The isolation of arboviruses from patient's low titer sera can be difficult. Here we compared the detection efficiency of Dengue (DEN), Yellow Fever (YF), Saint Louis Encephalitis (SLE), West Nile (WN), Ilheus (ILH), Group C (GC), Oropouche (ORO), Mayaro (MAY) and Venezuela Encephalitis Equine (VEE) viruses using a Modified Shell Vial Culture (MSVC) protocol to a Standard Cell Culture (SCC) protocol. First the MSVC and SCC protocols were compared using five dilutions for each of the following stock viruses: DEN-1, DEN-2, DEN-3, DEN-4, YF, SLE, WN, ILH, GC, ORO, MAY and VEE. Next, patients’ original sera from which viruses (DEN-1, DEN-2, DEN-3, YF, GC, ORO, MAY and VEE) had been previously isolated were compared by the two methods using five sera dilutions. In addition, seven sera that were positive for DEN-3 by RT-PCR and negative by SCC were processed by MSVC. The MSVC protocol was consistently 1-2 logs higher virus dilution more sensitive for virus detection than the SCC protocol for all stock Flaviviruses tested (DEN-1, DEN-2, DEN-3, DEN-4, YF, SLE, WN and ILH). MSVC was equal to or one log more sensitive for virus detection than SCC for the stock Bunyaviruses (GC and ORO). For the stock Alphavirus MAY, MSVC was equally or one log more sensitive for virus detection than SCC, while for VEE SCC was equally or one log more sensitive for virus detection than MSVC. MSVC was consistently one to two sera dilutions more sensitive than SCC for the detection of Flaviviruses from patients’ sera. Both methods were approximately equally sensitive for the detection of Bunyaviruses from patients’ sera and equal or one dilution less sensitive for the detection of Alphaviruses from patients’ sera. Additionally, MSVC detected DEN virus in five of seven DEN-3 RT-PCR positive, SCC negative patients’ sera.

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INTRODUCTION

The isolation of arboviruses from patient’s low titer sera can be difficult.

Shell vial culture centrifugation methods for virus isolation have been shown to be more sensitive than standard isolation methods for members of the Paramyxoviridae [1–4], Herpesviridae [5,6], Orthomyxoviridae [7] and Flaviviridae [8] families. DEN-2, Japanese Encephalitis (JE) and WN are the Flaviviridae viruses for which the Shell Vial method has been tested [9]. The original Shell Vial method has been adapted to 24 well tissue culture plates with centrifugation (Modified Shell Vial Culture (MSVC))[8].

We have compared the MSVC and standard cell culture (SCC) methods of virus detection for the Flaviviruses: DEN-1, DEN-2, DEN-3, DEN-4, YF, SLE, ILH and WN; the Bunyaviruses: GC, ORO and the Alphaviruses: MAY and VEE. The comparison was made using two cell lines, C636 and Vero, two incubation times, four and 10 days, with stock viruses and original patients’ sera from which viruses had previously been isolated.

METHODS

Stock viruses

DEN-1, DEN-2, DEN-3, DEN-4, YF, SLE, WN, ILH, GC, ORO, MAY and VEE viruses were propagated in Vero cells with Earle’s Modified Essential Medium with 100 units/ml Penicillin, 100 μg/ml Streptomycin, 1 mM Sodium Pyruvate and 2% (V/V) Fetal Bovine Serum at 37°C. Upon observation of cytopathic effects, media were collected, clarified by centrifugation at 40,000 g for five minutes and stored at −80°C until use. The Flaviviruses and Bunyaviruses were titered in BHK-21 cells; the Alphaviruses were titered in Vero cells.

Original sera

Sera were collected from febrile subjects and processed for virus isolation following the SCC method (1:5 inoculum, 10 day incubation, C636 and/or Vero culture) and stored at −80°C until use.

The SCC method

100 μl of each stock virus dilution (undiluted, 100, 10, 1 and 0.1 PFU) or serum dilution (1:5, 1:10, 1:50, 1:100 and 1:500) were inoculated in C636 and Vero cells propagated in T-25 cm² flasks. The cells were incubated at 28°C and 37°C for C636 and Vero cells, respectively, for 4 and 10 days. The cells were scraped off the flasks, transferred to 5 ml test tubes and collected by centrifugation at 450 g for 10 minutes. The cell pellets were reconstituted with PBS and spotted onto slides and tested by indirect immunofluorescent assay (IFA) using DEN, YF, SLE, WN, ILH, GC, ORO, MAY and VEE polyclonal antibodies.

