Journal of Virological Methods 197 (2014) 51–54

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

# Development of a Usutu virus specific real-time reverse transcription PCR assay based on sequenced strains from Africa and Europe☆

B. Nikolay[a,b,c], M. Weidmann[d], A. Dupressoir[e], O. Faye[a], C.S. Boye[c], M. Diallo[f], A.A. Sall[a,*]

[a] Unité des arbovirus et virus de fièvres hémorragiques, Institut Pasteur de Dakar, 34 Avenue Pasteur, Dakar, Senegal
[b] Université Cheikh Anta Diop Dakar, 24 Avenue Cheikh Anta Diop, Dakar, Senegal
[c] University Vienna, Dr. Bohr-Gasse 9/3, A-1030 Vienna, Austria
[d] Department of Virology, University Medical Center Goettingen, Kreuzbergring 57, D-37075 Goettingen, Germany
[e] CNRS UMR 8122, Institut Gustave Roussy, Villejuif 94805, France
[f] Unité d'entomologie médicale, Institut Pasteur de Dakar, Senegal

*Article history:*
Received 26 June 2013
Received in revised form 24 August 2013
Accepted 29 August 2013
Available online 13 September 2013

*Keywords:*
Usutu virus
Flavivirus
Real-time RT-PCR
Diagnosis
Standard RNA

A B S T R A C T

Usutu virus (USUV) has been isolated in several African and European countries mainly from mosquitoes and birds. However, previous benign and two recent severe cases of human infections point out the need of a tool for the identification of USUV in human samples. A published real-time reverse transcription (RT) PCR assay for the detection of USUV in human blood or cerebrospinal fluid does not take into account the genetic variability of USUV in different geographic regions. Therefore, this article presents a quantitative real-time RT-PCR assay based on sequences from Europe and Africa. Primers and probe were designed in conserved regions among USUV strains that differed from closely related flaviviruses. The specificity of the assay was investigated by testing 16 other flaviviruses circulating in Africa. The sensitivity was determined by testing serial dilutions of virus and RNA standard. Intra- and inter-assay coefficients of variation were evaluated by 10 reactions in a same and in different assays, respectively. The assay provides high analytical specificity for USUV and detection limits of 1.2 pfu/reaction for virus dilutions in L-15 medium or human serum and 60 copies/reaction for the RNA standard. The assay needs to be evaluated in a clinical context and integrated in standard diagnosis of flaviviral diseases.


Usutu virus, a flavivirus belonging to the Japanese encephalitis serocomplex of flaviviruses, has been isolated in several African and European countries mainly from mosquitoes and birds (Bakonyi et al., 2004, 2007; Hubálek et al., 2012; Nikolay et al., 2011; Tamba et al., 2010; Weissenbock et al., 2003). However, USUV infections of humans have been reported also from the Central African Republic and Burkina Faso, and recently two cases of neuroinvasive infections in immune-compromised patients in Italy confirmed the potential of USUV as human pathogen (Cavrini et al., 2009; Nikolay et al., 2011; Pecorari et al., 2009). As symptoms of flavivirus infections are unspecific, the occurrence of human cases emphasizes the need to develop a diagnostic tool for USUV, which can be integrated into routine diagnosis of flaviviral infections (Gaibani et al., 2010; Vazquez et al., 2011). In this regard, a USUV specific real-time reverse transcription (RT) PCR assay for the detection of USUV in human blood and cerebrospinal fluid was developed recently based on sequences from Austria and Hungary (Cavrini et al., 2011).

As the genetic variability of African USUV strains had not been taken into account (Nikolay et al., 2013), the universal application of this assay developed previously might be constrained. Therefore, to ensure detection of a maximum of genetically diverse USUV strains from distinct geographic regions, this article presents a quantitative real-time RT-PCR assay based on USUV sequences from Europe and Africa.

USUV sequences from South Africa, Senegal, Central African Republic, Austria, Hungary, and Italy (GenBank: KC754954, KC754955, KC754956, KC754957, AY453412, AY453411, EF206350, JF296698) were aligned to a USUV subtype strain (GenBank: KC754958) and closely related flaviviruses circulating in Africa as West Nile (GenBank: AY603654, DQ318019, DQ318020) but also Murray Valley Encephalitis (GenBank: NC000943, AF161266), and Saint Louis Encephalitis virus (GenBank: NC_007580). Primers and probe were designed in regions conserved among all USUV

*Abbreviations:* CV, coefficient of variation; *R*, correlation coefficient; USUV, Usutu virus.



