitized with specific anti-species capture antibody in tubes of preservative buffer solution.
ii. Twenty-five - color coded tubes containing species specific antibody-enzyme conjugate reagent.
iii. Twenty-five - tubes (non-color coded) containing color development buffer reagent.
iv. One vial of concentrated ABTS color reagent.
v. One vial of aqueous sodium fluoride stop solution.
vi. One vial of positive control solution (homologous species albumin).
vii. Disposable polypropylene pasteur pipettes - **NOT TO BE USED**.
viii. Product insert test kit instruction pamphlet.

b. Rainin Gilson Pipetman® (P-200) adjustable pipette and appropriate disposable pipette tips.
c. Calworth Stomacher®, Model 80.
d. Whirl-Pak® polyethylene bag, 6 oz size (7.5 x 17 cm).

21.23 Raw Sample Preparation

All types of raw meat and poultry product samples are prepared as follows:

a. Weigh out 1 gram of thawed, diced, raw sample product which is a homogeneous, representative portion of the whole sample.

b. Place in a 6 oz Whirl-Pak® bag.

c. Add 9 ml of distilled water.

d. Place the bag and it's contents in a Model 80 Calworth Stomacher® and stomach for a period of 60 seconds.

e. Allow the extract to settle for 2-3 minutes until a particle free liquid layer is formed in the top portion of the bag's contents. Use this upper liquid layer as the sample extract in the following test procedure.

21.24 Test Procedure

The following procedure is to be used, which represents minor modifications from the original product insert test kit instruction pamphlet. These procedural modifications are designed to improve the accuracy, precision and reproducibility of test results. The subsequent instructions represent the testing of 1 sample through 1 species test procedure. Obviously multiple samples and/or species tests may be performed simultaneously, as long as one is careful to keep track of reaction times, washing steps, various reagent steps, etc. relative to each given test sample.

a. Remove the appropriate color coded species Immunostick tube, antibody-enzyme conjugate reagent tube, and color development buffer tube (a set of 3) from refrigerated storage and allow to equilibrate to room temperature.

b. Label Immunostick caps and all tubes with appropriate sample identification codes.

c. Prepare the color development buffer reagent tube (non-color coded) for later use by adding 40 µl of ABTS concentrate to this tube, replace cap and mix in a gentle but complete manner.

d. Obtain the first color coded Immunostick tube, unscrew the cap and remove the immunostick-paddle, add 200 µl of prepared sample extract to the liquid in the tube, replace the immunostick-paddle in the tube and mix contents by rotating the cap rapidly 4-6 times and tighten the cap. DO NOT INVERT tubes to accomplish mixing at any stage in this procedure. Handle the paddle at all times only by it's attached cap, DO NOT TOUCH paddle with fingers.

e. Allow this tube to stand for 10 minutes at room temperature.

f. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for 10 seconds, then shake to remove excess water.

Note: Water dispensed from a squeeze bottle can also be used to carefully perform this wash step.

g. Place the washed immunostick-paddle into the second color coded tube of antibody-enzyme conjugate reagent, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

h. Allow this antibody-enzyme reagent tube to stand for 10 minutes at room temperature.

i. Remove the immunostick-paddle and wash the paddle and entire cap completely by placing it under a gentle stream of cold tap water for 30 seconds, then shake to remove excess water.

Note in step (f) above also applies here.

j. Place the washed immunostick-paddle into the final, non-color coded, tube of ABTS prepared (step c) color development buffer reagent, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

k. Allow the color development reagent tube to stand for 10 minutes at room temperature.

l. Add 200 µl of sodium fluoride stop solution to this color development tube, leave the paddle in, and mix well to stop the reaction.

m. Observe the above tube with the white paddle in it for the presence of any discernable green color in the solution or on the paddle surface. A green color indicates a positive test and the presence of the test species in the original meat sample. A colorless solution around the white paddle indicates a negative test and the absence of the test species in the sample.

All ELISA Immunostick positive species results which represent sample violations are to be confirmed by the traditional agar-gel immunodiffusion procedure as described in Part C.

21.241 Test Controls

The occasional use of positive and negative controls in performing this species screen test will ensure proper quality control and reliable test performance of this method. This should ALWAYS be done initially upon opening and placing into use a brand new kit package.

Each species test kit is supplied with a positive control vial (homologous species albumin solution) for this purpose. The negative control for any one particular species test kit may be obtained by using the positive control solution from any of the other heterologous species test kits: (eg. horse albumin solution should always give negative results in all other species kits except horse).

