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172-PT

PROFICIENCY TESTING 2013

BLUE TONGUE VIRUS (BTV)

Detection of BTV RNA in blood by real-time

Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

OPERATIONAL UNIT

COORDINATION OF VETERINARY DIAGNOSIS

EPIDEMIOLOGY AND RISK ASSESSMENT

(CVD-ERA)

DATE BEGIN PT: 22 APRIL 2013

DATE REPORT: 27 JUNE 2013

I. Introduction

Details relevant to the proficiency test (PT) are available in the Procedure PRO/2.5/01 'Beheer van de proficiency testen op het CODA-CERVA-Ukkel/Gestion des essais d'aptitude au CODA-CERVA-Uccle', which is summarized in the 'Manual for the participant'.

II. Aim

The aim of this PT was to evaluate the ability of the participating laboratories to identify the absence or presence of BTV RNA in blood of bovidae origin by RT-qPCR.

III. Materials and methods

III.1. Conduct of diagnostic tests

In the framework of this PT, predefined reference blood samples must be tested by means of RT-qPCR. The procedures for the RT-qPCR assays must be fully described in the SOPs of the participating laboratories.

III.2. Reference samples

Replicates of 6 reference blood samples of bovine origin, either free from detectable BTV RNA (n = 2; coded 'PT2013BLTVIRNB1' and 'PT2013BLTVIRNB2') or containing detectable BTV RNA (n = 4; coded 'PT2013BLTVIRPB1', 'PT2013BLTVIRPB2', 'PT2013BLTVIRPB3' and 'PT2013BLTVIRPB4') were used. In total, 80 aliquots were distributed to 4 participating laboratories. All participants received 20 aliquots: 3 aliquots of the reference blood samples PT2013BLTVIRNB1, PT2013BLTVIRNB2, PT2013BLTVIRPB1 and PT2013BLTVIRPB3, and 4 aliquots of the reference blood samples PT2013BLTVIRPB2 and PT2013BLTVIRPB4. The positions of the reference blood samples in the sent blocks were randomized for each participant (Table 3).

For each reference blood sample, a certificate containing the status of the sample (= 'golden standard') was made. The status of the reference blood samples was based on (i) the background of the samples and (ii) the results obtained during pre-verification, hereby using the LSI™ VetMAX™ BTV NS3 RT-qPCR kit from LSI (detecting all BTV serotypes) and 3 different in-house developed BTV RT-qPCR assays: one detecting all BTV serotypes, one detecting only BTV-1 and one detecting only BTV-8.
The 2 reference blood samples free from detectable BTV RNA, namely PT2013BLTVIRNB1 and PT2013BLTVIRNB2, were derived from 2 different BTV negative animals, whereas the reference blood samples containing detectable BTV RNA were obtained by spiking blood derived from BTV negative animals with either inactivated BTV-1 (PT2013BLTVIRPB2 and PT2013BLTVIRPB3) or BTV-8 (PT2013BLTVIRPB1 and PT2013BLTVIRPB4). Hereby, the viral load of BTV-1 was 100 times higher in PT2013BLTVIRPB3 compared to PT2013BLTVIRPB2, whereas the viral load of BTV-8 was 10 times higher in PT2013BLTVIRPB1 than in PT2013BLTVIRPB4. For each reference blood sample, the same qualitative result was obtained with the commercial and the in-house BTV non serotype specific RT-qPCR assay. In addition, the reference blood samples PT2013BLTVIRPB2 and PT2013BLTVIRPB3 tested positive in the BTV-1 specific RT-qPCR but negative in BTV-8 specific RT-qPCR, whereas the opposite was true for the reference blood samples PT2013BLTVIRPB1 and PT2013BLTVIRPB4. The reference blood samples PT2013BLTVIRNB1 and PT2013BLTVIRNB2 tested negative in both the BTV-1 specific and the BTV-8 specific RT-qPCR. In conclusion, the reference blood samples PT2013BLTVIRNB1 and PT2013BLTVIRNB2 were considered as BTV negative samples, and the reference blood samples PT2013BLTVIRPB1, PT2013BLTVIRPB2, PT2013BLTVIRPB3 and PT2013BLTVIRPB4 as BTV positive samples in RT-qPCR.

