Comparison of a reverse transcription-polymerase chain reaction assay and virus isolation for the detection of classical swine fever virus

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The authors evaluated the ability of a reverse transcription-polymerase chain reaction (RT-PCR) assay to detect classical swine fever virus (CSFV) in comparison with virus isolation and detection by an indirect immunoperoxidase assay (VI-IPA). To determine the specificity of the assay, samples from 60 spleens, 45 tonsils, ten submandibular lymph nodes, eight mesenteric lymph nodes and four kidneys, collected from pigs of various ages which had been slaughtered in abattoirs in Canada (a population free from CSFV), were tested. All the samples tested gave negative results by both VI-IPA and RT-PCR. A total of 20 samples were passaged in porcine kidney (PK) 15 cells and retested by both assays. All were found to be negative, giving a specificity of 100%.

To determine the analytical sensitivity of the assay, a similar comparative study was conducted, using CSFV grown in tissue culture and tonsil tissues from a CSFV-infected pig. For both infected tissues and tissue culture fluids, RT-PCR was ten times more sensitive than VI-IPA. Amounts as small as 0.6 infectious units per 100 mg of tissue were detected by RT-PCR, compared to 6 infectious units by VI-IPA. Similarly, RT-PCR could detect as little as 0.1 infectious unit per ml in tissue culture fluids, compared to one infectious unit per ml by VI-IPA.

To determine diagnostic sensitivity, three coded panels (two internal and one external), comprising 45 samples from 14 pigs, were tested. The diagnostic sensitivity of both RT-PCR and VI-IPA was found to be 100% for both internal panels. The results of the external panel, apart from two samples that were missed by both RT-PCR and VI-IPA, were found to be in total agreement. These two samples remained negative after amplification in PK15 cells. All the RT-PCR results were based on a single test whereas, for the VI-IPA results, positive results were obtained for five samples only after an amplification round in PK15 cells. Application of the RT-PCR assay for the diagnosis of CSFV would enable improved detection of the virus in a shorter time period.

Keywords

Introduction

Classical swine fever (CSF) (hog cholera) virus is an enveloped ribonucleic acid (RNA) virus which infects pigs. CSF virus (CSFV), together with Border disease virus and bovine viral diarrhoea virus, constitute the Pestivirus genus within the Flaviviridae family (19). The virus has an RNA genome of about 13,000 kilobases, which codes for approximately 4,000 amino acids. It is a non-polyadenylated RNA which is single-stranded and positively polarised (9).
The virus is responsible for a devastating disease of pigs, known as classical swine fever, which has inflicted major economic losses on the pig industry (8, 10, 16). The disease may run an acute, subacute, chronic or clinically non-apparent course. Mortality among the pig populations infected with the virus ranges from 100% in acute cases to none in non-apparent infections. The outcome of the disease depends on the CSFV strain involved as well as on the immune status, age and nutritional status of the animal (3).

Classical swine fever is a notifiable disease, which does not exist in many parts of the world, such as Canada (3). In countries where the disease does not exist, measures are taken to prevent its introduction. If, in spite of those measures, the disease is introduced through infected pigs or contaminated pig products, steps are taken to ensure that the infection is contained and eradicated. To ensure prompt and effective intervention if the disease is introduced, or to prevent the introduction of the disease itself, virus detection is essential. Clinical signs and lesions, used historically to monitor the disease, are no longer considered sufficient. Detection of antigens (1), antibodies (17) and isolation of the virus (12) are the most reliable methods employed to date. The detection of the CSFV genome has also been made possible with the advent of the polymerase chain reaction (PCR) technique for testing (13, 14). Owing to its sensitivity, specificity and speed, PCR offers definite advantages over conventional methods of detection. A reverse transcription-PCR (RT-PCR) for the detection of CSFV was developed in the laboratory of the authors (5) and has been partially evaluated for use as a diagnostic test (6). This report describes a systematic evaluation of the assay and assesses its use as a routine diagnostic test (4). The RT-PCR assay for the detection of CSFV has been previously described (5). The PCR primers are derived from the p125 gene of the allfort and Brescia strains (9, 11), i.e. CSFV-1 nucleotides 5067-5087 and 5'-GCTCCTGGTGGTTGAAACCTCGG-3', and CSFV-2 nucleotides 5554-5574, 5'-TGATGCTGTCACACAGGTGAA-3'. The PCR protocol was conducted as follows: denaturation at 94°C for one minute, followed by 59°C for one minute for primer annealing, and 72°C for two minutes for elongation. A total amount of 10 µl from each reaction mix was analysed in 2% agarose gel, stained with ethidium bromide and visualised by transillumination.

**Materials and methods**

**Tissue samples**

Samples were collected from pigs at abattoirs in Quebec, Canada (a population free from CSFV). Tonsils were harvested from pigs of different ages, as follows:

- 20 from market hogs
- 19 from adult pigs
- 4 from two-month-old pigs
- 2 from three-week-old pigs.

