CELL CULTURES FOR DIAGNOSIS OF ARBOVIRUS INFECTIONS IN LIVESTOCK AND WILDLIFE

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SUMMARY: Arboviruses can be isolated in serially propagated cells derived from various vertebrates and invertebrates. Cell cultures can be used for direct detection of antigen by fluorescent antibody and enzyme-linked immunosorbent assays, for nucleic acid hybridization, and for visualization of viruses with electron microscopy. Reagents for enzyme-linked immunosorbent assays for IgM and IgG antibodies, hemagglutination-inhibition, complement fixation, and serum dilution-plaque reduction neutralization tests can be prepared in cell cultures infected with these viruses. Thus, cell cultures can be used as laboratory hosts for essentially all isolation, identification, and serodiagnostic procedures for arboviruses. This paper outlines current methods for diagnosis of arbovirus infections in livestock and wildlife, describes certain of these techniques, and provides references for others.

Key words: cell cultures; arboviruses; serodiagnosis; virus isolation; wildlife; livestock.

I. INTRODUCTION

The term 'arbovirus," a contraction of the term '*ar*thropod-*bo*rne *virus*, denotes a virus maintained in nature in a biological transmission cycle between susceptible vertebrate hosts and hematophagous arthropods (they replicate in both). The International Catalogue of Arboviruses (12) lists more than 500 viruses, most of which have been categorized according to their antigenic relationships. Differences in antigenic, morphologic, biochemical, and genetic characteristics are used to separate the arboviruses into families, genera, serogroups, antigenic complexes, and viruses in an increasing order of relatedness. Many of the viruses listed in this catalogue do not meet the definition of an arbovirus, either because information is lacking regarding transmission by arthropods or because they definitely are not arthropod-borne. In this paper the term "arbovirus" denotes viruses causing documented biological infections of vectors and which are transmitted to vertebrates by the infected vector.

Arboviruses can be isolated in cell cultures or various laboratory animals. Either system can be used to prepare diagnostic reagents with which to perform serologic tests for antibody to arboviruses and for neutralization tests for antibody or for virus identification. This paper is limited to the use of cell cultures for the diagnosis of infections in livestock and wildlife with these viruses.

Few arboviruses have been associated with illness of livestock or wildlife (Table 1). Viremias in arbovirus infections are brief, generally no more than 1 to 3 d. Often viremia has passed by the time clinical signs appear. During the first few days after infection, however, viremia titers and the virus isolation rate may be very high.

The initial antibody response appears at the end of the viremic period; this may be coincidental or may quench the viremia. In either case, virus (antigen)-antibody complexing may impede both virus isolation and antibody determinations. The IgM fraction of the serum contains both neutralizing and hemagglutination-inhibiting antibodies early after infection. Later, IgG antibody appears, containing neutralizing, hemagglutination-inhibiting, and complement-fixing antibodies; IgG antibody may persist for years and perhaps for the life of the individual (1). As the titer and presence of anti-viral IgM antibody decreases, antiviral IgG antibody predominates coincidentally.

Antiviral antibody contained in the IgM fraction of serum seems to be antigenic complex-specific but not virus-specific in alphavirus (2) and certain flavivirus (15) infections but may be only serogroup-specific in infections caused by bunyaviruses and other viruses. Nevertheless, assays for IgM-class antibodies are valuable because their presence, even in a single serum specimen, provides presumptive evidence that the animal has been infected recently (3,4,17). The

TABLE 1

PRINCIPAL ARBOVIRUSES ASSOCIATED WITH CLINICAL SYNDROMES IN LIVESTOCK[a] OR WILDLIFE[b]

Virus	Clinical Syndromes				Biosafety Level[d]
	Encephalitis	Abortion	Fever	Systemic[c]	
eastern equine encephalitis	E, F		E		2V
western equine encephalitis	E		E		2V
Venezuelan equine encephalitis	E		E		3*V
Getah			E	E	3
Japanese encephalitis	E	S	E, S		2
Wesselsbron		O[e]		O	3*X
louping ill	E, F, S, O, B		O, B		3*X
Kyasanur Forest Disease			M	M	4
yellow fever			M	M	3*V
Rift Valley fever		O, C. B	O, C, B	O, C, B	3*VX
Akabane		O,[e] C,[e] B[e]	O,[f] C,[f] B[f]		3X
Nairobi sheep disease		O, C	O, C	O, C	X
bovine ephemeral fever			B	B	X
vesicular stomatitis			E, S, B	E,[g] S,[g] B[g]	2U
bluetongue		O,[e] B[e]	O, C, B, D	O,[g] C, D	2U
epizootic hemorrhagic disease			B, D	B, D	2U
African horse sickness			E	E	X
African swine fever			S	S	X

[a]Equines (E), Pheasants (F), Pigs (S), Sheep (O), Goats (C), and Cattle (B).

