**Multiplex real-time RT-PCR for detection and distinction of Spondweni and Zika virus**

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**Summary**

Zika (ZIKV) and Spondweni viruses (SPOV) are closely related mosquito borne flaviviruses in the Spondweni serogroup. The co-circulation and similar disease presentation following ZIKV and SPOV infection necessitates the development of a diagnostic tool for their simultaneous detection and distinction. We developed a one-step multiplex real-time RT-PCR (ZIKSPOV) to detect and distinguish between SPOV and ZIKV by utilizing a single primer set combined with virus specific hydrolysis probes. The ZIKSPOV assay was compared to published virus specific real-time RT-PCR assays and the limit of detection was comparable. The SPOV reference strain AR94 was detectable to 0.001 $TCID_{50}$ per PCR reaction, while African lineage ZIKV (MR 766) was detectable to 0.002 $TCID_{50}$ per reaction and Asian lineage ZIKV (H/PF/2013) to 0.05 $TCID_{50}$ per reaction. The ZIKSPOV assay did not detect other flaviviruses, indicative of its specificity for Spondweni serogroup. The ZIKSPOV assay is a useful addition to arbovirus diagnostic and surveillance tools in areas where ZIKV and SPOV are expected to co-circulate. Further evaluation is required to demonstrate the application of the assay for detection of ZIKV and SPOV in mosquito and human clinical samples.

**Keywords: Arbovirus; Zika virus; Spondweni virus; flavivirus; Flaviviridae; multiplex; *Aedes***

The *Flavivirus* genus (family *Flaviviridae*) comprises several arthropod-borne viruses that recently emerged or re-emerged as pathogens of great global public health importance (Holbrook, 2017). Zika virus (ZIKV), first isolated in 1947 in Uganda, has expanded its geographical range from Africa to other parts of the world following large outbreaks in the Yap islands in 2007, New Caledonia, French Polynesia, Isla de Pascua and the Cook Islands in 2013-2014, and the Americas in 2015 (Baronti et al., 2014; Campos et al., 2015; Dick et al., 1952a; Duffy et al., 2009; Dupont-Rouzeyrol et al., 2015; Fauci and Morens, 2016; Lanciotti et al., 2008; Tognarelli et al., 2016; Zanluca et al., 2015). Spondweni virus (SPOV) was first isolated in 1952 but was initially misidentified as a ZIKV strain, which resulted in the SA Ar 94 SPOV strain being recognized as the prototype after its isolation and identification from mosquitoes in 1955 in South Africa (Kokernot et al., 1957; Macnamara, 1954). Until recently considered to be endemic only to the African continent, a recent study detected SPOV viral RNA from *Culex quinquefasciatus* mosquitoes in Haiti (Sarah et al., 2018). To date, only six human cases of SPOV infection have been described, but this might be attributed to misdiagnosis and limited diagnostic assays specific for the detection of SPOV (Bearcroft, 1956; Draper, 1965; Macnamara, 1954; McIntosh et al., 1961; Wolfe et al., 1982). ZIKV has been isolated from *Aedes furcifer*, *Ae. aegypti*, *Ae. africanus* and *Ae. Luteocephalus* mosquitoes (Dick et al., 1952b; Lee and Moore, 1972 Marchette et al., 1969). Aedes *albopictus*, together with *Ae. aegypti*, forms an integral part in the global dissemination of Zika virus due to their abundance in tropical and sub-tropical regions worldwide (Grard et al., 2014; Kraemer et al., 2015). *Ae. circumluteolus* appears to be the preferred vector for SPOV but the virus' ecology remains poorly studied (McIntosh et al., 1961; McIntosh et al., 1972; Worth et al., 1961). The recent detection of SPOV outside Africa raises the concern that its global distribution might soon mimic that of Zika and dengue viruses.

