

Quality management in reference tests for the diagnosis of classical swine fever

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Summary

Inter-laboratory comparison tests for the diagnosis of classical swine fever (CSF) have been established by the national swine fever laboratories of European Union (EU) Member States. They provide a method of measuring both the quality of the results of diagnostic tests performed by laboratories and the competence with which they were performed. The objective is that all laboratories obtain the same result when investigating the same sample. This study evaluates the results of serological and virological reference tests for CSF (neutralisation test and virus isolation) performed over a period of three years.

The sensitivity of the serological diagnosis for the detection of CSF antibodies was very good and revealed a tolerance limit of the scored antibody titres of one dilution step. Results on the same sample in two consecutive years were similar. The variation of the scored antibody titres was larger when testing sera with a low CSF antibody titre. The interpretation of the antibody titres as 'CSF positive or negative' was only slightly altered by these variations. The backtitration of a neutralisation test (used as a control measure) is a more mathematical value which does not correlate directly with the biological system. Commercial CSF antibody enzyme-linked immunosorbent assays still display a lower sensitivity on individual samples compared to the reference neutralisation test.

Classical swine fever virus isolation was well established in all participating laboratories and caused very few problems. Specificity of CSF diagnosis by investigating CSF antibody and CSF virus negative sera was not problematic either.

In general, the reference tests for CSF diagnosis are well established in the EU. They are based on living systems, e.g. cells and virus, and consequently they have a different tolerance limit than pure mathematical values. What is important is that the interpretation of the test result is identical in all laboratories.

Keywords

Accreditation – Backtitration – Classical swine fever – Diagnosis – Neutralisation test – Quality management – Reference test – Tolerance limit – Virus isolation.

Introduction

Quality management systems (e.g. accreditation according to DIN EN ISO/IEC 17025 [3]) are becoming more and more important for veterinary diagnostic laboratories. In these systems one important factor is to validate test systems and compare results with other laboratories. This can be achieved by organising and/or participating in

inter-laboratory comparison tests, also known as 'proficiency testing'. The objective is that all laboratories obtain the same result when investigating the same sample.

The European Union (EU) Reference Laboratory (EURL) for classical swine fever (CSF) has been organising inter-

laboratory comparison tests since 1985 (6), long before the implementation of quality management systems became common among veterinary diagnostic laboratories. The participants are all national swine fever laboratories (NSFL) of EU Member States, including, since 1996, the NSFLs of most of the countries who have joined the EU recently (5). Three non-EU countries also participate regularly on a voluntary basis. The NSFLs are in charge of the CSF diagnosis in their country and should be able to perform the reference tests (1, 14), e.g. the neutralisation test (4, 11) for serological diagnosis, and virus isolation (8) so that a virological diagnosis can be made. Furthermore, they are responsible for the licensing of commercial test kits, i.e. antigen and antibody enzyme-linked immunosorbent assays (ELISAs) and they therefore need to perform the reference tests mentioned above to validate commercial ELISAs (2, 6).

The first inter-laboratory comparison tests in 1985 started with serological diagnosis by distributing five lyophilised pig sera with unknown antibody status to each of the participating laboratories. The test was extended in 1997 to include the detection of CSF virus (CSFV). In 2000 the number of sera was increased to six. The results obtained in the EURL and in those countries which participated regularly during the years 1999-2001 (a total of eighteen laboratories) will be evaluated here. The objective of this study is to give practical advice on tolerance limits and the interpretation of CSF reference tests for quality management. Statistical calculations will be published elsewhere (Floegel-Niesmann, in preparation).

Material and methods

In 1999 five coded pig sera were distributed to each participating laboratory (in 2000 and 2001 this number was increased to six). The serum panel usually included one negative pig serum, several CSF antibody positive pig sera, 'non CSF pestivirus' antibody positive pig sera and one serum containing CSFV (this was usually serum from a pig which had died of acute CSF, since international shipment of fresh organ samples from infected pigs is quite difficult).

All diagnostic tests available in the participating laboratory, or at least the reference tests, e.g. virus isolation and the neutralisation test (1, 2, 6), had to be performed. Apart from the CSF antibody titre, expressed as Neutralising Dosis₅₀ (ND₅₀ log₁₀), the laboratories were asked to provide information concerning cell culture, the test virus and the conjugates used. The samples were to be treated in the same way as routine diagnostic samples, thus making the result representative of a routine test. A certain number of repetitions was not requested. A period of three months was given to report the results to the EURL.

