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172-PT

PROFICIENCY TESTING 2013

Bovine Viral Diarrhea Virus (BVDV)

Detection of BVDV-specific antigens in bovine serum, blood (EDTA) and Ear notch samples by Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) and/or Enzyme Linked Immunosorbent Assay (ELISA)

OPERATIONAL UNIT
COORDINATION OF VETERINARY DIAGNOSIS
EPIDEMIOLOGY AND RISK ASSESSMENT
(CVD-ERA)

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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 BVDV-specific antigens in bovine serum, blood and ear-notch samples by RT-qPCR and/or antigen ELISA.

III. Materials and methods

III.1. Conduct of diagnostic tests

In the framework of this PT, predefined reference serum, blood and ear-notch samples must be tested by means of a BVDV RT-qPCR and/or a BVDV antigen ELISA. The procedures for these tests must be fully described in the SOPs of the participating laboratories.

III.2. Reference samples

All participants received 30 aliquots, namely 10 aliquots of each matrix: serum, bloed and ear-notch samples. Each matrix was sent in a different block (3 blocks in total) with reference samples (position 1-10).

III.2.1. Reference serum samples

Replicates of 5 reference serum samples of bovine origin, either free from detectable BVDV-specific antigens (n=2; coded 'PT2013BVDVIRNS1', 'PT2013BVDVIRNS2') or containing detectable BVDV-specific antigens (n=3; coded 'PT2013BVDVIRPS1', 'PT2013BVDVIRPS2' and PT2013BVDVIRPS3), were used.
In total, 90 aliquots of reference serum samples were distributed to 9 participating laboratories. All participants received 10 aliquots: 2 alliquots of each sample were distributed. The positions of the reference serum samples in the sent blocks were randomized for each participant (Table 10 and Table 13).

For each reference serum sample, a certificate containing the status of the sample (= 'golden standard') was made. The status of the reference serum samples was based on (i) the historical background of the animals and (ii) the results obtained by 2 different in-house developed BVDV RT-qPCR assays, the BVDV antigen test kit/serum plus ELISA from IDEXX and a BVDV-specific seroneutralisation assay (pre-verification).

The reference serum sample PT2013BVDVIRNS1 and PT2013BVDVIRNS2 were obtained from 2 different BVDV-free animals. In contrast, the reference serum sample PT2013BVDVIRPS1 was obtained from a sick newborn calve that was infected with BVDV type 2. This animal was seropositive using the BVDV-specific seroneutralisation assay. The reference sample PT2013BVDVIRPS2 was obtained from a calve that was classified as immunotolerant persistently BVDV-infected (IPI) animal. The reference sample PT2013BVDVIRPS3 was a 1/50 dilution of the serum PT2013BVDVIRPS2.

For each reference serum sample, the same qualitative result was obtained with both in-house developed BVDV RT-qPCR assays and the BVDV antigen ELISA kit from IDEXX.

After aliquoting the different reference serum samples, a homogeneity check was performed on 10 alliquots of each reference serum sample using an in-house developed BVDV RT-qPCR, hereby obtaining the same qualitative result for all aliquots of the same reference serum sample. Consequently, all reference serum samples were considered as reliable samples in order to evaluate the ability of laboratories to correctly identify the absence or presence of BVDV-specific antigens in bovine serum. In addition, all reference serum samples were tested once after the PT in order to confirm their stability and status (post-verification) using the same in-house developed BVDV RT-qPCR.

III.2.2. Reference blood samples

Replicates of 5 reference blood samples of bovine origin, either free from detectable BVDV-specific antigens (n=2; coded 'PT2013BVDVIRNB1' and 'PT2013BVDVIRNB2'') or containing detectable BVDV-specific antigens (n=3; coded 'PT2013BVDVIRPB1', 'PT2013BVDVIRPB2' and PT2013BVDVIRPB3), were used.
In total, 90 aliquots of reference blood samples were distributed to 9 participating laboratories. All participants received 10 aliquots: 2 alliquots of each sample were distributed. The positions of the reference blood samples in the sent blocks were randomized for each participant (Table 9 and Table 12).

For each reference blood sample, a certificate containing the status of the sample (= 'golden standard') was made. The status of the reference serum samples was based on (i) the historical background of the animals and (ii) the results obtained by 2 different in-house developed BVDV RT-qPCR assays, the BVDV antigen test kit/serum plus ELISA from IDEXX and a BVDV-specific seroneutralisation assay (pre-verification).

The reference blood sample PT2013BVDVIRNB1 and PT2013BVDVIRNB2 were obtained from the same animals (BVDV-free animals) of which the reference serum samples were obtained (PT2013BVDVIRNS1 and PT2013BVDVIRNS2). The reference blood samples PT2013BVDVIRPB1 and PT2013BVDVIRPB2 were obtained from 2 different calves classified as IPI. The reference blood sample PT2013BVDVIRPB2 is obtained from the same animal as the serum sample PT2013BVDVIRPS2. The reference sample PT2013BVDVIRPB3 was a 1/50 dilution of the blood sample PT2013BVDVIRPB2.

Using the in-house BVDV RT-qPCR the same qualitative result was obtained for all the reference blood samples. In contrast using the BVDV antigen ELISA the reference blood sample PT2013BVDVIRPB3 tested negative. Therefore the reference blood sample PT2013BVDVIRPB3 was considered to be negative.

After aliquoting the different reference blood samples, a homogeneity check was performed on 10 alliquots of each reference blood sample using an in-house developed BVDV RT-qPCR, hereby obtaining the same qualitative result for all 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 BVDV-specific antigens in bovine blood. In addition, all reference blood samples were tested after the PT in order to confirm their stability and status (post-verification) using the same in-house developed BVDV RT-qPCR.

III.2.1. Reference ear-notch samples

Replicates of 5 reference ear notch samples of bovine origin, either free from detectable BVDV-specific antigens (n=2; coded 'PT2013BVDVIRNE1' and 'PT2013BVDVIRNE2'') or containing detectable BVDV-specific antigens (n=3; coded 'PT2013BVDVIRPE1', 'PT2013BVDVIRPE2' and PT2013BVDVIRPE3), were used.
In total, 90 aliquots of reference blood samples were distributed to 9 participating laboratories. All participants received 10 aliquots: 2 alliquots of each sample were distributed. The positions of the reference blood samples in the sent blocks were randomized for each participant (Table 11 and Table 14).

For each reference ear notch sample, a certificate containing the status of the sample (= 'golden standard') was made. The status of the reference ear notch samples was based on (i) the historical background of the animals and (ii) the results obtained by 2 different in-house developed BVDV RT-qPCR assays, the BVDV antigen test kit/serum plus ELISA from IDEXX.

The reference ear notch sample PT2013BVDVIRNE1 and PT2013BVDVIRNE2 were obtained from the same animals (BVDV-free animals) of which the reference serum and blood samples samples were obtained (see above).
The reference ear notch samples PT2013BVDVIRPE1 and PT2013BVDVIRPE2 were obtained from the same calves classified as IPI (same animals as PT2013BVDVIRPB1 and PT2013BVDVIRPB2 respectively).
The reference ear notch sample PT2013BVDVIRPE3 was obtained from a different animal classified as IPI animal.

Using the in-house BVDV RT-qPCR and the antigen ELISA the same qualitative result was obtained for all the reference ear notch samples. After aliquoting the different ear notch samples, a homogeneity check was performed on 3 alliquots of each reference ear notch sample using an in-house developed BVDV RT-qPCR, hereby obtaining the same qualitative result for all aliquots of the same reference ear notch sample. Consequently, all reference ear notch samples were

considered as reliable samples in order to evaluate the ability of laboratories to correctly identify the absence or presence of BVDV-specific antigens in bovine ear notch samples. In addition, all reference ear notch samples were tested once after the PT in order to confirm their stability and status (post-verification) using the same in-house developed BVDV RT-qPCR.

An overview of the different reference serum, blood and ear notch samples and their assigned status for BVDV RT-qPCR and BVDV antigen ELISA is shown in Table 1.

