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Short communication

One-step RT-PCR for detection of Zika virus

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Abstract

Background: Zika virus (ZIKV) is an emerging mosquito-borne flavivirus circulating in Asia and Africa. Human infection induces an influenza-like syndrome that is associated with retro-orbital pain, oedema, lymphadenopathy, or diarrhea. Diagnosis of Zika fever requires virus isolation and serology, which are time consuming or cross-reactive.
Objective: To develop a one-step RT-PCR assay to detect ZIKV in human serum.
Study design: An assay targeting the envelope protein coding region was designed and evaluated for its specificity, detection limit, repeatability, and capacity to detect ZIKV isolates collected over a 40-year period from various African countries and hosts.
Results: The assay's detection limit and repeatability were respectively 7.7 pfu/reaction and 100% in serum and L-15 medium; none of 19 other flaviviruses tested were detected.
Conclusions: The assay is rapid, sensitive, and specific to detect ZIKV in cell culture or serum, but needs to be validated for diagnosis using clinical samples.
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Keywords: Zika virus; Detection; Diagnosis; RT-PCR

1. Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus (family Flaviviridae). The viral genome is a single-stranded positive RNA of 10,794 bases (Kuno and Chang, 2007). Human infection is characterized by an influenza-like syndrome that is associated with fever, headache, arthralgia, myalgia, malaise, anorexia, rash, asthenia, retro-orbital pain, oedema, lymphadenopathy, or diarrhea (Bearcroft, 1956; MacNamara, 1954; Simpson, 1964; Mondet, 2007). ZIKV was first isolated in 1947 from a monkey in Uganda (Dick et al., 1952) and has been known to circulate actively in East and West Africa and South-East Asia (Olson et al., 1981; McCrae and Kirya, 1982; Robert et al., 1986; Monlun et al., 1993; Akoua-Koffi et al., 2001; Nathan et al., 2001; Kilbourn et al., 2003). In April 2007, a Zika fever (ZF) outbreak occurred in the Yap States in Micronesia, resulting in 99 confirmed cases within 2 months (Bel, 2007). The outbreak showed that ZF is an emerging disease and emphasized the need for a reliable diagnostic tool, because the first cases were misdiagnosed as dengue infection. Routine laboratory diagnosis assays for ZF are based on virus isolation and serological methods (Fagbami, 1979). However, virus isolation is time consuming, whereas serological methods are limited by the need for acute and/or convalescent samples and by cross-reactions among flaviviruses. Since RT-PCR is widely used for flaviviruses detection (Kuno, 1998) and is rapid, sensitive, and specific in human clinical samples, we developed a one-step RT-PCR method to detect ZIKV.

Abbreviations: L-15, L-15 Leibovitz medium; ZF, Zika fever; ZIKV, Zika virus; RT-PCR, reverse transcriptase polymerase chain reaction; CRORA, WHO Collaborating Center for Arboviruses and Viral Hemorrhagic Fever; FBS, fetal bovine serum; RNA, ribonucleic acid; DTT, dithiothreitol; dNTPs, deoxynucleotide triphosphates; pfu, plaque-forming unit; TE, Tris–EDTA.

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Table 1
Zika strains used in this study

Reference	Host	Country	Year of isolation
ArD7117*	*Ae.luteocephalus*	Senegal	1968
ArD 9957*	*Ae.furcifer*	Senegal	1969
ArD30101	*Ae.luteocephalus*	Senegal	1979
ArD 30156*	*Ae.furcifer*	Senegal	1979
AnD 30332*	*Cercopithecus aethiops*	Senegal	1979
HD 78788	Human	Senegal	1991
ArD 127707	*Ae.furcifer*	Senegal	1997
ArD 127710	*Ae.taylori*	Senegal	1997
ArD 127984	*Ae.furcifer*	Senegal	1997
ArD 127987	*Ae.luteocephalus*	Senegal	1997
ArD 127988*	*Ae.furcifer*	Senegal	1997
ArD 127994*	*Ae.taylori*	Senegal	1997
ArD 128000*	*Ae.luteocephalus*	Senegal	1997
ArD 132912*	*Ae.dalzieli*	Senegal	1998
ArD 132915	*Ae.dalzieli*	Senegal	1998
ArD 141170	*Ae.dalzieli*	Senegal	2000
ArD 142623*	*Anopheles coustani*	Senegal	2000
ArD 149917	*Ae.dalzieli*	Senegal	2001
ArD 149810	*Ae.dalzieli*	Senegal	2001
ArD 149938	*Ae. dalzieli*	Senegal	2001
ArD 157995	*Ae. dalzieli*	Senegal	2001
ArD 158084	*Ae. dalzieli*	Senegal	2001
ArD 165522*	*Ae.vittatus*	Senegal	2002
ArD 165531*	*Ae.dalzieli*	Senegal	2002
ArA 1465*	*Ae.africanus*	Ivory Coast	1980
ArA 27096	*Ae.africanus*	Ivory Coast	1990
ArA 27101	*Ae.opok*	Ivory Coast	1990
ArA 27106	*Ae.luteocephalus*	Ivory Coast	1990
ArA 27290	*Ae.opok*	Ivory Coast	1990
ArA 27407	*Ae.africanus*	Ivory Coast	1990
ArA 27443	*Muci grahami*	Ivory Coast	1990
ArA 506/96*	*Ae.vittatus*	Ivory Coast	1996
ArA 975-99*	*Ae.aegypti*	Ivory Coast	1999
ArA 982-99	*Ae.vittatus*	Ivory Coast	1999
ArA 986-99	*Ae.furcifer*	Ivory Coast	1999
ArA 2718*	*Ae.luteocephalus*	Burkina Faso	1981
ArB 1362*	*Ae.africanus*	Central African Republic	1968

