Epstein-Barr virus has been implicated in nasopharyngeal carcinoma, Burkitt’s EBV EA.

detects a specific antibody response to VCA and does not cross react with associated with infectious mononucleosis (IM). The clinical syndrome of virus resides in the host for life.

recovery from primary infection over a period of 2 to 3 weeks; however, the reliable diagnosis.

cases, testing for EBV specific IgM antibodies is necessary to establish a

During primary acute phase EBV infection antibodies are produced against nuclear antigens (EBNA) are rarely present during the acute phase, but in most patients will experience symptoms of IM. In patients with a primary EBV infection, both IgG and IgM class antibodies are generally detectable with onset of symptoms. Anti-VCA IgM antibodies appear first, are first detectable at 2 to 4 weeks after primary infection, peak at the onset of symptoms and decline to undetectable levels within 2 to 3 months after the symptoms subside. Viral capsid antigen IgG antibody levels continue to rise, then decline to lower levels persisting for life.

Tests for heterophile antibody are commonly utilized for IM diagnosis. However, heterophile antibodies may be undetectable in children. In these cases, testing for EBV specific IgM antibodies is necessary to establish a reliable diagnosis.

Antibody detection by indirect immunofluorescence assay (IFA) using EBV-infected lymphocytes was first described by Henle, et al in 1966 and remains the reference method for EBV serologies. Both VCA and early antigens (EA) are found in infected lymphocytes. Therefore, when these cells are used as a test substrate, it is difficult to discriminate between fluorescence from anti-VCA and from anti-EA antibodies. However, using recombinant technology, Focus Diagnostics produced a cell substrate that expresses only VCA. As a result, the Focus Diagnostics’ RIFA test detects a specific antibody response to VCA.

TEST PRINCIPLE
The indirect immunofluorescent antibody (IFA) assay is a two stage "sandwich" procedure. In the first stage, the patient sera is diluted in Pretreatment Diluent. The Pretreatment Diluent is a buffered isotonic solution containing anti-human IgG which removes both free and complexed IgG from the sample. The diluted (pretreated) sera is added to appropriate slide wells in contact with the substrate and incubated. Following incubation, the slide is washed in buffered saline which removes unbound serum antibodies. In the second stage, each antigen well is overlaid with fluorescein-labeled antibody to human IgM. The slide is incubated allowing antigen-antibody complexes to react with the fluorescein labeled anti-human IgM. After the slide is washed, dried, and mounted, it is examined using fluorescence microscopy. Positive reactions appear as cells exhibiting bright apple-green cytoplasmic fluorescence against a background of red VCA negative control cells. Semi-quantitative endpoint titers are obtained by testing serial dilutions of positive specimens.

For EBV VCA IgM testing, Focus Diagnostics has developed a mammalian cell line expressing a recombinant viral capsid antigen (rVCA). The rVCA substrate is produced by 5 to 20% of the cells. The remaining cells are negative for VCA and serve as controls for non-specific reactivity.

MATERIALS SUPPLIED
Focus Diagnostics’ EBV VCA IgM RIFA test kit contains sufficient materials to perform 120 determinations.

rVCA IgM RIFA Substrate Slides
Ten slides of twelve wells each prepared with mammalian cells fixed onto each well. Approximately 5 to 20% of these cells express EBV recombinant viral capsid antigen with non-expressing cells serving as substrate control cells. Store sealed RIFA slide packets at 2 to 8°C. The sealed slides are stable until the date stated on the slide packet labels. To avoid condensation, allow the slides to warm to room temperature before opening the sealed packets.

VCA IgM RIFA Conjugate, 2.5 mL
One vial of fluorescein-labeled goat anti-human IgM, mu-chain specific. Contains Evans’ Blue counterstain with protein stabilizer and preservatives. Conjugate is stable at 2 to 8°C until the date stated on the label.