Table 1. The different reference serum, blood and ear-notch samples and their assigned status (NEG: negative; POS: positive; NI: non-interpretable) for BVDV RT-qPCR and BVDV antigen ELISA.

SAMPLE	MATRIX	STATUS	
		RT-qPCR	Ag ELISA
PT2013BVDVIRNS1	SERUM	NEG	NEG
PT2013BVDVIRNS2	SERUM	NEG	NEG
PT2013BVDVIRPS1	SERUM	POS	POS
PT2013BVDVIRPS2	SERUM	POS	POS
PT2013BVDVIRPS3	SERUM	POS	POS
PT2013BVDVIRNB1	BLOOD	NEG	NEG
PT2013BVDVIRNB2	BLOOD	NEG	NEG
PT2013BVDVIRPB1	BLOOD	POS	POS
PT2013BVDVIRPB2	BLOOD	POS	POS
PT2013BVDVIRPB3	BLOOD	POS	NEG
PT2013BVDVIRNE1	EAR NOTCH	NEG	NEG
PT2013BVDVIRNE2	EAR NOTCH	NEG	NEG
PT2013BVDVIRPE1	EAR NOTCH	POS	POS
PT2013BVDVIRPE2	EAR NOTCH	POS	POS
PT2013BVDVIRPE3	EAR NOTCH	POS	POS

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 or *failure* when the reported result does not match with the assigned status.

III.3.2. Level of agreement

The level of agreement achieved by the participating laboratories is expressed as the percentage of *success* for each of the 10 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 10 aliquots of reference samples for each matrix 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

LAB4, LAB6 and LAB9 participated in both PTs for RT-qPCR and Antigen ELISA. LAB1 and LAB7 only participated in the PT for antigen ELISA. LAB3, LAB5 and LAB8 only participated in the PT RT-qPCR.
The 10 aliquots of each reference sample (serum, blood and ear notch) were sent frozen (dry ice) to each of the participating laboratories by national courier and international courier on 4th of November 2013 (90 aliquots in total). All participants acknowledged receipt of the samples on the same day or 2 days later. Analyses were performed between 5th and 21st of November 2013 (Table 2).

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

Results were submitted to the operational unit CVD-ERA between 12th and 25th of November 2013. One laboratory hereby did not respected the deadline of 22nd of November 2013 for submission of the results (Table 2).
From one laboroatory no results were obtained. LAB2 did not sent any results to the PT provider.

Table 2. Overview of the dates on which (i) the reference serum and 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
		ELISA	RT-qPCR	
LAB1[1]	05/11/2013	14/11/2013	NA	22/11/2013
LAB2[2]	06/11/2013	NA	Not received	Not received
LAB3	05/11/2013	NA	06/11/2013	12/11/2013
LAB4	05/11/2013	06&07/11/2013	12/11/2013	18/11/2013
LAB5	04/11/2013	NA	18/11/2013	21/11/2013
LAB6	04/11/2013	05&06/11/2013	14/11/2013	19/11/2013
LAB7	04/11/2013	5/11/2013	NA	12/11/2013
LAB8	04/11/2013	NA	12&13/11/2013	20/11/2013
LAB9	04/11/2013	12&13/11/2013	21/11/2013	**25/11/2013**

Legend: NA = not applicable (test not performed). [1]LAB1 informed the PT provider on 13/11/2013 that sample ear notch 8 was empty; LAB1 was asked on 25/11/2013 to resubmit their results due to a transcription error for sample ear notch 9 (corrected version received on 02/12/2013).[2] LAB2 did not provide any results and were not considered in the evaluation.

IV.3. Compliance with the procedure

LAB3, LAB5, LAB7 and LAB9 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:

- **(i)** For the detection of BVDV-specific antigens by **RT-qPCR** (Tables 3, 4 and 5): for the matrix serum and blood all participating laboratories (LAB3,LAB4,LAB5,LAB6,LAB8 and LAB9) provided qualitative results that were in full agreement with the true status of the reference serum and blood samples (100% of agreement). For the matrix ear notch, 5 out of 6 laboroatories provided qualitative results that were in full agreement with the true status of the reference ear notch samples, whereas LAB8 misclassified 4 aliquots and hence reached 60% of agreement (Table 4).
- **(ii)** For the detection of BVDV-specific antigens by **antigen ELISA** (Tables 6, 7 and 8). For all matrices all participating laboratories (LAB1,,LAB4,LAB6,LAB7 and LAB9) provided qualitative results that were in full

agreement with the true status of the reference serum and blood samples (100% of agreement). LAB 4 did not participate for ear notch samples.

A quantitative data analysis was done by making box plots of the data for each matrix separately. A real quantitative analysis (k and h plots) was not possible since only 2 repeats of each sample were sent. This quantitative analysis is shown for educational purposes in Annex 1.

Table 3. RT-qPCR serum: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference serum samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference serum samples and 10 aliquots of reference blood samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR					
	3	**4**	**5**	**6**	**8**	**9**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
success	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)

Table 4. RT-qPCR Ear Notch: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference ear notch samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference ear notch samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR					
	3	**4**	**5**	**6**	**8**	**9**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (40.0)	0 (0.0)
success	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	**6 (60.0)**	10 (100.0)

Table 5. RT-qPCR Blood: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference blood samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference blood samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR					
	3	**4**	**5**	**6**	**8**	**9**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
success	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)

Table 6. ELISA serum: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference serum samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference serum samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR				
	1	**4**	**6**	**7**	**9**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
success	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)

Table 7. ELISA Ear Notch: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference ear notch samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference ear notch samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR			
	1	**6**	**7**	**9**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
success	9 (100.0)*	10 (100.0)	10 (100.0)	10 (100.0)
** LAB1 received 9 alliquots in stead of 10. Sample 8 was empty and PT provider was informed about this on 13/11/2013.*				

Table 8. ELISA Blood: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference blood samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference blood samples. Results are presented as absolute values and percentages (in parentheses).

	LABNR				
	1	**4**	**6**	**7**	**9**
failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
success	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)	10 (100.0)

IV.4.2. Variability among participating laboratories

(i) For the detection of BVDV-specific antigens by **RT-qPCR**, no variability between LAB3, LAB4, LAB5, LAB6 and LAB9 could be observed since these participants correctly identified all reference samples (all matrices). In contrast LAB8 misclassified all aliquots of the negative reference ear notch samples (PT2013BVDVIRNE1 and PT2013BVDVIRNE2). These ear notch samples were tested positive instead of negative. The other matrices were correctly identified by LAB8.

(ii) For the detection of BVDV-specific antigens by **antigen ELISA**, no variability between the participating labs could be observed since these laboratories correctly identified all reference samples (all matrices). .

For each participating laboratory, the obtained results and the assigned statuses for the reference serum and blood samples are shown in Table 9 (blood), 10 (serum) and 11 (ear notch) for RT-qPCR and in Tables 12 (blood), 13 (serum) and 14 (ear notch) for antigen ELISA.

Table 9. RT-qPCR blood: 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 the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive;.

