The Value of West Nile Virus RNA Detection by Real-time PCR in Urine Samples From Patients With Neuroinvasive Forms

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Abstract

Introduction Routine laboratory screening is based on the detection of WNV specific IgM and IgG in blood and cerebrospinal fluid. Confirmation is then classically applied RT-PCR in CSF, which often give negative results due to too short viraemia and late sampling. RT-PCR was applied for the first time for routine diagnosis purpose on urine samples.

Methods During 2018 outbreak in Tunisia, 107 patients presented WNV neurologic symptoms and were positive for WNV serology. Of them, 95 patients were sampled for urine and 35 were sampled for CSF. Qualitative RT-PCR was performed on both type of samples.

Results WNV RNA was detected in 50,5% of urine samples (48/95) and in 2,8% of CSF samples (1/35). WNV RNA was detectable from 1 to 41 day from symptom onset, however, positive urine rate was 53,1% during the first 10 days from symptom onset. The proportions of urine-positive and urine-negative samples, based on day of collection, showed no statistical difference ($p > 0,005$). Cycle threshold values ranged from 12 to 39, with no correlation with the day of collection. The lowest Ct value was detected for urine sampled on day 5 after symptom onset. A statistically significant difference was found between age groups of confirmed and non confirmed cases ($p < 0,001$).

Discussion/Conclusion Our study reports the use of RT-PCR on urine samples as a confirmatory diagnostic tool for WNV probable cases during an outbreak. Our findings underline the reliability, and rapidity of this confirmatory tool, even late and show its superiority on CSF investigation.

Introduction

West Nile virus (WNV) is an arthropod-borne virus that belongs to the *Flavivirus* genus in the *Flaviviridae* family and is serologically classified within the *Japanese encephalitis virus* antigenic complex (Fauquet et al., 2005). Phylogenetically, WNV is divided into eight lineages; only two, lineages 1 and 2, are recognized as pathogenic to humans (Pesko and Ebel, 2012). WNV life cycle is complex; it involves different local and migratory bird species as natural reservoir and amplifying hosts. It is maintained in nature through mosquitoes, mainly *Culex sp.*, that will ensure the transmission cycle between viremic birds and other susceptible hosts (Campbell et al., 2002). Human beings and equines represent accidental dead-end hosts (Blitvich, 2008).

Since its first isolation in 1937 from the blood of a febrile woman in the West Nile district of Northern Uganda (Smithburn et al., 1940), WNV expanded geographically in the Eastern hemisphere and caused sporadic outbreaks of benign human and equine infections in several countries in Africa, the Middle East, Europe and Asia (Blitvich, 2008). Later, in the 1990s, the Mediterranean Basin observed an alarming increase in the frequency and severity of these outbreaks (Murgue et al., 2001). In 1999, an outbreak of WNV human and equine infections was reported, for the first time, in the Western hemisphere in New York, USA (Nash et al., 2001). Nowadays, this virus is recognized as one of the most widespread arboviruses in the world (Kramer et al., 2008). In 2018, an unexpected increase of WNV cases was reported in Europe,

with a total of 1,503 human cases in the European Union. The highest increase compared to the previous transmission season was observed in Bulgaria (15-fold) followed by France (13,5-fold) and Italy (10, fold) (Burki, 2018; "ECDC-Epidemiological update," 2018). Tunisia also experienced an outbreak of WNV human infection from September to December 2018 ("Bulletin de veille et de riposte aux infections a virus West Nile en Tunisie 08/10/2018," ; "ECDC-Epidemiological update," 2018).