In addition to common symptoms such as fever, headache, nausea/vomiting, myalgia and arthralgia following ZIKV and SPOV infection, individuals might also develop conjunctivitis, vertigo, epistaxis, hematuria, hematospermia, aphthous ulcers, and maculopapular rash (Draper, 1965; McIntosh et al., 1961). ZIKV infection is also strongly associated with Guillain-Barre syndrome in adults, while microcephaly in infants has reinforced the need for rapid, accurate diagnostic assays (Brasil et al., 2016; de Araujo et al., 2016; do Rosario et al., 2016; Mlakar et al., 2016; Ventura et al., 2016). Serological cross-reaction between flaviviruses presents a major challenge to arbovirus diagnostic laboratories. The close genetic relationship between SPOV and ZIKV has likely led to misdiagnosis of some clinical cases in Africa where

both viruses are endemic (Haddow et al., 2016). Real-time RT-PCRs are available for the detection of ZIKV and SPOV individually (Lanciotti et al., 2008; McDonald *et al*., 2017). ZIKV and SPOV may co-circulate in specific regions, necessitating the development of a multiplex assay. We therefore aimed to develop a multiplex real-time RT-PCR to detect and distinguish SPOV and ZIKV for diagnostic and surveillance purposes.

Full genome sequences of SPOV (NC_029055 and DQ 859064) and ZIKV available from Genbank (NC_012532, KU 681082, KU 955594, KX 893855, KU 955593, KU 955591, KU 681081, KX 377335, KX 377336, KX 377337, KY 126351 and MF 692778) were aligned in BioEdit (v 7.0.5.3) using the "ClustalW multiple alignment" function. Multiple sets of possible primers and probes (not shown) were evaluated using AnnHyb (v4.946) to compare binding to SPOV, ZIKV and other flavivirus genomes. Secondary structure formation between primers, and heterodimer formation between primers, probes and primer-probe pairs were assessed using IDT OligoAnalyzer 3.1. The final primer and probe set, indicated in Table 1, performed best during the *in silico* evaluation process due to lower likelihood of secondary structure formation, and more specific binding to specific SPOV and ZIKV sequences. Degenerate nucleotides were incorporated at certain positions (indicated in bold in Table 1) in the primers and probes to account for variable nucleotide positions in the primer/probe binding regions of ZIKV and SPOV sequences. The primers and probes target the NS5 gene. The SPOV probe has a low affinity for ZIKV sequences within the primer-binding region, and *vice versa* ZIKV probe for SPOV sequences, and given stringent conditions, cross-reaction was expected to be limited. The primer and probe set was also preliminarily evaluated *in silico* against other medically important flaviviruses using AnnHyb (v 4.946). For most, the binding potential for either the primers, probes or both were relatively low, and in most cases, the probes would not bind within the PCR amplification region should amplification occur. The probes were labeled with FAM (SPOV) and HEX (ZIKV) dyes respectively, with BHQ-1 acting as quencher. The primer and probe sequences are also shown in supplementary Table S1, together with the respective primer and probe sites within sequences from recent and historical Zika virus isolates, to demonstrate primer and probe mismatches. The only concerning sequence is that from a Senegal mosquito isolate (KU955591) that has two forward primer mismatches and one probe mismatch. The effect of these could not be determined since we do not have access to this isolate.

**Table 1: ZIKSPOV multiplex real-time RT-PCR primers and probes**

| Description | Sequence 5'-3' | Genome position: *ZIKV | Genome position: #SPOV |
|---|---|---|---|
| ZIKSPOV F | TGG AG**Y** TAC TA**Y** GCC GC**M** | 7913-7930 | 7837-7854 |
| ZIKSPOV R | TGT GTA **K**CC T**Y**T CAC **Y**TC | 7966-7949 | 7890-7873 |
| SPOVP | FAM-CAT CTC AAA AGC AAG TCC TCG-BHQ1 | N.A | 7853-7873 |
| ZIKVP-2 | HEX-CAC CAT CCG CAA AGT **K**CA **R**G-BHQ1 | 7930-7949 | N.A |

* ZIKV Accession number [NC_012532]

# SPOV Accession number [NC_029055]

Nonstandard IUPAC nucleotide codes are indicated in bold font: Y (C or T); M (A or C); K (G or T); R (A or G).