Out of all the sera tested over the three years, six were chosen for evaluation in this study (they represented different categories: low-, medium- and high CSF antibody titres as well as sera containing CSFV). A survey of the CSF antibody titres of the sera is presented in Table I. In order to allow a laboratory to follow up its sensitivity over several years, two sera were included several times.

Table I
Neutralising antibody titres (log₁₀ ND₅₀) of sera chosen for evaluation as determined by the European Union Reference Laboratory for classical swine fever (1999-2001)

Year	CSFV used for neutralisation test	Serum					
		Z	Y	X	W	V	U
1999	CSF0902	1.3	1.9			< 0.7*	
	Homologous virus	1.6	2.5			< 0.7*	
2000	CSF0902	1.3-1.6	1.9	2.38			
	Homologous virus	1.6	2.5-2.8	3.1			
2001	CSF0902	1.6			1.17		0.7*
	Homologous virus	1.9			2.38		0.7*

CSFV: classical swine fever virus

* sera containing CSFV were included in 1999 and 2001

Test sera

Apart from a negative control serum, all the sera used for the inter-laboratory comparison tests were produced experimentally by inoculating pigs at the EURL. Classical swine fever virus isolates from recent epidemics in EU Member States were chosen to inoculate the pigs rather than older reference virus strains. All CSFV are listed in the database at the EURL (9). Classical swine fever 0123 was isolated from domestic pigs during an epidemic in Germany in 1995. Classical swine fever 0573 was isolated from domestic pigs and wild boar in Italy in 1998. Classical swine fever 0277 caused the epidemic in domestic pigs in 1997 in Germany, the Netherlands, Spain, Belgium and Italy. Data on the sera concerning the age of the pigs, the inoculum and the number of days post-infection when the serum was collected are presented in Table II. The sera were not diluted or treated in any other way other than being lyophilised for shipment.

Youden plot

The Youden plot (19) was chosen as a means of evaluating the scored CSF antibody titres. This method has been used previously (17) to evaluate inter-laboratory ELISA results for other diseases. It allows a laboratory to see whether an error is systematic (sensitivity too high or too low) or random. A result obtained by a laboratory on one sample was plotted with respect to the result it obtained on a similar sample. Scores in the upper right or lower left part

Table II
Data on the experimentally produced pig sera used in inter-laboratory comparison tests

Characteristic	Serum					
	Z	Y	X	W	V	U
Inoculum	CSF0123	CSF0123	CSF0123	CSF0573	CSF0123	CSF0277
Age of pig	Adult	Adult	Adult	Four months	Weaner	Weaner
Collection of serum (days post infection)	16	21	43	26	14	16
CSFV titre TCID ₅₀ /ml					10 ^{3.7}	10 ^{3.5}

CSFV: classical swine fever virus
 TCID: tissue culture infectious doses

of the chart indicate a systematic error because two values are out of limit. Scores in the lower right and upper left part of the chart indicate random errors, because only one score is out of the limit. In the Youden plot analysis the ND₅₀ log10 scored by the EURL is marked by a fat line and the tolerance limit of plus/minus one dilution step (twofold = 0.3 log10) is marked by thin lines. Sera with low, medium and high CSF antibody titres were chosen for evaluation by a Youden plot. Sera tested in two consecutive years were also evaluated in this way.

Results

Results of the inter-laboratory comparison test are evaluated each year at the annual meeting of NSFLs. Reports on these meetings are made available by the EU Commission, but the participating laboratories remain anonymous in order to guarantee confidentiality. Most laboratories used the neutralisation peroxidase-linked assay (4), but five laboratories used the neutralising immuno-fluorescence test (11). Porcine kidney 'PK15' cell cultures were used in all but two laboratories, which used sheep kidney 'SK6' cells instead. Reference virus strain CSFV 'Alfort 187' (7) now registered as CSF0902 (9) was used in all but one laboratory. A CSF antibody titre of 10 ND₅₀ (1 log10) is regarded as 'CSF antibody positive' according to EU legislation (1, 2, 6). This is the critical point in interpreting a result as either 'CSF antibody positive or negative'. Antibody titres in the neutralisation test are determined by using two-fold dilutions with an initial dilution of 1:5 (4).

