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Five years' experience of classical swine fever polymerase chain reaction ring trials in France

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Summary
Since 2004, the French National Reference Laboratory for classical swine fever (CSF) has conducted an annual proficiency test (PT) to evaluate the ability of local veterinary laboratories to perform real-time polymerase chain reaction (PCR) for CSF virus. The results of five years of testing (2004–2008) are described here. The PT was conducted under blind conditions on 20 samples. The same batch of samples was used for all five years. The number of laboratories that analysed the samples increased from four in 2004 to 13 in 2008. The results of the PT showed the following: cross-contamination between samples and deficiencies in RNA preparation can occur even in experienced laboratories; sample homogeneity should be checked carefully before selection; samples stored at –80°C for several years remain stable; and poor shipment conditions do not damage the samples with regard to detection of CSF virus genome. These results will enable redesign of the panel to improve the overall quality of the PT, which will encourage laboratories to check and improve their PCR procedures and expertise. This is an excellent way to determine laboratory performance.

Keywords
Classical swine fever – Inter-laboratory comparison test – Laboratory diagnosis – Pestivirus – Quality management – Real-time polymerase chain reaction – Reference laboratory.

Introduction

Classical swine fever (CSF) is a major disease of pigs and wild boar (*Sus scrofa*) and is responsible for heavy economic losses in cases of outbreaks in domestic pig herds. Accurate and rapid diagnosis is essential for its effective control and eradication. The causative agent is a single-stranded RNA virus belonging to the genus *Pestivirus* in the *Flaviviridae* family. The presence of CSF virus in a sample can be revealed by enzyme-linked immunosorbent assay (ELISA) antigen-capture, virus isolation in cell culture and the fluorescent antibody test. However, these techniques have several disadvantages: the ELISA lacks sensitivity and specificity, the fluorescent antibody test requires a high level of technical skill, and virus isolation involves a long delay in response and identification of revivable viruses only (3). Over the past decade, routine diagnosis has been transformed by the introduction of the reverse transcription polymerase chain reaction (RT-PCR), which enables detection of the viral genome in organs or blood samples (7). The rapidity, sensitivity and specificity of RT-PCR and real time RT-PCR (rRT-PCR) have improved diagnostic procedures considerably in local laboratories.

An RT nested-PCR assay adapted from the method described by McGoldrick *et al.* (12) has been used since 2002 by the French National Reference Laboratory (NRL) to monitor the real-time evolution of CSF outbreaks in wild boar in northern France, using analysis of hunted wild boar carcasses (15). The benefits of the monitoring programme are that, in addition to rapidly providing knowledge of virus spread, it allows the carcasses that test negative to be released for commercial trade. As a first step in speeding up the programme the French Ministry of Agriculture, in accordance with the French NRL, asked

manufacturers of test kits to develop commercial rRT-PCR kits to provide an easier and more rapid protocol for obtaining results from virology analyses. The NRL was responsible for the validation of the kits. Two commercial kits were approved officially, in 2004 and 2005 (11). The second step taken to reduce the delay in obtaining results from the analyses of hunted wild boar was to transfer the diagnostic procedures from the NRL, which is located in Brittany in western France, to local veterinary laboratories in northern France, close to the infected area. This was done in 2004. One year later, other local laboratories in different parts of France were asked to become proficient in performing rRT-PCR for CSF so as to be suitably prepared in case of outbreaks in domestic pigs. All these local laboratories were approved by the Ministry of Agriculture as part of a CSF PCR diagnosis network. As required by French law, a proficiency test (PT), in which local laboratories must participate, was implemented by the NRL to guarantee assay quality and to assess the performance of the network in this official system of diagnosis. To date, the NRL has carried out one PT each year. Other laboratories involved in CSF diagnosis, such as NRLs of neighbouring countries, have been invited to participate in this PT.

The aim of this article is to explain how the PT is organised, from batch sample design to the evaluation of participating laboratories. The paper presents the experience and knowledge gathered in this field over five years and draws some conclusions related to the implementation of an improved PT for genome detection in the future. The PT performed by the NRL to evaluate the performance of local veterinary laboratories differs from most other PTs described in the literature. The latter were in fact designed to evaluate a method (2, 17). The PT described here is similar to the PT organised by the CSF European Union Reference Laboratory (EURL) in Hanover for Member States of the European Union and some third countries (6).

Materials and methods

Organisation

The participating organisations were local veterinary diagnostic laboratories, the NRL, kit manufacturers and NRLs from other countries. Their participation was sought and, following agreement, they were asked to register.

Given that no specific standard existed for setting up a virology PT, the NRL proceeded as it had done for many years in CSF serology, by following the French standard AFNOR NF U 47-400 (1). The international guideline ILAC-G13:2000 (8) was adopted in 2007 and 2008 because the European Standard ISO/IEC 17043 (5) was not yet available.