Control testing may be performed in the following manner:

a. Remove the cap and Immunostick from a tube of an individual test series to be used for control testing.

b. Add 200 µl of negative or positive control solution to the liquid in the tube.

c. Replace the immunostick-paddle in the tube, mix contents by rotating the cap rapidly 4-6 times and tighten the cap.

d. Proceed with the remainder of the test procedure exactly as described above by continuing and completing steps e-m (Section 21.24; Test Procedure). Be sure to initially prepare an ABTS color development buffer reagent tube in the usual manner when you start your control tests.

21.25 Quality Control Procedures

a. Store all kit components at refrigerator temperature (4-8^{o} C) when not in use to preserve and maintain reactivity of immunoreagents.

b. Perform positive and negative control testing of an initially opened kit package and occasionally thereafter to insure proper test performance.

c. Observe the manufacturer's one year expiration date of all test kit components. Kits should not be used beyond the expiration date.

d. The concentrated ABTS color reagent solution tube should be observed over the kit shelf life. If this ABTS concentrate should start to turn a much darker shade of green than when it was originally received, this indicates decomposition, and a new tube of ABTS concentrate should be requested from the vendor.

e. All volumetric additions of sample extracts or reagents to the test procedure should be made only with the Rainin Gilson Pipetman® pipette instrument.

f. Kit components should be allowed to equilibrate to room temperature before commencing test procedure.

g. The 1 gram test sample used for extraction must be representative of the entire original sample in order to insure that test results accurately reflect the true composition of the original sample.

h. Preparation of the color development buffer reagent tube by the addition of ABTS concentrate (step c of Section 21.24, Test Procedure) should only be accomplished just prior to commencing the test procedure. Preparation of this reagent tube should not be done in advance (hours/days) because of the inherent chemical instability of ABTS in buffered substrate for extended time periods.

i. Accurate timings of washing and reaction steps should be performed.

j. Assure that all surfaces of the white immunostick-paddle and cap are adequately washed during the two timed wash steps.

k. Do not use hot or warm water for immunostick-paddle washing, only cold.

l. Since all reactions of this solid phase immunoassay occur on the surfaces of the white immunostick-paddle, it is very important not to touch the paddle surface with fingers or any other physical objects which might interfere with the immunoreaction.

m. When performing different species tests simultaneously on the same sample, be sure to maintain the proper continuity of color coded reagent tubes for each respective test species as you complete the test procedure. (eg. An anti-beef species immunostick (red color code) that has reacted with a beef sample extract if improperly placed in an anti-pork enzyme conjugate reagent tube (yellow color code), will produce a false negative result).

21.26 Technical Assistance

If any problems should arise during the performance of this species screen test or technical assistance is required on any aspect of the procedure, contact the following:

Dr. Richard P. Mageau
Microbiology Staff Officer
USDA, FSIS, OPHS, MD, EMIB
Washington, DC 20250
Telephone (202) 501-7600

21.27 Selected References

Anonymous. 1991. Commercial Immunostick Raw Meat Species Screening Kits; product insert instruction pamphlet.

Fukal, L. 1991. Review Article. Modern immunoassays in meat-product analysis. Die Nahrung 35(5):431-448.

PART C

21.3 (Confirmation) Agar Gel Immunodiffusion Test

21.31 Introduction

The final determination (confirmation) of an adulterant species of animal tissue in raw meat and poultry products is based upon the results of sample analysis by the agar-gel double immunodiffusion procedure presented in Part C. All presumptive positive violative results from the analytical methods in Part A or B are subjected to confirmation by agar-gel immunodiffusion before definitive compliance or legal actions are undertaken.

The agar-gel immunodiffusion procedure described in this section is based upon fundamental principles established previously by Ouchterlony, 1968, and modified for specific application and Agency use by Fugate and Penn, 1971. Agar-gel immunodiffusion is notable for it's qualitative ability to demonstrate similarities and resolve differences in related proteins based upon the formation of specific immunoprecipitin lines resulting from the diffusion of specific antigens and antibodies from wells or troughs cut into an agar matrix after they have reached their optimum proportions. As such, this procedure is ideally suited for meat species protein identification. In addition to being relatively easy to perform and providing results within a 24 hour period, the procedure also has the advantage of generally not being affected by the same factors which tend to produce false positive reactions in other immunoassays such as the Ring Precipitin test. If any false or "non specific" reactions should occur in a double immunodiffusion assay, it is possible to distinguish them from true positive reactions by carefully observing the immunoprecipitin pattern formed and it's relationship to known antigen extracts. The three basic types of reactions usually observed in double immunodiffusion assays are lines of identity, lines of partial identity and lines of non-identity. With a little practice and experience these types of reactions can be easily distinguished and their interpretation in relation to resolving the identity and/or relationships of similar proteins can be made in a definitive and reliable manner.