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
1	3	1	PT2013BVDVIRNB1	NEG	NEG	1
2	3	2	PT2013BVDVIRPB2	POS	POS	1
3	3	3	PT2013BVDVIRPB1	POS	POS	1
4	3	4	PT2013BVDVIRNB2	NEG	NEG	1
5	3	5	PT2013BVDVIRPB3	POS	POS	1
6	3	6	PT2013BVDVIRPB1	POS	POS	1
7	3	7	PT2013BVDVIRPB2	POS	POS	1
8	3	8	PT2013BVDVIRNB1	NEG	NEG	1
9	3	9	PT2013BVDVIRPB3	POS	POS	1
10	3	10	PT2013BVDVIRNB2	NEG	NEG	1
11	4	1	PT2013BVDVIRPB3	POS	POS	1
12	4	2	PT2013BVDVIRPB1	POS	POS	1
13	4	3	PT2013BVDVIRPB2	POS	POS	1
14	4	4	PT2013BVDVIRNB1	NEG	NEG	1
15	4	5	PT2013BVDVIRPB3	POS	POS	1
16	4	6	PT2013BVDVIRNB2	NEG	NEG	1
17	4	7	PT2013BVDVIRNB1	NEG	NEG	1
18	4	8	PT2013BVDVIRPB2	POS	POS	1
19	4	9	PT2013BVDVIRPB1	POS	POS	1
20	4	10	PT2013BVDVIRNB2	NEG	NEG	1
21	5	1	PT2013BVDVIRNB1	NEG	NEG	1
22	5	2	PT2013BVDVIRPB2	POS	POS	1
23	5	3	PT2013BVDVIRPB1	POS	POS	1
24	5	4	PT2013BVDVIRNB2	NEG	NEG	1
25	5	5	PT2013BVDVIRPB3	POS	POS	1
26	5	6	PT2013BVDVIRPB1	POS	POS	1
27	5	7	PT2013BVDVIRPB2	POS	POS	1
28	5	8	PT2013BVDVIRNB1	NEG	NEG	1
29	5	9	PT2013BVDVIRPB3	POS	POS	1
30	5	10	PT2013BVDVIRNB2	NEG	NEG	1

Table 9 (Continued)

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
31	6	1	PT2013BVDVIRPB3	POS	POS	1
32	6	2	PT2013BVDVIRPB1	POS	POS	1
33	6	3	PT2013BVDVIRPB2	POS	POS	1
34	6	4	PT2013BVDVIRNB1	NEG	NEG	1
35	6	5	PT2013BVDVIRPB3	POS	POS	1
36	6	6	PT2013BVDVIRNB2	NEG	NEG	1
37	6	7	PT2013BVDVIRNB1	NEG	NEG	1
38	6	8	PT2013BVDVIRPB2	POS	POS	1
39	6	9	PT2013BVDVIRPB1	POS	POS	1
40	6	10	PT2013BVDVIRNB2	NEG	NEG	1
41	8	1	PT2013BVDVIRPB3	POS	POS	1
42	8	2	PT2013BVDVIRPB1	POS	POS	1
43	8	3	PT2013BVDVIRPB2	POS	POS	1
44	8	4	PT2013BVDVIRNB1	NEG	NEG	1
45	8	5	PT2013BVDVIRPB3	POS	POS	1
46	8	6	PT2013BVDVIRNB2	NEG	NEG	1
47	8	7	PT2013BVDVIRNB1	NEG	NEG	1
48	8	8	PT2013BVDVIRPB2	POS	POS	1
49	8	9	PT2013BVDVIRPB1	POS	POS	1
50	8	10	PT2013BVDVIRNB2	NEG	NEG	1
51	9	1	PT2013BVDVIRNB1	NEG	NEG	1
52	9	2	PT2013BVDVIRPB2	POS	POS	1
53	9	3	PT2013BVDVIRPB1	POS	POS	1
54	9	4	PT2013BVDVIRNB2	NEG	NEG	1
55	9	5	PT2013BVDVIRPB3	POS	POS	1
56	9	6	PT2013BVDVIRPB1	POS	POS	1
57	9	7	PT2013BVDVIRPB2	POS	POS	1
58	9	8	PT2013BVDVIRNB1	NEG	NEG	1
59	9	9	PT2013BVDVIRPB3	POS	POS	1
60	9	10	PT2013BVDVIRNB2	NEG	NEG	1

Table 10. RT-qPCR serum: The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference serum samples (SAMPLE), the positions of the reference serum samples as placed in the block (LABPOSIT), and the status assigned by the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive;.

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
1	3	1	PT2013BVDVIRNS2	NEG	NEG	1
2	3	2	PT2013BVDVIRNS1	NEG	NEG	1
3	3	3	PT2013BVDVIRPS2	POS	POS	1
4	3	4	PT2013BVDVIRPS1	POS	POS	1
5	3	5	PT2013BVDVIRPS3	POS	POS	1
6	3	6	PT2013BVDVIRNS1	NEG	NEG	1
7	3	7	PT2013BVDVIRPS2	POS	POS	1
8	3	8	PT2013BVDVIRPS1	POS	POS	1
9	3	9	PT2013BVDVIRPS3	POS	POS	1
10	3	10	PT2013BVDVIRNS2	NEG	NEG	1
11	4	1	PT2013BVDVIRPS3	POS	POS	1
12	4	2	PT2013BVDVIRNS1	NEG	NEG	1
13	4	3	PT2013BVDVIRPS2	POS	POS	1
14	4	4	PT2013BVDVIRPS1	POS	POS	1
15	4	5	PT2013BVDVIRPS3	POS	POS	1
16	4	6	PT2013BVDVIRNS2	NEG	NEG	1
17	4	7	PT2013BVDVIRNS2	NEG	NEG	1
18	4	8	PT2013BVDVIRNS1	NEG	NEG	1
19	4	9	PT2013BVDVIRPS2	POS	POS	1
20	4	10	PT2013BVDVIRPS1	POS	POS	1
21	5	1	PT2013BVDVIRNS2	NEG	NEG	1
22	5	2	PT2013BVDVIRNS1	NEG	NEG	1
23	5	3	PT2013BVDVIRPS2	POS	POS	1
24	5	4	PT2013BVDVIRPS1	POS	POS	1
25	5	5	PT2013BVDVIRPS3	POS	POS	1
26	5	6	PT2013BVDVIRNS1	NEG	NEG	1
27	5	7	PT2013BVDVIRPS2	POS	POS	1
28	5	8	PT2013BVDVIRPS1	POS	POS	1
29	5	9	PT2013BVDVIRPS3	POS	POS	1
30	5	10	PT2013BVDVIRNS2	NEG	NEG	1

Table 10 (Continued)

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
31	6	1	PT2013BVDVIRPS3	POS	POS	1
32	6	2	PT2013BVDVIRNS1	NEG	NEG	1
33	6	3	PT2013BVDVIRPS2	POS	POS	1
34	6	4	PT2013BVDVIRPS1	POS	POS	1
35	6	5	PT2013BVDVIRPS3	POS	POS	1
36	6	6	PT2013BVDVIRNS2	NEG	NEG	1
37	6	7	PT2013BVDVIRNS2	NEG	NEG	1
38	6	8	PT2013BVDVIRNS1	NEG	NEG	1
39	6	9	PT2013BVDVIRPS2	POS	POS	1
40	6	10	PT2013BVDVIRPS1	POS	POS	1
41	8	1	PT2013BVDVIRPS3	POS	POS	1
42	8	2	PT2013BVDVIRNS1	NEG	NEG	1
43	8	3	PT2013BVDVIRPS2	POS	POS	1
44	8	4	PT2013BVDVIRPS1	POS	POS	1
45	8	5	PT2013BVDVIRPS3	POS	POS	1
46	8	6	PT2013BVDVIRNS2	NEG	NEG	1
47	8	7	PT2013BVDVIRNS2	NEG	NEG	1
48	8	8	PT2013BVDVIRNS1	NEG	NEG	1
49	8	9	PT2013BVDVIRPS2	POS	POS	1
50	8	10	PT2013BVDVIRPS1	POS	POS	1
51	9	1	PT2013BVDVIRNS2	NEG	NEG	1
52	9	2	PT2013BVDVIRNS1	NEG	NEG	1
53	9	3	PT2013BVDVIRPS2	POS	POS	1
54	9	4	PT2013BVDVIRPS1	POS	POS	1
55	9	5	PT2013BVDVIRPS3	POS	POS	1
56	9	6	PT2013BVDVIRNS1	NEG	NEG	1
57	9	7	PT2013BVDVIRPS2	POS	POS	1
58	9	8	PT2013BVDVIRPS1	POS	POS	1
59	9	9	PT2013BVDVIRPS3	POS	POS	1
60	9	10	PT2013BVDVIRNS2	NEG	NEG	1