* Corresponding author. Tel.: +221 338399223.
*E-mail addresses:* birgitnikolay@gmail.com (B. Nikolay), mweidma@gwdg.de (M. Weidmann), dupresso@igr.fr (A. Dupressoir), ofaye@pasteur.sn (O. Faye), cheikh.boye@ucad.edu.sn (C.S. Boye), asall@pasteur.sn (A.A. Sall).

0166-0934/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2013.08.039

**Table 1**
USUV strains and flaviviruses used for the test of specificity of real-time PCR assay.

| Reference number | Virus species | Host origin | Geographic origin | Year of isolation |
|---|---|---|---|---|
| SAAR1776 | Usutu | Cx. neavei | South Africa | 1959 |
| ArD19848 | Usutu | Cx. perfuscus | Kedougou (Senegal) | 1974 |
| HB81P08 | Usutu | Human | CAR | 1981 |
| ArD101291 | Usutu | Cx. gr. univittatus | Barkedji (Senegal) | 1993 |
| ArD192495 | Usutu | *Cx. neavei* | Barkedji (Senegal) | 2007 |
| ArB1803 | Subtype Usutu | *Cx. perfuscus* | CAR | 1969 |
| Dak Ar B 209 | Bagaza | *Culex sp.* | CAR | 1966 |
| Ar B 490 | Bouboui | *Anopheles paludis* | CAR | 1967 |
| An D 249 | Dakar Bat | *Scotiphilus sp.* | Senegal | 1962 |
| NGC 84850 | Dengue | Human | USA | 1944 |
| Ar D 14701 | Kedougou | *Aedes minutus* | Senegal | 1972 |
| An D 5443 | Koutango | *Tatera kempi* | Senegal | 1968 |
| ArB 472 | Ntaya | *Culex* sp. | CAR | 1967 |
| MK7148 | Sepik | *Mansonia septempunctata* | New Guinea | 1966 |
| An D 4600 | Saboya | *Tatera kempi* | Senegal | 1968 |
| SA Ar 94 | Spondweni | *Mansonia uniformis* | South Africa | 1955 |
| ArD 109325 | Uganda S | *Aedes furcifer* | Senegal | 1994 |
| ArB 4177 | Wesselsbron | *Rhipicephalus muhsamae* | CAR | 1982 |
| Eg101 | West Nile | Human | Egypte | 1951 |
| B965 | | Human | Uganda | 1937 |
| ArD 94343 | | *Cx. perfuscus* | Senegal | 1992 |
| FNV 281 | Yellow Fever | Human | Ghana | 1927 |
| Ar Y 276 | Yaounde | *Culex nebulosus* | Cameroon | 1968 |
| MR766 | Zika | Rhesus monkey | Uganda | 1947 |

strains and varying in other flaviviruses, and were evaluated with the primer probe test tool of Primer express® software (Applied Biosystems). The primers UsuFP (CAAAGCTGGACAGACATCCCTTAC) and UsuRP (CGTAGATGTTTTCAGCCCACGT) amplify a 103 bp sequence in the NS5 gene, detected by a dual labeled probe UsuP (6FAM-AAGACATATGGTGTGGAAGCCTGATAGGCA-TMR) (TIB Molbiol, Berlin, Germany).

RNA extractions were performed from 100 μl virus of 10-fold dilution spiked in L-15 medium or human serum, using the QIAamp viral RNA extraction kit (Qiagen, Heiden, Germany) following the providers' instructions. However, as a smaller volume of sample was used, the volumes of kit reagents were adjusted accordingly. The real-time RT-PCR assay was performed using an ABI 7500 cycler (Applied Biosystems, Foster City, US) and the Quantitect Probe RT-PCR kit (Qiagen, Heiden, Germany). Reactions were performed in 25 μl reaction volume containing 1 μl diluted RNA, 0.5 μM UsuFP and UsuRP, 0.2 μM of UsuP, 12.5 μl of 2× QuantiTect Probe RT-PCR Master Mix, and 0.25 μl of Quantitect RT Mix. The applied conditions were as follows: 15 min 50 °C, 15 min 95 °C and 40 cycles of 15 s 95 °C and 1 min 60 °C.