Although several different patterns of wells or troughs may be generally used in an agar-gel to perform double immunodiffusion reactions, the pattern ultimately employed is usually dependent upon the intended, specific application of the assay. Hvass, 1985, used a relatively simple, common, 7 well , circular pattern to differentiate raw meat species, while Fugate and Penn, 1971, used a

more complicated pattern consisting of 3 antisera troughs and 24 antigen extract wells. The latter was designed with the intention of demonstrating relationships among more than one species on a single plate and also to provide several identical reaction areas on the same plate showing the identity or non-identity relationship of an unknown meat species sample with known reference species tissue extracts. The concept of demonstrating several areas of identical results using several positive and negative controls within the same single reaction system provides almost irrefutable evidence in a court of law when applying this already well recognized immunodiffusion procedure to establish identity of a meat species in a case of fraudulent adulteration.

21.32 Equipment and Materials

a. Dish, Petri, plastic, 15 X 100 mm disposable
b. Pipettes, disposable, capillary, Pasteur type
c. Box, plastic, humidity chamber, or other air tight container used to maintain high humidity.
d. Cutter, agar-gel, or template pattern
e. Flask, side arm
f. Tubing, rubber or neoprene, high vacuum type
g. Tubing, brass (Cork borer), 5/32 x 1-3/4 inch (3.95 x 44.5 mm)
h. Applicators, wooden, cotton tipped
i. Pipettes, graduated, serological, assorted sizes
j. Dishes, staining (only if agar is to be dried and stained)
k. Slides, microscope, 1 x 3 inch (2.54 x 7.62 cm);(only if agar is to be dried and stained)
l. Filter paper, Whatman No. 1 and No. 42
m. Pans, plastic, 6 x 12 x 6 inch (15.2 x 30.5 x 15.2 cm), or other suitable containers (used only if agar is to be air dried and stained).
n. Assorted laboratory flasks, beakers, tubes, etc.

Clean all glassware, rinse in distilled water and heat a minimum of two hours at 200°C in a dry heat oven to eliminate contamination from prior use.

21.33 Reagents

a. Normal saline, (0.85 percent sodium chloride solution):

Dissolve 8.5 g NaCl in 1000 ml distilled water.

b. Buffered saline (0.85 percent sodium chloride solution, pH 7.2 phosphate buffered):

To 1000 ml normal saline, add 1.25 ml stock phosphate buffer solution. Adjust pH to 7.2 if required.

c. Phosphate buffer stock solution - pH 7.2:

Dissolve 34 g monobasic potassium phosphate (KH_2PO_4) in 500 ml distilled water. Adjust pH to 7.2 with 1 normal sodium hydroxide (NaOH), (requires approximately 175 ml). Dilute to 1000 ml with distilled water. Store under refrigeration.

d. Agar, 1.0 percent (Oxoid Purified Agar, L28):

To 99 ml buffered saline, add 1.0 g purified agar. Heat with constant stirring until agar is melted. Filter hot agar through glass wool or several thicknesses of cheese cloth.

Dispense into screw cap flasks or tubes and sterilize by autoclaving for 20 minutes at 15 pounds pressure. Cool agar to 49-50°C and add 1.0 ml of stock merthiolate solution (1:100) per 100 ml melted agar, to give a final concentration of 1:10,000. Tighten caps (airtight) and store until needed. Remelt when needed in boiling water bath. (Agar can be stored for extended periods of time if caps are airtight and no desiccation or growth occurs).

e. Tissue extracts from known animal species:

Cut muscle tissue collected from animals (known species) into 10 g portions and freeze until needed. To 10 g of ground or finely diced tissue, add 30 ml normal saline and stomach for specified times as shown in Table 1 Section 21.17. Let stand a minimum of 90 minutes. Decant liquid and filter through Whatman No. 42 filter paper. Use immediately. (note Section Quality Control of key reagents or procedures).