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The MSVC method
100 ul of each stock virus dilution (undiluted, 100, 10, 1 and 0.1 PFU) or serum dilution (1:5, 1:10, 1:50, 1:100 and 1:500) were inoculated in C636 and Vero cells propagated in 24 wells plates. The plates were centrifuged at 680 g for 30 minutes and incubated at 33°C and 37°C for C636 and Vero cells, respectively, for 4 and 10 days. The cells were scraped off the plates, transferred to 5 ml test tubes and collected by centrifugation at 450 g for 10 minutes. The cell pellets were reconstituted with PBS and spotted onto slides and tested by IFA using DEN, YF, SLE, WN, MAY and VEE polyclonal antibodies.

RESULTS AND DISCUSSION
For all stock Flaviviruses tested by the two methods, the MSVC method consistently required one to two logs less virus for virus detection in C636 cells than the SCC method (Table 1). The required amount of virus for detection by MSVC in Vero cells ranged from two logs less, to equal that, required by the SCC method. For the Bunyaviruses, in both cell lines, the MSVC method required equal, or one log less, virus than SCC for virus detection. The results are mixed for the Alphaviruses. In both cell lines, MAY like the Bunyaviruses, the MSVC method required equal, or one log less, virus than SCC for virus detection. With VEE, MSVC required equal, or one log more, virus for virus detection than the SCC method (Table 1).

The two methods were compared for virus detection from sera from which virus had been isolated following the SCC method. For the Flavivirus sera (table 2, sera 1–18) MSVC consistently required one to two dilutions less sera for virus detection than SCC. Additionally, the MSVC method identified virus in four sera that were negative for virus detection by SCC after four days of incubation (table 2; Vero cells serum 7; C636 cells sera 13, 15 and 17). Virus could not be re-isolated from three sera (table 2, sera 10, 11 and 12) by SCC while virus was detected after only four days of incubation by MSVC. For the Bunyaviruses, in both cell lines, the MSVC method required equal, or one dilution less, serum for virus detection than SCC (table 2, sera 19–27). For the Alphavirus sera, MSVC required more serum for virus detected after four days of incubation for three of seven specimens in C636 culture (table 2, sera 28, 30 and 31) than SCC. However, after ten days of incubation the two methods required equal amounts of sera for virus detection for six of the seven sera. One serum (# 34) was negative for virus detection by MSVC but positive by SCC, in both cell lines.

To further compare the two methods, seven SCC negative, DEN-3 RT-PCR positive sera were assayed by MSVC. MSVC identified virus in five of seven sera after 10 days of incubation in C636 culture while SCC detected virus in three of the sera (table 2, sera 35–41).

The SCC method maybe less reproducible for virus detection than MSVC. DEN virus could not be detected in three sera (table 2, sera 10, 11 and 12) from which the viruses were originally isolated by SCC and three SCC negative, DEN-3 RT-PCR positive sera were SCC positive for DEN virus (table 2, sera 35, 40 and 41). Possibly those sera have low DEN virus titers that are at or near the limit of virus detection by SCC. However, all six specimens were positive for DEN virus by MSVC.

MSVC is a rapid and efficient method for the isolation of Flaviviruses (DEN-1, DEN-2, DEN-3, DEN-4, YF, SLE, WN and ILH), Bunyaviruses (ORO and GC) and the Alphavirus MAY. The SCC method could be more suitable for the isolation of the Alphavirus VEE. For studies that involve the isolation of arboviruses the utilization of both C636 MSVC and Vero SCC may maximize virus isolation.

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The study protocol was approved by the Naval Medical Research Center Institutional Review Board (Protocol NMCRD.2000.0006) in

| Table 1. Comparison of stock virus detection by SCC and MSVC. |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| VIRUS          | Genera          | C636 Cells Lowest concentration virus detected (PFU) | Vero Cells Lowest concentration virus detected (PFU) |
|                |                 | Day 4 SCC | Day 4 MSVC | Day 10 SCC | Day 10 MSVC | Day 4 SCC | Day 4 MSVC | Day 10 SCC | Day 10 MSVC |
| DEN 1 (16007)  | Flavivirus      | 100       | 1         | 10         | 0.1        | 1          | 0.1        | 10         | 1          |
| DEN 2 (16681)  | Flavivirus      | 4.7x10⁴   | 10        | 100        | 0.1        | 4.7x10⁴    | 100        | 4.7x10⁴    | 0.1 |
| DEN 3 (IQD 1728) | Flavivirus     | 100       | 0.1      | 10         | 0.1        | 3.0x10³    | 100        | 100        | 1          |
| DEN 4 (1036)   | Flavivirus      | 10        | 0.1      | 10         | 0.1        | 100        | 10         | 1          | 0.1        |
| YF (17D)       | Flavivirus      | 3.2x10⁵   | 1         | 10         | 0.1        | 10         | 1          | 1          | 0.1        |
| SLE (CDC)      | Flavivirus      | 1         | 0.1      | 1          | 0.1        | 0.1        | 1          | 0.1        | 0.1        |
| WNV (CDC)      | Flavivirus      | 10        | 0.1      | 0.1        | 0.1        | 0.1        | 0.1        | 0.1        | 0.1        |
| ILHEUS (ATCC)  | Flavivirus      | 10        | 0.1      | 100        | 0.1        | 1          | 0.1        | 0.1        | 0.1        |
| ILHEUS (FSE 0800) | Flavivirus     | 100       | 0.1      | 10         | 0.1        | 0.1        | 1          | 0.1        | 0.1        |
| GROUP C (IQU 1719) | Bunyavirus   | 1         | 0.1      | 0.1        | 0.1        | 0.1        | 0.1        | 0.1        | 0.1        |
| OROPOUCHE 172  | Bunyavirus      | 10        | 1         | 10         | 1          | 10         | 1          | 10         | 10         |
| MAYARO (TRVL15537) | Alphavirus   | 10        | 1         | 10         | 1          | 10         | 1          | 10         | 10         |
| VEE (TC 83)    | Alphavirus      | 0.1       | 1         | 0.1        | 0.1        | 0.1        | 0.1        | 0.1        | 0.1        |