WNV infection in humans is generally symptomless; however, up to 20% of infected patients may have influenza-like syndrome and less than 1% develop severe neuroinvasive disease such as meningitis, encephalitis, or acute flaccid paralysis (Hayes et al., 2005; Hart et al., 2014). These severe symptoms affect especially elderly and immunocompromised people in whom infection is sometimes fatal (Arnold et al., 2005; Koch et al., 2021; Mada et al., 2020; Ravindra et al., 2004). Treatment is currently non-specific providing supportive measures in intensive care units (Diamond, 2009). Laboratory diagnosis of WNV infection is classically based on a first screening by detecting IgM and IgG antibodies specific to WNV in serum samples using Enzyme Linked Immunosorbent Assay (ELISA) techniques (Barzon et al., 2015). Given the quite long incubation period of WNV infection, these serological markers are generally positive when clinical symptoms appear and patients having negative IgM are discarded. In contrast, patients with positive IgM will be considered as "Probable cases" and, due to possible cross reaction with other flaviviruses, WNV infection is to be confirmed by direct detection of the virus or seroneutralisation of the cytopathic effect (Barzon et al., 2015; "National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention. West Nile virus in the United States: guidelines for surveillance, prevention, and control. 4th revision. "). Direct diagnosis is classically based on virus detection in blood and CSF samples (Barzon et al., 2015; "National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention. West Nile virus in the United States: guidelines for surveillance, prevention, and control. 4th revision. "). However, as viremia and virorachia are too short, molecular detection of WNV RNA in these clinical samples has generally been of limited diagnostic value (Tilley et al., 2006; Busch et al., 2005; Lindsey et al., 2019; Koch et al., 2021). Recent studies revealed high viral load and longer viral shedding in urine samples of acutely infected patients and suggested that detection of WNV RNA in urine could represent a more effective tool to confirm WNV infection (Barzon et al., 2015, 2013a, 2013b; Magurano et al., 2012; Niedrig et al., 2018; Pacenti et al., 2020; Papa et al., 2014; Tonry et al., 2005).

In Tunisia, the first outbreak of WNV infection occurred in 1997 and counted 111 cases with neurological symptoms and 8 deaths (Triki et al., 2001). Two other outbreaks occurred in 2003 (112 cases and 9 deaths) and 2012 (86 cases with 12 deaths) (Bouatef et al., 2010). A national program for WNV surveillance was established in 2010 and consisted in the systematic notification and virological investigation of suspect patients, especially those with neurological symptoms, mainly meningitis and/or encephalitis. According to the National guidelines, serum and urine samples are to be collected from all suspect cases. A first screening for WNV-specific IgM antibodies by ELISA tests in serum samples is done in one of five designated referral laboratories, in the south, center and north regions of the country and IgM antibodies positive cases are considered as "Probable cases". Confirmation using molecular tests is centralized in the National Reference Laboratory. In the present paper, we report the results of the

molecular laboratory investigations during the WNV human infection outbreak in Tunisia 2018 with a particular emphasis on the utility of the detection of WNV RNA in urine samples and its usefulness as a confirmatory tool for probable cases.

Materials And Methods

Sample collection

Urine and CSF samples were collected from 107 patients, positive to anti-WNV IgM (n = 33) or having sero-converted to anti-WNV IgG (n = 74) and classified accordingly as probable cases for WNV infection (Table 1). For three patients, more than one urine was obtained. The patients are originated from different governorates in Tunisia. Serological screening for WNV IgM and IgG was performed using the commercial enzyme immunoassays ELISA from Euroimmun®, Germany. The tests were carried out according to the manufacturers’ instructions and a patient was classified as "Probable WNV case" when WNV IgM were positive together with IgG. Patients with positive IgM and negative IgG were re-sampled and retained as probable cases only in case of IgG sero-conversion. Urine and CSF samples were referred for molecular confirmation of WNV infection to the Laboratory of Clinical Virology in Pasteur Institute of Tunis, which acts as the National Reference Centre for Arboviruses, Tunisia. The vast majority of cases (80%) were received between October and mid-November 2018. The samples were accompanied with an information sheet that includes demographic and clinical information.

Table 1
Specimen collected from the studied population

Specimen	Number of probable cases
Urine and CSF	23
Urine	72
CSF	12
Total	107

Molecular tests

Urine and CSF samples were tested for the presence of WNV RNA by reverse transcription real time PCR (RT-PCR). RNA was extracted from 140µL of CSF or urine using the QiAmp mini kit (QIAGEN®, Germany). The extracted RNA was then used in a real time reverse transcription PCR test targeting a 92-nucleotides-long sequence in the highly conserved 3'-non-coding region of the genome, as previously described (Tang et al., 2006). The RT-PCR reaction was performed using the Invitrogen™ SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Germany) in the Applied Biosystems™ 7500 real-time PCR machine. The reaction mixture contained a total volume of 20µL including: 10µL of 2X buffer, 0.25µL Rnasin (Promega®, USA), 0.8µL of forward primer (FW: 5'- AAGTTGAGTAGACGGTGC TG -3'; nt 10533 to