To obtain an RNA standard, primers UsuUP (GGAAGACCAGGAGGAAGGACAC) and UsuDP (CAAAACCCTGTCCTCCTGGAC) (TIB Molbiol, Berlin, Germany) were designed to amplify the entire NS5 gene (2715 bp). RNA of the USUV reference strain SAAR1776 was extracted using QIAamp viral RNA extraction kit, and reverse transcribed using Superscript II kit (Invitrogen, Carlsbad, USA) and pdN6 random primers (Roche, Mannheim, Germany), following the provider's instructions. The resulting complementary DNA was amplified using Go-Taq PCR kit (Promega, Madison, USA) with PCR conditions as follows: 5 min 94 °C, 45 cycles of 1 min 94 °C, 1 min 54 °C and 2.5 min 72 °C, and 10 min 72 °C. The obtained PCR product was cloned into vector pGEM-T (Promega, Madison, USA), following the provider's instructions, and sequenced with standard primers M13 FP (TGTAAAACGACGGCCAGT) and M13 RP (CAGGAAACAGCTATGACC) to exclude sequence errors and to identify the orientation of the insert. The insert was transcribed using SP6 polymerase (Roche, Mannheim, Germany) at 37 °C for 2 h, followed by 2 min inactivation at 62 °C. DNA was removed using the DNA-free kit (AMBION), following the provider's instructions. Samples were tested by RT-PCR and PCR for the complete removal of DNA. Subsequently, the transcribed RNA was quantified using RiboGreen kit (Invitrogen, Carlsbad, USA), following the provider's instructions, and the copy number was calculated.

The sensitivity of the assay was determined by testing five USUV strains circulating in Africa. The specificity was tested with a USUV subtype strain and 18 isolates of 16 different flaviviruses (three different lineages were tested for WNV) (Table 1). Prior to the real-time RT-PCR assay, RNA isolations were tested by RT-PCR using Superscript II reverse transcriptase (Invitrogen, Carlsbad USA) with pdN6 random primers (Roche, Mannheim, Germany) followed by PCR with the primer pair FD3-FU1 as described previously for flavivirus amplification (Kuno et al., 1998). The detection limit of the real-time RT-PCR assay was evaluated by serial 10-fold dilutions of USUV reference strain SAAR1776 in L-15 medium (GibcoBRL, Grand Island, NY, USA) and human serum. Additionally, the detection limit was analyzed by 10-fold dilutions of RNA standard. Intra-assay