Youden plot analysis

In 1999 the sera evaluated were serum Z, with a low antibody titre, and serum Y, with a medium antibody titre (Fig. 1). One participating laboratory scored in the lower

left field, revealing a systematic error in sensitivity. Two laboratories scored in the upper right field. Most laboratories scored within the tolerance limit of one dilution step.

In 2000, the sera evaluated were serum Z, with a low antibody titre, and serum X, with a high antibody titre (Fig. 2). One laboratory had a random error scoring in the upper left field. Three laboratories scored in the upper right field. The majority of laboratories scored within the tolerance limit of one dilution step.

In 2001, the sera evaluated were serum W and serum Z, both with low CSF antibody titres (Fig. 3). It is noteworthy that in this case the tolerance limit of one dilution step reaches underneath the threshold of 1 log10, where the serum is interpreted as CSF antibody positive (1, 2, 6). One laboratory failed on serum Z by dropping below 1 log10 ND₅₀, but scored serum W correctly. Three laboratories had a reduced sensitivity for serum W, scoring antibody titres below 1 log10 ND₅₀, whereas serum Z was still interpreted as CSF antibody positive (≥ 1 log10 ND₅₀). Two laboratories scored in the upper right field. In general the results from evaluation of two sera with a low CSF antibody titre are much more wide-ranging across the chart than compared to the sera with medium and high antibody titres (Figs 1 and 2).

In 1999 and 2000 the results obtained for serum Y with a medium CSF antibody titre were evaluated (Fig. 4). One laboratory had a reduced sensitivity in 1999, but scored correctly in 2000. Only two laboratories scored below the tolerance limit.

In 2000 and 2001 the results obtained for serum Z with a low antibody titre were evaluated (Fig. 5). In 2001 one laboratory had a reduced sensitivity, with its score dropping below the threshold of 1 log10 ND₅₀ and two laboratories scored below the tolerance limit, but still above the threshold. No laboratory had a reduced sensitivity over both years, e.g. there were no scores in the lower left field. In comparison with serum Y, used in 1999 and 2000, the scores here are much more wide-ranging across the chart.

Internal controls

When evaluating the influence of the backtitration (the real amount of test virus used, determined as tissue culture infectious doses₅₀ [TCID₅₀]) the majority of laboratories scored 100 TCID₅₀ (6, 14). However, the scored CSF antibody titres varied within the tolerance limit indicated above, although the backtitration was identical (Fig. 6). If a higher TCID₅₀ in the backtitration was detected the antibody titre scored was still within this tolerance limit.

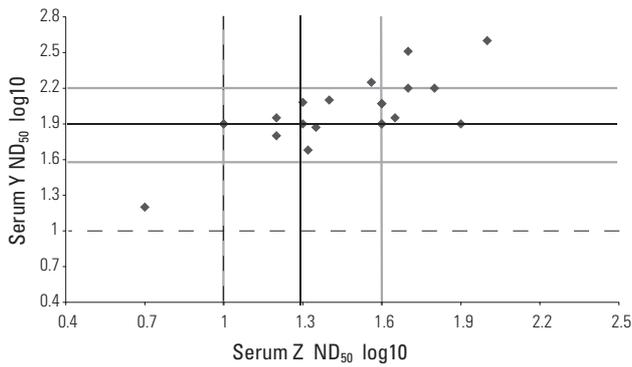


Fig. 1
Youden plot analysis of the results scored on two classical swine fever antibody positive sera with different ND₅₀ log₁₀ against CSF0902: serum Z against serum Y in 1999

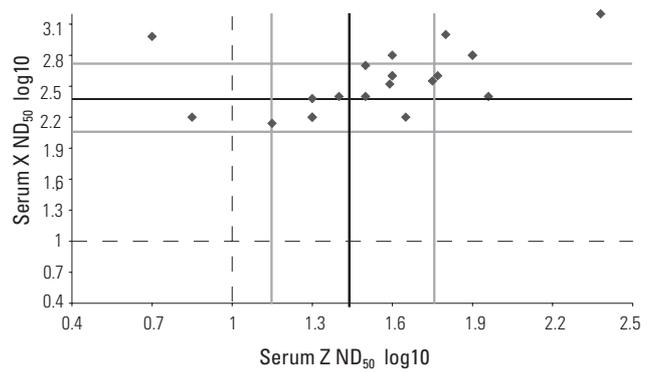


Fig. 2
Youden plot analysis of the results scored on two classical swine fever antibody positive sera with different ND₅₀ log₁₀ against CSF0902: serum Z against serum X in 2000