After aliquoting the different reference blood samples, a homogeneity check was performed on 10 aliquots of each reference blood sample. The homogeneity check was performed using the in-house developed BTV non serotype specific RT-qPCR assay. For all reference blood samples, the same qualitative result was obtained for all 10 aliquots of the same reference blood sample. Consequently, all reference blood samples were considered as reliable samples in order to evaluate the ability of laboratories to correctly identify the absence or presence of BTV RNA in blood of bovine origin. In addition, 3 aliquots of each reference blood sample were tested after the PT using the in-house developed BTV non

serotype specific RT-qPCR assay in order to confirm the stability and status of the reference blood samples (post-verification).

III.3. Classification of results, level of agreement and threshold for qualification

III.3.1. Classification of results

Results provided by the participating laboratories are categorized as *success* when the reported result matches with the assigned status (positive result when the reference sample is truly positive, negative result when the reference sample is truly negative) or *failure* when the reported result does not match with the assigned status (positive result when the reference sample is truly negative, negative result when the reference sample is truly positive, non-interpretable result when the reference sample is truly negative or positive).

III.3.2. Level of agreement

The level of agreement achieved by the participating laboratories is expressed as the percentage of *success* for the 20 aliquots of reference samples used for this PT.

III.3.3. Threshold for qualification

Following the procedure, a participating laboratory is only qualified if the level of agreement for the 20 aliquots of reference samples is at least 90%.

IV. Results

For confidentiality reasons, the participating laboratories are quoted anonymously and the concordance table is safely kept at the operational unit CVD-ERA of CODA-CERVA.

IV.1. Transfer and start of the analyses of the reference samples

The 20 aliquots of reference blood samples were sent frozen (dry ice) to each of the 4 participating laboratories by national courier on 22nd of April 2013 (80 aliquots in total). All laboratories acknowledged receipt of the samples on the same day. Analyses were performed between 23rd and 26th of April 2013 (Table 1).

IV.2. Dates at which results were returned to the operational unit CVD-ERA

Results were submitted to the operational unit CVD-ERA between 26th of April and 3rd of May 2013. All participants hereby respected the deadline of 3rd of May 2013 for submission of the results.

Table 1. Overview of the dates on which (i) the reference blood samples were received and analyzed by the participating laboratories, and (ii) the obtained results were submitted to the operational unit CVD-ERA of CODA-CERVA.

Laboratory	Reference samples received	Start of analysis	Submission of the results (Excel file)
LAB1	22/04/2013	26/04/2013	03/05/2013
LAB2	22/04/2013	24/04/2013	02/05/2013
LAB3	22/04/2013	23/04/2013	26/04/2013
LAB4	22/04/2013	24/04/2013	02/05/2013

IV.3. Compliance with the procedure

All participating laboratories have provided a duly dated and signed copy of the results.

IV.4. Qualitative data analysis

IV.4.1. Level of agreement

Qualitative data analysis showed that all 4 participating laboratories provided qualitative results that were in full agreement with the assigned status of the reference blood samples and hence reached 100% of agreement (Table 2).

A quantitative data analysis (box plots) is shown for educational purposes in Annex 1.

Table 2. Agreement between results generated by the participating laboratories (LABNR) and the status of the reference blood samples assigned by CODA-CERVA. All participating laboratories received 20 aliquots of reference blood samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR			
	1	**2**	**3**	**4**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
success	20 (100.0)	20 (100.0)	20 (100.0)	20 (100.0)

IV.4.2. Variability among participating laboratories

Since all participants reached 100% of agreement for the detection of BTV RNA in reference blood samples, no variability between participating laboratories could be observed at the qualitative data level.

For each participating laboratory, the obtained results and the assigned statuses for the reference blood samples are shown in Table 3.

Table 3. The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference blood samples (SAMPLE), the positions of the reference blood samples as placed in the block (LABPOSIT), and the status assigned by CODA-CERVA (STATUS). NEG: negative; POS: positive.