N.A. = not applicable

Virus stocks were cultured in VeroE6 cells. Passage histories and other information are summarized in Table 2. Viral RNA was extracted from virus stocks using the QIAamp® RNA Viral kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. VeroE6 culture supernatant of virus stocks (140µL) were added to AVL lysis buffer and RNA was eluted in 60µL volumes and tested immediately or stored at -20°C until further testing.

Real-time RT-PCR reactions were performed using the Quantitect® probe RT-PCR kit (Qiagen, Valencia, CA, USA) and LightCycler 96® Real-Time PCR System (Roche, Mannheim, Germany). Initial optimization involved varying $MgCl_2$ concentrations and cycling conditions. The final PCR conditions were as follows: amplifications were carried out in 25µL reaction mixtures containing 5µL of the target virus RNA, 12.5µL of 2 x Quantitect Probe RT-PCR Master mix (plus additional $MgCl_2$ to achieve a final concentration of 5mM per reaction volume), 0.25µL Quantitect enzyme mix, 1µM of both the forward and reverse primers and 0.2µM of both ZIKV and SPOV probes. Cycling conditions were as follows: reverse transcription was performed at 50°C for 30 minutes, followed by 95°C for 15 minutes. Amplification consisted of 2-step cycling with a total of 45 cycles; denaturation at 95°C for 15 seconds and combined annealing and extension at 55°C for 60 seconds.

The ability of the assay to detect different isolates was determined by testing 14 SPOV and three ZIKV isolates (Table 2) using the optimized ZIKSPOV multiplex RT-PCR. The prototype Spondweni (AR 94) and Zika (MR 766 – African lineage) virus strains, as well as an Asian

