Detection of West Nile virus RNA (lineages 1 and 2) in an external quality assessment programme for laboratories screening blood and blood components for West Nile virus by nucleic acid amplification testing

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Background. A second Italian External Quality Assessment Programme was run in 2011 to assess the performance of Blood Transfusion Centres in detecting West Nile virus RNA in plasma.

Materials and methods. Each participant received two panels containing negative samples and samples positive for West Nile virus lineages 1 and 2, some of which with a viral concentration close to or below the 95% limit of detection of the respective commercial nucleic acid amplification test assay: the PROCLEIX WNV Assay or the Cobas TaqScreen West Nile Virus Test.

Results. Eleven laboratories took part in the External Quality Assessment Programme. All of them correctly identified the positive samples with a viral concentration above the 95% limit of detection. No false positive results or pre-/post-analytical errors were observed.

Discussion. The External Quality Assessment Programme run in 2011 allowed participants to assess the performance of the nucleic acid amplification test methods applied in their seasonal routine screening of blood donations. The results confirm the 95% limit of detection reported by the test kits' manufacturers for both West Nile virus lineages.

Keywords: WNV, RNA, EQAP, NAT, plasma.

Introduction

West Nile virus (WNV), an arbovirus belonging to the Flaviviridae family, is transmitted by the bite of an infected mosquito which becomes infected after biting infected wild birds, the primary hosts of the virus. Most wild birds can act as amplifying hosts as they can sustain an infectious viraemia for 1 to 4 days after exposure. Humans, horses and other mammals are considered incidental or dead-end hosts. In fact, the virus in mammals is usually not sufficient to be transmitted back to the mosquito, thereby ending the transmission cycle. In humans the infection is usually asymptomatic (80%)[1,2]. WNV symptomatic infection ranges from mild febrile syndrome, termed West Nile fever, to neuro-invasive disease (WNND) and possibly death. The risk of this disease increases with age and appears to be significantly higher in immunocompromised individuals, especially in organ transplant recipients. Person to person transmission does not occur during occasional contact. However, WNV can be transmitted to recipients of blood transfusions and organ transplants obtained from asymptomatic donors.

In recent years, an increasing incidence of WNV infection in humans has been reported in Europe and neighbouring countries[3]. With regards to Italy, human cases of WNV infection were identified in the last 3 years in the north-eastern part of the country[4] (suggesting that WNV has become endemic in this area) while veterinary and entomological surveillance identified the circulation of WNV also in other areas of Italy. A National Plan for Surveillance of human WNND has been implemented by the Italian Ministry of Health since 2010, which recommended that the activities be carried out in the period when the risk of WNV infection is higher[5]. In 2011, the National

Blood Centre, taking into account the 2010 risk assessment of WNV transmission by transfusion of blood and blood components, identified the period in which WNV NAT screening of blood donors had to be introduced (15 July-15 November 2011, later extended to 30 November 2011 due to the exceptional heat in the autumn months), as well as the areas in which this testing had to be implemented: the provinces of Bologna, Ferrara, Modena, Mantua, Rovigo, Venice and Vicenza (all provinces in the regions of Emilia-Romagna, Lombardy, and Veneto)[6]. Subsequently, it was decided that nucleic acid amplification testing (NAT) for WNV had to be extended also to an "affected area", namely an area in which a human case of WNND occurs or a WNV-NAT-positive donor is confirmed. Thus, when notification of human cases of WNND were reported in the late summer of 2011, WNV NAT was also implemented in the provinces of Treviso, Belluno, Udine, Oristano and Nuoro (which are in the regions of Veneto, Friuli Venezia Giulia, and Sardinia). Furthermore, the province of Ancona implemented WNV NAT after the first human case of WNV infection was identified. This measure was taken on a voluntary basis as the infected subject was not a donor.

In Italy, WNV NAT is performed using the Cobas TaqScreen West Nile Virus Test on Cobas s 201 system (Roche Molecular Systems Inc., Branchburg, NJ, USA) or PROCLEIX WNV Assay on Procleix TIGRIS system (Gen-Probe Inc. and Novartis Diagnostics, San Diego, CA, USA) on mini-pools of six donations (to be converted to single-donation testing in an "affected area") or on single donations, respectively.