	LABNR	LABPOSIT	SAMPLE	STATUS	RESULT	SUCCESS
1	1	1	PT2013BLTVIRPB2	POS	POS	1
2	1	2	PT2013BLTVIRPB1	POS	POS	1
3	1	3	PT2013BLTVIRNB2	NEG	NEG	1
4	1	4	PT2013BLTVIRPB2	POS	POS	1
5	1	5	PT2013BLTVIRPB3	POS	POS	1
6	1	6	PT2013BLTVIRPB3	POS	POS	1
7	1	7	PT2013BLTVIRPB1	POS	POS	1
8	1	8	PT2013BLTVIRNB1	NEG	NEG	1
9	1	9	PT2013BLTVIRNB2	NEG	NEG	1
10	1	10	PT2013BLTVIRPB1	POS	POS	1
11	1	11	PT2013BLTVIRPB2	POS	POS	1
12	1	12	PT2013BLTVIRPB4	POS	POS	1
13	1	13	PT2013BLTVIRNB1	NEG	NEG	1
14	1	14	PT2013BLTVIRPB4	POS	POS	1
15	1	15	PT2013BLTVIRPB3	POS	POS	1
16	1	16	PT2013BLTVIRPB4	POS	POS	1
17	1	17	PT2013BLTVIRPB4	POS	POS	1
18	1	18	PT2013BLTVIRNB1	NEG	NEG	1
19	1	19	PT2013BLTVIRNB2	NEG	NEG	1
20	1	20	PT2013BLTVIRPB2	POS	POS	1
21	2	1	PT2013BLTVIRPB3	POS	POS	1
22	2	2	PT2013BLTVIRPB1	POS	POS	1
23	2	3	PT2013BLTVIRNB1	NEG	NEG	1
24	2	4	PT2013BLTVIRNB2	NEG	NEG	1
25	2	5	PT2013BLTVIRPB1	POS	POS	1
26	2	6	PT2013BLTVIRPB2	POS	POS	1
27	2	7	PT2013BLTVIRPB4	POS	POS	1
28	2	8	PT2013BLTVIRNB1	NEG	NEG	1
29	2	9	PT2013BLTVIRPB4	POS	POS	1
30	2	10	PT2013BLTVIRPB3	POS	POS	1
31	2	11	PT2013BLTVIRPB4	POS	POS	1
32	2	12	PT2013BLTVIRPB4	POS	POS	1
33	2	13	PT2013BLTVIRNB1	NEG	NEG	1
34	2	14	PT2013BLTVIRNB2	NEG	NEG	1
35	2	15	PT2013BLTVIRPB2	POS	POS	1
36	2	16	PT2013BLTVIRPB2	POS	POS	1
37	2	17	PT2013BLTVIRPB1	POS	POS	1
38	2	18	PT2013BLTVIRNB2	NEG	NEG	1
39	2	19	PT2013BLTVIRPB2	POS	POS	1
40	2	20	PT2013BLTVIRPB3	POS	POS	1

(Table 3 - CONTINUED)

	LABNR	LABPOSIT	SAMPLE	STATUS	RESULT	SUCCESS
41	3	1	PT2013BLTVIRPB2	POS	POS	1
42	3	2	PT2013BLTVIRPB4	POS	POS	1
43	3	3	PT2013BLTVIRNB1	NEG	NEG	1
44	3	4	PT2013BLTVIRPB4	POS	POS	1
45	3	5	PT2013BLTVIRPB3	POS	POS	1
46	3	6	PT2013BLTVIRPB4	POS	POS	1
47	3	7	PT2013BLTVIRPB4	POS	POS	1
48	3	8	PT2013BLTVIRNB1	NEG	NEG	1
49	3	9	PT2013BLTVIRNB2	NEG	NEG	1
50	3	10	PT2013BLTVIRPB2	POS	POS	1
51	3	11	PT2013BLTVIRPB2	POS	POS	1
52	3	12	PT2013BLTVIRPB1	POS	POS	1
53	3	13	PT2013BLTVIRNB2	NEG	NEG	1
54	3	14	PT2013BLTVIRPB2	POS	POS	1
55	3	15	PT2013BLTVIRPB3	POS	POS	1
56	3	16	PT2013BLTVIRPB3	POS	POS	1
57	3	17	PT2013BLTVIRPB1	POS	POS	1
58	3	18	PT2013BLTVIRNB1	NEG	NEG	1
59	3	19	PT2013BLTVIRNB2	NEG	NEG	1
60	3	20	PT2013BLTVIRPB1	POS	POS	1
61	4	1	PT2013BLTVIRPB4	POS	POS	1
62	4	2	PT2013BLTVIRPB4	POS	POS	1
63	4	3	PT2013BLTVIRNB1	NEG	NEG	1
64	4	4	PT2013BLTVIRNB2	NEG	NEG	1
65	4	5	PT2013BLTVIRPB2	POS	POS	1
66	4	6	PT2013BLTVIRPB2	POS	POS	1
67	4	7	PT2013BLTVIRPB1	POS	POS	1
68	4	8	PT2013BLTVIRNB2	NEG	NEG	1
69	4	9	PT2013BLTVIRPB2	POS	POS	1
70	4	10	PT2013BLTVIRPB3	POS	POS	1
71	4	11	PT2013BLTVIRPB3	POS	POS	1
72	4	12	PT2013BLTVIRPB1	POS	POS	1
73	4	13	PT2013BLTVIRNB1	NEG	NEG	1
74	4	14	PT2013BLTVIRNB2	NEG	NEG	1
75	4	15	PT2013BLTVIRPB1	POS	POS	1
76	4	16	PT2013BLTVIRPB2	POS	POS	1
77	4	17	PT2013BLTVIRPB4	POS	POS	1
78	4	18	PT2013BLTVIRNB1	NEG	NEG	1
79	4	19	PT2013BLTVIRPB4	POS	POS	1
80	4	20	PT2013BLTVIRPB3	POS	POS	1