**Table 2: Virus stock information.**

| Stock virus (isolate) | Source | Year | Locality | Passage history | Stock virus titer $TCID_{50}$/mL | ZIKSPOV RT-PCR Ct value | GenBank accession |
|---|---|---|---|---|---|---|---|
| SPOV (AR94) | *Mansonia uniformis* | 1955 | Tete pan, KZN, SA | MB#11; Vero#2 | $1 \times 10^{4.5}$ | 19.36 | KX227370 |
| SPOV (AR2238) | *Aedes circumluteolus* | 1959 | Ndumu, KZN, SA | MB#2; Vero#2 | $1 \times 10^{4.0}$ | 19.15 | MH829610 |
| SPOV (AR2239) | *Aedes circumluteolus* | 1959 | Ndumu, KZN, SA | MB#3; Vero#2 | $1 \times 10^{4.0}$ | 19.91 | MH829611 |
| SPOV (AR1266) | *Aedes circumluteolus* | 1958 | Ndumu, KZN, SA | MB#2; Vero#2 | $1 \times 10^{3.75}$ | 19.95 | MH829607 |
| SPOV (AR2203) | *Mansonia africana* | 1959 | Ndumu, KZN, SA | MB#3; Vero#2 | $1 \times 10^{4.25}$ | 20.04 | MH829609 |
| SPOV (AR1081) | *Eretmapodites silvestris* | 1958 | Ndumu, KZN, SA | MB#3; Vero#2 | $1 \times 10^{4.25}$ | 19.55 | MH829602 |
| SPOV (AR1077) | *Aedes circumluteolus* | 1958 | Ndumu, KZN, SA | MB#2; Vero#2 | $1 \times 10^{4.25}$ | 19.80 | MH829601 |
| SPOV (AR1086) | *Aedes circumluteolus* | 1958 | Ndumu, KZN, SA | MB#3; Vero#2 | $1 \times 10^{4.5}$ | 19.90 | MH829604 |
| SPOV (AR1168) | *Mansonia africana* | 1958 | Ndumu, KZN, SA | MB#3; Vero#2 | $1 \times 10^{4.0}$ | 19.03 | MH829606 |
| SPOV (AR1084) | *Mansonia africana* | 1958 | Ndumu, KZN, SA | MB#1; Vero#2 | $1 \times 10^{4.0}$ | 20.25 | MH829603 |
| SPOV (AR3061) | *Aedes fowleri/fryeri* | 1960 | Lumbo, Mozambique | MB#2; Vero#2 | $1 \times 10^{4.25}$ | 18.47 | MH829612 |
| SPOV (AR2164) | *Aedes circumluteolus* | 1959 | Ndumu, KZN, SA | MB#2; Vero#2 | $1 \times 10^{4.25}$ | 17.41 | MH829608 |
| SPOV (H127) | *Homo sapiens* | 1958 | Laboratory infection, SA | MB#2; Vero#2 | $1 \times 10^{4.0}$ | 18.85 | MH829613 |
| SPOV (AR1163) | *Mansonia africana* | 1958 | Ndumu, KZN, SA | MB#2; Vero#2 | $1 \times 10^{2.25}$ | 23.25 | MH829605 |
| ZIKV (MRS-OPY-Martinique-PaRi-2015) | *Homo sapiens* | 2015 | Martinique | C6-36#1; Vero#1 | $1 \times 10^{6.22}$ | 15.67 | KU647676 |
| ZIKV (H/PF/2013) | *Homo sapiens* | 2013 | French Polynesia | C6-36#1; Vero#4 | $1 \times 10^{7.16}$ | 12.81 | KJ776791 |
| ZIKV (MR766) | *Macaca mulatta* | 1947 | Uganda | C6-36#1; Vero#5 | $1 \times 10^{3.82}$ | 20.39 | DQ859059 |
| Dengue 1 (Hawaii-prototype) | *Homo sapiens* | 1944 | Hawaii | Vero#11 (previous unknown) | $1 \times 10^{2.67}$ | > 45* | KM204119 |
| Dengue 2 (NGC) | *Homo sapiens* | 1944 | Papua New Guinea | Vero#11 (previous unknown) | $1 \times 10^{2.43}$ | > 45 | KM204118 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dengue 3 (H87) | *Homo sapiens* | 1956 | Philippines | Vero#11 (previous unknown) | $1 \times 10^{1.47}$ | > 45 | KU050695 |
| Dengue 4 (H241) | *Homo sapiens* | 1956 | Philippines | Vero#11 (previous unknown) | $1 \times 10^{5.2}$ | > 45 | KR011349 |
| West Nile virus (SA93/01) | *Homo sapiens* | 2001 | South Africa | Vero#5 | $1 \times 10^{6.75}$ | > 45 | EF429198 |
| Wesselsbron virus (H177) | *Homo sapiens* | 1955 | Simbu, KZN, SA | Vero#5 | $1 \times 10^{6.75}$ | > 45 | JX423785 |
| Usutu virus (AR1776) | *Culex neavei* | 1959 | Ndumu, KZN, SA | MB#6; Vero#5 | $1 \times 10^{5.2}$ | > 45 | EU074021 |

* not detected

lineage ZIKV (H/PF/2013), were additionally tested in the ranges of 0.001 to 31,622 $TCID_{50}$/mL (AR94), 0.0002 to 6,606 $TCID_{50}$/mL (MR 766) and 0.014 to 14,454,398 $TCID_{50}$/mL (H/PF/2013) in half-log increments and in triplicate to determine the limit of detection. These virus dilutions were also tested with previously published virus specific primer and probe sets to compare the multiplex assay to established monoplex tests: SPOV specific (McDonald et al., 2017) and ZIKV specific (Lanciotti et al., 2008), respectively. The analytical specificity of the assay was evaluated by testing other flaviviruses of medical importance as summarized in Table 2. A cycle threshold value of 40 was arbitrarily set as cut-off for positive/negative interpretation of test results.