Table 11. The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference ear notch samples (SAMPLE), the positions (numbers) of the reference ear notch samples as provided for the laboratories (LABPOSIT), and the status assigned by the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive;

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
1	3	1	PT2013BVDVIRPE2	POS	POS	1
2	3	2	PT2013BVDVIRNE1	NEG	NEG	1
3	3	3	PT2013BVDVIRPE3	POS	POS	1
4	3	4	PT2013BVDVIRNE2	NEG	NEG	1
5	3	5	PT2013BVDVIRPE1	POS	POS	1
6	3	6	PT2013BVDVIRPE3	POS	POS	1
7	3	7	PT2013BVDVIRNE2	NEG	NEG	1
8	3	8	PT2013BVDVIRPE1	POS	POS	1
9	3	9	PT2013BVDVIRNE1	NEG	NEG	1
10	3	10	PT2013BVDVIRPE2	POS	POS	1
11	4	1	PT2013BVDVIRPE1	POS	POS	1
12	4	2	PT2013BVDVIRPE3	POS	POS	1
13	4	3	PT2013BVDVIRNE2	NEG	NEG	1
14	4	4	PT2013BVDVIRPE1	POS	POS	1
15	4	5	PT2013BVDVIRNE1	NEG	NEG	1
16	4	6	PT2013BVDVIRPE2	POS	POS	1
17	4	7	PT2013BVDVIRPE2	POS	POS	1
18	4	8	PT2013BVDVIRNE1	NEG	NEG	1
19	4	9	PT2013BVDVIRPE3	POS	POS	1
20	4	10	PT2013BVDVIRNE2	NEG	NEG	1
21	5	1	PT2013BVDVIRPE2	POS	POS	1
22	5	2	PT2013BVDVIRNE1	NEG	NEG	1
23	5	3	PT2013BVDVIRPE3	POS	POS	1
24	5	4	PT2013BVDVIRNE2	NEG	NEG	1
25	5	5	PT2013BVDVIRPE1	POS	POS	1
26	5	6	PT2013BVDVIRPE3	POS	POS	1
27	5	7	PT2013BVDVIRNE2	NEG	NEG	1
28	5	8	PT2013BVDVIRPE1	POS	POS	1
29	5	9	PT2013BVDVIRNE1	NEG	NEG	1
30	5	10	PT2013BVDVIRPE2	POS	POS	1

Table 11 (Continued)

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
31	6	1	PT2013BVDVIRPE1	POS	POS	1
32	6	2	PT2013BVDVIRPE3	POS	POS	1
33	6	3	PT2013BVDVIRNE2	NEG	NEG	1
34	6	4	PT2013BVDVIRPE1	POS	POS	1
35	6	5	PT2013BVDVIRNE1	NEG	NEG	1
36	6	6	PT2013BVDVIRPE2	POS	POS	1
37	6	7	PT2013BVDVIRPE2	POS	POS	1
38	6	8	PT2013BVDVIRNE1	NEG	NEG	1
39	6	9	PT2013BVDVIRPE3	POS	POS	1
40	6	10	PT2013BVDVIRNE2	NEG	NEG	1
41	8	1	PT2013BVDVIRPE1	POS	POS	1
42	8	2	PT2013BVDVIRPE3	POS	POS	1
43	8	3	PT2013BVDVIRNE2	**NEG**	**POS**	**0**
44	8	4	PT2013BVDVIRPE1	POS	POS	1
45	8	5	PT2013BVDVIRNE1	**NEG**	**POS**	**0**
46	8	6	PT2013BVDVIRPE2	POS	POS	1
47	8	7	PT2013BVDVIRPE2	POS	POS	1
48	8	8	PT2013BVDVIRNE1	**NEG**	**POS**	**0**
49	8	9	PT2013BVDVIRPE3	POS	POS	1
50	8	10	PT2013BVDVIRNE2	**NEG**	**POS**	**0**
51	9	1	PT2013BVDVIRPE2	POS	POS	1
52	9	2	PT2013BVDVIRNE1	NEG	NEG	1
53	9	3	PT2013BVDVIRPE3	POS	POS	1
54	9	4	PT2013BVDVIRNE2	NEG	NEG	1
55	9	5	PT2013BVDVIRPE1	POS	POS	1
56	9	6	PT2013BVDVIRPE3	POS	POS	1
57	9	7	PT2013BVDVIRNE2	NEG	NEG	1
58	9	8	PT2013BVDVIRPE1	POS	POS	1
59	9	9	PT2013BVDVIRNE1	NEG	NEG	1
60	9	10	PT2013BVDVIRPE2	POS	POS	1

Table 12. Antigen ELISA blood: 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 the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive;.

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
1	1	1	PT2013BVDVIRNB1	NEG	NEG	1
2	1	2	PT2013BVDVIRPB2	POS	POS	1
3	1	3	PT2013BVDVIRPB1	POS	POS	1
4	1	4	PT2013BVDVIRNB2	NEG	NEG	1
5	1	5	PT2013BVDVIRPB3	NEG	NEG	1
6	1	6	PT2013BVDVIRPB1	POS	POS	1
7	1	7	PT2013BVDVIRPB2	POS	POS	1
8	1	8	PT2013BVDVIRNB1	NEG	NEG	1
9	1	9	PT2013BVDVIRPB3	NEG	NEG	1
10	1	10	PT2013BVDVIRNB2	NEG	NEG	1
11	4	1	PT2013BVDVIRPB3	NEG	NEG	1
12	4	2	PT2013BVDVIRPB1	POS	POS	1
13	4	3	PT2013BVDVIRPB2	POS	POS	1
14	4	4	PT2013BVDVIRNB1	NEG	NEG	1
15	4	5	PT2013BVDVIRPB3	NEG	NEG	1
16	4	6	PT2013BVDVIRNB2	NEG	NEG	1
17	4	7	PT2013BVDVIRNB1	NEG	NEG	1
18	4	8	PT2013BVDVIRPB2	POS	POS	1
19	4	9	PT2013BVDVIRPB1	POS	POS	1
20	4	10	PT2013BVDVIRNB2	NEG	NEG	1
21	6	1	PT2013BVDVIRPB3	NEG	NEG	1
22	6	2	PT2013BVDVIRPB1	POS	POS	1
23	6	3	PT2013BVDVIRPB2	POS	POS	1
24	6	4	PT2013BVDVIRNB1	NEG	NEG	1
25	6	5	PT2013BVDVIRPB3	NEG	NEG	1
26	6	6	PT2013BVDVIRNB2	NEG	NEG	1
27	6	7	PT2013BVDVIRNB1	NEG	NEG	1
28	6	8	PT2013BVDVIRPB2	POS	POS	1
29	6	9	PT2013BVDVIRPB1	POS	POS	1
30	6	10	PT2013BVDVIRNB2	NEG	NEG	1

Table 12 (Continued)

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
31	7	1	PT2013BVDVIRNB1	NEG	NEG	1
32	7	2	PT2013BVDVIRPB2	POS	POS	1
33	7	3	PT2013BVDVIRPB1	POS	POS	1
34	7	4	PT2013BVDVIRNB2	NEG	NEG	1
35	7	5	PT2013BVDVIRPB3	NEG	NEG	1
36	7	6	PT2013BVDVIRPB1	POS	POS	1
37	7	7	PT2013BVDVIRPB2	POS	POS	1
38	7	8	PT2013BVDVIRNB1	NEG	NEG	1
39	7	9	PT2013BVDVIRPB3	NEG	NEG	1
40	7	10	PT2013BVDVIRNB2	NEG	NEG	1
41	9	1	PT2013BVDVIRNB1	NEG	NEG	1
42	9	2	PT2013BVDVIRPB2	POS	POS	1
43	9	3	PT2013BVDVIRPB1	POS	POS	1
44	9	4	PT2013BVDVIRNB2	NEG	NEG	1
45	9	5	PT2013BVDVIRPB3	NEG	NEG	1
46	9	6	PT2013BVDVIRPB1	POS	POS	1
47	9	7	PT2013BVDVIRPB2	POS	POS	1
48	9	8	PT2013BVDVIRNB1	NEG	NEG	1
49	9	9	PT2013BVDVIRPB3	NEG	NEG	1
50	9	10	PT2013BVDVIRNB2	NEG	NEG	1

Table 13. Antigen ELISA serum: The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference serum samples (SAMPLE), the positions of the reference serum samples as placed in the block (LABPOSIT), and the status assigned by the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive;.