Asterisks (*) indicate Strain sequences of strains used to design the primer pair ZIKENVR and ZIKENVF shown in Fig. 1. Strains sequenced with the ZIKENVR and ZIKENVF primers to confirm amplification of ZIKV are underlined.

2. Material and methods

2.1. Viruses

ZIKV and flavivirus isolates used in this study were supplied by the WHO Collaborating Center for Arboviruses and Viral Hemorrhagic Fever (CRORA) at Institut Pasteur de Dakar (Tables 1 and 2). Viral stocks were prepared using AP61 continuous cell lines and infection progression was monitored using indirect immunofluorescence assay (Digoutte et al., 1992).

2.2. RNA extraction

RNA was extracted from ZIKV isolates and flaviviruses stocks using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) according to the manufacturer's recommendations.

2.3. Primer design

The envelope protein coding regions of ZIKV isolates were sequenced following an amplification step with a nested RT-PCR protocol with outer primer pairs Unifor ($5'_{1171}$TGGGGNAAYSRNTGYGGNYTNTTYGG$_{1197}3'$) and Unirev ($5'_{2178}$CCNCCHRNNGANCCRAARTCC-CA$_{2155}3'$) and inner pairs Mounifor2 ($5'_{1209}$GGRDRMD-TBKWSAYVTGYGCNAWRTT$_{1235}3'$) and Mounirev2 ($5'_{2094}$CCNATNSWRCTHCCHKHYYTRWRCCA$_{2068}3'$) (Gaunt, personal communication). Primer positions indicated herein are based on ZIKV strain MR-766 sequence (AY632535). To design specific primers for ZIKV detection, sequences obtained for the ZIKV envelope protein coding region and sequences of other flaviviruses available in Genbank were aligned using Clustal X (Thompson et al., 1997). Regions showing highest homology among ZIKV isolates and highest divergence with other flaviviruses were