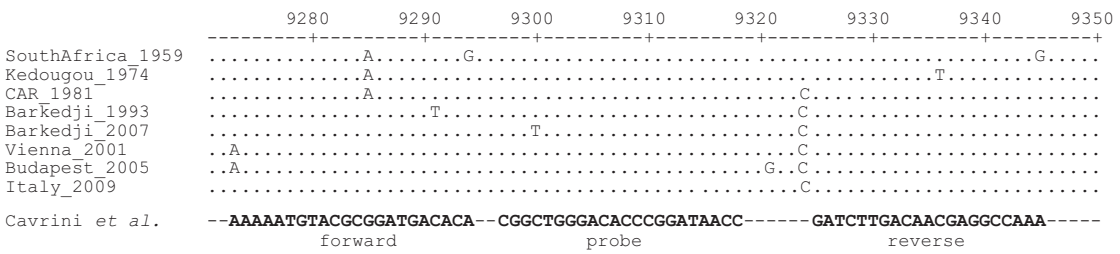


**Fig. 1.** Sequence alignment of USUV sequences from South Africa, Senegal, CAR, Austria, Hungary, and Italy with primers and probe used in real-time PCR assay of Cavrini et al. (2011). The indicated nucleotide positions refer to the genome sequence of USUV reference strain from South Africa (SAAR1776).

```
                        10190      10200      10210      10220      10230      10240      10250      10260
                       ---------+---------+---------+---------+---------+---------+---------+---------+
SouthAfrica_1959       ..........................................................................................
Kedougou_1974          .......C..................................................................................
CAR_1981               ..........................................................................................
Barkedji_1993          ..........................................................................................
Barkedji_2007          ..........................................................................................
Vienna_2001            ..........................................................................................
Budapest_2005          ..........................................................................................
Italy_2009             ...T......................................................................................

Primers & probe        --------CAAAGCTGGACAGACATCCCTTAC-------------AAGACATATGGTGTGGAAGCCTGATAGGCA-----
                                    forward                                    probe

CAR_1969(subtype) .G..C..C..G........G..AG.T..C..T.....A...A.A........T.....C..C..TT....T.....CA. G
WNV(AY603654)     .......GG.G.AA....GT...G....A...T.A..A..A.....G.....C........C...T....T.....AA..
WNV(DQ318019)     .A......G.G..G....GT..TG.T..A...T.T..A...A.A........T........C..TT....C.....A...
WNV(DQ318020)     .A......G.G..G....GT..TG.T..A...T.T..A...A.A........T........C..TT....C.....A...
MVEV(NC000943)    ..A.G...TCCGA......G..AG....A..TGTA......A.A........C.....C.....T..C.....G..CA.G
MVEV(AF161266)    ..A.G...TCCGA......G..AG....A..TGTA......A.A...... ..C.....C.....T..C.....G..CA.G
SLEV(NC007580)    .A...T.GGCGGCT....AC.....T..A...CTA..A...A.A..G.....C.............T..C..A..AA.G

                        10270     10280     10290
                       ---------+---------+---------+-----
SouthAfrica_1959       ..................................
Kedougou_1974          ..................................
CAR_1981               ..................................
Barkedji_1993          ..................................
Barkedji_2007          ..................................
Vienna_2001            ................................A..
Budapest_2005          ................................A..
Italy_2009             ................................A..

Primers & probe        ---------ACGTGGGCTGAAAACATCTACG----
                                   reverse

CAR_1969(subtype) G....T.....A........G........T..A..
WNV(AY603654)     G.C..A..C........A.........C.G.TA..
WNV(DQ318019)     ..C..C..C..T...........T...C.T.T...
WNV(DQ318020)     ..C..C..C..T...........T...C.T.T...
MVEV(NC000943)    ..CA.......A........G...........C..
MVEV(AF161266)    ..CA.......A........G...........C..
SLEV(NC007580)    ..CA.A..C..C........G...........TC.
```

**Fig. 2.** Primers and probe design in conserved regions of USUV genome. Primers and probe are located in a highly conserved region of the NS5 gene. This region is variable among closely related flaviviruses including the USUV subtype strain CAR_1969. The indicated nucleotide positions refer to the genome sequence of the USUV reference strain from South Africa (SAAR1776).