V. Discussion

The purpose of this PT was to assess the performances of the participating laboratories when analyzing reference blood samples of bovidae origin for the detection of BTV RNA by RT-qPCR.

All participating laboratories provided qualitative results that were in full agreement with the assigned status of the reference blood samples (100% of agreement) (Table 2 and Table 3).

Two different RNA extraction kits were used: the QIAamp Viral RNA mini kit from Qiagen by LAB1 (batch 142.317.644) and LAB4 (batch 139.314.796), and the Nucleospin 96 Virus Core kit from Macherey-Nagel by LAB2 (batch 1206/001) and LAB3 (batch 1203/001). Also different RT-qPCR assays were used by the participating laboratories: LAB2 and LAB3 used an in-house developed BTV non serotype specific BTV RT-qPCR, while LAB1 and LAB4 used the ADIAVET® BTV REALTIME kit from Adiagene (2 batches: 35K2TR171 and 35K2TR180). All used RT-qPCR assays were able to detect RNA from all BTV serotypes.

VI. Conclusions

According to the procedure currently in force, the performance of a participating laboratory is satisfactory if at least 90% of the results provided by this laboratory is in agreement with the status of the reference blood samples assigned by CODA-CERVA (see III.3.3.). Consequently, all participants achieved a satisfactory performance for the detection of BTV RNA in blood of bovine origin by RT-qPCR.

Head CVD-ERA

Yves Van der Stede

Appendix

Name of the participating Laboratories

Association Régionale de Santé et d'Identification Animales (ARSIA) (Mons, Belgium)

Laboratoire de Médecine Vétérinaire de l'Etat (LMVE) (Grand Duchy of Luxemburg)

Veterinary and Agrochemical Research Center (CODA-CERVA), Unit Vesicular and Exotic Diseases (Ukkel, Belgium)

Veterinary and Agrochemical Research Center (CODA-CERVA), Unit Virological Platform (Ukkel, Belgium)

Annex 1: Quantitative data analysis

Besides qualitative data analysis (positive, negative or non-interpretable result), also quantitative data analysis was performed using the statistical software program R (box plots).

When comparing the quantitative results obtained by RT-qPCR, it should be noted that the Ct or Cp values are not normalized with the internal controls. In addition, modifiable factors such as extraction protocol, PCR machine and calculation of Ct or Cp values are not taken into account. Therefore, for RT-qPCR, calculation of Mandel's h- and k-statistics and ANOVA are not included in the report (only box plots).

In the framework of the PT, the virus was heat-inactivated in order to provide the participating laboratories with non-infectious material. Noteworthy, heat inactivation can result in damage of the genomic material of the virus. The degree of genomic damage can be influenced by a number of factors such as segment inherent sequence stability and stabilizing interactions (e.g. with proteins). Consequently, heat-induced genomic damage may differently influence RT-qPCR assays targeting different genomic segments.

The quantitative data analysis in this report was not used to evaluate the participants in this PT, but should only be considered as educational information for the participants in order to evaluate their performance and/or to standardize their different diagnostic tests.

I. Box plots

Box plots of the Ct or Cp values per reference blood sample and per participating laboratory were made using the statistical software R, and are shown in Figure 1.