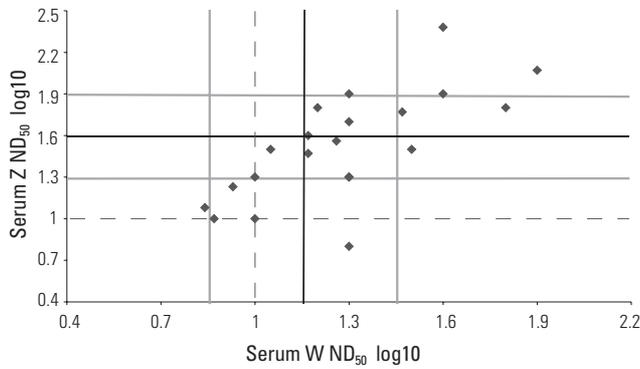


Fig. 3
Youden plot analysis of the results scored on two classical swine fever antibody positive sera with different ND₅₀ log₁₀ against CSF0902: serum W against serum Z in 2001

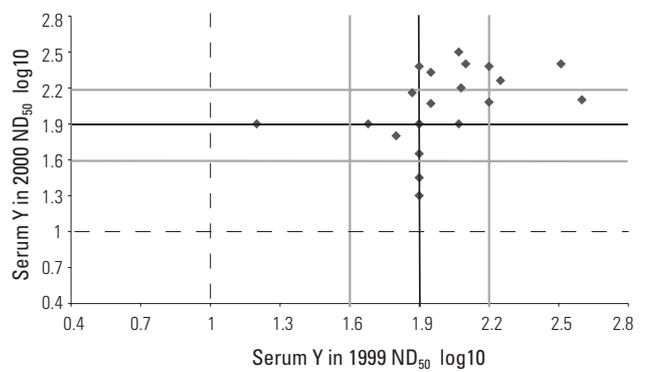


Fig. 4
Youden plot analysis for the results scored on the same classical swine fever antibody positive serum in two consecutive years: serum Y in 1999 and 2000

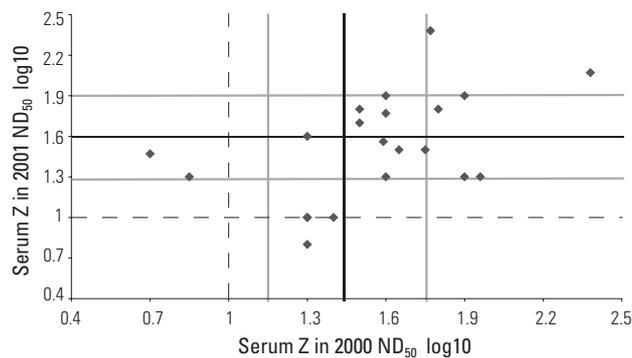
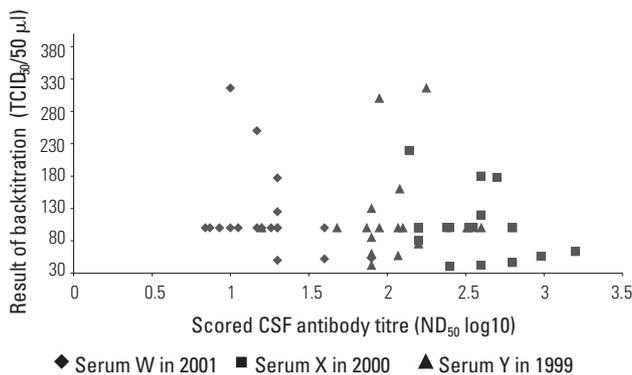


Fig. 5
Youden plot analysis for the results scored on the same classical swine fever antibody positive serum in two consecutive years: serum Z in 2000 and 2001

The antibody titre scored by the European Union Reference Laboratory (EURL) is marked by fat lines. The deviation of one dilution step above and below the antibody titre scored by the EURL is marked by thin lines. A score within these thin lines is within the variation of one dilution step. The dotted lines mark the threshold for interpretation of the antibody titre as classical swine fever (CSF) antibody positive or negative (ND₅₀ 1 log₁₀)

◆ Score of one laboratory on both sera



TCID: tissue culture infections doses

Fig. 6

The correlation of the backtitration and the scored classical swine fever (CSF) antibody titre evaluated on serum W, serum X and serum Y, one serum in each year

When a lower TCID₅₀ was detected the antibody titre score rose. Overlooking the influence of the backtitration on the scored CSF antibody titres of serum Z in three consecutive years (Fig. 7), CSF antibody titres from 0.7-2 log₁₀ ND₅₀ were scored, although the backtitration (100 TCID₅₀) was identical.