Participating laboratories received a panel of samples and had nine days to analyse them and return their results. This period was adopted to mimic routine conditions. Within one month, the results of the PT were communicated to the participating laboratories in a report that had been approved by the PT advisory board and discussed with the laboratory correspondent (a local laboratory veterinarian who specialised in PCR methods).

If the results obtained by the French local veterinary laboratories were as expected, they were submitted to the French Ministry of Agriculture for official approval. If they failed, the reasons for the failure were discussed with the NRL, and the laboratory usually took part in a second session within the same year and under the same conditions as the first session. Only the results of the first sessions are presented here.

Parameters tested in the proficiency test

To assess the sensitivity, specificity and repeatability of the technique implemented routinely in their laboratory, participating laboratories had to analyse 20 samples under blind conditions. The number of samples was selected to ensure adequate checking of the protocol characteristics at a reasonable cost. Sensitivity was assessed using nine positive samples, repeatability was checked by analysis of one of the positive samples that was considered to be at the minimal required detection level, repeated five times (samples 12 to 16), and specificity was assessed using eight negative samples. In addition, two random samples, chosen from among the 20 samples, were included in the panel to prevent collusion between participants.

Samples

Each batch consisted of samples of cell suspensions or organs of pigs which mimicked field samples (Table I). The same panel of samples was also analysed by the manufacturers of each kit for approval of each new batch of kits (data not shown).

The positive cell suspensions were obtained by amplifying different strains of CSF virus in PK15 cell cultures; subsequently they were harvested and diluted after three cycles of freezing and thawing. All strains used in the PT were chosen from the NRL collection to represent different genogroups. Field isolates from the last two outbreaks in French wild boar were included in the panel: samples 12 to 16 contained the Moselle strain isolated from the Thionville outbreak that was similar to the Rostock strain, and sample 18 contained the Bas-Rhin strain, which is similar to the Uelzen strain and is responsible for the current outbreak in northern France (15). The samples were diluted to 10^{-1} (sample 20) and

Table I

Characterisation of samples used in the proficiency test between 2004 and 2008: expected qualitative result in polymerase chain reaction for internal positive control and for the classical swine fever gene of interest, together with the rating and scoring system used in the assessment of these tests as described by Depner *et al.* (4)

The scores varied in accordance with how difficult a sample was to diagnose: correct and false test results for samples ‘pp’ and ‘nn’ were scored respectively as 1 and –3, correct and false test results for samples ‘p’ and ‘n’ were scored respectively as 2 and –2, correct and false test results for sample ‘b’ were scored respectively as 3 and –1. The random score was calculated as: (correct result score + wrong result score)/2. Sample 11a was used in 2004 and 2005 and sample 11b was used in 2008

Sample number	Type of sample	Expected PCR results		Ring test analysis			
		IPC	CSF	Difficulty rating	Correct result score	Wrong result score	Random score
1	Mesenteric lymph node of pig 2-20/07/04	+	–	nn	1	–3	–1
2	Spleen of specific-pathogen-free pig 8,315	+	–	nn	1	–3	–1
3	Spleen of conventional pig 1-20/07/04	+	–	nn	1	–3	–1
4	Spleen of specific-pathogen-free pig 8,330	+	–	nn	1	–3	–1
5	Inactivated border disease strain Aveyron	+	–	n	2	–2	0
6	Inactivated border disease strain I4E and porcine reproductive and respiratory syndrome strain I5C	+	–	n	2	–2	0
7	Inactivated border disease I4E, PRV II3M and PPV I2B strains	+	–	n	2	–2	0
8	Spleen of pig number 8,399 infected with Eystrup strain and sampled 48 h post infection	+	+	pp	1	–3	–1
9	Kidney of pig number 8,399 infected with Eystrup strain and sampled 48 h post infection	+	–/+	b	3	–1	1
10	Spleen of pig number 8,420 infected with Eystrup strain and sampled 24 h post infection	+	+	p	2	–2	0
11a	Mesenteric lymph node of pig 8,361 infected with Eystrup and sampled 48 h post infection	+	+	pp			
11b	Minimum essential medium Eagle	–	–	n			
12 to 16	Moselle CSFV strain (genogroup 2.3) dilution 10^{-4}	–	+	p	2	–2	0
17	Alfort CSFV strain (genogroup 1.1) dilution 10^{-4}	–	+	p	2	–2	0
18	Bas-Rhin CSFV strain (genogroup 2.3) dilution 10^{-4}	–	+	p	2	–2	0
19	Eystrup CSFV strain (genogroup 1.1) dilution 10^{-4}	–	+	p	2	–2	0
20	Paderborn CSFV strain (genogroup 2.1) dilution 10^{-1}	–/+	+	pp	1	–3	–1
Total score of the panel					**33**	**–36**	**–6**

CSF: classical swine fever
CSFV: classical swine fever virus
IPC: internal positive control
PCR: polymerase chain reaction
PPV: porcine parvovirus
PRV: pseudorabies virus
b: borderline sample, difficult to detect
n: CSF-negative sample
nn: negative sample, free of pestivirus genome
p: CSF= positive sample
pp: CSF= positive sample, very easy to detect

10^{-4} (samples 12 to 19). The samples were expected to be free of porcine cell RNA at a dilution of 10^{-4}, which constituted the internal positive control (IPC) of the two kits. For biosafety reasons, even though all the participating laboratories had protected facilities, the samples were inactivated by the addition of β-propiolactone (1/4,000) and checked for inactivation by virus isolation on PK15 cells. The liquid samples were divided into identical 250 µl aliquots and coded with a random number so that testing would be performed blind.