VCA IgM Positive Control, 0.30 mL
One vial of human serum bottled at screening dilution. Contains preservatives. Stable at 2 to 8°C until the expiration date stated on the label. Do not use if cloudy, discoloration or other indications of bacterial contamination are present. Allow to warm to room temperature just prior to use. Do not pretreat. Repeated freezing and thawing is deleterious and should be avoided.

EBV Negative Control, 0.25 mL
One vial of human serum bottled at screening dilution. Contains preservatives. Stable at 2 to 8°C until the expiration date stated on the label. Do not use if cloudy, discoloration or other indications of bacterial contamination are present. Allow to warm to room temperature just prior to use. Do not pretreat or dilute. Repeated freezing and thawing is deleterious and should be avoided.

IgM Pretreatment Diluent, 12 mL
One vial of PBS containing goat monospecific antiserum to human IgG, with preservatives. Stable at 2 to 8°C until the expiration date stated on the bottle label. Allow to warm to room temperature before using.
specimen. Therefore, pretreatment of the serum to remove free and complexed IgG antibody is strongly recommended. Prepare 1:20 screening dilutions of patient sera as follows: mix 5 µL of patient serum with 95 µL IgM Pretreatment Diluent in microcentrifuge tubes or a microtiter plate; and, allow at least 5 minutes for immunoprecipitation reaction to occur. The diluted sample may be used as is, or may be centrifuged to clear precipitate from serum. The precipitate will not interfere with the assay.

Where it is necessary to determine endpoint titers, use PBS to serially dilute the pretreated specimens.

**TEST PROCEDURE**

1. Remove slides from cold storage. To avoid condensation, allow slides to reach room temperature before opening slide packets.
2. Apply 10 µL of VCA IgM Positive Control, as bottled at screening dilution, to the appropriate slide well. Use PBS to serially dilute the Positive Control 32-fold beyond the bottled dilution. Apply 10 µL of each serial dilution to an appropriate slide well.
3. Apply 10 µL of EBV Negative Control, as bottled at screening dilution, to the appropriate well. Do not dilute the Negative Control.
4. For each patient sample to be tested, add approximately 10 µL of the diluted/pre-treated sample (see Specimen Pretreatment, above) to an appropriate slide well. Make notations to later identify each well when reading the results.
5. Incubate slide(s) in a humid chamber for 90 ± 2 minutes at 35 to 37°C.
6. Remove slides from the humid chamber and gently rinse each slide with a stream of PBS. Do not aim the stream of PBS directly at the slide wells. Rinse one row at a time to avoid mixing of specimens. Wash slides by submerging the rinsed slides into Coplin or slide staining jars containing PBS for 10 minutes.
7. Dip the washed slides briefly in distilled or purified water, and allow the slides to air dry.
8. Add approximately 10 µL VCA IgM RIFA® Conjugate to each slide well.
9. Incubate slides in a humid chamber for 30 ± 2 minutes at 35 to 37°C.
10. Repeat wash steps 6 and 7.
11. Place a few drops of Mounting Medium on the slide and cover with a 24 x 50 mm coverslip. Remove any air bubbles and excess Mounting Medium with absorbant paper.
12. View wells at a final magnification of 200X on a properly equipped fluorescence microscope. For optimum fluorescence, read slides the same day the assay is performed. If this is not possible, store in the dark at 2 to 8°C up to 24 hours.

**QUALITY CONTROL**

Each run (each time a slide, or group of slides, is processed) should include both Positive and Negative controls.

1. The Positive Control should endpoint (1+fluorescence) at 16 fold beyond the bottled dilution. However, due to differing laboratory conditions, including equipment, the endpoint may range from 8 to 32 fold beyond the bottled dilution.
2. The Negative Control should be negative at the bottled dilution. All of the cells should appear orange to red in color.

If controls do not exhibit these results, patient test results should be considered invalid and the assay repeated.

INTERPRETATION OF TEST RESULTS

Microscope optics, light source condition and type will determine overall fluorescent intensity and endpoint titers. Read control wells first during every run to ensure correct interpretation.