f. Antisera:

Undiluted anti-horse, beef, pork, sheep, chicken and turkey species serum, or others as may be required.

g. Tissue extract-(unknown samples to be confirmed):

Extract unknown tissue(s) as in (e) above, using 25 g tissue and 75 ml normal saline.

h. Staining solution:

Dissolve 2 g acid fuchsin in 500 ml absolute methyl alcohol; add 400 ml distilled water and 100 ml glacial acetic acid.

i. Destaining solution:

To 500 ml absolute methyl alcohol, add 400 ml distilled water and 100 ml glacial acetic acid.

j. Acidified Distilled Water:

To 1000 ml distilled water, add 0.2 ml glacial acetic acid.

k. Mounting fluid:

A commercially available material for mounting cover slips permanently.

21.34 Preparation of Agar-Gel Immunodiffusion Plates

a. Agar Plate Preparation.

Remelt purified agar prepared above and dispense 18-20 ml into the 15 x 100 mm plastic petri dishes. Allow to solidify and refrigerate for a minimum of 30 minutes. Store no more than 2 weeks under refrigeration in a high humidity atmosphere. Do not use plates showing desiccation or microbial growth. (Note: Quality Control Section 21.35)

b. Cutting Pattern of Wells and Troughs.

Remove the plates from refrigeration and cut the desired pattern by one of the two methods described below:

i. Use a gel cutting tool which has the proper well and trough cutting tubes and knife edges permanently

embedded in a fixture such as plexiglas or other solid substance.

Figure 1 illustrates one such tool. Align the tool carefully on the agar surface to obtain a perpendicular cut, then press down firmly to cut the agar.

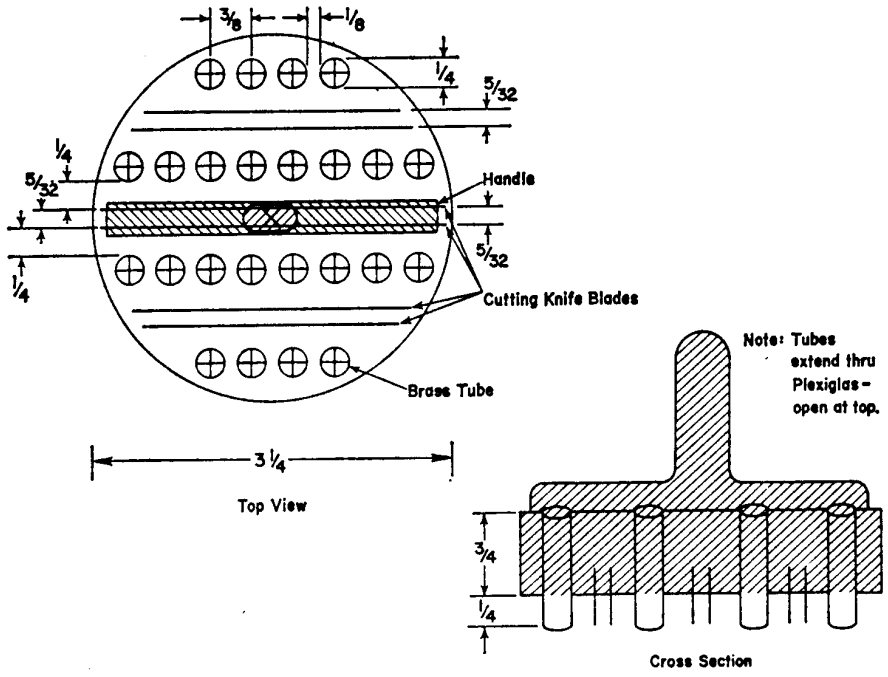

FIG. 1 - Cutting tool used to cut pattern of wells and troughs in agar-gel. (Fugate and Penn, 1971)

ii. Using a pattern of the desired arrangement drawn on graph paper, center the plate over the pattern, agar side up. Press a metal tube of acceptable diameter, connected to a vacuum source by a vacuum tube and side arm flask, through the agar at the indicated places on the pattern. Then cut the troughs with a razor blade or scalpel along the lines of the pattern; or use a tool fashioned with two blades or

knife edges the correct distance apart, and with a downward motion cut the agar.