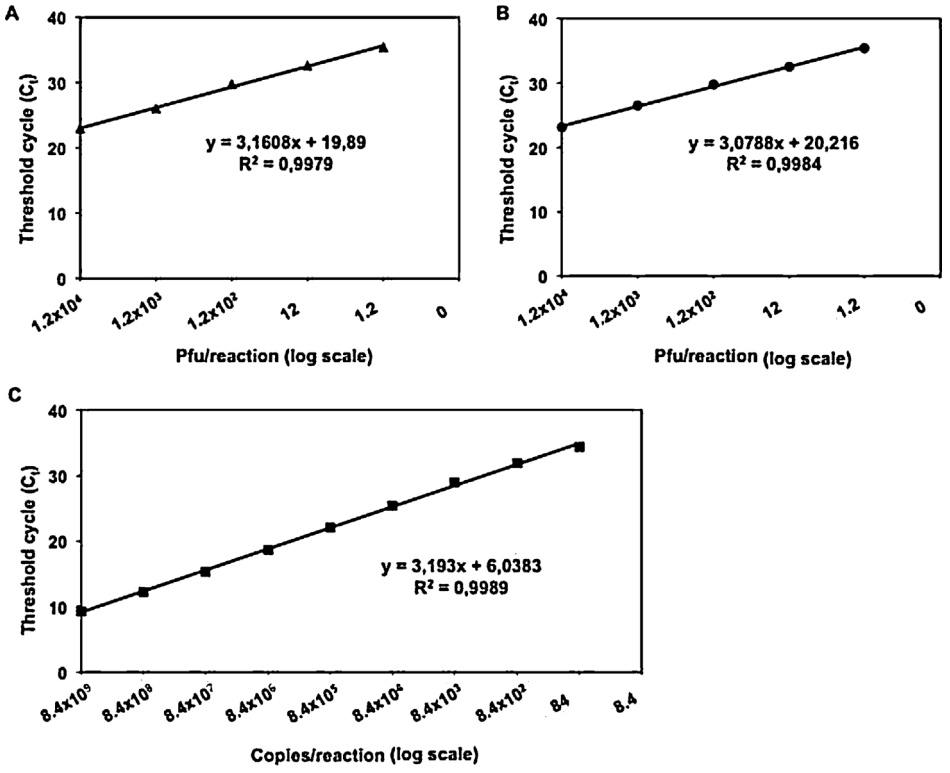


**Fig. 3.** Serial 10-fold dilutions of virus in L-15 medium (A) and human serum (B), as well as of RNA standard (C) have been tested in the real-time RT-PCR assay. Tested dilutions ranged from $1.2 \times 10^4$ to 0 pfu/reaction for virus in L-15 medium and serum, and from $8.4 \times 10^9$ to 8.4 copies/reaction for the RNA standard.

coefficient of variation (CV) was evaluated by 10 reactions in a same assay, inter-assay CV by 10 reactions in different assays. Repetitions were performed in the same laboratory by the same person.

As demonstrated in Fig. 1, the alignment of primers and probes of the previously developed real-time RT-PCR assay (Cavrini et al., 2011) with the sequences of USUV isolates from Africa shows sequence variations mainly in the region of the forward primer. The position of this primer in a non-conserved region might lead eventually to problems in USUV detection of strains circulating in Africa. Therefore, primers and probe were designed in a conserved region of USUV genomes from South Africa, Senegal, the Central African Republic, Austria, Hungary, and Italy taking into account other flaviviruses circulating in Africa to ensure analytical USUV specificity. The alignment of sequences of five USUV strains, the USUV subtype, and closely related flaviviruses together with the location of the newly designed primers is shown in Fig. 2. In the real-time RT-PCR assay, these primers and probe were able to detect five different USUV isolates from Africa. Furthermore, when testing 18 strains of 16 other flaviviruses (Table 1) and the USUV subtype strain, no amplification was observed. The RNA of these flaviviruses was tested previously successfully by RT-PCR with the primers pair FD3-FU1.

The detection limit for USUV in L-15 medium, as well as in human serum, was 1.2 pfu/reaction, corresponding to 600 pfu/ml. Furthermore, the detection limit was tested with a RNA standard containing the entire NS5 gene and was 60 copies/reaction. Fig. 3 shows the threshold cycles (Ct) for serial 10-fold dilutions of virus in L-15 medium (Fig. 3A) and human serum (Fig. 3B) and for dilutions of the RNA standard (Fig. 3C). The correlation coefficients ($R$) of the 10-fold dilution series were of 0.998 for virus dilutions in L-15 and human serum and of 0.999 for dilutions of RNA standard. Efficiencies ranged from 106% to 111%. The intra-assay and inter-assay CV for the detection limits were of 0.8 –1.1% and of 1.6–2%, respectively.

This real-time RT-PCR assay was adapted for the identification of genetically diverse USUV strains demonstrated by its ability to detect all tested USUV isolates from Africa including an isolate from an infected human in the Central African Republic. Furthermore, it has a high analytical specificity for USUV tested with the most abundant flaviviruses circulating in Africa and a USUV subtype strain. The detection limits of 1.2 pfu/reaction or 60 copies of RNA standard/reaction indicate a good analytical sensitivity. Taking into account the reported cases of human USUV infections and the recent information about USUV genetic variability in Africa, an application of this universal USUV specific real-time RT-PCR assay should be recommended in flavivirus identification. Especially in geographic regions with demonstrated USUV and WNV circulation, where highly specific tools for the identification of USUV are necessary, this assay might be highly valuable (Gaibani et al., 2010; Vazquez et al., 2011). However, even though the primers design in conserved regions of the USUV genome suggests that the assay can detect European USUV strains, its evaluation with European isolates is necessary prior to its integration into routine diagnosis of flaviviral diseases. Moreover, an evaluation in a clinical context with samples of more infected individuals will be necessary.