Positive organs were obtained from infected pigs, which were bred and infected in protected facilities at the French National Agency for Food, Environmental and Occupational Health and Safety (Anses). Details of the infection and the sampling protocol are given in Table I. Organs were selected when they tested positive on rRT-PCR and negative for virus isolation. The selected organs were divided into identical 100 mg portions and coded randomly in the same way as the liquid samples.

Cell suspensions that were CSF-negative were obtained in the same way but with strains of other viruses, such as porcine reproductive and respiratory syndrome virus, border disease virus and pseudorabies virus (Table I). Negative organs were sampled from non-infected pigs. Sample 11a was removed from the panel in 2006 because it had been depleted. Thus, there was no sample 11 in 2006 or 2007 and it was replaced in 2008 by sample 11b, which was a sample of minimum essential medium (MEM, Eagle with L-Glutamine, Lonza, Belgium), a liquid buffer that contained no biological material.

The samples were prepared in 2004 and stored at –80°C in the NRL facilities. All the samples were sent in dry ice by special delivery service to the participating laboratories, as stipulated in the biosafety regulations of the International Air Transport Association.

Quality control of samples

The quality of the samples and their preparation were verified by the NRL. Given that quality assurance according to standard international guideline ILAC-G13:2000 (8) was adopted in 2007, the homogeneity and stability of non-negative samples was checked fully in 2008. Homogeneity was verified by calculating the mean and standard deviation (SD) of the results obtained by each manufacturer with their own kit in previous years. Stability was checked by calculating the mean and SD of results from triplicates of each non-negative sample analysed by the NRL before shipment. Given that the two available kits provide qualitative results, homogeneity was considered to be acceptable if the mean cycle threshold value (Ct) was below the threshold and if the SD multiplied by 1.96 was less than 3 Ct. According to the Student's *t* table, this indicates that variation in the Ct, with an alpha risk of 0.05, was below 3, which was estimated to be the usual variation in the results for these PCRs. Stability was considered to be acceptable if the mean Ct of the triplicates was within the mean Ct calculated for homogeneity ± 3.12 SD. According to the Student's *t* table, this indicates that the Ct of these three samples, with an alpha risk of 0.05, did not differ from the mean Ct calculated for homogeneity.

A parallel experiment was carried out on samples other than those included in the PT to check the influence of storage conditions during shipment. Three eight-week-old specific-pathogen-free pigs were inoculated with 5 ml of 10^6 median tissue culture infective doses ($TCID_{50}$) of the Eystrup CSF strain (4 ml by the nasal and 1 ml by the oral route) and slaughtered two days post inoculation. The pigs were bled on the day of inoculation and just before euthanasia. The spleens of the pigs were sampled immediately after death, except that one pig (number 13) was exsanguinated, kept in the necropsy room at 20°C and sampled 29 h after death. The samples were collected without any particular handling precautions with respect to RNAse, to mimic field conditions. The spleen and blood samples were kept for several days at –20°C, 4°C and 22°C and analysed 0, 1, 2, 5, 13 and 19 days post collection. After 19 days kept at –20°C, four pieces of the frozen spleen of pig 8 were analysed. One of the samples was thawed and refrozen once before analysis, another twice, another three times and the other four times. The RNA was extracted with a commercial kit (RN-Easy; Qiagen, Venlo, the Netherlands) and CSF diagnosis was performed by rRT-PCR (Taqvet PPC®; Laboratoire Service International, Lyons, France), both according to the manufacturer's instructions.

In 2010, the NRL verified the accuracy of the minimal required level of detection (in the repeated samples, 12 to 16) by comparison of the mean Ct value to the minimal required detection level determined by the EURL for CSF in 2009.