Remove the agar plugs in the wells with a metal tube connected to a vacuum source. Experience will dictate how to avoid tearing the agar surrounding the wells. Remove the trough plugs with an applicator stick which has one end shaved to present a shovel edge. Gently push the applicator stick to the dish bottom and guide it along the cut, raising the strip of agar as a plow would.

Remove the remaining agar in the wells and troughs with a cotton tipped applicator very carefully so as to not tear the surrounding agar surface.

c. Sealing Wells and Troughs

Hold the plate at a 45^{o} angle and, with a Pasteur pipette, place a thin layer of agar on the floor of each well and trough, sealing the bottom edges of the cut agar to the plate. Do not add an excess of agar. Repair torn wells or troughs in a similar way; if necessary, refill the well or trough and recut it. Caution: An overfilled well will distort the agar and the reaction bands.

d. Preparation of Tissue Extracts: (Protein antigens)

Using the desired known animal species muscle tissue, prepare saline extracts as described in Reagents Section 21.33 e. and g. Do the same for unknown tissue that is to be analyzed. (Note: Quality Control, Section 21.35)

e. Charging the Wells

Mark the outside of the plate to identify the location and contents of each well and trough. Using a Pasteur capillary pipette, partially fill the wells with the known and unknown extracts, maintaining a concave meniscus. Overfilling to form a convex meniscus will interfere with diffusion and may cause wells to overflow. Always place the extract of the unknown between known antigens of two different species. Like antigens will form continuous reactant bands in the agar media, and unlike antigens will form discontinuous bands (See Figure 2).

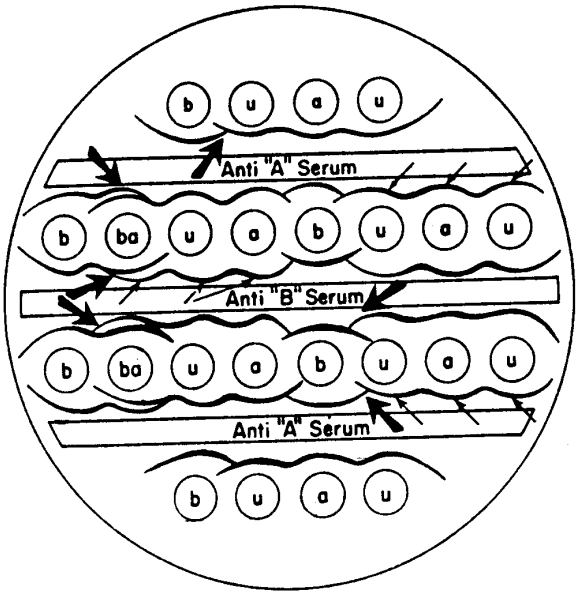

FIG. 2 - Precipitin pattern resulting from heterologous antigen-antisera reactions: a, antigens derived from species A; b, antigens derived from species B; u, antigens derived from unknown; ⇒ , lines of partial identity; → , lines of identity. Although atypical, the above pattern results when all antigens react with antisera used. The identification of unknown antigen u is accomplished by lines of identity formed with antigen a. Both a and u form lines of partial identity with lines formed by antigen b, which is indicated by a spur reaction. It can be concluded that antigen u is derived from species A and is similar but not identical to species B. (Fugate and Penn, 1971)

f. Charging of Troughs:

Fill troughs with the antisera. Use one plate to determine two species only. (e.g., beef and sheep, or beef and horse, etc.). Use the top and bottom troughs for one antiserum, and the center trough for the other. (See Figure 2).

g. Incubation and Observation:

Replace the plate covers and allow the plates to remain at room temperature for 1 1/2 to 2 h. Refill the wells and troughs with the appropriate antigens and antisera. Line the bottom of an airtight chamber with wet filter paper or cotton. Incubate the plates in this high humidity chamber at room temperature for 18 to 24 h. To read the plates, direct a light source parallel to the agar surface, i.e., from the side of the plate, and hold the plate over a dark black background. The reactant bands will appear white on a grey surface. If the bands are not fully developed, refill the wells and troughs, and continue incubation in the chamber for an additional 24 to 48 h under refrigeration.

Following incubation, remove the plates from the humidity chamber, discard the remaining reactants and gently wash the plates under a stream of distilled water. Use a soft cotton applicator to remove any film from the agar surface and precipitated matter from the wells and troughs. Dry the bottom of the petri dish with a soft laboratory tissue and observe the plate for reaction bands. Position the plate in alignment with the worksheet (Figure 3) and draw the reaction bands observed on the plate onto the worksheet.