The multiplex ZIKSPOV assay was able to detect all 14 SPOV isolates, collected from South Africa and Mozambique over a period of six years (1955 – 1960), as well as the prototype African lineage ZIKV isolate and two Asian lineage viruses (Table 2). Half-log dilutions of stock SPOV and ZIKV viruses were extracted and tested in triplicate, and the limit of detection determined as the lowest titer per reaction volume yielding positive results in all three replicates. The ZIKSPOV multiplex was able to detect SPOV AR94 to 0.001 $TCID_{50}$ per RT-PCR reaction volume, compared to the monoplex McDonald assay which was able to detect 0.0003 $TCID_{50}$ (Figure 1A). The ZIKSPOV multiplex was able to detect African lineage ZIKV MR766 to 0.002 $TCID_{50}$ per RT-PCR reaction volume, compared to the monoplex Lanciotti assay which was able to detect 0.0007 $TCID_{50}$ (Figure 1B). The ZIKSPOV multiplex was able to detect Asian lineage ZIKV H/PF/2013 to 0.05 $TCID_{50}$ per RT-PCR reaction volume, compared to the monoplex Lanciotti assay which was able to detect 0.02 $TCID_{50}$ (Figure 1C). The limit of detection per reaction of the ZIKSPOV assay translates to 0.1, 0.2 and 1.4 $TCID_{50}$ per mL of SPOV AR94, ZIKV MR766 and H/PF/2013 culture supernatant respectively. The ZIKSPOV RT-PCR assay was specific for the detection of SPOV and ZIKV and did not detect other mosquito borne flaviviruses of medical importance such as West Nile virus, the four dengue viruses (serotype 1-4), Wesselsbron virus, and Usutu virus (Table 2).

