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Diagnostic Validation of the RealStar® Zika Virus Reverse Transcription Polymerase Chain Reaction Kit for Detection of Zika Virus RNA in Urine and Serum Specimens

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Abstract. With the Zika virus outbreak in South America starting in 2015 and its potential to cause malformation of the fetus in infected women, the need for diagnostic methods became obvious. Until now, only limited data are available on the diagnostic performance of commercial kits. Here, we present data comparing the RealStar® Zika Virus RT-PCR Kit 1.0 for detection of Zika virus from 208 serum and urine samples collected in French Guiana with a reference method. Of these, 114 samples tested positive with the RealStar® Kit and 111 with the reference method.

Diagnosis of Zika virus (ZIKV) infections is difficult due to unspecific and often mild symptoms.[1] The emergence of ZIKV in South America in 2015 with a high number of infections and the rare but nevertheless imminent severe cases prompted World Health Organization (WHO) to declare an official emergency status.[2,3] Several real-time reverse transcription polymerase chain reaction (RT-PCR) protocols have been published and commercial kits have been made available for detection of ZIKV RNA.[4,5] The RT-PCR method which is probably most widely used for the detection of ZIKV was published by Lanciotti and others in 2008 (Lanciotti E PCR).[4] This study has been carried out to assess performance characteristics of a commercial kit, the RealStar® Zika Virus RT-PCR Kit 1.0 (and the identical version the RealStar Zika Virus RT-PCR Kit U.S.; altona Diagnostics GmbH, Hamburg, Germany), for ZIKV detection in serum and urine of Zika suspected cases in comparison with the Lanciotti E PCR. Analytical data on the RealStar Zika Virus RT-PCR Kit are also presented. The kit was Communauté européenne marked for in vitro diagnostics in February 2016 and received the Food and Drug Administration Emergency Use Authorization in May 2016.

Samples involved in this study were selected among clinical specimens received as part of routine diagnostic and expertise activities of the arboviruses National Reference Center (NRC) in French Guiana in 2016. A total of 208 clinical specimens, 103 sera and 105 urines were selected, all collected from 153 patients exhibiting symptoms of ZIKV infection between the first and the eighth day following the onset of disease. Paired serum and urine samples were available for 53 patients (Supplemental Table 1).

RNA was extracted from 140 µL serum or urine using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, adding the internal control template form the RealStar Kit during the lysis step. Extracted RNA was tested in parallel with the RealStar Zika Virus RT-PCR Kit 1.0 according to the manufacturer's instructions and with the Lanciotti E PCR (primers and probe ZIKV 1086, ZIKV 1162c, ZIKV 1107-FAM) using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The Lanciotti E PCR was run with the same reagents (proprietary development of altona Diagnostics GmbH) using the same RNA volumes as used for the RealStar kit and with the following cycling conditions: 55°C for 15 minutes; 95°C for 2 minutes; 45 cycles at 94°C for 15 seconds and 60°C for 60 seconds.

A very good correlation between cycle threshold (C_t) values obtained with both assays was observed and the overall performance of the RealStar Zika Virus RT-PCR Kit compared with the Lanciotti E PCR is very good, with diagnostic sensitivity and specificity of respectively 95.5% (confidential interval 95% (CI 95): 91.6–99.3%) and 91.8% (CI 95: 86.3–97.2%)(Table 1).

After single testing of the samples with both methods, 13 samples showed a discrepant result. All discrepant samples had a high C_t value (> 35.7, corresponding to a low viral load). The discrepant samples were retested. After repetition, four samples remained positive only with the RealStar Zika Virus RT-PCR Kit. For two of the four samples, a paired sample from the same patient was available which was tested positive with both methods and confirmed that the patient indeed was ZIKV positive (Supplemental Table 1, patients 80 and 126). Furthermore, these four samples have been selected among samples of patients for whom a diagnosis of ZIKA infection had been done by the NRC with Lanciotti E PCR positive results obtained on both serum and urine samples. The positive results obtained from previous RNA extractions showed high C_ts corresponding to low viral load.

It is noteworthy that the combined use of both serum and urine samples increases the sensitivity of Zika diagnostic as well as the correlation of both PCR assay results through the decrease of stochastic detections associated to low viral loads: whatever test used, only one discrepant Zika diagnostic, positive with the RealStar Zika Virus RT-PCR Kit and negative with the Lanciotti E PCR, was observed among the 53 patients for which paired serum and urine samples were available.