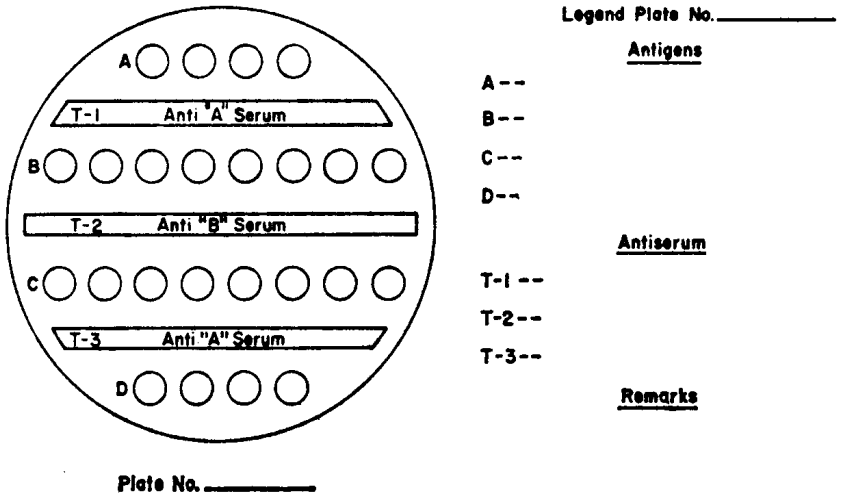

FIG. 3 - Worksheet showing well and trough arrangement and antigen-antisera placement. (Fugate and Penn, 1971)

h. Interpretation of Precipitin Reactions

Interpretation of results depends upon lines formed with known and unknown antigens. Figure 4 (A) illustrates an identity line, i.e., the precipitin line that forms when the antigens are identical. Figure 4 (B) shows partial identity lines, i.e., the lines that form when extracts contain similar but not identical proteins which react with the same antiserum. Figure 2 (page 21-26) illustrates a typical reaction with an unknown and 2 known antigens, showing lines of identity and partial identity. Since unknown antigen u forms a continuous wave pattern with known antigen a, lines of identity form. The lines formed by known antigen b appear as spurs of those formed by antigen a and u, and are typical lines of partial identity.

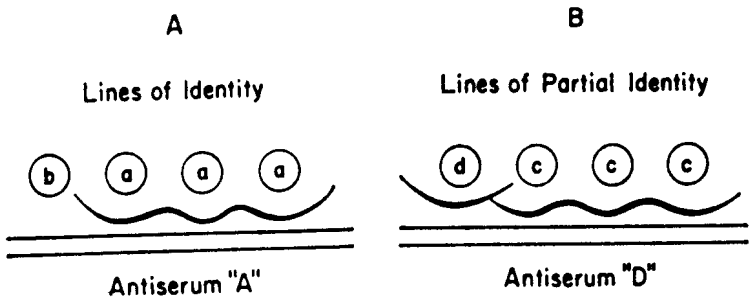

FIG. 4 - Precipitin lines of identity and partial identity. A, lines of identity formed with homologous antigen-antiserum only (antigen a vs. antiserum A); B. lines of partial identity formed when similar antigens react with the same antiserum. Note the typical spur formed, indicating lines of partial identity. (Antigen c and d are similar but not identical). (Fugate and Penn, 1971).

Figure 2 also illustrates the pattern of precipitin lines formed when the sample contains tissue antigens from 2 species (wells ba). In the majority of cases, the antisera will not react with heterologous antigens and lines of partial identity do not form. This occurs when the animal species are closely related (such as bovine and ovine).

Figure 5 illustrates areas containing identical antigen alignment. Four of the 6 areas have antigens reacting with antiserum A and 2 of the 4 areas are in position to react with antiserum B. The 2 remaining areas (2 and 4) are control as well as indicative sites. The mixtures of antigens a and b in wells marked ba are in position to react with both antisera and illustrate precipitin lines that occur when the sample contains tissues from both species.