[b]Grouse (F), Monkeys (M), and Deer (D).

[c]Includes one or more of the following syndromes: hepatitis with jaundice, edema, vomiting, rash, drop in milk production, depression, muscle stiffness or necrosis, lameness and coronitis, excessive salivation, anorexia, respiratory disease, hemorrhagic enteritis, epistaxis, hemorrhage.

[d]Biosafety level (*see* reference 12): V indicates that personnel should be vaccinated with this virus, should have demonstrable antibody to the virus, and that without such vaccination the next higher containment level is recommended; * indicates that work with these viruses at containment level 3 requires HEPA filtration of all exhaust air prior to discharge to the outside; X indicates restricted by U. S. Department of Agriculture regulations or policy; U indicates that exotic but not indigenous viruses and subtypes within these serogroups are restricted by U. S. Department of Agriculture regulations or policy (including but not limited to vesicular stomatitis Alagoas, bluetongue serotypes not found in the U.S., and Ibaraki viruses, respectively).

[e]Congenital arthrogryposis-hydranencephaly.

[f]Adults.

[g]Vesicular lesions common.

laboratory diagnostic procedures given below do not assume that the animal tested is sick or not sick; they are the same in either instance.

II. MATERIALS[a]

A. Equipment

Water-jacketed CO_2 incubator, model 3314 Forma Scientific[1]

Centrifuge, model PR-10, International Equipment[2]

Water baths (37°, 56° C) model 270, Precision Scientific Group[3]

Biological safety cabinet, model NU-407-624, NuAire[4]

Spectrophotometer, Titertek Multiskan MC, Flow[5]

Plate washer, Titertek Microplate washer 20, Flow[6]

Single and multichannel pipettors, Titertek, Eflab Oy[7]

B. Chemicals

Tween 20, P-1379, Sigma[8]

ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonate), 506402 Kirkegaard and Perry Laboratories[9]

Ethanol, 0821 Midwest Grain[10]

Sodium carbonate (Na_2CO_3), 3604 Baker[11]

Sodium bicarbonate ($NaHCO_3$), 3506-5[11]

Sodium phosphate (NaH_2PO_4), 3818-1,[11]

Disodium phosphate (Na_2HPO_4), 3828-1[11]

Sodium chloride (NaCl), S-271, Fisher[12]

Medium 199 containing Hanks' balanced salts solution, 400-1200 GIBCO[13]

Fetal bovine serum, A-1110-L, HyClone[14]

Antibiotics solution: penicillin-streptomycin, 600-5140[13]

Gentamicin sulfate, 17-5182 Whittaker[15]

Fungizone, 43760, Squibb[16]

Trypsin, 840-7250[13]

Trypan blue, 630-5250, Life Technologies[17]

C. Supplies

Pipette tips, ML-6RS, Analytic Lab Accessories[18]

Disposable 96-well microtiter plates, U-shaped, 001-010-2401, Dynatech[19]

Disposable 96-well microtiter plates, flat-bottom, 3595, Costar[20]

Antispecies IgM, Organon Teknika[21]

Mouse antiviral antibody[22]

Antimouse IgG peroxidase conjugate, 23347[21]

12-Well, Teflon-coated spot slides, 10-111, Cel-Line[23]

1-Liter roller bottles, 25130, Corning[24]

6-Well plastic trays, 35 × 10 mm, 3406[20]

25-cm² Flasks, 4-3013-3, Becton Dickinson[25]

III. SOLUTIONS

Overlay medium for observing plaques

A. Solution A: 100 ml 10× concentrated Medium 199, 50

[a] Use of trade names and commercial sources is for identification only and does not imply endorsement by the U. S. Public Health Service, the U. S. Department of Health and Human Service, Yale University, or the U. S. Department of Agriculture.

ml heated fetal bovine serum, 2 ml antibiotics mixture; Q. S. to 453 ml with sterile distilled water.