Reading the Slides
Positive cells should be clearly distinct from the rVCA-negative background cells. Due to the unique nature of this recombinant substrate, it is common to observe reduced fluorescent intensity near the cell's center. In many cases, positive cells will have a target-like appearance with bright intense fluorescence over the majority of the cell and a dimmer central area. This staining pattern is expected and should be interpreted as positive. Use control wells to become familiar with positive and negative staining patterns and relative intensity.

Read the fluorescent intensity of cells on each well, and grade the fluorescence as follows:

- **2 to 4+**: Moderate to intense apple-green fluorescence of 5 to 20% of cells.
- **1+**: Definite, but dim fluorescence equivalent to that observed for the Positive Control at its reference endpoint titer.
- **Negative**: No fluorescence or fluorescence equal to that observed in the Negative Control well.

Interpreting the Patient Specimen Results
The reciprocal of the highest serum dilution that exhibits definite (1+) apple-green fluorescence in 5 to 20% of the cells is termed the serum endpoint titer.

<table>
<thead>
<tr>
<th>Endpoint Titer</th>
<th>Interpretation</th>
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<tr>
<td>≥1:20</td>
<td>VCA IgM endpoint titers of 1:20 and greater are considered presumptive evidence of recent or current infection by EBV.</td>
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<tr>
<td>&lt;1:20</td>
<td>VCA IgM endpoint titers less than 1:20 suggest that the patient does not have an acute EBV infection. This may be found in patients with either no history of EBV infection (such individuals are thus susceptible to infectious mononucleosis) or those with onset of infection more than 3 months previous to the specimen collection date.</td>
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Non-specific Fluorescence
Naturally occurring cellular antigens expressed on the host cell surface may react with some patient sera. These cellular antigens are common to all cells; therefore, these sera are detected by the fact that they react with all or most of the substrate cells rather than just the 5 to 20% rVCA positive cells. However, if the VCA titer exceeds the anti-cell titer, these sera may still be tested. In cases where the result is uninterpretable, a second serum specimen and/or an alternate methodology may be required.

LIMITATIONS

1. Anti-VCA IgM is detectable for a very finite period following onset of primary EBV infection. Approximately 2 to 3 months post onset anti-VCA IgM will have decreased to levels undetectable by IFA and will remain undetectable thereafter.
2. Samples obtained too early during primary infection may not contain detectable antibodies. If EBV infection is suspected, a second sample should be obtained 10 to 21 days later and tested in parallel with the original sample to look for seroconversion.
3. Paired sera must be assayed concurrently.
4. All results from this and other EBV serologies must be correlated with clinical history and other data available to the attending physician.
5. When used according to instructions, the IgM Pretreatment Diluent has been shown to inactivate up to 7700 mg/dL of IgG antibody in human serum.

EXPERIMENTAL VALUES

Individuals that do not have a current infection, or have not experienced a primary EBV infection within the previous 2 to 3 months will be negative for VCA IgM antibody by RIFA®.

104 samples from a normal (asymptomatic) population were tested for EBV VCA IgM antibodies using the Focus Diagnostics EBV VCA RIFA® IgM test: 73 samples were from a blood bank in Tennessee, 16 samples were from a blood bank in Florida, and the remaining 15 samples were from healthy laboratory workers in Cypress, CA. At the 1:20 screening dilution, all sera were found negative for EBV VCA IgM antibodies.

SPECIFIC PERFORMANCE CHARACTERISTICS

Sensitivity and Specificity
An outside investigator evaluated the Focus Diagnostics EBV VCA RIFA® IgM kit sensitivity and specificity using 128 characterized serum samples: all sera were characterized using a complete panel of EBV markers (VCA IgG and IgM, EA, and EBNA). All tests were performed in accordance with established kit protocols, including using controls.