Analytical methods

The participating laboratories had to work under routine conditions. Either of the two officially approved commercial kits could be used: Taqvet® or Adiavet® (Adiagène, Saint-Brieuc, France). Briefly, both commercial kits were ready-to-use 'real-time' one-step RT-PCR assays. Both tests were based on an initial reverse transcription of the CSF virus RNA into complementary DNA (cDNA), followed by cDNA amplification by PCR using primers specific for CSF virus and TaqMan probes labelled with FAM (fluorescein) dye. The mix also contained primers and probes labelled with VIC dye, which is specific to porcine cell RNA (the IPC), so that RNA extraction, reverse transcription and further DNA amplification could be checked. For each sample, the laboratories had to provide a Ct value for the gene of interest (CSF) and a Ct value for the IPC. It should be noted that the IPC could be negative in cell suspensions that were centrifuged and these could be considered to be almost non-cellular samples. The kits were designated A and B in the results to preserve confidentiality. One of the approved RNA extraction kits for these rRT-PCRs had to be used, i.e. RN-Easy or Total RNA isolation Nucleospin RNA II (Macherey-Nagel EURL, Hoerdt, France). All reagents had to be used according to the manufacturer's instructions. The same methods were used to characterise the samples as described above.

Analysis of results from the participating laboratories

The laboratories were judged both on their general conduct throughout the test and on their qualitative results. The following procedure was adopted. First, the laboratories were evaluated on criteria such as the time taken to respond, acknowledgement of sample receipt, accurate reporting of sample number, etc. Secondly, coherence between the Ct value and the conclusion submitted by the laboratory was checked, on the basis of the threshold values of the kit. Thirdly, the criteria of the method were evaluated. For sensitivity, the qualitative results of non-negative samples had to be positive. For repeatability, the repeated sample had to give the same qualitative results. For specificity, the qualitative results of the CSF genome-free samples had to be negative.

Other errors were simply pointed out and the laboratory was responsible for dealing with them. The Ct values obtained for the IPC were examined. They had to be below the threshold for organ samples and above the threshold for non-cellular samples. The quantitative Ct values were evaluated. To assess the accuracy of the analyses performed by the laboratories, the mean and SD of the Ct values obtained by all participants for each non-negative sample in each year were calculated and compared with the result for each participant. Reproducibility was determined from the coefficient of variation (CV), which was calculated by dividing the SD by the mean of the Ct values of replicate samples and multiplying the result by 100. Repeatability was determined in the same way, using the results for the repeated sample.

Finally, the qualitative results were subjected to the statistical analysis proposed by Depner *et al.* (4). All the samples (except sample 11, which did not provide consistent results over the five years) were qualified with letters and scores for correct or false results, with regard to the difficulty of producing a correct diagnosis. The annotation 'pp' corresponds to a positive sample that was very easy to detect (early Ct value, usually below 32) for which the correct result score for this sample is 1, the false result score is –3. The annotation 'p' corresponds to a positive sample (virus genome positive, Ct value usually between 32 and 38) for which the correct result score is 2 and the false result score is –2. The annotation 'b' corresponds to a borderline sample that was difficult to detect (late Ct value, usually above 38) for which the correct result score is 3, and the false result score is –1. The annotation 'n' corresponds to a negative sample (other pestivirus genomes) for which the correct result score is 2 and the false result score is –2. The annotation 'nn' corresponds to a negative sample (no virus genome) for which the correct result score is 1 and the false result score is –3. Sample 9, a kidney from a pig infected with the Eystrup strain and sampled 48 h post infection, was qualified as 'b', because of the late Ct values in the preliminary PCR performed by the NRL. Each sample was also assigned a random score, representing the mean score when test results are chosen at random, that was calculated by adding the 'correct result' and 'false result' scores and dividing by 2. These scores were used to calculate a total percentage score for each laboratory:

P = (total score obtained – random score) / (maximum total score – random score) × 100%

The maximum and random scores for the panel are shown in Table I.

Results

Sample quality

Homogeneity was considered to be acceptable for each non-negative sample except sample 9. The SD was 1.79 and 1.98 for spleen samples 8 and 10, respectively, and the SD varied between 0.76 and 1.71 for non-cell suspensions. For sample 9, the results were either positive or negative and the SD, calculated on positive results, was 3.7. Stability was considered to be acceptable in 2008 (data not shown).

Except for spleens that were kept at 22°C, the Ct values for the gene of interest remained almost the same from collection to 19 days post collection (Table II). The variation was less than 3 Ct, measured on the same run, between the first and last results, even if the organ was not sampled immediately after death. For spleens stored at 22°C, the results differed depending on the pigs. In one, the Ct value was similar to that obtained when analysed on day 0. In another, the Ct value was obtained later. Endogenous RNA was more degraded than CSF viral RNA at room temperature. Indeed, the Ct values for the IPC were obtained later on day 13 than on day 5. Freezing and thawing cycles had no effect on the results: the Ct values obtained from the spleen of pig 8 were respectively 31, 30, 30 and 30 after one, two, three and four freezing and thawing cycles for the gene of interest and 21, 20, 21 and 20 for the IPC.

For a given run, the mean Ct value for the repeated samples (i.e. 12 to 16, dilution 10^{-4} of the Moselle strain, considered as the minimal required detection level of the NRL) was 32.3, and the Ct values for the two minimal detection levels required by the EURL (16) were 33.4 and 35.4.