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
1	1	1	PT2013BVDVIRNS2	NEG	NEG	1
2	1	2	PT2013BVDVIRNS1	NEG	NEG	1
3	1	3	PT2013BVDVIRPS2	POS	POS	1
4	1	4	PT2013BVDVIRPS1	POS	POS	1
5	1	5	PT2013BVDVIRPS3	POS	POS	1
6	1	6	PT2013BVDVIRNS1	NEG	NEG	1
7	1	7	PT2013BVDVIRPS2	POS	POS	1
8	1	8	PT2013BVDVIRPS1	POS	POS	1
9	1	9	PT2013BVDVIRPS3	POS	POS	1
10	1	10	PT2013BVDVIRNS2	NEG	NEG	1
11	4	1	PT2013BVDVIRPS3	POS	POS	1
12	4	2	PT2013BVDVIRNS1	NEG	NEG	1
13	4	3	PT2013BVDVIRPS2	POS	POS	1
14	4	4	PT2013BVDVIRPS1	POS	POS	1
15	4	5	PT2013BVDVIRPS3	POS	POS	1
16	4	6	PT2013BVDVIRNS2	NEG	NEG	1
17	4	7	PT2013BVDVIRNS2	NEG	NEG	1
18	4	8	PT2013BVDVIRNS1	NEG	NEG	1
19	4	9	PT2013BVDVIRPS2	POS	POS	1
20	4	10	PT2013BVDVIRPS1	POS	POS	1
21	6	1	PT2013BVDVIRPS3	POS	POS	1
22	6	2	PT2013BVDVIRNS1	NEG	NEG	1
23	6	3	PT2013BVDVIRPS2	POS	POS	1
24	6	4	PT2013BVDVIRPS1	POS	POS	1
25	6	5	PT2013BVDVIRPS3	POS	POS	1
26	6	6	PT2013BVDVIRNS2	NEG	NEG	1
27	6	7	PT2013BVDVIRNS2	NEG	NEG	1
28	6	8	PT2013BVDVIRNS1	NEG	NEG	1
29	6	9	PT2013BVDVIRPS2	POS	POS	1
30	6	10	PT2013BVDVIRPS1	POS	POS	1

Table 13 (Continued)

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
31	7	1	PT2013BVDVIRNS2	NEG	NEG	1
32	7	2	PT2013BVDVIRNS1	NEG	NEG	1
33	7	3	PT2013BVDVIRPS2	POS	POS	1
34	7	4	PT2013BVDVIRPS1	POS	POS	1
35	7	5	PT2013BVDVIRPS3	POS	POS	1
36	7	6	PT2013BVDVIRNS1	NEG	NEG	1
37	7	7	PT2013BVDVIRPS2	POS	POS	1
38	7	8	PT2013BVDVIRPS1	POS	POS	1
39	7	9	PT2013BVDVIRPS3	POS	POS	1
40	7	10	PT2013BVDVIRNS2	NEG	NEG	1
41	9	1	PT2013BVDVIRNS2	NEG	NEG	1
42	9	2	PT2013BVDVIRNS1	NEG	NEG	1
43	9	3	PT2013BVDVIRPS2	POS	POS	1
44	9	4	PT2013BVDVIRPS1	POS	POS	1
45	9	5	PT2013BVDVIRPS3	POS	POS	1
46	9	6	PT2013BVDVIRNS1	NEG	NEG	1
47	9	7	PT2013BVDVIRPS2	POS	POS	1
48	9	8	PT2013BVDVIRPS1	POS	POS	1
49	9	9	PT2013BVDVIRPS3	POS	POS	1
50	9	10	PT2013BVDVIRNS2	NEG	NEG	1

Table 14. The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference ear notch samples (SAMPLE),), the positions (numbers) of the reference ear notch samples as provided for the laboratories (LABPOSIT), and the status assigned by the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive;

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
1	1	1	PT2013BVDVIRPE2	POS	POS	1
2	1	2	PT2013BVDVIRNE1	NEG	NEG	1
3	1	3	PT2013BVDVIRPE3	POS	POS	1
4	1	4	PT2013BVDVIRNE2	NEG	NEG	1
5	1	5	PT2013BVDVIRPE1	POS	POS	1
6	1	6	PT2013BVDVIRPE3	POS	POS	1
7	1	7	PT2013BVDVIRNE2	NEG	NEG	1
8	1	8	PT2013BVDVIRPE1	POS		.
9	1	9	PT2013BVDVIRNE1	NEG	NEG	1
10	1	10	PT2013BVDVIRPE2	POS	POS	1
11	6	1	PT2013BVDVIRPE1	POS	POS	1
12	6	2	PT2013BVDVIRPE3	POS	POS	1
13	6	3	PT2013BVDVIRNE2	NEG	NEG	1
14	6	4	PT2013BVDVIRPE1	POS	POS	1
15	6	5	PT2013BVDVIRNE1	NEG	NEG	1
16	6	6	PT2013BVDVIRPE2	POS	POS	1
17	6	7	PT2013BVDVIRPE2	POS	POS	1
18	6	8	PT2013BVDVIRNE1	NEG	NEG	1
19	6	9	PT2013BVDVIRPE3	POS	POS	1
20	6	10	PT2013BVDVIRNE2	NEG	NEG	1
21	7	1	PT2013BVDVIRPE2	POS	POS	1
22	7	2	PT2013BVDVIRNE1	NEG	NEG	1
23	7	3	PT2013BVDVIRPE3	POS	POS	1
24	7	4	PT2013BVDVIRNE2	NEG	NEG	1
25	7	5	PT2013BVDVIRPE1	POS	POS	1
26	7	6	PT2013BVDVIRPE3	POS	POS	1
27	7	7	PT2013BVDVIRNE2	NEG	NEG	1
28	7	8	PT2013BVDVIRPE1	POS	POS	1
29	7	9	PT2013BVDVIRNE1	NEG	NEG	1
30	7	10	PT2013BVDVIRPE2	POS	POS	1

Table 14 (Continued)

	LABNR	LABPOS	SAMPLE	STATUS	STATUS	SUCCESS
31	9	1	PT2013BVDVIRPE2	POS	POS	1
32	9	2	PT2013BVDVIRNE1	NEG	NEG	1
33	9	3	PT2013BVDVIRPE3	POS	POS	1
34	9	4	PT2013BVDVIRNE2	NEG	NEG	1
35	9	5	PT2013BVDVIRPE1	POS	POS	1
36	9	6	PT2013BVDVIRPE3	POS	POS	1
37	9	7	PT2013BVDVIRNE2	NEG	NEG	1
38	9	8	PT2013BVDVIRPE1	POS	POS	1
39	9	9	PT2013BVDVIRNE1	NEG	NEG	1
40	9	10	PT2013BVDVIRPE2	POS	POS	1

V. Discussion

The purpose of this PT was to assess the performances of the participating laboratories when analyzing individual reference serum, blood and ear notch samples of bovine origin for the detection of BVDV-specific antigens by RT-qPCR and/or antigen ELISA.