Participation in External Quality Assessment Programmes (EQAP) for a laboratory performing screening tests on human blood and blood components is considered important in the context of Quality Assurance[7] to ensure that its system works efficiently, not only with respect to the analytical phase but also to the whole process, namely from receipt/storage of the samples to data transcription on the reporting sheet. Based on this consideration, in 2010 the National Blood Centre and the National Centre for Immunobiologicals Research and Evaluation (CRIVIB) of the Italian National Institute of Health (ISS) organised a NAT EQAP study for blood transfusion centres performing WNV NAT on blood donations[8]. A second NAT EQAP for WNV was organised in 2011 to provide continuous support for participating blood transfusion centres in monitoring the quality of their analytical performance and the competence of their operators.

Materials and methods

Participants

The 2011 EQAP involved ten Italian blood transfusion centres located in Emilia Romagna, Lombardy, Marche, Sardinia and Veneto which are authorised at a regional level to perform routine WNV RNA screening. In addition, one foreign blood transfusion centre from Greece agreed to participate in the Italian EQAP.

All participating laboratories were asked to complete an agreement form and a questionnaire on the NAT methods applied. A code was assigned to each participant.

Nucleic acid amplification testing methods

Two commercially available NAT assays for the detection of WNV RNA in plasma were used by the EQAP participants: the Cobas TaqScreen West Nile Virus Test on Cobas s 201 system and the PROCLEIX WNV Assay on Procleix TIGRIS system.

Both assays were validated by the respective manufacturer for analytical sensitivity, using dilution series of WNV lineage 1 reference material. This sensitivity, expressed as the 95% limit of detection (LOD), is about 40 copies/mL for the Cobas TaqScreen WNV Test and about 10 copies/mL for the PROCLEIX WNV Assay (as reported in the respective packaging inserts).

With respect to WNV lineage 2, the Cobas TaqScreen WNV Test was shown to have a 95% LOD of about 5 copies/mL while in the case of the PROCLEIX WNV Assay, a 95% LOD of approximately 20 copies/mL was estimated on the basis of reported raw data[9,10].

Among the 11 participating laboratories, five used the PROCLEIX WNV Assay, five used the Cobas TaqScreen WNV Test and one used both NAT kits.

Negative and positive samples

The EQAP negative samples were prepared from commercial solvent/detergent-treated plasma that tested negative for hepatitis C, hepatitis B and human immunodeficiency viruses and WNV by NAT. The EQAP positive samples were obtained by spiking this negative plasma with the following materials, all heat-inactivated:

- WNV-RNA ISS 0109, an Italian reference preparation of WNV lineage 1 (1,000 copies/mL). This was obtained from a secondary standard supplied by Roche which had been calibrated against the Health Canada reference preparation (HC-SC WNV Nat Ref 001/03)[11];
- WNV lineage 2, obtained from the supernatant of WNV-infected cell cultures. The viral concentration of the supernatant was estimated to be about 3-5x10^6 copies/mL of WNV RNA. Before use, the supernatant was diluted 2.44 log in negative human plasma to a final concentration of about 10-20,000 copies/mL;
- Usutu virus (USUV)[12], obtained diluting the supernatant of USUV-infected Vero E6 cell cultures to a final concentration of about a 1x10^6 copies/mL.

West Nile virus panels

Two panels of 15 vials each were designed for the EQAP 2011.

Panel 1 consisted of: (i) seven samples of WNV lineage 1 (WNVL1), two containing 360 copies/mL, three containing 60 copies/mL and two containing 10 copies/mL; (ii) six samples of WNV lineage 2 (WNVL2), two containing about 20,000 copies/mL, two containing about 3,300 copies/mL and two containing about 550 copies/mL; (iii) one WNV negative plasma sample; and (iv) one sample negative for WNV but positive for USUV.

