SPECIES DOUBLE DIFFUSION TEST (OUCHTERLONY)

10.1 PURPOSE

10.1.1: To determine the species of origin in cases when animal blood is suspected.

A. Theory

This test utilizes an Ouchterlony plate that consists of a petri dish containing a blue agarose gel with sample wells punched into the agarose.

When a sample extract (suspected dog blood, for example) is placed into a well in the gel of the plate, and an antiserum containing antibodies (for example, dog antiserum) is placed in an adjacent well, these samples diffuse out of their respective wells and into the gel.

If the antibodies in the antiserum recognize the antigens in the sample when the two diffusion fronts meet, then antigen/antibody complexes will form, creating a contrasting white precipitin band in the gel. This band can be visualized between the wells.

B. Limitations

1. Antisera may cross react with other species.

2. This test does not confirm the presence of blood.

3. An incorrect antigen-to-antibody ratio may result in a false positive or false negative reaction. A 1:1 antigen/antibody ratio is optimal.

4. A large quantity of sample may be necessary when utilizing animal antisera.

10.1.2: To prepare Ouchterlony plates for species determination and to perform quality control on prepared reagents or purchased antisera/sera.

10.1.3: To determine if the animal antiserum cross-reacts with other species.

10.2 RESPONSIBILITY

10.2.1: Forensic Science Examiners (however titled) from the Division of Scientific Services who have been trained in the discipline of the species double diffusion test procedure according to FB SOP-26 (Training Manual and Checklist).

10.2.2: Forensic Science Examiners in the Forensic Biology Unit. Ordering information is maintained in a log book and/or electronically in the Forensic Biology Unit. New chemicals and reagents are purchased according to GL-6 (Purchasing). For additional information, refer to the Biological Inventory located in Appendix 3.

10.3 SAFETY

Use appropriate measures for the proper handling of biohazardous materials, Ouchterlony plates, Trypan Blue and sodium azide according to GL-2 (Safety Manual) and the Safety Data Sheets.

10.4 DEFINITIONS

A. PBS: Phosphate Buffered Saline
B. QRW: Quality Record Worksheet (Appendix 1)

10.5 TEST PROCEDURE

This procedure will be performed at the discretion of the examiner, with input from the Unit Lead(s) when applicable, based on the submitting agency requests, case information and the condition of the evidence.

10.5.1: Materials

A. Ouchterlony plates and punch
B. Animal antisera (anti-dog, -cat, -deer, etc.)
C. Known animal bloodstain extracts or thawed sera controls
D. PBS
E. 0.5% ammonia
F. Disposable pipets or micropipette and tips
G. Centrifuge tubes
H. Light source

10.5.2: Procedure

A. Preparing extracts:
   1. Extract a portion of the questioned sample in a centrifuge tube with 30-50µl of PBS until the extract is straw colored or darker in appearance. If necessary, the sample can be extracted on a shaker at room temperature or overnight at 4°C.

   2. 0.5% ammonia may be used in place of PBS to extract aged samples or samples that are difficult to extract.

B. Each plate must contain a positive control and negative reagent blank control as an intermediate check. Reagent QC is always conducted prior to use on case samples.

1. Known bloodstain extracts, used as positive controls for animal antisera, should be made fresh for each use with the same extraction solution used for the questioned samples.

2. Frozen aliquots of animal sera may be thawed and used as positive controls for animal antisera. Discard after use or refreeze (avoid repeated freezing and thawing).

3. PBS or 0.5% ammonia may be used as a negative reagent blank control according to the extraction solution used.

4. Record the lot number(s) of extraction solution(s) and animal standards used, if applicable, on the Ouchterlony Quality Record Worksheet (FBQR-08). Record the lot number(s) of extraction solution(s) used for questioned samples on the General Reagent Sheet (FB QR-09).

C. Using the punch, create wells in the agarose in a pattern according to the Ouchterlony Quality Record Worksheet (FBQR-08) in section 10.6.3.I below. Remove the excess punched agarose from each area to form the well.

D. Show placement of the controls, questioned samples and antiserum/antisera on the schematic diagram of the Ouchterlony Quality Record Worksheet (FBQR-08).

E. Using a pipette, fill the wells in the Ouchterlony plate with the controls, questioned samples and antiserum/antisera according to the diagram. Avoid air bubbles or overflowing the wells.

F. Allow the plate to sit upright and level at room temperature until all samples have diffused into the agarose.

G. Turn the plate upside down and allow the plate to stand overnight (12-16 hrs) at 4°C.

H. Examine the plate for precipitin lines using back lighting and record the results on the schematic diagram of the Ouchterlony Quality Record Worksheet (FBQR-08).