10552), 0.8µL of reverse primer (RV: 5'- AGACGGTTCTGAGGGCTT AC -3'; nt 10625 to 10606), 0.3µL of probe (5'-FAM-CTCAACCCCAGGAGGACTGG-TAMRA-3'; nt 10560 to 10579), 0.05µL of ROX, 0.8µL Enzyme and 5µL of extracted viral RNA. The thermal cycling consisted of 45°C for 15 min for RT, 95°C for 2 min, and 40 cycles of 95°C for 15s, 57°C for 5s and 60°C for 30s. The Tunisian WNV strain RNA from the 1997 outbreak (PaH001, GenBank Genome Accession: AY268133) was used as positive control.

Statistical analyses

Descriptive and analytical statistics were performed using the IBM SPSS 19.0 statistical package. Categorical variables were expressed as frequencies and percentages, while quantitative variables were reported by median, minimal and maximal values. Pearson's chi-square test was used in order to compare proportions. Student t-test was used in order to compare 2 quantitative variables. Statistical significance was defined as a two-tailed *p*-value of less than 0.05.

Results

On the 95 investigated urine samples, 48 (50,5%) were tested positive by RT-PCR. In contrast, on the 35 investigated CSF, only one (2,8%) was detected positive in a patient from whom a urine sample was also collected and revealed positive. The results of RT-PCR in the 23 patients having both urine and CSF samples are showed in Fig. 1.

Figure 2 shows the timing of urine and CSF collection with their RT-PCR results in the 107 patients included in the present study (Fig. 2.A and Fig. 2.B, respectively). Viruria was detectable for studied patients from 1 up to 41 days after symptom onset. During the first 10 days after symptom onset, 53,1% of total collected urine were positive (34/64). In contrast, only 33,3% were positive during the next 10 days (7/21). The proportions of urine-positive and urine-negative samples, based on day of collection, were statistically similar ($p > 0,005$) (Table 2). Later, WNV RNA in urine was detectable until 41 days after symptom onset. For the three patients who had more than urine sample, WNV RNA was detectable as follow: i) day 3(Ct value 24) and day 9 (Ct value 30); ii) day 4 (Ct value 28) and day 5 (Ct value 37) and iii) day 11 (Ct value 39), day 17 (Ct value 38) and day 41 (Ct value 37).

Table 2
Results of urine WNV RNA detection by RT-PCR according to demographic and clinical features and days after symptom onset

		TOTAL	WNV Urine + (n = 48)	WNV Urine – (n = 47)
Age	Median [min, max]	51 [3,85]	61 [3,85]	30 [3,84]
	< 60 n (%)	58 (61)	21 (43,7)	37 (78,7)
	≥ 60 n (%)	37 (39)	27 (56,3)	10 (21,3)
Sex	Sex ratio	1,2	1,8	0,8
	Male n (%)	51 (53,7)	31 (60,8)	20 (39,2)
	Female n (%)	44 (46,3)	17 (38,6)	27 (43,7)
Clinical presentation	Encephalitis n (%)	36 (37,9)	22 (61,1)	14 (38,9)
	Meningitis n (%)	44 (46,3)	18 (40,9)	26 (59,1)
	Other* n (%)	15 (15,7)	8 (53,3)	7 (46,7%)
Days after symptom onset	Median [min, max]	8 [1,41]	6 [1,41]	9 [3,26]
	0 to 10 n (%)	64 (67,3%)	34 (53,1%)	30 (46,9%)
	11 to 20 n (%)	21 (22,2%)	7 (33,33%)	14 (66,66%)
	> 21 n (%)	10 (10,5%)	7 (0,7%)	3 (0,3%)
Other: Guillain-barré syndrom, convulsion, paraparesis, fever, vomiting, headache, dizziness or coma				

Overall, the Ct values for urine samples ranged from 12 to 39, the mean Ct value is 33 ± 6 . Low Ct values less than 20, and so high viral load, were obtained for three urine samples collected on day 5 after symptom onset (Ct value 12, 18 and 19) (Fig. 3). High Ct values more than 35 were obtained since day 3 after symptom onset. The Ct values of positive urine samples did not show significant correlation with number of days after symptom onset (correlation coefficient $r = 0,255$ CI95% [-0,020; 0,493]).