B. Solution B: 500 ml sterile distilled water, 10 gm agarose or Noble agar; autoclave 15 min.

1. Single overlay (with neutral red)

30 ml 7.5% $NaHCO_3$, 300 ml solution A, 8 ml 1:300 neutral red, 147 ml solution A, 15 ml diethylaminoethyl dextran (DEAE; not necessary when agarose is used instead of Noble agar); Q. S. to 1000 ml with solution B.

2. Double overlay

First overlay (without neutral red) contains 30 ml 7.5% $NaHCO_3$, 470 ml solution A, 15 ml DEAE; Q. S. to 1000 ml with solution B. Second overlay (with neutral red) contains 30 ml 7.5% $NaHCO_3$, 270 ml solution A, 4 ml 1% neutral red, 181 ml solution A, 15 ml DEAE; Q. S. to 1000 ml with solution B. Maintain ingredients without agar at 37° C and ingredients with agar and final mixture at 44° C. Mix ingredients just before use.

3. Phosphate buffered saline (PBS) (*pH 7.2) 10×*

Add 6.65 g NaH_2PO_4, 35 g Na_2HPO_4, and 255 g NaCl to 2 liter of sterile, distilled water. Shake well after addition of each salt. Add another liter of water and allow to stand (with occasional shaking) until all salts are dissolved. Dilute 1:10 (with distilled water) to prepare working PBS solution as needed.

IV. PROCEDURE

A. Safety

When attempting virus isolations, safety precautions should be used to avoid possible laboratory-acquired infection (7). Wearing rubber or plastic gloves and a laboratory coat and using aseptic techniques are sufficient precautions for handling Biosafety Level (BL) 1 or BL 2 viruses. For viruses that pose special hazards to laboratory workers (e.g., BL 3 agents), special conditions are required for physical containment. For viruses that pose extreme hazards to laboratory workers or may cause epidemic disease (BL 4 agents), maximum containment is needed. Questions regarding potentially hazardous specimens should be directed to a specialty laboratory. Exposure to aerosols always should be minimized or eliminated by using biological safety cabinets and closed systems for grinding tissues, by using blunt-ended needles for collecting and transferring liquids, and by providing appropriate ventilation systems for filtering or otherwise scrubbing or cleaning exhaust air. For BL 4 viruses, personnel showers, disinfecting for individual decontamination, and using germ-free-type isolators (class 3 cabinets) are recommended. Both in the field and in the laboratory, specimens should be assumed to contain virus and be potentially dangerous.

Vaccines against eastern, western, and Venezuelan equine encephalitis, yellow fever, Japanese encephalitis, and Rift Valley fever viruses are available and should be used to protect high-risk groups of humans, including laboratory workers. For information regarding vaccines not otherwise available, contact the Division of Vector-Borne Viral Diseases, Centers for Disease Control, P. O. Box 2087, Fort Collins, CO 80522. Certainly laboratory workers should be monitored both clinically and serologically for evidence of laboratory infections. In this way prior infections can be documented and potential future infections can be prevented.

B. Processing specimens

1. Process whole blood, serum, or tissue samples for virus isolation immediately, or store on dry ice or in another suitable deep-freezing unit (−70° C). Use air-tight or adequately sealed tubes or screw-cap vials, avoiding contact with CO_2. Bluetongue viruses are inactivated quickly when exposed to temperatures 0° to −20° C, particularly when the medium in which they are frozen does not contain a stabilizing substance. Therefore, maintain bluetongue viruses at 4° C and ship on wet ice, keeping the specimen insulated from the ice. To maintain bluetongue viruses dependably, suspend in a medium containing buffered lactose peptone. Macerate tissue in the medium appropriate to the cell culture used, as a 10% suspension, and clarify by centrifugation at 600 $\times g$ for 20 min.
2. When serum samples are to be tested only for antibody, they can be shipped and stored at ambient temperatures, unless they are contaminated with microorganisms or will be in transit for long periods. Although preservation of infectious virus may not be critical for antigen detection, specimens should still be shipped and stored at low temperatures to prevent further degradation of proteins.

C. Selection of cell culture system

Because arboviruses vary widely in individual biologic characteristics, no single virus isolation system suffices. In general, these viruses may be isolated by inoculating appropriate cell cultures with serum, whole blood, or with clarified suspensions of macerated clinical specimens.