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### Table 2. Comparison of virus detection in sera by SCC and MSVC.

| Serum # | SCC (C6/36 1:5 isolate) | RT-PCR | C636 Cells Highest dilution virus detected | Vero Cells Highest dilution virus detected |
|---------|--------------------------|---------|--------------------------------------------|-------------------------------------------|
|         |                          | Day 4 SCC | Day 4 MSVC | Day 10 SCC | Day 10 MSVC | Day 4 SCC | Day 4 MSVC | Day 10 SCC | Day 10 MSVC |
| 1       | D1                       | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 2       | D1                       | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 3       | D1                       | 1:50     | 1:100      | 1:100      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 4       | D2                       | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 5       | D2                       | 1:10     | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 6       | D3                       | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 7       | D3                       | 1:100    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 8       | D3                       | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 9       | D3                       | 1:5      | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 10      | D3                       | NEG      | 1:100      | NEG        | 1:500       | NEG       | NEG        | NEG         | NEG         |
| 11      | D3                       | NEG      | 1:100      | NEG        | 1:500       | NEG       | NEG        | NEG         | NEG         |
| 12      | D3                       | NEG      | 1:5       | NEG        | 1:500       | NEG       | NEG        | NEG         | NEG         |
| 13      | D3                       | NEG      | 1:5      | NEG        | 1:500       | NEG       | NEG        | NEG         | NEG         |
| 14      | D3                       | 1:50     | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 15      | D3                       | NEG      | 1:100      | NEG        | 1:500       | NEG       | NEG        | NEG         | NEG         |
| 16      | D3                       | 1:100    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 17      | D3                       | NEG      | 1:10      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 18      | YF                       | 1:100    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 19      | GC                       | 1:50     | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 20      | GC                       | 1:100    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 21      | GC                       | 1:50     | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 22      | ORO                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 23      | ORO                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 24      | ORO                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 25      | ORO                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 26      | ORO                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 27      | ORO                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 28      | MAY                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 29      | MAY                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 30      | MAY                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 31      | VEE                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 32      | VEE                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 33      | VEE                      | 1:500    | 1:500      | 1:500      | 1:500       | 1:500     | 1:500      | 1:500       | 1:500       |
| 34      | VEE                      | 1:50     | NEG        | 1:5        | NEG         | 1:50      | NEG        | 1:10        | NEG         |
| 35      | VEE                      | NEG      | D3         | NEG        | 1:5         | 1:50      | NEG        | NEG         | NEG         |
| 36      | VEE                      | NEG      | D3         | NEG        | 1:5         | 1:50      | NEG        | NEG         | NEG         |
| 37      | VEE                      | NEG      | D3         | NEG        | 1:5         | 1:50      | NEG        | NEG         | NEG         |
| 38      | VEE                      | NEG      | D3         | 1:10       | NEG         | 1:50      | NEG        | NEG         | NEG         |
| 39      | VEE                      | NEG      | D3         | 1:10       | NEG         | 1:50      | NEG        | NEG         | NEG         |
| 40      | VEE                      | NEG      | D3         | 1:50       | NEG         | 1:50      | 1:10       | 1:500       | 1:500       |
| 41      | VEE                      | NEG      | D3         | 1:10       | 1:500       | 1:100     | 1:500      | 1:500       | 1:500       |

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compliance with all applicable Federal regulations governing the protection of human subjects.

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**Author Contributions**

Conceived and designed the experiments: TK EC. Performed the experiments: EC. Analyzed the data: TK EC. Wrote the paper: TK EC.

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