To determine the analytical sensitivity of the RealStar kit, in vitro transcript containing the ZIKV target region based on strain MR766 was quantified photometrically and diluted in half-logarithmic steps. These in vitro transcript dilutions were tested in replicates of 24 with the RealStar Zika Virus Kit. The hit rate was determined to calculate the 95% limit of detection (LoD95) by probit analysis. The calculated LoD95 is 0.61 copies/µL eluate (95% confidence interval: 0.39–1.27 copies/µL). RNA of the ZIKV strains MR766, MR_OPY_Martinique_PaRi_2015, and H/PF/2013 (European Virus Archive, Marseille, France) was tested for reactivity with the RealStar kit and showed comparable signals. In vitro transcript of strain ArD142623, which has the lowest sequence

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TABLE 1
Performance of the RealStar Zika Virus RT-PCR Kit compared with the Lanciotti E PCR

Cases		Lanciotti E PCR Positive	Lanciotti E PCR Negative	Lanciotti E PCR Total
RealStar Zika Virus RT-PCR Kit	Positive	106	8	114
	Neg	5	89	94
	Total	111	97	208

CI = confidence interval. Sensitivity: 95.5% (CI 95: 91.6–99.3%). Specificity: 91.8% (CI 95: 86.3–97.2%).

identity to any other ZIKV strain in the target region, was synthesized and also positively tested for reactivity. This indicates a high probability to detect all circulating ZIKV variants with the RealStar kit.

Potential cross-reactivity of the RealStar kit was tested with RNA of closely related virus species and with pathogens causing similar symptoms. No cross-reactivity was detected for Chikungunya virus, Dengue virus (types 1–4), West Nile virus, Japanese encephalitis virus, St. Louis encephalitis, Murray Valley encephalitis, Sudan virus, Marburg virus, Zaire ebolavirus, *Plasmodium falciparum*, and Parvovirus B19 (data not shown). With high levels of Usutu virus RNA extracted directly from cell culture supernatant, a slight cross-reactivity with reduced fluorescence compared with ZIKV amplification was observed.

To show equivalency of different real-time PCR instruments, 20 replicates of samples containing RNA at a concentration of 3 × LoD95 were tested. The ABI Prism® 7500 SDS and 7500 Fast SDS (Thermo Fisher, Waltham, MA), CFX96 Real-Time PCR Detection System and CFX96 Deep Well Real-Time PCR Detection System (Bio-Rad, Hercules, CA), LightCycler® 480 Instrument II (Roche, Basel, Switzerland), Rotor-Gene® 6000 (Corbett), and Rotor-Gene Q 5/6 plex Platform (QIAGEN, Hilden, Germany) have shown to perform equally well in conjunction with the kit.

The data presented here suggest that the RealStar Zika Virus RT-PCR Kit is a useful tool for ZIKV detection in urine and serum with similar performance compared with the Lanciotti E PCR. In a recent publication by L'Huillier and others, the RealStar Zika Virus Kit was also compared with the Lanciotti E and in addition to the pre-membrane protein PCR.[4] L'Huillier and others came to the conclusion that the RealStar Kit and the Lanciotti PCR methods are of similar sensitivity and specificity.[6] Nevertheless, we present more data on paired serum and urine samples (53 compared with 25) and more positive samples in general (111 compared with 65). In agreement with the work by L'Huillier, we observed a low virus load in the samples reflected by the high Ct values generated by both assays. It is noteworthy mentioning that L'Huillier reports a very high rate of equivocal specimens with the Lanciotti PCR methods in due to an unidentified technical issue; we also repeatedly faced unexplained false positivities with the same method in our laboratory during the early phase of the outbreak but not within the framework of this study.

The viremia in patients infected with ZIKV is in general relatively low and of short duration.[7] This makes comparison of methods difficult as the viral load in patient samples is often close to the limit of detection and positive PCR results might occur randomly between replicate testing of the same sample. The low viral load has a direct impact on the interpretation of negative PCR results. Negative PCR results do not exclude ZIKV infection or presence of the virus in the specimen. To increase the possibility of ZIKV detection, testing of paired serum and urine samples is highly recommended and serology should also be used to diagnose Zika fever as also recommended by WHO and other public health authorities.[7–9]

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