1. For viruses that replicate in mosquitoes, use cell lines derived from mosquitoes (C6/36 cells from *Aedes albopictus*, AP-61 cells from *Aedes pseudoscutellaris*, or TR-248 cells from *Toxorhynchites amboinensis*) for virus isolation.
2. For isolation of viruses from ticks, culicoids, or tissues of livestock or wildlife, use cell lines such as African green monkey kidney (Vero or LLC-MK_2), porcine kidney (PS or PK-2), and baby hamster kidney (BHK-21), or primary cell cultures from tissues of domestic chicken or duck embryos.

D. Propagation of cell cultures

1. Grow stock cell cultures to monolayer in 1-liter (490 cm^2) sterile plastic roller bottles with Medium 199 containing Hanks' balanced salt solution, 5 to 10% fetal bovine serum, 1.24 g $NaHCO_3$/liter, and antibiotics.
2. Each week exchange growth medium for a maintenance medium with the above formulation but with $NaHCO_3$ increased to 2.2 g/liter.
3. Transfer stock cell cultures weekly using a brief exposure to trypsin.

4. Seed fresh sterile stock bottles at a concentration of about 100 000 cells/ml.
5. Seed each flat-bottom well of 6-well plastic trays with about 400 000 cells; adjust cell volume if using other configurations, such as 24- or 96-well containers.
6. Maintain the cells in Medium 199 as described above with 2.2 g $NaHCO_3$/liter.
7. Incubate cell culture for 4 d at 37° C in a humidified atmosphere containing 5% CO_2:95% air.
8. Use the cultures for virus isolation or neutralization tests when the cells form monolayers.

E. Virus isolation:
1. Inoculate 0.1 to 0.2 ml of undiluted and 1:10 diluted serum, or 10 and 1% suspensions of supernatant fluids from clarified macerated tissues, on confluent monolayer cultures of the appropriate cells grown in 25-cm^2 plastic flasks, in tubes, or in small roller bottles. Drop the inoculum onto the center of the monolayer, agitate, and allow to adsorb for about 45 min at room temperature before washing the cell sheet twice with PBS.
2. Add medium containing balanced salt solution, 5 to 10% heated (56° C for 30 min) fetal bovine serum, and appropriate supplements.
3. Maintain cultures at temperatures and conditions appropriate to the cell type. For example, maintain Vero cells at 37° C and C6/36 cells at 28° C. Supernatant fluids from cell cultures that do not show cytopathic effects (CPE) within 10 d after inoculation are passed once more in the same cell line, using 1 ml of supernatant fluid.

F. Plaque assay
1. Grow cells to monolayer in 6-well plastic cluster plates, using for nutrient the same basal medium described previously but supplemented with agar (Table 2).
2. Inoculate cells with virus by dropping virus suspension onto surface of monolayer.
3. For viruses that form plaques within a few days, a single agar overlay with neutral red can be used. For viruses that do not form plaques for a week or more, a double agar overlay, with neutral red in the second overlay, can be used. The virus to be isolated determines the technique. When in doubt, use the double overlay technique; examine the plates or flasks against a black background, and when plaques are observed apply the second overlay with neutral red. Reincubate the cultures at the appropriate temperature after applying the second overlay, and examine for plaques daily thereafter.

G. Harvesting the isolate
1. After CPE or plaques are observed, harvest the supernatant fluids or plaques, respectively, into medium containing 20% heated fetal bovine serum.
2. Freeze at −70° C for later passage in cell cultures.
3. To produce working seed or stock virus for use in neutralization tests and other procedures, inoculate additional cell cultures using fluid medium with 10% fetal bovine serum; observe daily. When CPE involves 75 to 100% of the cells, harvest the cells and the supernatant fluid by shaking the flasks vigorously or by scraping. Clarify the suspension by low speed centrifugation, and store frozen in multiple aliquots.