For the sensitivity study, only primary acute phase serum samples (IgM+, IgG+, EA+/−, and EBNA−) were selected. Of the 31 acute phase serum samples, the Focus Diagnostics EBV VCA RIFA® IgM kit found all 31 samples positive, demonstrating 100% sensitivity. (see Table 1)

For the specificity study, EBV negative (all EBV markers negative) and EBV Latent/Convalescent phase (IgM−, IgG+, EA+/−, and EBNA−) serum samples were selected. Of the 69 IgM negative serum samples (9 EBV negative, 60 EBV Latent/Convalescent), the Focus Diagnostics EBV VCA RIFA® IgM kit found all 69 samples negative, demonstrating 100% specificity. (see Table 1)

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Sensitivity and Specificity</td>
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<tr>
<td>Number of Samples: 100 (31 positives, 69 negatives)</td>
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<tr>
<td>Characterized Sera</td>
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<td>+</td>
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<td>Focus</td>
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Sensitivity.............100%............(31/31) .............. 95% CI = 89.8% to 100%
Specificity.............100%............(69/69) .............. 95% CI = 94.8% to 100%

Method Comparison
Method Comparison studies were conducted off-site by an independent investigator and at Focus Diagnostics by in-house investigators. All testing was performed at each site according to each assay's respective package insert, including using controls.

Using 128 serum samples, an outside Investigator compared the Focus Diagnostics’ EBV VCA IgM RIFA® test to 2 currently marketed EBV VCA IgM kits: 1 IFA and 5 ELISA kits. Positives and negatives, for the purpose of the method comparison, were defined as those samples receiving concurrence from 4 of 6 kits. Equivocal results, those samples that did not receive concurrence from 4 of 6 kits, could not be defined and were not included in the method comparison study.

Using 109 serum samples determined by a licensed commercial reference laboratory EBV VCA IgM positive and 147 serum samples characterized by Focus Diagnostics as EBV latent/convalescent phase (VCA IgM−, EBNA+), Focus Diagnostics conducted an in-house method comparison, comparing the Focus Diagnostics EBV VCA RIFA® IgM kit to 2 currently marketed EBV VCA IgM IFAs.

In the combined results of the two method comparison studies, the Focus Diagnostics EBV VCA IgM RIFA® test demonstrated 98% relative sensitivity, 100% relative specificity, and an overall correlation of 99% (371/374). (see Table 2)
TABLE 2
Method Comparison: Summary of In-house and Off-site Studies
Number of Samples: 374 (158 positives, 216 negatives)
Consensus Results

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<tbody>
<tr>
<td>Focus</td>
<td>155</td>
<td>0</td>
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<tr>
<td>Diagnostics</td>
<td>3</td>
<td>216</td>
</tr>
</tbody>
</table>

Relative Sensitivity .... 98% ...... (155/158) ...... 95% CI = 94.5% to 99.6%
Relative Specificity .... 100% ...... (216/216) ...... 95% CI = 98.3% to 100%

Cross-reaction
Cross reaction studies were conducted using 35 samples with IgM antibodies against either cytomegalovirus, herpes simplex virus, or varicella-zoster virus. None of the 35 samples tested positive using the Focus Diagnostics EBV VCA IgM RIFA® test. This finding suggests that the presence of IgM antibodies against other human herpes viruses does not result in cross-reactions to VCA in the Focus Diagnostics test.

Reproducibility
Inter-assay reproducibility was established by testing 35 known VCA IgM positive samples using three lots of Focus Diagnostics’ EBV VCA IgM RIFA® slides. Each sample was titered to endpoint and the fluorescent intensity scored for each slide lot. All titers were within one two-fold dilution and the fluorescent intensities were within one order of magnitude. Intra-assay reproducibility was assessed using three positive samples titered to endpoint ten times each on the same lot of slides. All endpoints were within one two-fold dilution. Fluorescent intensities remained the same throughout. These results demonstrate the Focus Diagnostics’ EBV VCA IgM RIFA® test’s excellent inter- and intra-assay reproducibility.

REFERENCES

This package insert is available in French, German, Italian, and Spanish at www.focusdx.com, and is available in other languages from your local distributor.