Figure 2.B illustrates WNV RNA detection in CSF for all studied patient having CSF (n = 35). Virorachia was detectable for only one patient sampled on day 3, Ct value 36. This patient was also confirmed in urine sample collected on day 5, Ct value 38. None of CSF obtained after 3 days after symptom onset was positive, including the 17 confirmed patients having been confirmed on urine samples (Fig. 2.B). No CSF was collected after 9 days after symptom onset.

Table 2 outlines the results of WNV RNA detection from probable WNV cases having urine samples (n = 95), according to demographic and clinical features and days after symptom onset. A statistically significant difference was found between age groups of confirmed and non confirmed cases ($p < 0,001$). Incidence of confirmed cases increases with age over than 60-year-old (56,3%, $p < 0,001$). Confirmed cases were mainly men (64.6%), however, no significant association was found between RT-PCR positivity and patient's sex ($p > 0,005$). Most of probable cases presented with encephalitis or meningitis (n = 80/95, 84,2%). Some cases presented with other symptoms such as Guillain-barré syndrom, convulsion, paraparesis, fever, vomiting, headache, dizziness or coma. The proportions between confirmed and non confirmed cases according to clinical manifestations were not statistically significant ($p > 0,001$).

Discussion/conclusion

The present paper reports the results of routine testing of WNV RNA by RT-PCR for the diagnosis of patients with probable WNV infection, enrolled during the 2018 Tunisia outbreak. Confirmation of WNV infection by detection of WNV RNA in urine samples of symptomatic patients was possible for 50,5% of probable cases, while WNV RNA detection in CSF was much less frequent (2,8%). Patients were mainly men 64,6% and incidence of confirmed cases increases with age over than 60-year-old (56,3%, $p < 0,001$). Similar age and gender distribution of WNV neuro-invasive disease was reported (Jean et al., 2007; Koch et al., 2021; Mada et al., 2020).

Urine is, nowadays, becoming an attractive alternative sample for the detection of several pathogens that may infect humans, since it may be obtained easily, quickly, with high volume, in serial samples, and with no exposure to potential infectious risk for the health care staff comparing to veinopuncture. Urine samples are used for the detection of Cytomegalovirus genome for newborns with congenital cytomegalo-infection, of Zika Virus genome for patients having neuro-invasive disease in endemic regions, of Mycobacterium tuberculosis genome for individuals with active pulmonary tuberculosis, of Chlamydia trachomatis genome for persons screened for sexually transmitted infections and of Polyomaviruses genome for renal post-transplanted patient (Jorge et al., 2020; Marangu et al., 2015; Meyer, 2016; Nicloux et al., 2020; Xu et al., 2021). WNV RNA was detected in urine samples of an infected patient with encephalitis for the first time in 2005, 8 days after symptom onset (Tonry et al., 2005). Subsequent studies reported that WNV RNA is detected more frequently, with higher viral load and for longer period in patients' urine samples than in plasma or CSF (Barzon et al., 2013a; Magurano et al., 2012; Niedrig et al., 2018; Pacenti et al., 2020; Papa et al., 2014). For that reasons, it was proposed in recent international guidelines to include the use of urine samples as suitable alternative diagnostic sample comparing to serum and CSF ("Piano Nazionale integrato di sorveglianza e risposta al virus della West Nile – 2016";

"Mission COREB nationale. Infection à West-Nile virus (VWN) : repérer et prendre en charge un patient suspect."). However, published reports on the results of WNV detection in urine samples as part of routine diagnostic are still scarce.