Table 2
Flavivirus strains used in this study

Flavivirus species	Reference	Host	Country	Year of isolation
Dengue 1	ArA 15120	*Ae.aegypti*	Ivory Coast	1985
Dengue 2	ArD 63334	*Ae.furcifer*	Senegal	1989
Dengue 2	ArA 6894	*Ae.aegypti*	Burkina Faso	1986
Dengue 2	ArA 29982	*Aedes luteocephalus*	Ivory Coast	1992
Dengue 2	ArD 140 875	*Ae.furcifer*	Senegal	1999
Dengue 2	ArD 140 884	*Ae.luteocephalus*	Senegal	1999
Dengue 2	ArD 141 069	*Ae.furcifer*	Senegal	1999
Dengue 2	ArD 141 070	*A.luteocephalus*	Senegal	1999
Dengue 2	ArD 141073	*A.taylori*	Senegal	1999
Dengue 2	ArD 142 774	*Aedes fircifer*	Senegal	1999
Dengue 4	HD 38549	Human	Senegal	1983
Yellow fever	ArA 408/78	*Ae.luteocephalus*	Ivory Coast	1978
Yellow fever	HA 016/97	Human	Liberia	1997
Yellow fever	ArD 149213	*A.luteocephalus*	Senegal	2000
Yellow fever	ArD 149 214	*Ae.furcifer*	Senegal	2000
West Nile	AF260968	Human	Egypt	1951
West Nile	M12294	Human	Uganda	1937
Usutu	ArD 130317	*Culex perfuscus*	Senegal	1998
Ss. Usutu	ArB 1803/69	*Cx.perfuscus*	Central African Republic	1969
Bagaza	ArB 209	*Culex sp*	Central African Republic	1966
Bouboui	ArB 490	*Anopheles paludis*	Central African Republic	1967
Dakar Bat	AnD 249	*Scotiphilus* sp.	Senegal	1962
Kedougou	ArD 14701	*Aedes minutus*	Senegal	1972
Koutango	AnD 5443	*Tatera kempi*	Senegal	1968
Ntaya	ArB 472	*Culex* sp.	Central African Republic	1967
Uganda S	ArD 109325	*Ae.furcifer*	Senegal	1994
Saboya	AnD 4600	*Tatera.kempi*	Senegal	1968
Sepik	MK7148	*Mansonia septempunctata*	New Guinea	1966
Spondweni	SA Ar 94	*Mansonia uniformis*	South African Republic	1955
Wesselsbron	ArB 4177	*Rhipicephalus muhsamae*	Central African Republic	1982
Yaounde	ArY 276/68	*Culex nebulosus*	Cameroon	1968

selected to design ZIKENVF and ZIKENVR primers for ZIKV genome detection.

2.4. One-step RT-PCR amplification

RT-PCR was carried out using TITAN one-tube RT-PCR kit (Boehringer Mannheim Biochemicals)-with 22.8 μl RNA extracted from 50 μl of ZIKV samples and eluted in 50 μl of Tris EDTA (TE) buffer and 500 ng of each primer. Amplification was performed in a 9700 GeneAmp PCR Thermocycler (Applied Biosystem, Foster City, USA) using the following procedure: 1 cycle at 50°C for 30 min and 95°C for 2 min, and 35 cycles at 95°C for 20 s, 55°C for 20 s, and 68°C for 30 s, followed by a final elongation step at 68°C for 7 min. Amplicons were visualized on 2% ethidium bromide stained agarose gel.

2.5. Specificity and detection limit of the RT-PCR assay

The specificity of the assay was evaluated using various ZIKV isolates and flaviviruses described in Tables 1 and 2. In addition, flaviviruses were tested with VD8/EMF1 primers as described by Pierre et al. (1994) in order to confirm the presence of RNA.

The detection limit of the assay using primers ZIKENVF and ZIKENVR was evaluated by testing serial 10-fold dilutions of viral suspension in human sera previously tested negative for mosquito-borne flaviviruses and Leibovitz 15 medium (L-15) supplemented with 10% fetal bovine serum (FBS).

The intra-assay repeatability was measured by testing 10 times the 10-fold dilutions series during the same run.

3. Results

3.1. Selection of primers and evaluation of their specificity

ZIKVENVF (5′-GCTGGDGCRGACACHGGRACT-3′) and ZIKVENVR (5′-RTCYACYGCCATYTGGRCTG-3′) primers were designed for one-step RT-PCR and hybridized respectively to positions 1538–1558 and 1902–1883 of the ZIKV genome sequence AY632535 (Fig. 1). The one-step RT-PCR assay detected viral RNA from all 37 ZIKV strains (Table 1, Fig. 2). As a further confirmation, the 364 bp amplicons from five isolates were sequenced and showed 92% to 99% nucleotide similarity with ZIKV Uganda 1947