Results from the participating laboratories

The same batch was analysed for five years between 2004 and 2008. The number of laboratories that participated ranged from four in 2004 to 13 in 2008 (see Table III). The NRL and a kit manufacturer also participated every year. The other participants were local veterinary laboratories. In 2007 and 2008, one and then two European NRLs also took part. During the five years, 21 panels were analysed with kit A and 26 panels with kit B.

During the same period, two laboratories were cautioned because they sent their results after the deadline, two laboratories did not return the attestation sheet stating that the assays had been done in their laboratory, and one had to be reminded to register. Two laboratories drew a false conclusion with regard to the Ct value and the threshold of the kit.

Seven true positive samples were analysed to test sensitivity in 47 assays. Four laboratories found 14 false negative results, 12 of which were on cell suspensions (Table IV). Two laboratories had several false negatives in the same batch, and one laboratory had five negative

Table II
Evolution of the cycle threshold value, according to storage temperatures, in blood and spleen of pigs sampled immediately after death, except for pig 13, sampled 29 hours after death
Cycle threshold (Ct) values are given for the gene of interest (classical swine fever) and the internal positive control (in brackets)

Sample	Storage temperature	Pig identification	Ct values					
			0 dps	1 dps	2 dps	5 dps	13 dps	19 dps
Blood	–20°C	08		39 (23)	39 (23)	39 (23)	37 (22)	38 (23)
		13		36 (23)	37 (25)	38 (23)	35 (22)	35 (23)
	4°C	08		39 (21)	37 (21)	37 (22)	37 (23)	39 (25)
		13		35 (21)	35 (21)	35 (22)	35 (23)	34 (24)
	22°C	08	38 (20)	37 (21)	37 (21)	38 (23)	37 (27)	36 (27)
		13	35 (20)	34 (21)	34 (21)	36 (22)	35 (26)	36 (28)
		36	36 (20)					
Spleen	–20°C	08		31 (18)	31 (19)	30 (18)	30 (18)	31 (21)
		36		32 (20)	32 (19)	31 (18)	31 (18)	30 (18)
		13		31 (22)	31 (22)	34 (25)	31 (24)	
	4°C	08	29 (18)	32 (18)	30 (19)	31 (19)	30 (21)	
		36		31 (17)	32 (18)	31 (18)	31 (19)	31 (21)
		13			32 (23)	32 (22)	33 (24)	32 (25)
	22°C	08	31 (18)	30 (18)	29 (19)	31 (20)	31 (23)	36 (26)
		36	31 (18)	31 (18)	32 (19)	32 (20)	32 (24)	32 (25)
		13			31 (23)	38 (30)	ND	ND

dps: days post sampling ND: not done

Table III
Distribution in each year of laboratories according to the score calculated using the statistical analysis proposed by Depner *et al.* (4)

Year	Number of laboratories	
	Participating	With a score less than 100%
2004	4	2
2005	7	3
2006	11	0
2007	12	5
2008	13	8

Table IV
Results of detection of classical swine fever genome using commercial kits A and B in organ and cell suspensions
Number of false positive (F+) results or false negative (F–) results (with kit used in brackets) in 2005 and 2008. There were no false negative or positive results in 2004, 2006 and 2007

Laboratory	Type of sample	2005	2008
A	Organ		1 F+ (B)
	Cell suspension		1 F+ (B)
B	Organ	1 F+ (B)	
C	Organ		3 F+ (B)
D	Cell suspension		3 F– (A)
E	Cell suspension	1 F– (A)	
F	Organ	1 F– (A)	
	Cell suspension	8 F– (A)	
G	Organ		1 F– (A)

results for the repeated sample. For sample 9, 86% of the results were positive with kit A and 35% were positive with kit B. Only one laboratory had difficulties with repeatability for samples 12 to 16 in 2008. One repetition out of five was found to be negative.

Seven samples negative for the CSF virus genome were analysed to test the specificity in 47 assays. Three laboratories found six false positive results, five of which were from organ samples (Table IV). One laboratory had three false positive results in the same batch.

The IPC for samples of pure cell suspensions was found to be positive in 75% and 99% of the results with kit A and kit B, respectively. The IPC of cell suspension samples diluted to 10^{-1} was found to be positive in 14% and 69% of the results with kit A and kit B, respectively. The IPC of cell suspension samples diluted to 10^{-4} was found to be positive in 4% and 14% of the results with kit A and kit B, respectively.

The Ct values for samples 8, 10, 12 to 16, and 17 to 20 are presented in Table V. The CV varied between 0.0% and 8.9%. The intra-laboratory CV, calculated each year for each laboratory on the repeated samples 12 to 16, varied between 0.0 and 7.9 (data not shown).

The scores obtained by the laboratories, calculated according to the method of Depner *et al.* (4), were between 68% and 100%. The number of laboratories scoring less than 100% in any given year is shown in Table III.