For the detection of BVDV-specific antigens by RT-qPCR, five out of six participating laboratories (LAB3, LAB4,LAB5,LAB6 and LAB9) provided qualitative results that were in full agreement with the true status of the reference samples for all matrices (100% agreement), whereas LAB8 misclassified all 4 aliquots of the negative ear notch samples PT2013BVDVIRNE1 and PT2013BVDVIRNE2 (60% of agreement). Different RNA extraction kits were used: MagVet Universal Isolation Kit from LSI by LAB4 and LAB8, QIAamp Viral RNA mini kit from Qiagen by LAB5, LAB6 and LAB9, QIAamp RNA blood mini kit from Qiagen by LAB4. LAB3 used Magnamax 96 Viral RNA Isolation kit (LSI life technologies). LAB3 tested the ear notch samples also with 'fast lysis buffer' that allows the execution of the RT-PCR without RNA extraction. The results are plotted in the boxplot in Annex 1 (LAB 3.1).
LAB5 used an in-house developed BVDV RT-qPCR while the other participating laboratories used a commercially available BVDV RT-qPCR: LAB6 and LAB8 used the VetMax BVDV screening test from LSI (batch B12S-099) and LAB 3 used the VetMax BVD4ALL from LSI (Lot001). LAB 4 and LAB9 used the TaqVet BVDV Screening Kit from LSI (batch B12S-097).

For the detection of BVDV-specific antigens by antigen ELISA, all participating laboratories (LAB1, LAB4, LAB6, LAB7 and LAB9) provided qualitative results that were in full agreement with the true status of the reference samples for all matrices (100% agreement). The reference blood sample PT2013BVDVIRPB3 was reported as negative by all participants. As. All participating laboratories used the BVDV antigen test kit/serum plus ELISA from IDEXX, but 3 different batches were used: batch B411 (LAB1,LAB7 and LAB4), batch B761 (LAB6) and batch B051 (LAB9). All participating laboratories used the short incubation protocol.

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 samples assigned by the BVDV reference laboratory of CODA-CERVA (see III.3.3.). Consequently, all participating laboratories achieved a satisfactory performance for the detection of BVDV-specific antigens by ELISA in serum, blood and ear notch samples.

LAB3, LAB4, LAB5, LAB6 and LAB9 achieved a satisfactory performance for the detection of BVDV-specific antigens in reference serum, blood and ear notch samples by RT-qPCR, whereas LAB8 achieved only a satisfactory performance for the detection of BVDV-specific antigens by RT-qPCR in reference serum and blood samples. LAB8 did not obtain the required 90% of agreement for RT-qPCR in ear notch samples.

Head CVD-ERA
Yves Van der Stede

Appendix

Name of the participating laboratories

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) (Niort, France)

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

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

Dierengezondheidszorg Vlaanderen (DGZ) (Torhout + Lier, Belgium)

Idexx Laboratories (IDEXX) (Switzerland AG)

Idexx Laboratories (IDEXX) (Montpellier SAS)

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

Laboratoire Service International (LSI) (Lissieu, France)

Veterinary and Agrochemical Research Center (CODA-CERVA), (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 programs R (box plots) and SAS 9.2 (summary statistics).

During this PT only 2 alliquots of each sample (serum, blood and ear notch) were provided. Therefore the analysis in this PT was limited to perform the boxplots in R and this for the RT-qPCR and antigen ELISA separately for each matrix. 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.
For the antigen ELISA, the normalized OD values, calculated according to the instructions of the PT provider, were used.

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: RT-qPCR and antigen ELISA for serum, blood and ear notch samples

For the **RT-qPCR serum, blood and ear notch reference samples** box plots of the Ct or Cp values per reference sample and per participating laboratory were made using the statistical software R and are shown in Figure 1, Figure 2 and Figure 3, respectively.

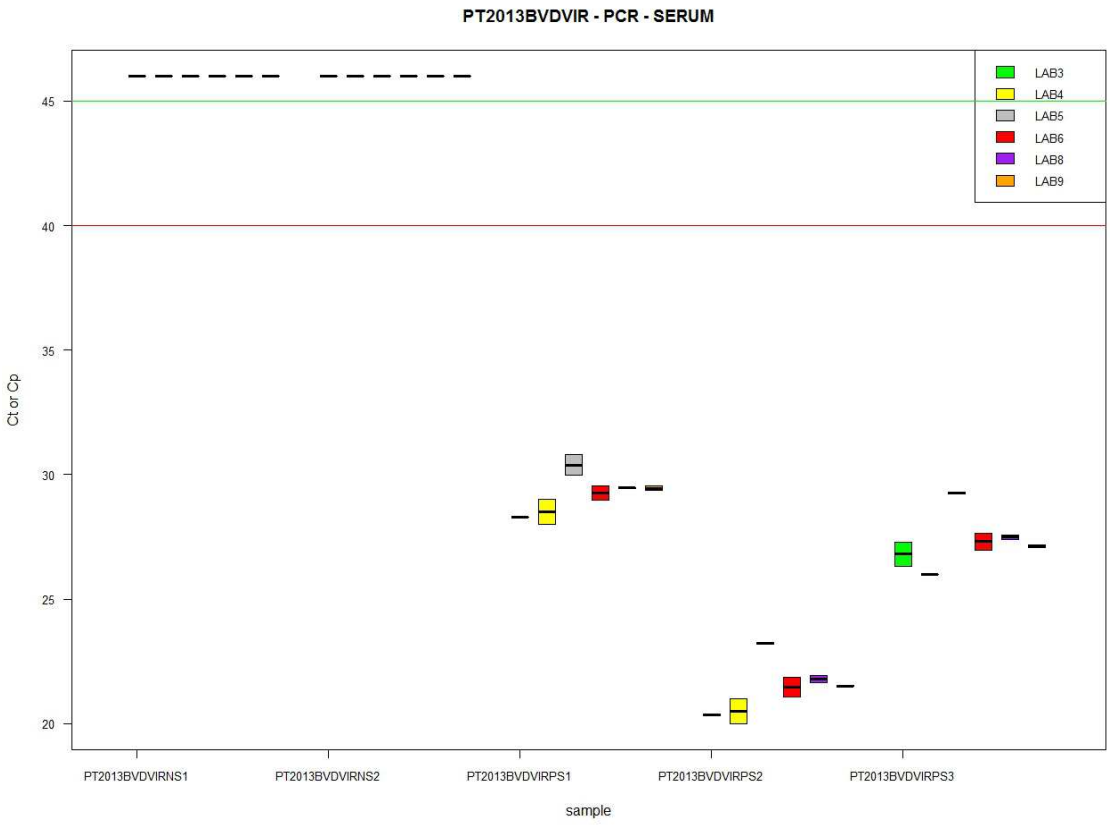

Figure 1. Box plots showing the Ct or Cp values per reference serum and per participating laboratory. Box plots represent the minimum value and the maximum value (only 2 alliquots per sample) and the median value (line). Cut-off values for the different RT-qPCR assays are shown in red (40) and green (45). A default Ct or Cp value of 45 was assigned to negative results, according to the corresponding RT-qPCR.