Classical swine fever antibody enzyme-linked immunosorbent assay

Classical swine fever antibody ELISAs were performed on serum Z, which had a low antibody titre, for three consecutive years (Fig. 8). Different commercial ELISAs as

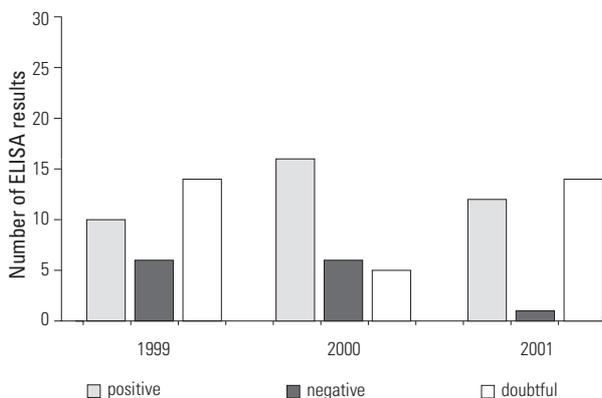
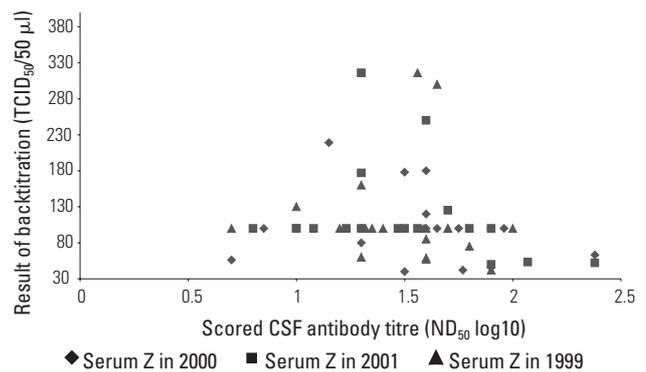


Fig. 8

Results of classical swine fever antibody enzyme-linked immunosorbent assay (ELISA) (commercial and homemade) obtained on serum Z in three consecutive years.

A total of 30 ELISAs were performed in 1999 and 27 were performed in both 2000 and 2001. Results were evaluated as positive, doubtful and negative



TCID: tissue culture infections doses

Fig. 7

The correlation of the backtitration and the scored classical swine fever (CSF) antibody titre evaluated on serum Z in three consecutive years

well as ‘homemade’ ELISAs were used and some laboratories performed more than one ELISA. The results give a general overview of the performance of ELISAs versus the reference test (neutralisation test). According to general laboratory experience, the CSF antibody ELISAs are known to be less sensitive than the neutralisation test. The number of correct positive results increased from 33% in 1999 to 59% in 2000 and dropped again to 44% in 2001. In 1999 46% of the sera were scored doubtful instead of clearly positive by the ELISA. In 2000, the number of doubtful results decreased to 18%, but increased again in 2001 to 51%. In contrast, the number of false negative results decreased significantly between 2000 and 2001 from 22% to 0.3%.

Detection of classical swine fever virus

Serum samples including CSFV were distributed in 1999 and in 2001. All but three laboratories detected the CSFV by virus isolation (data not shown). One laboratory accidentally inactivated the sample on arrival, another laboratory scored another sample as CSFV positive. Although the CSFV titre was determined, it could not be interpreted correctly, because the environmental conditions in which the samples were transported were not identical. Classical swine fever antigen ELISAs were not performed on a regular basis, there were therefore not enough data available for evaluation. Diagnostic polymerase chain reaction (PCR) was performed on a voluntary basis during the three years of the tests. The results of an inter-laboratory comparison test using PCR have been published elsewhere (16).

Each year, a 'pestivirus antibody negative' serum was included and all but one laboratory in 2001 scored this negative control correctly (data not shown).