Include a copy of this worksheet in the case jacket, file the original in the designated notebook and/or electronically.

I. If no precipitin line is observed, re-examine the plate for up to 48 hours.

J. If the controls do not give the appropriate results, the test is considered to have failed. Review the test procedure and repeat the test if the quantity of sample allows for retesting. If the controls still do not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.

K. Record the results on the appropriate QRW.

L. A second qualified examiner will observe/confirm the results and initial/date the appropriate QRW.

10.5.3: Results and Conclusions

A. Positive

1. A white precipitin line between a sample well and the antiserum well indicates a positive result.

2. Suggested Report Wording:

a.

Testing Performed	Results	Conclusion
Immunological - Species	Positive	(Animal) origin indicated

b. *[ ] gave a positive(s) result with an immunological species test utilizing anti-[ ] antiserum.*

B. Negative

1. No white precipitin line between a sample well and the antiserum well indicates a negative result.

2. Suggested Report Wording:

a.

Testing Performed	Results	Conclusion
Immunological - Species	Negative	(Animal) origin not detected

b. *An immunological species test utilizing anti-[ ] antiserum was performed on [ ]. Biological material of [ ] origin was not detected with this test.*

C. Inconclusive

1. A white precipitin line between a sample well and the antiserum well could not be determined.

2. Suggested Report Wording:
   a.

Testing Performed	**Results**	**Conclusion**
Immunological - Species	Indeterminate	Inconclusive[1]
Comment: [1]Due to an indeterminate result and/or substrate interference, this test was determined to be inconclusive.		

   b. *An immunological species test utilizing anti-[ ] antiserum was performed on [ ]. Due to indeterminate results and/or substrate interference, this/these test(s) was/were determined to be inconclusive.*

3. Record the reason a result is determined to be inconclusive on the appropriate QRW.

D. Failed

1. The controls did not give the appropriate results.

2. If there is not enough sample to repeat the test then no conclusion is possible.

3. Suggested Report Wording:
   a.

Testing Performed	**Results**	**Conclusion**
Immunological - Species	Failed Test	No conclusion possible

   b. *An immunological species test utilizing anti-[ ] antiserum was performed on [ ]. Due to the failure of this/these test(s), no conclusion(s) is/are possible.*

4. Record the reason the test failed on the appropriate QRW.

10.6 PREPARATION/QC PROCEDURE

Ouchterlony plates will be prepared as needed.

10.6.1: Phosphate buffered saline

Materials

A. Phosphate Buffered Saline tablets 1tablet
B. dH_2O 200ml
C. Stock bottle

Procedure

A. Dissolve tablets in dH_2O.
B. Place in a stock bottle.
C. Record the required information on the Ouchterlony Reagent Log Sheet.
D. Discard after six (6) months.

10.6.2: 1% Agarose, 0.25% Trypan Blue

Materials

A. Phosphate buffered saline 100mL
B. Type I agarose 1.00g
C. Trypan blue 0.0125g
D. Sodium azide ~0.025g
E. Sterile petri dishes (50x9mm)
F. Serological pipets (10ml)
G. Controls: Known positive control(s) and negative reagent blank(s)

Procedure

A. Heat 0.0125g of Trypan blue in 50mL PBS to dissolve (do not boil).

B. In a separate container, heat 1g of agarose in 50mL PBS until dissolved. Re-measure and add dH_2O to volume as needed.

C. Add Trypan blue solution to agarose solution. Heat solution to boiling.

D. Add approximately 0.025g of sodium azide.

E. Swirl to mix thoroughly.

F. Using a serological pipet, add 4mL of agarose media to each sterile petri dish and swirl gently to spread agarose over the bottom of the plate. Avoid the formation of bubbles.

G. Allow to cool, then cover with lids and place in the refrigerator in a zip lock bag, inverted to prevent condensation.

H. Test each new lot with the appropriate antisera and corresponding controls as needed before use according to the test procedure, Ouchterlony Quality Record Worksheet (FBQR-08) and the Ouchterlony Reagent Log Sheet. Record the required information.

I. If the appropriate results are not obtained, review the procedure and repeat the test with a 2nd plate.

J. If the appropriate results are still not obtained, discard, make new plates and retest. If the new plates still do not yield the appropriate results, then inform the Unit Lead to try to determine the root cause.

K. If the plates are acceptable for use, store inverted in the refrigerator in a zip lock bag labeled with the lot # (date of preparation), control date and examiner's initials.