Table V
Quantitative results obtained for positive samples. Calculated cycle threshold value of the polymerase chain reaction for classical swine fever (classical swine fever, gene of interest)

Sample		Kit A				Kit B				
		2005	2006	2007	2008	2004	2005	2006	2007	2008
8: spleen of pig number 8,399 infected with Eystrup strain and sampled 48 h post infection	n	4	5	6	6	4	3	6	6	7
	m	29.0	31.7	30.5	30.8	30.6	29.3	30.9	29.7	30.2
	sd	0.8	1.8	1.8	1.6	2.2	1.5	1.2	0.9	0.6
	CV	2.8	5.6	5.8	5.1	7.1	5.2	4.0	3.1	2.1
10: spleen of pig number 8,420 infected with Eystrup strain and sampled 24 h post infection	n	3	5	6	5	4	3	6	6	7
	m	34.3	37.6	35.8	36.6	35.6	33.7	35.3	35.4	35.7
	sd	3.1	1.2	1.7	1.6	0.7	1.2	1.7	1.0	1.7
	CV	8.9	3.2	4.7	4.3	2.1	3.4	4.9	2.8	4.6
12 to 16: Moselle CSFV strain (genogroup 2.3) dilution 10^{-4}	n	4	5	6	6	4	3	6	6	7
	m	35.6	33.0	33.7	35.0	35.6	34.1	34.5	35.6	35.7
	sd	0.8	0.6	0.5	1.1	0.9	0.5	0.6	0.8	0.6
	CV	2.3	1.9	1.5	3.1	2.5	1.6	1.8	2.2	1.8
17: Alfort CSFV strain (genogroup 1.1) dilution 10^{-4}	n	2	5	6	6	4	3	6	6	7
	m	30.5	34.0	34.7	35.1	37.1	35.0	35.4	36.0	35.0
	sd	0.7	1.5	1.8	2.4	1.3	0.0	1.9	1.0	1.2
	CV	2.3	4.3	5.3	6.9	3.5	0.0	5.4	2.7	3.4
18: Bas-Rhin CSFV strain (genogroup 2.3) dilution 10^{-4}	n	3	5	6	6	4	3	6	6	7
	m	34.0	34.6	35.6	35.2	36.3	35.3	34.0	35.2	35.6
	sd	2.6	1.0	2.4	2.6	1.0	2.3	1.4	1.1	1.2
	CV	7.8	3.0	6.7	7.4	2.7	6.5	4.2	3.2	3.4
19: Eystrup CSFV strain (genogroup 1.1) dilution 10^{-4}	n	3	5	6	6	4	3	6	6	7
	m	30.7	32.1	33.4	32.4	35.4	34.0	33.3	34.7	34.0
	sd	2.1	1.2	2.0	1.9	1.7	0.0	0.9	0.6	0.8
	CV	6.8	3.6	6.1	6.0	4.8	0.0	2.7	1.6	2.4
20: Paderborn CSFV strain (genogroup 2.1) dilution 10^{-1}	n	4	5	6	6	4	3	6	6	7
	m	23.3	23.8	24.3	23.8	25.8	24.7	24.6	25.2	25.1
	sd	1.0	1.0	1.8	0.9	1.8	0.6	1.2	0.8	1.2
	CV	4.1	4.3	7.4	3.9	7.2	2.3	5.1	3.1	4.9

CSFV: classical swine fever virus
CV: coefficient of variation
m: mean of cycle threshold values
n: number of participating laboratories
sd: standard deviation

Negative results for sample 9 (low positive spleen) were responsible for 13 of the 15 scores of 89%.

Discussion

The laboratories that obtained negative results for expected positive samples had, each time, a problem with the extraction procedure. After discussing the protocols employed, all laboratories obtained the correct results, except for laboratory G, which repeated its mistake and required a third panel of organs to solve its problem (data not shown). False negative samples caused by poor extraction technique are difficult to detect on cell suspensions, which are centrifuged and are almost non-cellular samples. Indeed the IPC, which is a control that aids the detection of PCR inhibition or extraction errors, is commonly negative and cannot reveal a problem with extraction. It can be assumed that obtaining a negative IPC in routine analyses of cellular samples would prompt the laboratory to repeat the assay. It should be stressed that even if cell suspensions are easier to prepare than infected organs for the PT, organs must be included in the batch, especially when this type of IPC, internal to the porcine cell, is used. More generally, the different categories of matrix analysed under the conditions of field diagnosis need to be represented in the batch of samples, to check the efficacy of the laboratory's extraction procedure for each biological matrix. For this reason, blood samples, which are used in the epidemiological surveillance of French domestic pigs sampled in slaughterhouses, will be added to the next panel.