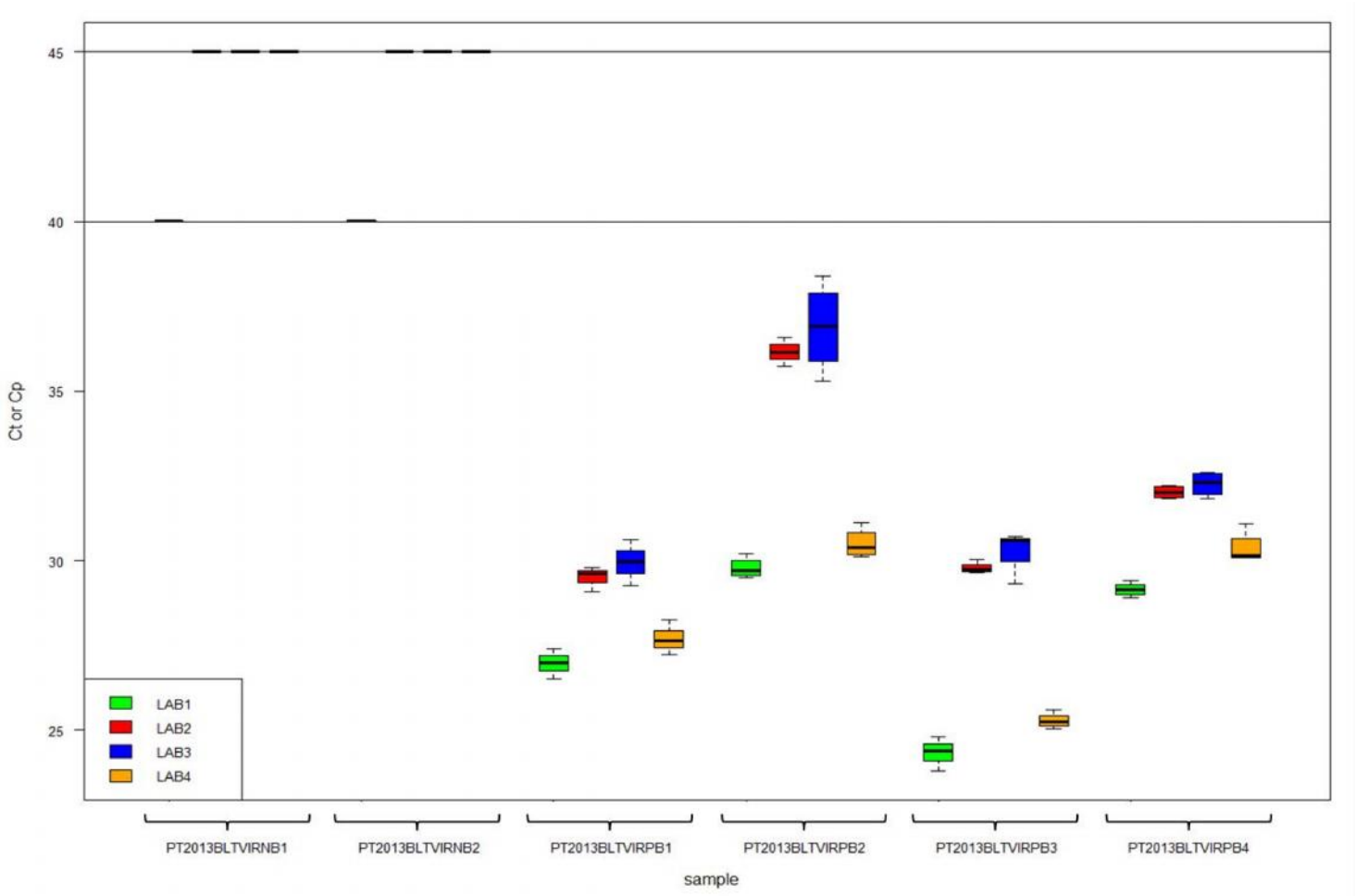

Figure 1. Box plots showing the Ct or Cp values per reference blood sample and per participating laboratory. Box plots represent the minimum value, the maximum value, the median, the lower (25%) and upper (75%) quartile, and possible outliers per sample and per laboratory. Cut-off values for the different RT-qPCR methods are shown in black: 40 for LAB1, 40-45 for LAB2 and LAB3, and 45 for LAB4. A default Ct or Cp value of 40 or 45 was assigned to negative results, according to the corresponding RT-qPCR assay. LAB2 and LAB3 used an in-house developed BTV non serotype specific BTV RT-qPCR, whereas LAB1 and LAB4 used the ADIAVET® BTV REALTIME kit from Adiagene (different batches).