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                  1538                    1558
ArA506            GCTGGGGCAG ACACCGGAAC T
ArA975            .......... .......... .
ArB1362           .......... .......... .
ArA1465           .....A..G. ....A..G.. .
ArA2718           .....T.... ....T..... .
ArD7117           .....T.... ....T..... .
ArD9957           .....C.... ....T..... .
ArD30101          .....T.... ....T..... .
ArD30156          .....T.... ....T..... .
AnD30332          .....T.... ....T..... .
ArD127988         .....T.... ....T..... .
ArD127994         .....T.... ....T..... .
ArD128000         .....T.... ....T..... .
ArD132912         .....A..G. ....A..G.. .
ArD142623         .....A..G. ....A..G.. .
ArD165522         .....T.... ....T..... .
ArD165531         .....T.... ....T..... .
AF372422          .......... ....T..... .
AY632535          .......... .......... .
AF372416NTA       .G.TCA.... CAGGAAATTG G
AF372421SAB       CA...T.AGA CTGA.AT.TG A
AF298808DEN1      T.G..A..TA CA..GTC.CA A
AF231721DEN1      T.G.....TT CA..ATCCCA G
S64849DEN1        T.G.....TT CA..ATCCCA A
AY702039DEN2      C.C..A..G. ....ACA.GG A
AB194882DEN2      C.C..A..G. .T.AACA.GA G
L10042DEN2        C.C..A..G. ....ACA.GG A
AF231718DEN2      C....A.... ....ACA.GG G
AF231720DEN2      C....A.... ....ACA.GG G
M93130DEN3        AT.TCAAGCA GACT.CCCCA A
L11430DEN3        T.A..A..TA CA..A.A... A
L11433DEN3        T.A..A..TA CAG.A.A.GT A
AF231722DEN4      ..A..A.... ....GTC.GA A
U18431DEN4        ..A..A.... ....ATC.GA G
S66064DEN4        ..A..A.... ....ATC.GA A
U89338YF          AGC..CAGT. GTGGG.TGTG G
AY502949YF        AGC..AAGT. G.GGG.TGTG G
AY572535YF        AGC..AAGT. G.GGG.TGTG G
AY495573YF        AG...AAGT. G.GGG.TGTG G
U54798YF          AGC..TAGC. G.GGG.TGTG G
AF312554YF        AG...AAGT. G.GGT.TGTG G
U17066YF          AG...AAGT. G.GGG.TGTG G
U23574YF          AG...AAGT. G.GGG.TGTG G
U23572YF          AGC..TAGC. G.GGG.TGTG G
AY033391WN        AG..CT.G.A GT..T.TGTG G
AF260967WN        ..A..CT..A TG.TAC.TGG C
AY428527WN        AG..CT.G.A GT..T.TGTG G

Primer ZIKENVF    GCTGGDGCRG ACACHGGRAC T
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                  1883                 1902
ArA506            CAGCCCAGAT GGCGGTGGAC
ArA975            .......... ..........
ArB1362           .......... ..........
ArA1465           .......A.. ...A.....T
ArA2718           .......... ......A...
ArD7117           .......... ......A...
ArD9957           .......... ..........
ArD30101          .......... ..........
ArD30156          .......... ..........
AnD30332          .......... ..........
ArD127988         .......... ..........
ArD127994         .......... ..........
ArD128000         .......... ..........
ArD132912         .......A.. ...A.....T
ArD142623         .......A.. ...A.....T
ArD165522         .......... ......A...
ArD165531         .......... ......A...
AF372422          .......... ..........
AY632535          ...T...... ..........
AF372416NTA       ATCT.A.TGA CAGCAAATCT
AF372421SAB       GCCG..GATG ATGCT.CAGG
AF298808DEN1      TTTT.G.CCC AAGATGAA.A
AF231721DEN1      TTTT.G.CCC AAGATGA..A
S64849DEN1        TTTT.G.CCC AAGATGA..A
AY702039DEN2      TTTGAG.T.A CAGAT.T.GA
AB194882DEN2      TTTGAG.T.A T.GAT.T.GA
L10042DEN2        TTTGAG.T.A T.GAT.T.GA
AF231718DEN2      TTTGAG.TCA T.GAC.T.GA
AF231720DEN2      TTTGAG.TCA T.GAC.T.GA
M93130DEN3        TTCT...CGG A.GATG.ACA
L11430DEN3        TTT.T.CACA .AG.A.....
L11433DEN3        TTT.T..ACG .AG.A.....
AF231722DEN4      ATAGAG.T.A .AGATGT..A
U18431DEN4        ATAGAG.T.A .AGATGTA.A
S66064DEN4        A.TAGAGATA A.A.A..TGA
U89338YF          GT.ATAGT.G CTGATGATCA
AY502949YF        GT.ATAGT.G CTGATGATCA
AY572535YF        GT.ATAGT.G CTGATGATCA
AY495573YF        GT.ATAGT.G CTGATGATCA
U54798YF          GT.ATAGTGG CTGATGATCA
AF312554YF        GTAATGGTGG CTGATGATCA
U17066YF          GT.ATAGT.G CTGATGATCA
U23574YF          GT.ATAGT.G CTGATGATCA
U23572YF          GT.ATAGTGG CTGATGATCA
AF260967WN        TG.GA.GA.G .C...GTAGA
AY428527WN        T.T.T.GTCA .TG.C.TC.T
AY033391WN        T.T.T.GTCA .TG.C.TC.T

Primer ZIKENVR    GTCRGGTYTA CCGYCAYCTR
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Fig. 1. Alignment of annealing regions of ZIKENVR and ZIKENVF for Zika virus and selected flavivirus strains.