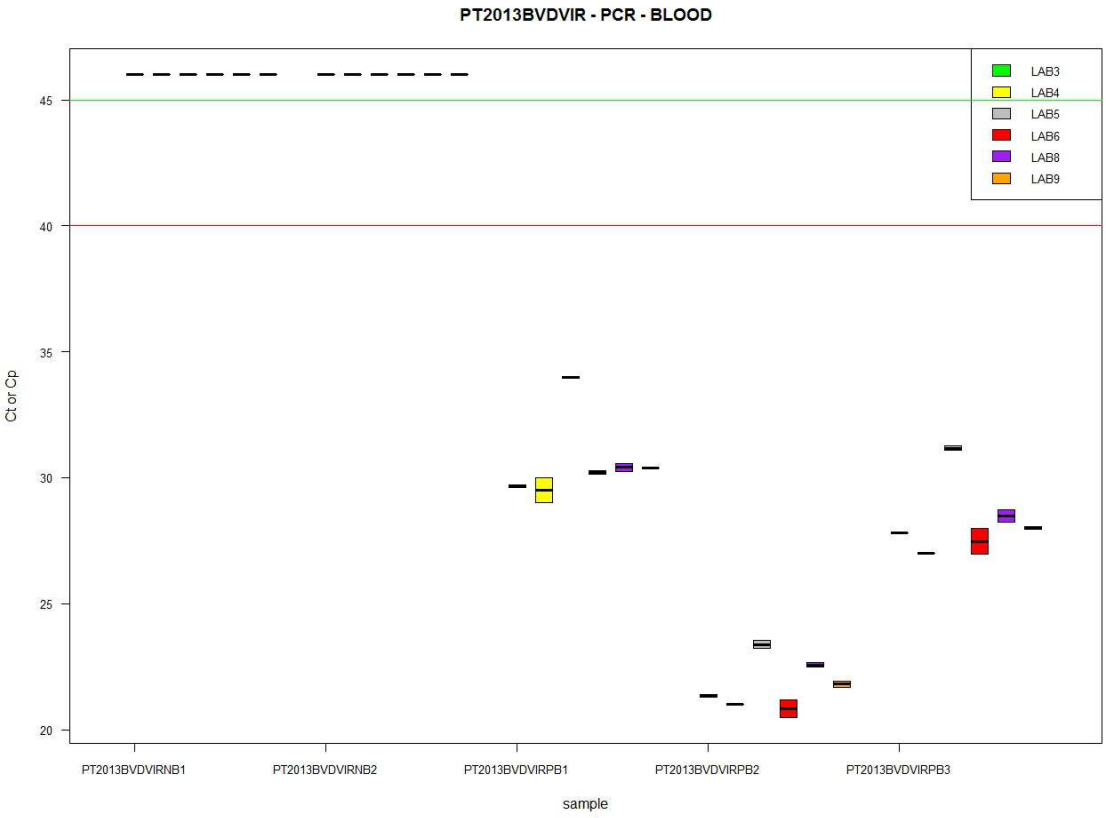

Figure 2. Box plots showing the Ct or Cp values per reference blood sample and per participating laboratory. Box plots represent the minimum value and the maximum value (only 2 alliquots per sample) and the median value (line). Cut-off values for the different RT-qPCR assays are shown in red (40) and green (45). A default Ct or Cp value of 45 was assigned to negative results, according to the corresponding RT-qPCR.

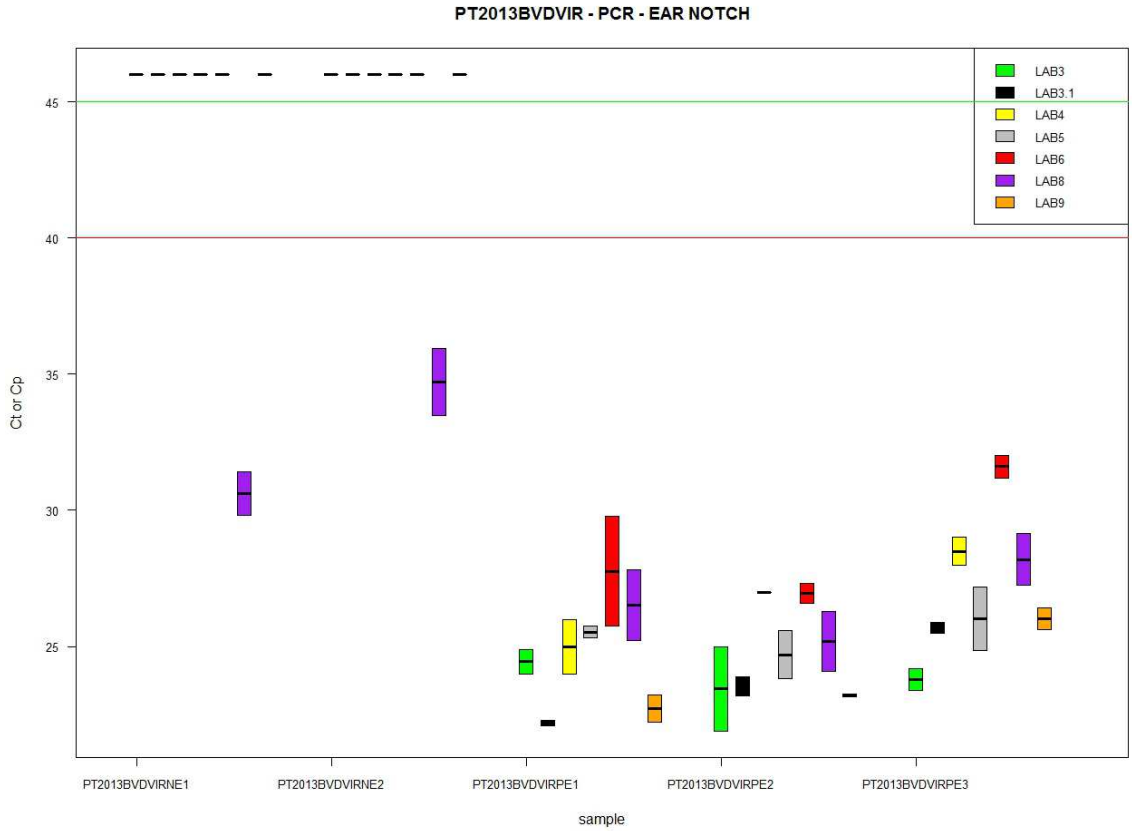

Figure 3. Box plots showing the Ct or Cp values per reference ear notch sample and per participating laboratory. Box plots represent the minimum value and the maximum value (only 2 alliquots per sample) and the median value (line). Cut-off values for the different RT-qPCR assays are shown in red (40) and green (45). A default Ct or Cp value of 45 was assigned to negative results, according to the corresponding RT-qPCR. For LAB 3 the results of the obtained Ct (Cp) values using the fast lysis buffer on ear notch samples (instead of the RNA extraction) are shown accordingly (LAB3.1).

For the **antigen ELISA** , **<u>serum, blood and ear notch reference samples</u>** box plots of the corrected/normalized OD values per reference sample and per participating laboratory were made using the statistical software R and are shown in Figure 4, Figure 5 and Figure 6, respectively.