## Acknowledgement

BN was awarded of a scholarship by the Austrian Federal Ministry for Science and Research (BMWF). The work was supported by the Federal Ministry of Education and Research (BMBF), Grant Number 01KI0710, “Research on Zoonotic Infectious Diseases” program, “Emerging arthropode-borne viral infections in Germany: Pathogenesis, diagnostics and surveillance”, Institut Pasteur de Dakar and European Union grant HEALTH.2010.2.3.3-3 Project 261391 EuroWest Nile (http://eurowestnile.isciii.es/ewn).

## References

Bakonyi, T., Gould, E.A., Kolodziejek, J., Weissenbock, H., Nowotny, N., 2004. Complete genome analysis and molecular characterization of Usutu virus that emerged in Austria in 2001: comparison with the South African strain SAAR-1776 and other flaviviruses. Virology 328, 301–310.

Bakonyi, T., Erdelyi, K., Ursu, K., Ferenczi, E., Csorgo, T., Lussy, H., Chvala, S., Bukovsky, C., Meister, T., Weissenbock, H., Nowotny, N., 2007. Emergence of Usutu virus in Hungary. J. Clin. Microbiol. 45, 3870–3874.

Cavrini, F., Gaibani, P., Longo, G., Pierro, A.M., Rossini, G., Bonilauri, P., Gerundi, G.E., Di Benedetto, F., Pasetto, A., Girardis, M., Dottori, M., Landini, M.P., Sambri, V., 2009. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August–September. Euro Surveill., 14.

Cavrini, F., Pepa, M.E., Gaibani, P., Pierro, A.M., Rossini, G., Landini, M.P., Sambri, V., 2011. A rapid and specific real-time RT-PCR assay to identify Usutu virus in human plasma, serum, and cerebrospinal fluid. J. Clin. Virol. 50, 221–223.

Gaibani, P., Pierro, A.M., Cavrini, F., Rossini, G., Landini, M.P., Sambri, V., 2010. False-positive transcription-mediated amplification assay detection of West Nile virus in blood from a patient with viremia caused by an Usutu virus infection. J. Clin. Microbiol. 48, 3338–3339.

Hubálek, Z., Rudolf, I., Čapek, M., Bakonyi, T., Betášová, L., Nowotny, N., 2012. Usutu virus in Blackbirds (*Turdus merula*), Czech Republic, 2011–2012. Transbound. Emerg. Dis, http://dx.doi.org/10.1111/tbed.12025.

Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N., Cropp, C.B., 1998. Phylogeny of the genus Flavivirus. J. Virol. 72, 73–83.

Nikolay, B., Diallo, M., Boye, C.S., Sall, A.A., 2011. Usutu virus in Africa. Vector Borne Zoonotic Dis. 11, 1417–1423.

Nikolay, B., Dupressoir, A., Firth, C., Faye, O., Boye, C.S., Diallo, M., Sall, A.A., 2013. Comparative full length genome sequence analysis of usutu virus isolates from Africa. Virol. J. 10, 217.

Pecorari, M., Longo, G., Gennari, W., Grottola, A., Sabbatini, A., Tagliazucchi, S., Savini, G., Monaco, F., Simone, M., Lelli, R., Rumpianesi, F., 2009. First human case of Usutu virus neuroinvasive infection, Italy, August–September 2009. Euro Surveill., 14.

Tamba, M., Bonilauri, P., Bellini, R., Calzolari, M., Albieri, A., Sambri, V., Dottori, M., Angelini, P., 2010. Detection of Usutu virus within a West Nile Virus Surveillance Program in Northern Italy. Vector Borne Zoonotic Dis. 11, 551–557.

Vazquez, A., Jimenez-Clavero, M., Franco, L., Donoso-Mantke, O., Sambri, V., Niedrig, M., Zeller, H., Tenorio, A., 2011. Usutu virus: potential risk of human disease in Europe. Euro Surveill., 16.

Weissenbock, H., Kolodziejek, J., Fragner, K., Kuhn, R., Pfeffer, M., Nowotny, N., 2003. Usutu virus activity in Austria, 2001–2002. Microbes Infect. 5, 1132–1136.