Panel 2 consisted of: (i) eight WNVL1 samples: one containing 360 copies/mL, four containing 100 copies/mL, two containing 60 copies/mL and one containing 10 copies/mL; (ii) six WNVL2 samples: one each containing about 100 copies/mL,16 copies/mL and 3 copies/mL and three containing <1 copy/mL; and (iii) one WNV negative plasma sample.

Negative and positive samples were distributed in 2.5 mL-frozen aliquots. Before distribution to participants, panels were tested by an external laboratory using the PROCLEIX WNV Assay in order to confirm the negativity/positivity of the samples.

Study design

Participants were sent Panel 1 in September 2011 and Panel 2 in October 2011 and were asked to test them using their routine NAT kit for WNV RNA without further dilution of the samples. Results had to be reported, on the dedicated EQAP website, in a qualitative way, i.e. either positive or negative. After submission of the results, the system immediately confirmed their correctness. The proficiency criteria adopted to evaluate the participants in the EQAP were that the 360 and 100 copies/mL-WNVL1 samples and the 20,000, 3,300, 550 and 100 copies/mL-WNVL2 samples had to be correctly detected based on the 95% LOD of the NAT kit used and that no false-positive results were allowed for the two WNV-negative samples.

The other positive samples with WNV concentrations at or below the 95% LOD of either or both methods were excluded from the evaluation of the results considering that participants could miss the target as a consequence of the random distribution of the virus in the plasma. The sample negative for WNV but positive for USUV was also excluded from the evaluation as its primary role was to investigate potential cross-reactivity.

In case of a discrepancy between the value reported by the participant and the expected result, the EQAP Coordinator could assist the concerned laboratory in finding the root cause of the error and to determine whether it was a pre-analytical phase error (e.g. numbering of the sample before extraction), a post-analytical phase error (e.g. interpretation/transcription of the results), or an unspecified analytical error.

Shipment

Samples were shipped on dry ice and participants were asked to check the integrity of the parcel, the presence of dry ice and the status of the samples immediately upon receipt.

Results

The WNV RNA EQAP 2011was run between September and November 2011. In each shipment, a total of 12 panels were distributed to the 11 participants. One laboratory using both NAT kits requested two panels.

Table I reports the overall results obtained in the present EQAP with both NAT kits.

With respect to WNVL1 samples, the 360 copies/mL-samples were correctly detected by all participants (100% positivity rate) in the three independent runs. All laboratories also correctly identified the 100 copies/mL-samples in the four

Table I - Overall results obtained in the present EQAP.

		Cobas TaqScreen WNV Assay							PROCLEIX WNV Assay						
	Laboratory code Copies/mL	**1**	**2**	**4**	**5**	**7**	**12**	**Total (%)**	**2**	**3**	**6**	**8**	**10**	**11**	**Total (%)**
WNVL1 samples	360	3/3	3/3	3/3	3/3	3/3	3/3	18/18 (100%)	3/3	3/3	3/3	3/3	3/3	3/3	18/18 (100%)
	100	4/4	4/4	4/4	4/4	4/4	4/4	24/24 (100%)	4/4	4/4	4/4	4/4	4/4	4/4	24/24 (100%)
	60	4/5	4/5	5/5	5/5	5/5	5/5	28/30 (93.3%)	5/5	5/5	5/5	5/5	5/5	5/5	30/30 (100%)
	10	1/3	3/3	0/3	1/3	0/3	1/3	6/18 (33.3%)	3/3	3/3	3/3	3/3	3/3	3/3	18/18 (100%)
WNVL2 samples	20,000	2/2	2/2	2/2	2/2	2/2	2/2	12/12 (100%)	2/2	2/2	2/2	2/2	2/2	2/2	12/12 (100%)
	3300	2/2	2/2	2/2	2/2	2/2	2/2	12/12 (100%)	2/2	2/2	2/2	2/2	2/2	2/2	12/12 (100%)
	550	2/2	2/2	2/2	2/2	2/2	2/2	12/12 (100%)	2/2	2/2	2/2	2/2	2/2	2/2	12/12 (100%)
	100	1/1	1/1	1/1	1/1	1/1	1/1	6/6 (100%)	1/1	1/1	1/1	1/1	1/1	1/1	6/6 (100%)
	16	1/1	1/1	1/1	1/1	1/1	1/1	6/6 (100%)	1/1	1/1	1/1	1/1	1/1	1/1	6/6 (100%)
	3	1/1	1/1	1/1	0/1	1/1	1/1	5/6 (83.3%)	1/1	0/1	1/1	0/1	0/1	1/1	3/6 (50%)
	<1	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0%)	0/3	0/3	0/3	0/3	0/3	0/3	0/18 (0%)
USUV samples	10^6	1/1	1/1	1/1	1/1	1/1	1/1	6/6 (100%)	1/1	1/1	1/1	1/1	1/1	1/1	6/6 (100%)
Negative samples	0	0/2	0/2	0/2	0/2	0/2	0/2	0/12 (0%)	0/2	0/2	0/2	0/2	0/2	0/2	0/12 (0%)