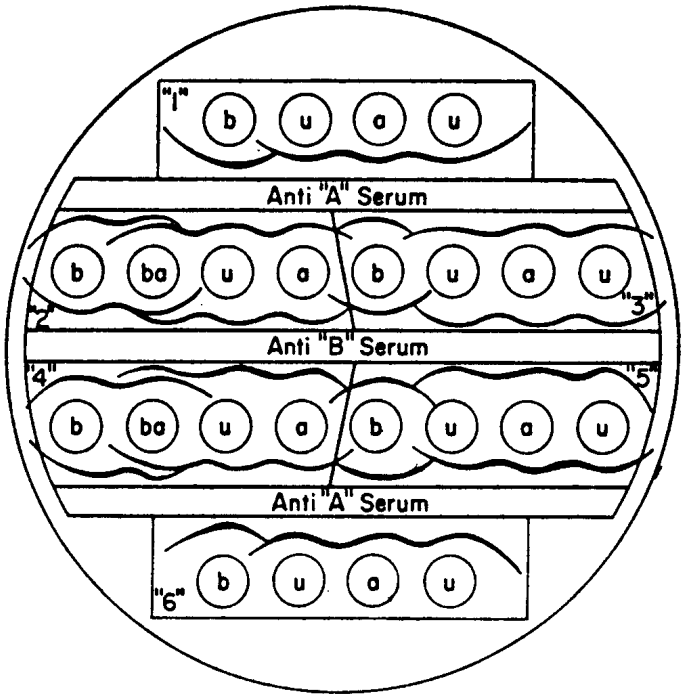

FIG. 5 - Position and reaction sites (6 areas) each consisting of 4 antigen wells. With the exception of areas 2 and 4, antigen placement is identical in each area. Areas 2 and 4 utilize one well each for a mixture of the 2 known antigens (ba), and illustrate precipitin reactions when sample consists of tissues from both species. All areas, except 1 and 6, are positioned to react with both antisera. Interpretation of results from areas 1, 3, 5, and 6 should correlate. Lines enclosing areas indicate portion of plate mounted on slides for preservation. (Fugate and Penn, 1971)

i. Staining Reaction Bands

To keep a permanent record, dialyze to remove free proteins and salts, then dry, stain, and prepare a mount under a cover slip, as follows:

Flood the plate with 500 to 1000 ml pH 7.2 buffered saline in a plastic pan. Replace with fresh buffer twice daily for three days, then once daily for two more days. Finally replace with acidified distilled water and let stand overnight.

Drain off the acidified distilled water, and cut a block of the reaction areas from the agar, and place it onto a 1 x 3 inch (2.54 x 7.62 cm) marked glass slide. Cover the block with a strip of filter paper, and dry in the incubator to a very thin film. Wash gently with a cotton applicator wetted with distilled water to remove adhering bits of the filter paper. Stain the films in acid fuchsin staining solution for 10 minutes. Remove the excess stain and rinse in destaining solution for a period of 15-20 minutes using 2-3 changes, until the agar is clear. Allow the slides to dry, then mount under cover slips with mounting fluid.

j. Photographic Recording of Reaction Bands

One of the easiest methods to obtain a permanent record of the immunodiffusion reaction is to photograph the entire unstained plate. Although there are many ways to achieve this, one of the easiest and quickest is to use a Cordis Immunodiffusion Camera. This is an instrument with preset optics, light source and Polaroid Camera which uses Polaroid Type 084 or 107 black and white film packs. The plate is placed in the instrument, the shutter is tripped, the film tab is pulled from the camera and within 25 seconds an excellent quality black and white print of the immunodiffusion reaction is produced.

21.35 Quality Control Procedures

a. Tissue Extracts from Known Species:

It is extremely important to establish the authenticity of these reference tissues before they are used, since

the basis for the types of immunodiffusion reactions obtained with unknown tissue extracts in the agar gel immunodiffusion test depends upon the use of known species tissue extracts.

b. Prepared Agar Gel Immunodiffusion Plates:

It is usually convenient to prepare a large number of plates at one time for future needs. Care must be taken to prevent deterioration of these plates during storage in the refrigerator. It has been found most useful to stack about 10 plates together in double or triple, air tight, tightly sealed plastic bags. Any plates showing microbial contamination, desiccation, or salt crystal formation should not be used as they will adversely effect the formation of immunoprecipitin lines.

c. The Specific Anti-species Sera: Sera used in the immunodiffusion procedure should always be initially checked for their proper reactivity against known, authentic reference tissues prior to their routine use as a diagnostic reagent.

21.36 Selected References

Fugate, H. G., and S. R. Penn. 1971. Immunodiffusion technique for the identification of animal species. J. Assoc. Off. Anal. Chem. 54:1152-1156.

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