1. The plates are acceptable for use when a positive result is obtained between an antiserum and its corresponding positive control (blood or serum) and when a negative result is obtained between the antiserum and the blank buffer/negative control.

2. A second qualified examiner will observe/confirm the results and initial/date the Ouchterlony Quality Record Worksheet (FBQR-08) and the Ouchterlony Reagent Log Sheet.

L. Discard any unused plates after six (6) months or sooner if any bacterial/fungal growth is noted or dehydration of the gel occurs.

M. Discard/replace chemicals according to the manufacturer’s expiration dates or according to 21.4.3.E in FB SOP-21 (General Chemical and Reagent QC).

Manufacturer’s expiration dates with only month and year indicated (i.e. 04/2014) expire the last day of the month noted.

10.6.3 Evaluating Antisera

Materials

A. Ouchterlony plates
B. Animal antisera (anti-dog, -cat, -deer, etc.)
C. Known bloodstain extracts or thawed sera controls (include a human bloodstain)
D. PBS
E. 0.5% ammonia
F. dH_2O
G. Disposable pipets or micropipette and tips
H. Centrifuge tubes

Procedure

A. If the antiserum to be tested is lyophilized, reconstitute according to manufacturer's specifications.

B. Prepare the following samples for the detection of cross reactivity with animal antisera according to the test procedure.
   1. Human bloodstain extract
   2. Corresponding animal control bloodstain and/or thawed serum
   3. All available animal bloodstain extracts and thawed sera

C. Test the antisera according to the test procedure. Record the results and other appropriate information on the Ouchterlony Quality Record Worksheet (FBQR-08), see examples under section I below.

   A second qualified examiner will observe/confirm the results and initial/date the Ouchterlony Quality Record Worksheet (FBQR-08).

D. An antiserum exhibits cross reactivity when it yields a positive result with a sample other than its corresponding positive control. If cross reactivity occurs, consideration will be taken to determine if the antiserum is acceptable for use.

E. If the appropriate results are not obtained and the antiserum is determined to be unsuitable for use, review the procedure and replace the antiserum as needed.

F. If the antiserum is determined to be acceptable for use, store as follows:
   1. Antiserum received in a lyophilized state:
      Aliquot 50ul volumes of the reconstituted antiserum into centrifuge tubes labeled with the antiserum type and lot #. Store in the freezer in a zip lock bag labeled with the antiserum, lot #, date received, date reconstituted and examiner's initials.

Store additional bottles with lyophilized antiserum in the freezer. Label each bottle with the date received and examiner's initials. Re-titrate lyophilized antiserum after thawing and reconstituting as above.

2. Antiserum received in a liquid state:

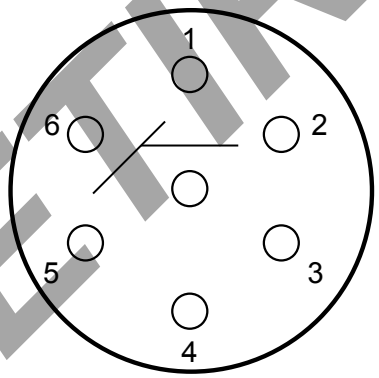
   Aliquot 50ul volumes into centrifuge tubes labeled with the antiserum type. Store in the freezer in a zip lock bag labeled with the antiserum, lot #, date received and examiner's initials.

G. Thawed antiserum may be stored in the refrigerator until consumed. Discard if a decrease in activity or bacterial growth is observed.

H. Avoid repeated freezing and thawing of the antiserum.

I. Ouchterlony Worksheet example:

C - anti-Dog
1 -dog serum #55983
2 -cat
3 -chicken
4 -cow
5 -deer
6 -human blood



αSerum
Lot #/Exp: 02727 / 04/20

Final Date: 11/01/19

Confirmation
(Initial/Date): KJL 11/01/19

10.7 REFERENCES

A. Ouchterlony, O., 1948a, “Antigen-antibody reactions in gels," Acta. Pathol. Microbiol. Scand. 26 (1949), 507.
B. Ouchterlony, O. “Antigen-antibody reaction in gels”, Ark. Kemi. Mineral Geol. 26B (14).
C. Ouchterlony, O. 1949b. Antigen-antibody reactions in gels II. Factors determining the site of the precipitate. Ark. Kemi. 1:43-48.
D. Ouchterlony, O. 1949c. Antigen-antibody reactions in gels III. The time factor. Ark. Kemi. 1:55-59.

E. Ouchterlony, O. 1968. Handbook of Immunodiffusion and Immuno-electrophoresis, Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan.

F. GL-2 (Safety Manual)

G. GL-6 (Purchasing)

H. Safety Data Sheets