independent runs (100% positivity rate) while the 60 copies/mL-samples were correctly detected by the Cobas TaqScreen WNV Test with a positivity rate of 93.3% and by the PROCLEIX WNV Assay with a positivity rate of 100% in the five independent runs. Finally, the 10 copies/mL-samples were correctly detected by the Cobas TaqScreen WNV Test with a positivity rate of 33.3% and by the PROCLEIX WNV Assay with a positivity rate of 100%.

With respect to WNVL2 samples, all laboratories correctly identified the 20,000, 3,300, 550, 100 and 16 copies/mL-samples (100% positivity rate). In the case of the 3 copies/mL-sample, the positivity rate was 83.3% for the Cobas TaqScreen WNV Test and 50.0% for the PROCLEIX WNV Assay. All three samples containing <1 copy/mL tested negative.

Both EQAP negative samples were correctly identified by all EQAP participants. Finally, all the EQAP samples negative for WNV RNA but positive for USUV were identified as positive by both NAT kits, indicating the presence of cross-reactivity.

Discussion

Participation in EQAP, in addition to being a requirement for the accreditation and certification of laboratories, represents a good opportunity for a laboratory to verify the correctness of its analytical results and to compare its performance with that of other laboratories. In fact, through the evaluation of the results obtained in these studies, a laboratory can evaluate the effectiveness of its quality system and estimate the ability of the analytical procedures (in terms of methods, equipment and reagents) of generating results that meet the parameters and the technical specifications of the assay. Finally, each participant can detect possible weaknesses in the pre- and post-analytical steps of their processes.

Here we present the results of the EQAP for WNV NAT organised in 2011 for blood transfusion centres that perform WNV RNA screening of blood donations. In a previous EQAP for WNV NAT, carried out in 2010, only lineage 1 was used to prepare the positive samples of the panels. In the present study, reference

materials representing both WNV lineages were used: an Italian reference preparation of lineage 1 with a well-defined viral concentration (expressed in copies/mL as it was calibrated against the Health Canada preparation) and lineage 2 material obtained from a diluted WNV supernatant with a final concentration of 10-20,000 copies/mL. The inclusion of lineage 2 in this second EQAP proved to be an appropriate choice as this lineage, initially isolated in humans only in Greece[13], has since been reported also in Italy. In particular, in late summer 2011, an autochthonous case of human WNV infection, clinically identified as fever of unknown origin and caused by a lineage 2 strain, was identified in Ancona[14], prompting the implementation of WNV NAT in this province on a voluntary basis.

The results obtained for WNVL1 samples confirm what had already been observed in the previous EQAP. In fact, samples containing 360 and 100 copies/mL were correctly detected as positive in 100% of the cases by both NAT assays. Furthermore, for samples containing 60 and 10 copies/mL, positivity rates of 100% were observed for the PROCLEIX WNV Assay while positivity rates of 93.3% and 33.3%, respectively, were obtained by the Cobas TaqScreen WNV Test. In both cases, the results were as expected, i.e. in line with the 95% LOD stated by the test kits' manufacturers (about 10 copies/mL for the PROCLEIX WNV Assay and about 40 copies/mL for the Cobas TaqScreen WNV Test). Furthermore, these results confirm the viral concentration assigned to the Italian WNV Reference Preparation ISS 0109 (1,000 copies/mL) used as the starting material for the preparation of the WNVL1 samples.