Detection of WNV RNA in urine samples by RT-PCR was introduced for the first time in Tunisia during the 2018 outbreak; that's why, not all probable cases were sampled for urine samples. Regardless, we found that it allows confirmation of 50,5% of probable cases (48/95). Previous studies such one conducted in northeastern Italy in 2012, one in Greece in 2013 and another in Hungary in 2014, revealed positivity rates in urine samples of 43,8% (14/32), 40% (14/35) and 27,3% (3/11), respectively (Barzon et al., 2013a; Papa et al., 2014; Nagy et al., 2016). We also found that WNV RNA detection was more effective in urine samples than in CSF which confirms previous findings (Barzon et al., 2015, 2013a, 2012; Pacenti et al., 2020). While 50,5% of urine samples from our patient were positive, only one CSF, sampled on day 3 after symptom onset, was positive. These findings confirm how fleeting viraemia is and underlies the superiority of detecting virus RNA in urine samples.

Barzon et al. Pacenti et al. found that viral shedding in urine samples starts within the first week after symptom onset then lasts up to 30 days (Barzon et al., 2012; Pacenti et al., 2020). In our series, WNV RNA could be detected in urine samples from 1 up to 41 days after symptom onset. This extends further the possibility to confirm WNV infection even late. No relationship was observed between the time after symptom onset and the WNV RNA load in urine samples. Otherwise, viraemia was detectable even for years after primo infection suggesting chronic renal infection (Baty et al., 2012; Murray et al., 2010). For these reasons, RT-PCR positive results should be interpreted in conjunction with clinical and serological results.

In this series, WNV RNA was not detected in 49,5% patients (n = 47) having positive IgM and IgG. Negative results should be interpreted by caution for many reasons. First, as it was experimentally reported by Tonry et al. that WNV RNA excretion is intermittent (Tonry et al., 2005), one unique negative urine sample should to exclude WNV infection, theoretically. In this series, viral excretion kinetic in urine samples could not be studied as serial samples were not systematically obtained. Second, positive serology results are not always reliable. IgM may cross react with other flaviviruses such as arboviruses like dengue virus, Zika virus and Japanese encephalitis virus, leading to false positive results (Barzon et al., 2015; Khan et al., 2018). In fact, several commercial kits are available nowadays, targeting antigens from recombinant proteins, virus-like particles or lysates of infected cells (Barzon et al., 2015). It was suggested that sequence similarity of structural proteins of flaviviruses, mainly of the immunodominant envelope protein, may lead to cross reacting antibodies (Mansfield et al., 2011). Thus, it is recommended to exclude the presence of other flaviviruses antibodies, especially those co-circulating in the same geographic area (" National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention. West Nile virus in the United States: guidelines for surveillance, prevention, and control. 4th revision. "). In addition, many studies reported that IgM may persist until 30 to 90 days after primary infection and even longer in safe blood donors, until 200 days (Prince et al., 2005). Thus, their presence in the absence of any other confirmation by RT-PCR may indicate a past infection. The rest of probable cases requires absolute confirmation by seroneutralisation, which will be the only laboratory method to confirm WNV IgM positivity in the absence of virus RNA detection by RT-PCR (Barzon et al., 2015).

Conclusion

As an emerging disease, WNV neuroinvasive infection cases should be diagnosed and confirmed quickly and effectively. In addition to the detection of WNV IgM specific antibodies as the front line screening assay, this study underlies the role of detecting WNV RNA on urine samples of acute infected patients. Our findings demonstrated that this represents a helpful tool for confirmation of probable WNV cases.

Declarations

Statements

All papers must contain the following statements after the main body of the text and before the reference list:

Acknowledgement

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Statement of Ethics

Authors state that subjects (or their parents or guardians) have given their written informed consent and this study obtained the ethical approval from the Bio-Medical Ethics Committee of the Pasteur Institute of Tunis, Tunisia.

Conflict of Interest Statement

The authors have no conflicts of interest to declare

Funding Sources

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Figures

Figure 1

Comparison between WNV RNA detection in urine and CSF samples for the 23 probable cases having both samples.

Figure 2

A: Number of WNV RNA positive and negative urine samples based on the day after symptom onset for 95 probable cases having at least one urine sample. B: Number of WNV RNA positive and negative CSF samples based on the day after symptom onset for 35 probable cases having CSF sample.

Figure 3

Relationship between Ct values in urine samples and days after symptom onset for 48 confirmed cases (52 positive urines).