H. Virus titrations
1. Prepare serial 10-fold dilutions of virus in PBS (pH 7.2) containing 5% heated fetal bovine serum, or in another diluent containing sufficient protein to protect the virus from the effects of light, heat, vibrations, and other deleterious conditions. For titration of virus to be used in neutralization tests in which virus and antibody are mixed, add equal volumes of virus and normal serum (i.e. no neutralizing antibody to the virus being titrated) before dropping on the cells.
2. Drop 0.1 ml of each dilution onto the center of a separate well containing monolayer cultures of cells. Rock the plates gently back and forth to spread the virus-containing fluid uniformly on the monolayer.
3. Incubate the plates for 45 to 60 min at 28° to 37° C in a humidified atmosphere containing 5% CO_2:95% air.
4. Overlay each well with nutrient medium containing 1% agar.
5. If the overlay contains neutral red contrast stain (*see* above), examine the cells daily for plaques. When plaques are observed, count them daily until the numbers of plaques per well no longer increase.
6. Theoretically, there should be one-tenth as many plaques in wells inoculated with a given dilution as in wells inoculated with the next lower dilution (next higher concentration). In practice, only a rough approximation of the theoretical number is found. However, by carefully diluting and dispensing virus dilutions, the laboratory worker can often come close to the expected proportional number of plaques.
7. When a double overlay (two additions of medium with agar, the second of which contains neutral red) is used, examine the cells daily after the addition of the second overlay; count the plaques and record numbers as described above. The twofold difference in dilution must be considered when making final calculations.

I. Virus identification
1. Use sucrose-acetone extracted antigens for hemagglutination and hemagglutination-inhibition (8) and for complement-fixation (6) tests. Prepare here these antigens from clarified supernatant fluids of virus-infected cell cultures using the method originated by Clarke and Casals (8). Alternatively, virus may be grown in cell cultures, substituting 2.5% bovine albumin Fraction V for bovine serum, and the clarified supernatant fluids used in hemagglutination-inhibition and complement-fixation tests without sucrose acetone extraction.
2. Identify viruses by testing such antigens against

polyclonal hyperimmune mouse ascitic fluids (22). Use serum dilution-plaque reduction neutralization (PRN) tests to identify the virus type. Use monoclonal antibodies selected for epitope specificity as reagents for virus identification and characterization (11). During epizootics, these tests have practical and immediate applications.

J. Neutralization test

Serum dilution-plaque reduction neutralization tests in cell cultures (13) are definitive for identifying virus types and subtypes. When a virus isolate is neutralized by antibody prepared against a reference virus, compare the antibody titer to the unidentified virus with the antibody titer to the homologous titer; differences between the two titers indicate differences between the viruses. Perform the test as follows:

1. Examine cells to be used microscopically to confirm growth to monolayers, and inspect for microbial contamination.
2. Remove a vial of stock virus from the freezer and dilute serially 10-fold to contain approximately 200 plaque forming units (pfu)/0.1 ml. Any suitable isotonic preparation (PBS, Medium 199, balanced salt solution, etc.) supplemented with 5% fetal bovine serum and antibiotics can be used as diluent.
3. When testing only a few specimens for antibody, dispense them into clean glass or plastic tubes and dilute 1:5. When testing many specimens or a single serum for antibody titer, prepare serum dilutions in microtiter plates. Make twofold dilutions of the specimens, beginning at 1:5 or other appropriate dilution, using microtiter loops or multichannel pipettors.
4. Heat specimens 30 min at 56% C to inactivate nonspecific neutralizing substances. Heating is easily done when specimens are in tubes but can be done when serum samples are in microtiter plates by floating the plates in a water bath.
5. Add approximately 200 pfu of virus to an equal 0.1 ml volume of 1:5 serum dilution. The mixture contains 100 pfu of virus/0.1 ml and the serum dilution is 1:10.
6. Controls consist of virus suspensions containing about 200, 20, and 2 pfu/0.1 ml diluted with an equal volume of normal serum to contain about 100, 10, and 1 pfu/0.1 ml.
7. Incubate test mixtures 18 h at 4° C. Other times and temperatures of incubation, including 1 h at 37° C, have been used, but overnight incubation in the refrigerator is the most convenient and maximizes the size of the test one person can handle.
8. After incubation, drop 0.1 ml of the virus-serum mixture onto the center of a well containing the cell monolayer. Gently rock the plate containing inoculated cells to spread the inoculum uniformly over the monolayer, and incubate 45 min at 37° C to allow adsorption of virus to cells.
9. Without removing the inoculum, overlay cells with medium containing agar. Allow the agar to solidify at room temperature (about 20 to 30 min), invert the plate and incubate at 37° C.
10. Examine plates daily for appearance of plaques; these are counted, and the numbers are recorded. Determine neutralization titers as the highest dilution of immune reagent (patient serum, hyperimmune ascitic fluid, monoclonal antibody, etc.) that inhibits at least 90% of the plaques as compared with control titrations.