Regarding WNVL2 samples, a wide range of dilutions were used (from 2.44 log to 8.67 log, corresponding to 10-20,000 copies/mL to <1 copy/mL, respectively) as the concentration of the starting material of lineage 2 was not well defined and the two NAT kits had proven to have a different analytical sensitivities, in terms of 95% LOD, toward this lineage. It is worth noting that these 95% LOD values are confirmed by the results obtained on WNVL2 samples in the present EQAP: the Cobas TaqScreen WNV Test appears to be slightly more sensitive than the PROCLEIX WNV Assay towards WNV lineage 2 (3 copies/mL-sample: 83% positivity rate *vs.* 50% positivity rate, respectively). This observation should be further supported by other collaborative studies in which multiple isolates of lineage 2 are included.

As stated by the test kit's manufacturer, the Cobas TaqScreen WNV Test has broad cross-reactivity to other flaviviruses such as Japanese encephalitis virus (JEV), Murray Valley encephalitis virus, Kunjin virus and St. Louis encephalitis virus. With respect to the PROCLEIX WNV Assay, false-positive results have only be described for the Kunjin virus and not for other flaviviruses from the JEV serogroup[10]. A recent study by Sambri *et al.* found that the PROCLEIX WNV Assay was cross-reactive with USUV in plasma samples while no false-positive results were observed testing the same plasma samples with the Cobas TaqScreen WNV Test[15]. Using plasma samples spiked with serial dilutions of USUV, it was possible to estimate that the PROCLEIX WNV Assay detects USUV in plasma when the viral concentration is a median tissue culture infective dose (TCID50) of at least $1x10^6$. This observation prompted us to include USUV in the 2011 EQAP panels. Interestingly, our EQAP results show that actually both NAT kits could detect USUV in the WNV-negative sample (containing about $1x10^6$ copies/mL). To investigate this finding further, we organised a small blind study in which the three participating laboratories received a panel made of plasma samples spiked with serial dilutions of USUV (starting concentration, about $1x10^6$ copies/mL) ranging from 1:2 to 1:32. One participant tested the panel with both NAT kits, one with only the PROCLEIX WNV Assay and the last one with only the Cobas TaqScreen WNV Test. The results confirmed that both NAT kits can detect the USUV genome although with different sensitivities: up to the 1:32 dilution (approximately equal to 32,000 copies/mL) for the PROCLEIX WNV Assay and between 1:16 and 1:32 (approximately between 62,500 and 32,000 copies/mL) for the Cobas TaqScreen WNV Test.

Based on the proficiency criteria adopted in the present EQAP, the outcome was favourable for all participants. Finally, it should be noted that there were no problems of cross-contamination or carry over. This good performance, and in particular the ability of the two NAT kits to detect very low concentrations of WNV (less than 100 copies/mL for WNVL1 and 16 copies/mL for WNVL2) is reassuring, especially considering that the viral load of WNV in plasma samples is reported to be between $1.1x10^2$ and $7.6x10^3$ copies/mL[16]. It is worth noting that in a recent EQAP

for WNV RNA addressed to diagnostic laboratories, 68% of the participants were able to detect $1.2x10^3$ copies/mL of lineage 1 virus while 73% (mean) of the participants were able to detect $1x10^5$ to $1x10^6$ copies/mL of lineage 2[17]. As pointed out by the authors, these results reflect the fact that the majority of participants used in-house polymerase chain reaction methods. In fact, a positivity rate of 100% was observed for both lineages with a commercial assay. This observation highlights the importance of using validated NAT assays with a well-defined sensitivity.

In conclusion, the WNV NAT 2011 EQAP confirmed the results obtained with the previous WNV NAT EQAP and, by adding lineage 2, provided participants with an excellent opportunity to evaluate the performance of the NAT assays used for the screening of blood donations and blood components with respect to both WNV lineages.

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