Discussion

Classical swine fever is an OIE List A disease, and as it is highly contagious the laboratory diagnosis has to be performed as fast as possible in order to start eradication measures. This means that the test systems in the diagnostic laboratories have to produce reliable results. The sensitivity and specificity of tests used for CSF diagnosis should be known (2) in order to choose the correct sample size and to avoid false negative or false positive results. The tolerance limits of a test result should be evaluated in a sensible way. If they are too tight, unnecessary repetitions and valuable loss of time may occur.

The interpretation of results scored in the neutralisation test was mostly correct, although the value of the scored CSF antibody titres varied. The variation between the CSF antibody titres scored for a single serum was greater in sera with a low CSF antibody titre, indicating that this kind of sera is the most difficult for diagnosis. As the pig population in the EU Member States is CSF negative and no vaccination is performed, possible CSF antibody titres due to a CSF outbreak will most likely be in the lower range, because the infection is recent (13). However, the majority of laboratories interpreted the samples correctly as CSF antibody positive (1 log₁₀ ND₅₀ and above). Systematic errors and false negative results were rare. No laboratory had consecutive sensitivity problems over three years. Several laboratories had scores in the upper right field. For the diagnosis of CSF antibodies this would indicate an increased sensitivity rather than a systematic error. Scores in the lower left field are the critical ones here.

A tolerance limit of CSF antibody titres of one dilution step has so far been the norm in the experience of most laboratories (unpublished findings). The data evaluated here indicate that a tolerance limit of one dilution step in the scored result is the minimum value. As a control of the actual test virus titre used in the neutralisation test, a backtitration is performed. The test virus titre should be 100 TCID₅₀/50 µl. A calculated tolerance limit of 30-300 TCID₅₀ had been fixed artificially (4). Our data shown that although the backtitration was more than twofold higher in some cases the scored CSF antibody titre did not drop significantly. In cases, where the backtitration was below 100 TCID₅₀ the scored CSF antibody titres were slightly higher. There was no linear correlation between the scored antibody titre and the backtitration. It can be concluded that the allowable tolerance limit of the backtitration is rather wide. Inclusion of a CSF antibody positive control serum might be even more useful, because the scored antibody titres (results) can be

compared. Thus far, CSF0902 (7) has been used by most laboratories as a test virus for the neutralisation test. The CSF antibody titres scored with CSF0902 on the sera here were considerably lower than the CSF antibody titres scored with the recent homologous CSFV. This raises the question of whether CSF0902 is still a suitable reference strain for the neutralisation test or whether a more recent field virus isolate might be more appropriate. On the other hand, CSF0902 is well characterised and adapted and easy to handle in cell culture, whereas field virus isolates are a more heterogenic material and show more variation in cell culture growth. Furthermore, CSF0902 can easily be recognised as a laboratory contaminant (if suspected) by genetic typing (15). This is particularly important in countries where CSF has not occurred for a very long time (18).

There has been extensive evaluation of ELISAs over the last decade (12), especially with regard to sensitivity and specificity. The commercial and 'homemade' CSF antibody ELISA used in these tests improved over the three years. However, at present they are still less sensitive on individual samples than the neutralisation test, which is still the reference test for CSF serology. The specificity and the use of CSF antibody ELISA on a herd basis has not been evaluated here.

The performance of CSFV isolation was very satisfactory. Only one false positive result and one false negative result were reported. It can be concluded that this reference test does work reliably in all laboratories. The problem of autolysed samples, which is unfortunately common with samples from wild boar (10), could not be simulated for this trial.

In general, the two reference tests for CSF diagnosis, i.e. virus isolation and the neutralisation test, do not cause problems in the NSFLs of EU Member States. Both tests rely on living systems, e.g. cells and virus, which cannot be as well standardised as a chemical reagent. This is a crucial point that has to be kept in mind when implementing quality management systems.

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Gestion de la qualité des épreuves de référence pour le diagnostic de la peste porcine classique

G. Floegel-Niesmann & V. Moennig

Résumé

Les laboratoires nationaux de référence des États membres de l'Union européenne (UE) pour la peste porcine classique (PPC) ont mis en place des essais de comparaison interlaboratoires pour le diagnostic de cette maladie. Ces essais constituent une méthode permettant d'évaluer à la fois la fiabilité des résultats des épreuves diagnostiques réalisées par les laboratoires et les compétences liées à leur réalisation. Ils visent à obtenir des résultats identiques pour un même échantillon. L'étude évalue les données des épreuves sérologiques et virologiques de référence employées pour la PPC (test de neutralisation et isolement viral) sur une période de plus de trois ans.