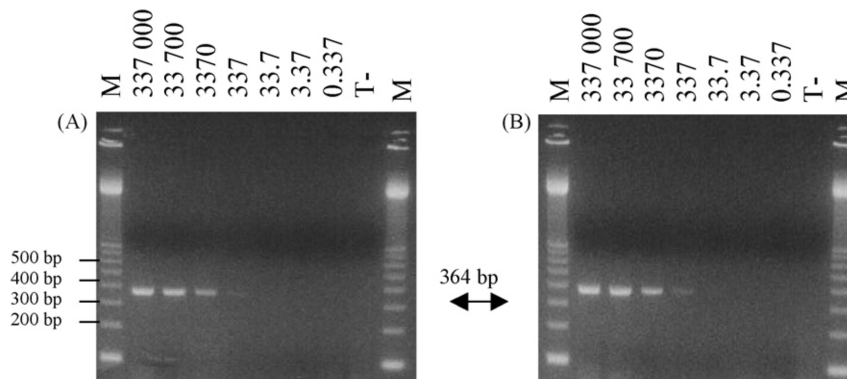

Fig. 2. Agarose gel electrophoresis of products of RT-PCR assay sensitivity in (A) L-15 medium and (B) human serum in pfu/ml. T, negative control; M, molecular weight marker (Amersham100 pair Base-Pair Ladder, GE Healthcare, UK).

strain MR-766 sequence (AY632535) by BLAST analysis (Altschul et al., 1990).

In addition, no amplification was observed with any of the 31 isolates of 19 flaviviruses tested (Table 2), although they contained RNA, as confirmed by the amplification with mosquito-borne consensus primer pairs VD8/EMF1 (Pierre et al., 1994) (data not shown).

3.2. Detection limit of the one-tube RT-PCR assay

To evaluate the detection limit, two ZIKV isolates, ArD165531 and ArD142623, were selected to consider sequence variability in the primer binding sites. Tenfold serial dilutions of a 3.37×10^5 pfu/ml ZIKV initial stock were prepared in L-15 medium containing 10% FBS or in human serum previously tested negative by RT-PCR for mosquito-borne flaviviruses. The detection limit of the assay was found to be 7.7 pfu/reaction in human serum and L-15 medium (Fig. 2), corresponding to a titer of 337 pfu/ml for both ZIKV isolates. The intra-assay repeatability of the detection limit in human serum and L-15 was assessed by testing 10 samples, each containing 3370 pfu/ml, 337 pfu/ml, or 33.7 pfu/ml. The assay detected 10 out of 10 (100%) dilutions in L-15 and in sera with 3370 pfu/ml and 337 pfu/ml; no dilutions in samples with 33.7 pfu/ml were detected.

4. Discussion

RT-PCR has been successfully used for identification and diagnosis of arboviruses (Scaramozzino et al., 2001). We have developed and evaluated a rapid, sensitive, and specific RT-PCR assay for the detection of ZIKV in L-15 medium and human serum. To our knowledge, this is the first published report describing a one-tube RT-PCR for specific detection of ZIKV.

The analytical specificity of the assay was evaluated using RNA from 37 ZIKV isolates and from 31 isolates of 19 related flaviviruses. Amplicons of the expected size and sequences were observed only from the ZIKV samples, indicating the specificity of this assay.

The detection limit of 337 pfu/ml is similar to that reported for flaviviruses assays (Morita et al., 1991; Brown et al., 1994) and is low enough to detect ZIKV viraemia ranging from 10^3 pfu/ml to 10^6 pfu/ml in natural human infection (Simpson, 1964; Weinbren and Williams, 1958; Bearcroft, 1956). This detection limit eliminated the need for a nested PCR. In addition, the high repeatability (100%) of the assay in L-15 medium and human serum demonstrated its robustness. Moreover, the one-step RT-PCR assay was easier and faster than virus isolation and serological methods. Since ZF clinical presentation is not very specific, the RT-PCR assay presented herein should improve distinguishing ZIKV from other arboviruses (e.g., dengue and chikungunya) in areas where they are endemic and cocirculating, as in south-eastern Senegal (Monlun et al., 1993). In this regard, further studies with samples from naturally infected ZF patients should better define the kinetics of viremia and anti-ZIKV IgM and IgG antibodies at a very early phase of infection.

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