K. Antigen detection

Direct detection of viruses and antigens in clinical specimens can provide a rapid diagnosis but is less sensitive than methods requiring replication of the virus (amplification of antigens) and does not yield a virus isolate. For early detection of virus antigens in mosquito cell cultures inoculated with clinical materials, immunofluorescence techniques can be used to identify yellow fever virus (21) and other viruses in less than 72 to 96 h, long before the appearance of CPE. The indirect fluorescent antibody (IFA) test has been used to identify arboviral antigens in intrathoracically inoculated *Toxorhynchites* mosquitoes (20) and in cell cultures infected with arboviruses (19). When viruses cause no cytopathology in cells infected with them, fluorescent antibody techniques are invaluable for making an early diagnosis.

L. Immunofluorescent antibody assay

1. Inoculate cell cultures with the clinical specimen and incubate until CPE, involving about 50% of the cells, appear.
2. Expose cells briefly to trypsin, shake into suspension, and mix with an equal volume of uninfected cells.
3. Drop about 20 μl of this suspension onto each spot of a 12-spot slide, air dry and fix the slide in cold (−20° C) acetone, and store it at −70° C.
4. To test the cells for viral antigen, remove a slide from the freezer, allow it to thaw, and air dry it at ambient temperature.
5. Add 20 μl of primary (antiviral) antibody to each spot, incubate the slide at 37° C for 30 min in a humidified chamber, and wash it in PBS, pH 7.2 to 7.4.
6. After air drying, stain the cells with fluorescein-conjugated antibody to the species in which the primary antibody was made. Add trypan blue to the conjugate at a concentration of 1:2000 as a counterstain, if desired. Optimal concentrations of both the first and second (conjugated) antibodies are determined by prior titrations with control antigens (virus-infected cell cultures). Controls include infected and uninfected cell cultures and cell cultures incubated with normal mouse ascitic fluid or another appropriate reagent. Nonspecific reactivity may be reduced by prior adsorption of the primary and secondary antiserum reagents with normal tissue.
7. Examine stained slides with a fluorescence microscope fitted with appropriate filters and objective. Score test results semiquantitatively according to the intensity of fluorescence and the percentage of fluorescing cells per field.

M. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA), using capture antibodies charcterized by high avidity for antigen (polyclonal, physically separated IgM, or monoclonal antibodies) and nucleic acid hybridization (5), are increasingly used for direct detection of viral antigens or viral nucleic acid in cell cultures infected with viruses, in clinical specimens, in tissues from wild vertebrates, and in mosquitoes, ticks, and other arthropods. Clinical specimens from patients infected with many arboviruses contain quantities of viral antigen sufficient to be detected by ELISA; such viruses include Rift Valley fever (16), yellow fever (14), Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis, Wesselsbron, Kyasanur Forest disease, louping ill, vesicular stomatitis New Jersey, and other viruses. Therefore, these methods should be used in epizootic situations, in which time is a critical element.

The antigen capture ELISA may be done as follows:

1. Add 75 μl of capture antibody at an optimal dilution determined by titration (usually 1:500 to 1:2000) in carbonate-bicarbonate buffer, pH 9.6, to the solid-phase support, polystyrene microtiter plate. Allow antibody to bind for 3 h at 22 or 37° C or overnight at 4° C.
2. Wash the plate repeatedly with PBS containing 0.05% Tween 20. Add 50 μl of the clinical sample. Include virus-positive and -negative controls in each assay.
3. After optimal incubation (usually 16 h at 22 to 45° C, depending on the virus system) and washing, add 50 μl polyclonal or monoclonal detecting antibody (double sandwich method). When polyclonal or monoclonal antibody of the same species is used for both capture and detection, the detecting antibody must be conjugated to an enzyme (or radionuclide); if antibody prepared in another species is used, it should be followed by conjugated anti-species-detecting antibody (50 μl) and substrate (75 μl).
4. Record color change as optical density, using a spectrophotometer. If incubations and washings between the additions of reagents were adequate, the results can be read as the ratios of test values to control values. Usually, ratios greater than or equal to 2.0 are considered positive for IgM antibody. If the optical density of the control (background) is exceedingly low, such ratios will be artificially high; common sense should be applied in these situations.