Both PCR kits had previously been demonstrated to be highly specific (11), and any false positive results on samples free of the CSF viral genome were likely to be due to cross-contamination between samples, as already described (13, 14). The samples were prepared while taking great precautions to avoid cross-contamination, i.e. intensively decontaminating the facilities and using separate preparation times for each sample. Thus it is assumed that any contamination occurred while the assays were being run in local laboratories. France is CSF-free for domestic pigs, and wild boar samples from the outbreak in northern France were analysed in only two laboratories. One of them gave a false positive result. It can be assumed that the other laboratory facilities are free of CSF virus and any cross-contamination can only occur between the PT samples or with the internal positive control. Laboratory C had three false positive results in the same batch in 2008, after several years of practice. These results confirm the need, even among trained staff, to preclude this sort of accident by introducing a negative control between samples during the assay (14). However, it should be noted that the viral genome load of some positive PT samples is higher than under field conditions. This also underlines the need to include several negative samples in the panel.

Unexpected positive results were observed for the IPCs. The cell suspensions were centrifuged and diluted and were expected to be free of cellular RNA, which constitutes the IPC of the kits. However, the Ct value for the IPCs of the diluted samples was often positive. Initially, the positive IPCs were assumed to have resulted from cross-contamination, but after several sessions it was evident that cellular RNA was present in the samples, even if the IPCs were more often negative at higher dilution. A sample of MEM was introduced in 2007 to test the ability of the laboratories to detect genome-free samples. Some laboratories were surprised by this kind of sample, which mimicked an inhibited sample. However, laboratories are sometimes confronted with dubious samples, such as swabs with no biological material, and must be able to investigate them. In 2008, laboratory F found a positive IPC for this sample, which confirmed the importance of including an MEM sample in a panel analysed with this kind of PCR, in which the IPC is derived from the sample.

Several bioethical issues had to be considered during the preparation of the panel for the PT. Positive organ samples were not easy to obtain because, in accordance with biosafety regulations, only samples negative on viral isolation should be sent to participants. The organs of an infected animal can be selected at the very beginning and very end of viraemia. At these stages, the spleens of animals are positive for PCR with some strains of CSF, but negative on viral isolation (11). However, it is very difficult to determine the exact time of occurrence of these stages of infection and the best time for euthanasia. It is also not possible, for ethical reasons, to kill a huge number of pigs in order to select the right organ. One solution is to use the 'Chinese' vaccination strain (9) to infect the pigs. This strain does infect pigs but the infection does not spread between animals (10), so biosafety is not a problem. However, this vaccine strain is also hard to detect in blood or organs owing to the low level of replication in pigs. An alternative solution, as performed initially in chemical PTs, is to crush the negative organs, spike them with an inactivated strain and freeze them. This option will be tested in the next panel.

The assay carried out by the NRL to investigate appropriate storage conditions demonstrated the good stability of the samples with regard to detection of the CSF virus genome. These results have been confirmed by others (13, 18). Thus, the PCR results were unaffected even if the recommended transport temperature was not respected. Although the aim of this PT was not to determine the assigned values of the samples but rather the laboratory's capacity to perform an rRT-PCR for CSF virus under good conditions, the participating laboratories like to be aware of the performance of the other participants and the assigned value was determined for their information. The results of these five successive PTs demonstrate the excellent reproducibility of the laboratories. The Ct values are constant and show little variation. These results also indicate that samples stored at –80°C for five years are very stable and that this kind of panel can be prepared in advance for use over several years. However, if the batches are prepared in advance, the stability of the samples must be checked before shipping.

Sample 9, from the kidney of a pig infected with a small quantity of viral genome, revealed a problem with detection. This sample, probably because the viral genome load was too low, gave 55% negative results and 45% positive results. At batch conception this sample was expected to be positive, but it was then considered to be inconstant and the negative results were not considered false results. The assay carried out by the NRL on storage conditions eliminated the hypothesis of genome degradation and the calculation for the estimation of homogeneity, included for the last session, confirmed a problem with homogeneity. Sample homogeneity, which gives a consistent result, whether positive or negative, needs to be checked meticulously when an organ is selected. This also shows that it serves no purpose to include a sample in a batch if its results are not reliable. Finally, this example underlined the limits of detection of both kits.

Comparison of the Ct values obtained for the repeated sample (samples 12 to 16, dilution 10^{-4} of the Moselle strain) and for the sample considered as the minimum required detection level determined by the EURL in Hanover (16) showed that the threshold determined by the NRL fits with the threshold of the EURL.

Many laboratories did not obtain a score of 100% using the scoring system of Depner *et al.* (4) because of the difficulty in detecting the viral genome in sample 9, which was caused by its very low viral load. This scoring system provides a rapid assessment of the overall ability of a laboratory to perform a technique. In this case, it demonstrates the generally satisfactory performance level of the participating laboratories but also highlights the difficulties encountered by each laboratory, even those used to implementing this kind of assay, in maintaining a top level of performance in a technique that requires considerable rigor and precision. Laboratories can be assisted in checking the absence of contamination by the inclusion of internal negative controls.