Avec une limite de tolérance équivalente à une dilution pour les titres des anticorps étudiés, la sensibilité du diagnostic sérologique pour la détection des anticorps vis-à-vis de la PPC s'est avérée très bonne. Un même échantillon a produit des résultats identiques deux années de suite. Bien qu'une variation plus importante du titre des anticorps évalués ait été constatée dans les sérums possédant un titre plus faible en anticorps vis-à-vis de la PPC, cette variation n'a pas réellement affecté l'interprétation des données et leur classement en résultats positifs ou négatifs. Le titrage en retour d'un test de neutralisation (employé en guise de contrôle) constitue une valeur à caractère plus mathématique ne présentant aucune corrélation directe avec le système biologique. Les méthodes de dosage immuno-enzymatique des anticorps vis-à-vis de la PPC disponibles sur le marché sont systématiquement moins sensibles au niveau des échantillons individuels que le test de neutralisation de référence. La procédure d'isolement du virus de la PPC était maîtrisée par tous les laboratoires participant à l'étude et n'avait donné lieu qu'à très peu de problèmes. De même, la spécificité du diagnostic de la PPC par la recherche d'anticorps vis-à-vis de la PPC et de sérums négatifs pour le virus de la PPC n'a jamais été un problème.

Les épreuves de référence mises en œuvre dans le diagnostic de la PPC sont généralement bien établies au sein de l'UE. Elles se fondent sur des systèmes vivants (cellules et virus, par exemple) et présentent dès lors une limite de tolérance différente de celle des modèles purement mathématiques. Il importe avant tout d'obtenir une interprétation identique des résultats des analyses par l'ensemble des laboratoires.

Mots-clés

Accréditation – Épreuve de référence – Diagnostic – Gestion de la qualité – Isolement viral – Limite de tolérance – Peste porcine classique – Test de neutralisation – Titrage en retour.



Gestión de la calidad de las pruebas de referencia para el diagnóstico de la peste porcina clásica

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Resumen

Los Laboratorios Nacionales para la Peste Porcina Clásica (PPC) de los Estados Miembros de la Unión Europea (UE) elaboraron pruebas comparativas interlaboratorios para el diagnóstico de esta enfermedad. Esas pruebas constituyen un método de medida de la calidad de los resultados de las pruebas de diagnóstico efectuadas por los laboratorios y, también, de la competencia con que se las realiza. Su objetivo consiste en que todos los laboratorios obtengan el mismo resultado en las pruebas realizadas en la misma muestra. En este estudio se evalúan los resultados de las pruebas serológicas y virológicas de referencia (prueba de neutralización y aislamiento del virus) para la PPC efectuadas durante un lapso de tres años.

La sensibilidad del diagnóstico serológico para detectar la presencia de anticuerpos contra la PPC resultó muy buena y reveló un límite de tolerancia de los títulos del anticuerpo determinados utilizando una sola dilución. Los resultados obtenidos en la misma muestra, en dos años consecutivos, eran similares. La variación de los títulos del anticuerpo detectados era superior cuando se hicieron pruebas serológicas con un título inferior del anticuerpo contra la PPC. Esas variaciones no alteraban significativamente la interpretación de los títulos de anticuerpos como "positivos o negativos". La retrovaloración de un test de neutralización (empleado como medida de control) es un valor más matemático, que no guarda una correlación directa con el sistema biológico. Si se los compara con las pruebas de neutralización de referencia, los tests de inmunoabsorción enzimática para detectar anticuerpos contra la PPC que se comercializan actualmente siguen teniendo una sensibilidad inferior en las muestras individuales.

Todos los laboratorios participantes utilizan el aislamiento del virus de la PPC sin mayores problemas. La especificidad del diagnóstico de la PPC mediante la investigación sobre anticuerpos contra la PPC y los sueros negativos al virus tampoco provocó dificultades.

Por lo general, las pruebas de referencia para el diagnóstico de la PPC están bien establecidas en la Unión Europea. Se basan en sistemas vivos, es decir, en células y virus, y por consiguiente sus límites de tolerancia difieren de los valores matemáticos puros. Lo que importa, es que la interpretación de sus resultados sea idéntica en todos los laboratorios.

Palabras clave

Acreditación – Aislamiento de virus – Diagnóstico – Gestión de la calidad – Límite de tolerancia – Peste porcina clásica – Prueba de neutralización – Prueba de referencia – Retrovaloración.



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