N. Other methods for detecting virus

Recent advances in recombinant DNA technology have made it possible to use nucleic acid probes for diagnosing viral infections and for detecting viral genetic material in tissues from other sources and in body fluids. In these assays, virus-specific nucleic acids are bound to a solid phase and detected with radiolabeled DNA (17) or RNA probes (18). Gel electrophoresis of double stranded RNA of bluetongue and epizootic hemorrhagic disease of deer has been used effectively for identification and diagnosis. These techniques could provide rapid and exquisitely specific methods for identification of viral RNA in tissues from animals, reservoir hosts, and arthropod vectors, and to identify specific nucleotide sequences in viral genetic material. However, the technique is not yet widely accepted for use with arboviruses because of low sensitivity with some viruses. Recently developed biotinylated probes may obviate the need for radiolabeled probes (9,10). Undoubtedly, DNA and RNA hybridization will become a rapid diagnostic method and an epidemiologic tool.

Fourfold or greater increases or decreases in antibody titers between acute- and convalescent-phase serum samples from a patient provide serodiagnostic confirmation of infection. The PRN test with paired serum samples is the standard by which other serodiagnostic tests are judged. However, such tests require multiple specimens collected days to weeks apart. When rapidity of diagnosis is not critical or infection with closely related viruses confounds interpretation of results, PRN tests are invaluable.

1. The IgM antibody capture ELISA may be performed as follows:
   a. Dilute commercially available anti-species IgM antibody in carbonate buffer and coat wells of a microtiter plate with 75 μl; incubate the plate overnight at 4° C.
   b. Add 50 μl of 1:100 diluted test serum to each well; incubate the plate for 1 h at 37° C.
   c. Add to each well 50 μl of viral antigen prepared by clarifying supernatant fluid from virus-infected cell cultures; incubate the plate for 1 h at 37° C.
   d. Add 50 μl of antiviral (secondary) antibody; incubate the plate for 1 h at 37° C.
   e. If the secondary antibody has been conjugated to an enzyme, 75 μl of substrate is added. Record the intensity of the color as optical density, using a spectrophotometer.

   If incubations and washings between the addition of each reagent were adequate, the results can be read as the ratio of test value to control value; usually ratios greater than or equal to 2.0 are considered positive for IgM antibody. Serum samples can be tested at single dilutions or serially diluted twofold and tested at each dilution.

   A double sandwich ELISA, using a tertiary (detecting) anti-species IgG antibody conjugated to an enzyme that reacts with the secondary (antiviral) antibody can also be used. Whether testing only a few or many serum samples for IgM antibody to many viruses the double sandwich ELISA is preferred because enzyme-conjugated antibody preparations are not needed for each of the antiviral reagents.

V. DISCUSSION

Cell lines derived from mosquitoes have such advantages over laboratory animals as being easy to contain and reducing the risk of aerosol production. Because they are highly stable and have growth optima at lower temperatures than do mammalian cells they may be taken to the field, inoculated with specimens,

and returned to the laboratory weeks later, after amplifying the virus that was sought. However, cell lines derived from arthropods have the disadvantage in some cases of not producing CPE. Specific, secondary steps are required for recognition of the presence of virus in the culture. Because it is much easier to maintain serially propagated cell lines than to prepare primary cell cultures, our laboratories principally use Vero cells for virus isolation but also use mosquito cells when possible.

Because of antigenic cross-reactivities between closely related arboviruses, the most confimatory test is virus isolation from the affected host. This can be done by using cell cultures alone or in combination with antigen detection in host tissues or in virus-infected cell cultures by using ELISA, IFA tests, electron microscopy, and nucleic acid hybridization. Electron microscopy and immunofluorescence techniques require skilled technicians and expensive microscopes. These needs, the possible subjectivity of the results, and the lack of sensitivity of IFA tests are additional drawbacks.

IgM ELISA using antigen produced in infected cell cultures is the primary method for rapid detection of antibody indicative of recent infection. Other tests, such as neutralization, hemagglutination-inhibition, and complement fixation, may be required for final identification of the etiologic agent of infection. Viral antigens and stock viruses used in all of these tests may be produced in cell cultures. In some instances, laboratory animals, such as baby mice for most arboviruses and embryonated hen's eggs for bluetongue viruses, will also give satisfactory results and often prove superior to cell cultures. However, because of the large quantity of reagent produced, safety of production, and convenience, cell cultures are usually more satisfactory than laboratory animals for producing such reagents.

VI. REFERENCES

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The authors thank Dr. Thomas P. Monath, Division of Vector-Borne Viral Diseases, Centers for Disease Control, Fort Collins, CO, for his constructive comments and editorial assistance.

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