The PT also confirmed the good specificity and sensitivity of the two commercial kits, which produced consistent results throughout the five years, even with different batches. This finding was also confirmed by other results, obtained by the NRL and the manufacturers, when the same panel was analysed for the approval of new batches (data not shown). These results and the calculated CV also showed that the Ct values obtained in different runs were consistent and reproducible.

Conclusion

In conclusion, several goals can be attained by organising a PT every year in the manner described herein. Laboratory participation is essential, for both local and national establishments, and regardless of whether they practise the technique routinely or only occasionally. The laboratories are thus able to analyse positive samples, which is rare in CSF-free countries, and to check their procedures, because the PT has to be performed under routine conditions. Local French laboratories are required by French law to participate, in order to obtain approval. Moreover, all laboratories with quality management systems have to validate their diagnostic tests by taking part in PTs. This PT also enabled the NRL to learn more about the CSF diagnosis network, and about the staff of the local laboratories and their way of working. Even if the PT does not correspond exactly to the regulatory conditions, because the samples have no history, it shows how the different laboratories would perform when faced with a similar situation and determines the reliability of their results.

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Essais inter-laboratoires d’aptitude pour l’utilisation de la réaction de polymérisation en chaîne pour détecter la peste porcine classique : une expérience de cinq ans en France

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Résumé

Depuis 2004, le Laboratoire national de référence français pour la peste porcine classique (PPC) réalise annuellement un essai inter-laboratoires d’aptitude (EILA) visant à évaluer l’aptitude des laboratoires vétérinaires locaux à utiliser la réaction de polymérisation en chaîne (PCR) en temps réel pour détecter le virus de la PPC. Les auteurs présentent les résultats de cet EILA sur une période de cinq ans (de 2004 à 2008). L’EILA a porté sur 20 échantillons testés en aveugle. La même série d’échantillons a été utilisée durant les cinq années. Le nombre de laboratoires participant à l’EILA est passé de 4 en 2004 à 13 en 2008. Les EILA ont

fait ressortir les conclusions suivantes : le risque de contamination croisée entre échantillons et la mauvaise préparation des ARN existe, y compris dans les laboratoires expérimentés ; le choix des échantillons doit être précédé d'une vérification rigoureuse de leur homogénéité ; les échantillons stockés à une température de –80 °C restent stables pendant plusieurs années ; les conditions de transport médiocre n'endommagent pas les échantillons pour ce qui concerne la capacité de détection du virus de la PPC. Ces résultats permettront de concevoir un nouvel éventail d'échantillons de manière à améliorer la qualité globale de l'EILA, ce qui encouragera les laboratoires à vérifier et à améliorer leurs procédures et leurs compétences en matière de PCR. Il s'agit d'une excellente méthode pour évaluer les performances des laboratoires.

Mots-clés
Diagnostic de laboratoire – Essai inter-laboratoires d'aptitude – Gestion de la qualité – Laboratoire de référence – Peste porcine classique – Pestivirus – Réaction de polymérisation en chaîne en temps réel.

Cinco años de experiencia de pruebas interlaboratorios en Francia con la reacción en cadena de la polimerasa para detectar la peste porcina clásica

F. Pol, M. Le Dimna & M.-F. Le Potier

Resumen
Desde 2004, el Laboratorio de Referencia francés para la peste porcina clásica (PPC) viene realizando cada año una prueba de competencia destinada a evaluar la aptitud de los laboratorios veterinarios locales para aplicar la técnica de reacción en cadena de la polimerasa (PCR) en tiempo real para la detección del virus de la PPC. Los autores exponen los resultados de las pruebas de competencia efectuadas durante cinco años (2004–2008), en condiciones de anonimato y utilizando un mismo lote de 20 muestras. El número de laboratorios que analizaron las muestras fue en aumento, desde cuatro en 2004 hasta trece en 2008. Del proceso se desprende lo siguiente: la contaminación cruzada entre muestras y las deficiencias en la preparación del ARN pueden darse incluso en laboratorios avezados; conviene comprobar cuidadosamente la homogeneidad de las muestras antes de la selección; las muestras conservadas varios años a –80°C se mantienen estables; y el envío en condiciones deficientes no afecta a las muestras por lo que respecta a la posibilidad de detectar en ellas el virus de la PPC. Estos resultados servirán para redefinir la composición de la batería de muestras y mejorar así la calidad general de las pruebas de competencia, lo que a su vez alentará a los laboratorios a comprobar y perfeccionar sus protocolos y competencias técnicas para la realización de PCR. Se trata de un medio excelente para evaluar la competencia de los laboratorios.

Palabras clave
Diagnóstico de laboratorio – Gestión de la calidad – Laboratorio de referencia – Peste porcina clásica – Pestivirus – Pruebas de comparación entre laboratorios – Reacción en cadena de la polimerasa en tiempo real.

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