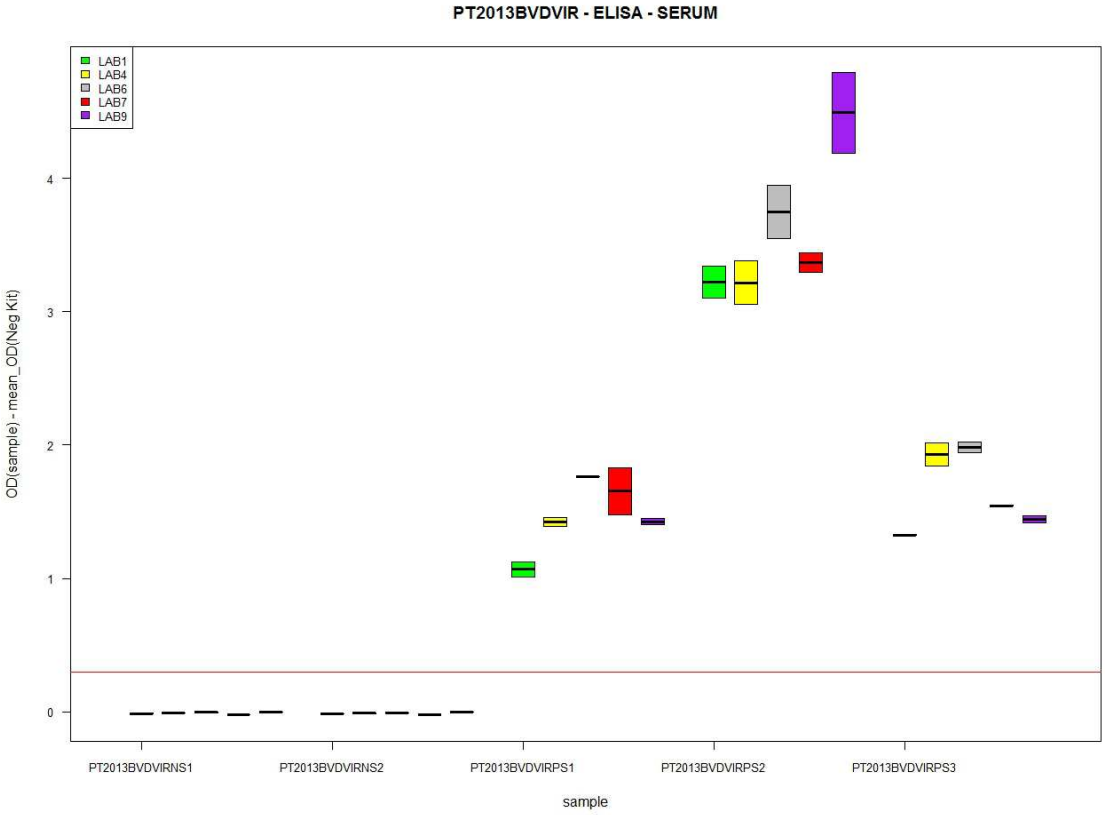

Figure 4. Box plots showing the normalized OD values per reference serum sample and per participating laboratory. Box plots represent the minimum value and the maximum value (only 2 alliquots per sample) and the median value (line).. The cut-off value of 0.3 is shown in red.

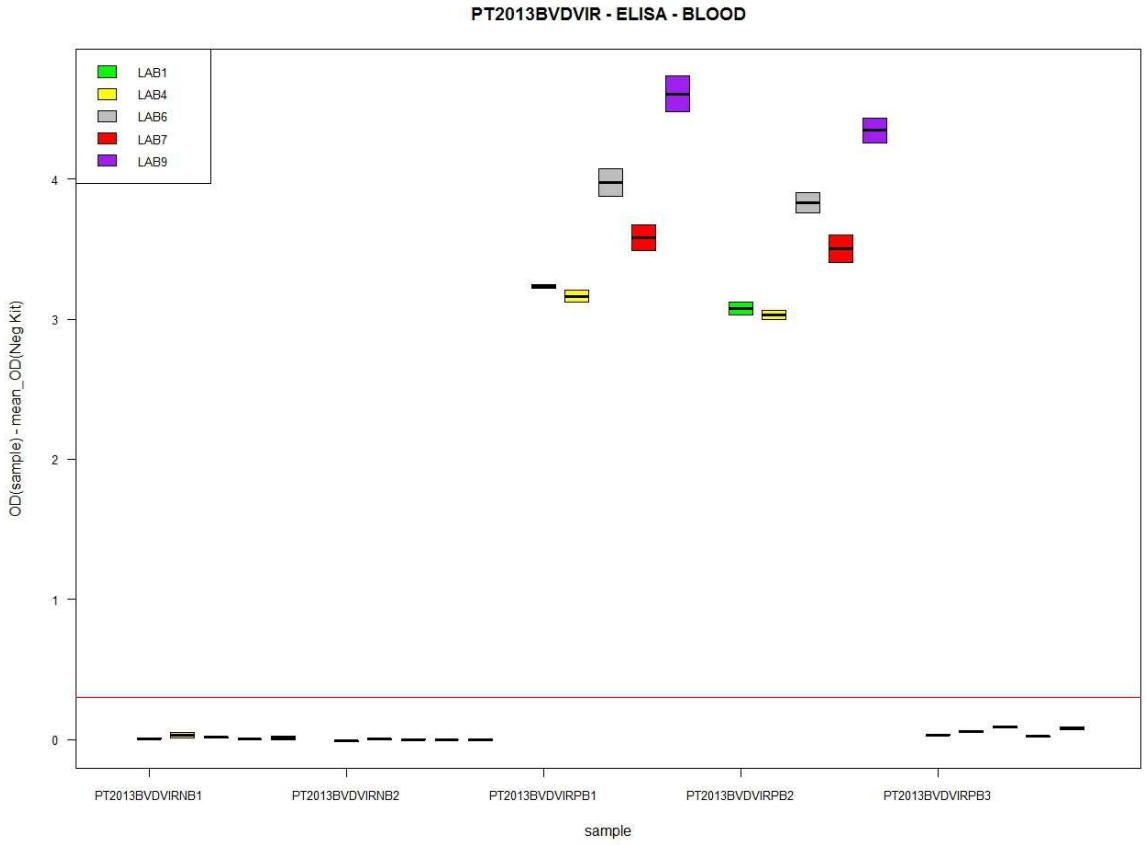

Figure 5. Box plots showing the normalized OD values per reference blood sample and per participating laboratory. Box plots represent the minimum value and the maximum value (only 2 alliquots per sample) and the median value (line).. The cut-off value of 0.3 is shown in red. The sample PT2013BVDVIRPB3 (1/50 dilution of PT2013BVDVIRPB2) was detected negative by all laboratories.

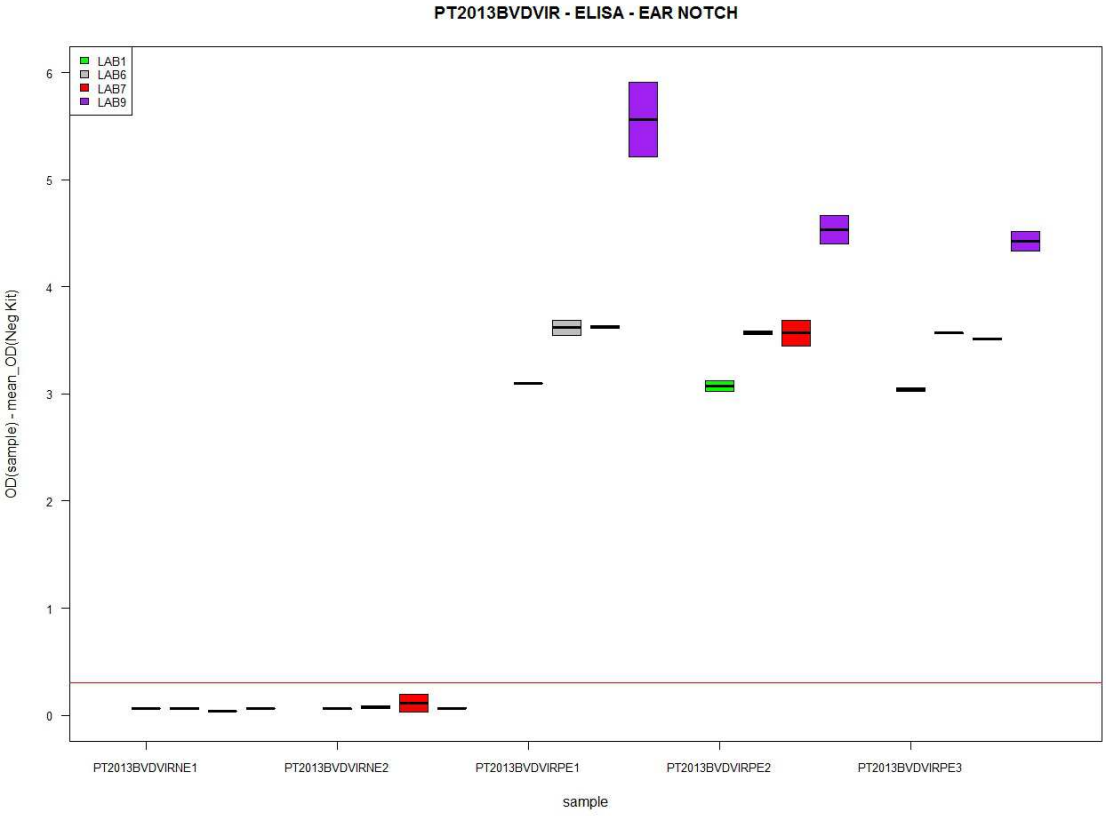

Figure 6. Box plots showing the normalized OD values per reference ear notch sample and per participating laboratory. Box plots represent the minimum value and the maximum value (only 2 alliquots per sample) and the median value (line). The cut-off value of 0.3 is shown in red.