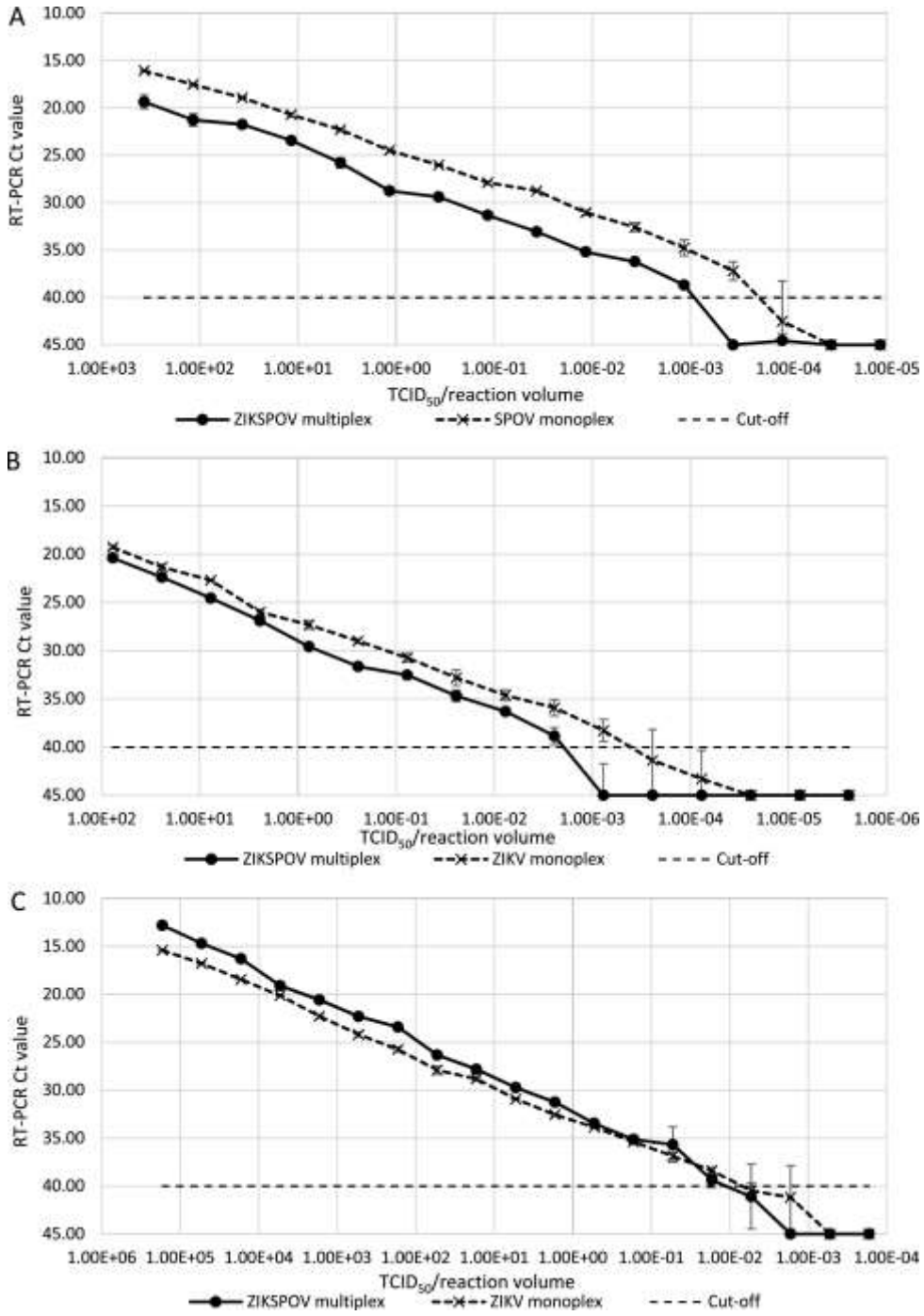


**Figure 1.** Comparison of the limit of detection of the ZIKSPOV multiplex RT-PCR with SPOV and ZIKV specific RT-PCR assays. Figure 1A shows the comparison of detection of SPOV AR94 using the ZIKSPOV RT-PCR and SPOV specific RT-PCR assays. Figure 1B shows the comparison of detection of ZIKV MR766 African lineage using the ZIKSPOV RT-PCR and ZIKV specific RT-PCR assays. Figure 1C shows the comparison of detection of ZIKV H/PF/2013 Asian lineage using the ZIKSPOV RT-PCR and ZIKV specific RT-PCR assays. Error bars indicate cycle threshold standard deviation from the means. The cut-off value is indicated with a dotted vertical line at a Ct value of 40.

Laboratory experiments described here confirm the sensitivity and specificity of the ZIKSPOV real-time RT-PCR. The assay was able to detect 14 SPOV isolates collected over a period of 6 years from South Africa and Mozambique, as well as one African and two Asian lineage ZIKV isolates. The same assay, however, failed to detect related mosquito borne flaviviruses that might also co-circulate with ZIKV and SPOV, suggesting a high level of specificity. The ZIKSPOV assay was shown to be only slightly less sensitive (not more than $10^{0.5}$ $TCID_{50}$) than monoplex ZIKV or SPOV specific real-time RT-PCR assays, which was not unexpected from a multiplex format. This slight decrease in the limit of detection is, however, likely of very little or no clinical relevance, and is outweighed by the advantages of a single-tube multiplex assay that is able to detect viral loads well below 1 $TCID_{50}$ per PCR reaction. A previous study found a mean viral load in Zika patient blood of $5x10^4$ RNA copies/mL of blood (range: $1x10^2$–$2x10^6$) and $2x10^4$ RNA copies/mL of urine (range: $4x10^2$–$8x10^4$) (Corman et al., 2016). Viral load, particularly in blood, dependent on time of collection post onset.

The ZIKSPOV multiplex assay developed in this study could represent a valuable addition to the battery of assays performed in arbovirus reference and research laboratories in Africa and beyond. In particular the assay could find use not only in vector surveillance studies where ZIKV and/or SPOV are expected to be circulating, but also in human surveillance or as part of a differential diagnostic testing algorithm of patients with acute febrile illness of unknown cause. Our study highlights the potential of this assay, but further evaluation and validation using patient clinical material or field mosquito samples are needed to provide more confidence in its use as a diagnostic tool. Patient clinical material from SPOV acutely infected individuals might, however, be difficult to obtain and such analysis might therefore have to rely on laboratory generated materials from animal models. The assay would also need to be evaluated for its ability to detect a more representative range of ZIKV isolates that are currently known to circulate, including West African strains.

**Data availability**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Author contributions**

RR performed laboratory experiments, data analysis, interpretation and co-wrote the first draft of the manuscript.

WM provided financial support, data interpretation and reviewed the manuscript.

JTP provided financial support and reviewed the manuscript.

PJVV conceptualized the study, performed laboratory experiments, data analysis, and interpretation, and co-wrote the first draft of the manuscript.

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