Spleens were harvested, as follows:

- 45 from sows
- 13 from market hogs
- 2 from three-week-old pigs.

Submandibular and mesenteric lymph nodes were collected from adult pigs. To generate coded tissue panels, samples were collected from eight- to ten-week-old piglets infected with the *Standard* strain at eight to eleven days post-infection, and from a piglet infected with the *United Kingdom* (UK) isolate at 23 days post-infection (6, 20), and stored at -70°C until use. Pigs were infected with BAI Nervous or New South Wales strains of CSFV and euthanased at eight and eleven days post-infection, respectively, then left at room temperature for six hours to mimic field conditions. Tissues were removed and left at 4°C for 48 hours to simulate transportation to the laboratory, before being stored at -70°C.

**Preparation of panels and sample processing**

Three coded panels were evaluated. Two of these panels, consisting of 25 samples in total, were set up in the laboratory of the authors and the third, containing 20 samples from pigs naturally infected with CSFV, was provided by the National Veterinary Services Laboratories in Ames, Iowa, United States of America. Tissue samples were weighed, minced and homogenised in Dulbecco’s minimum essential medium (DMEM) to produce a 10% (weight/volume) emulsion.

**Nucleic acid isolation**

Total RNA was isolated as previously described (6). In brief, samples were digested with proteinase K at 37°C for two hours for tissue culture samples and at 56°C overnight for tissue. This was followed by phenol-chloroform-isooamyl alcohol extraction and ethanol precipitation at -20°C. The precipitate was dried and re-suspended in 20 µl of water.

**Reverse transcription-polymerase chain reaction and analysis of product**

The RT-PCR assay for the detection of CSFV has been previously described (5). In brief, the PCR primers are derived from the p125 gene of the allfort and Brescia strains (9, 11), i.e. CSFV-1 nucleotides 5067-5087 and 5'-GCTCCTGGTGGTTGAAACCTCGG-3', and CSFV-2 nucleotides 5554-5574, 5'-TGATGCTGTCACACAGGTGAA-3'. The PCR protocol was conducted as follows: denaturation at 94°C for one minute, followed by 59°C for one minute for primer annealing, and 72°C for two minutes for elongation. A total amount of 10 µl from each reaction mix was analysed in 2% agarose gel, stained with ethidium bromide and visualised by transillumination.

**Analysis by restriction endonucleases**

Aliquots of 10 µl of the amplified RT-PCR mixtures were digested with the restriction enzyme Ava II. The resulting fragments were electrophoresed and visualised as described above.

**Virus isolation and immunoperoxidase assay**

Suspensions of porcine kidney (PK) 15 cells were inoculated with 50 µl of 10% tissue homogenate and seeded in duplicate into 96-well plates. The cells were then incubated at 37°C for 48 hours in a 5% CO₂ incubator. The cells were subsequently fixed with 20% acetone for ten minutes and CSFV antigens were detected using a monoclonal antibody, WH303 (4). CSFV-seronegative mouse ascites fluid was applied to the
duplicate set of wells to act as a control. The cells were then washed. Bound anti-CSFV antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, and subsequent use of enzyme substrate, hydrogen peroxide and chromogen, 3-amino-9-ethylcarbazole (1).

Results

Specificity of classical swine fever virus reverse transcription-polymerase chain reaction

A total of 127 abattoir samples from different tissues – namely: tonsils, spleens, submandibular lymph nodes, mesenteric lymph nodes and kidneys – were tested by virus isolation and immunoperoxidase assay (VI-IPA) and by RT-PCR. All samples tested gave negative results by both assays. Subsequently, 20 of those samples were chosen at random and passaged in PK15 cells before being retested to ensure that they were true negatives. The harvested cell culture fluids were all found to be negative by both assays.

Analytical sensitivity of the assay

The analytical sensitivity of the assay in relation to VI-IPA was determined by contrasting the detection of CSFV nucleic acid by RT-PCR with that of CSFV antigen by VI-IPA. These tests were performed on tonsil tissues collected from a pig infected with the Standard strain, and on CSFV grown in cell culture (Tables I and II). Serial dilutions of infected tissue culture fluids were made in DMEM and subsequently tested for viable virus by VI-IPA. The RT-PCR assay was conducted on nucleic acid isolated from these dilutions. The results are shown in Table I. In regard to RT-PCR assays, positive results were obtained from infected tissue culture fluids diluted up to 10^5-fold. At the same dilution, VI-IPA gave negative results. The samples which gave positive results by VI-IPA were diluted up to 10^3-fold, ten-fold less than in the case of RT-PCR. Taking into consideration the dilution factor, RT-PCR could detect amounts as small as 0.1 infectious particle per ml of fluid, whereas 1 infectious unit per ml could be detected by VI-IPA. Similar experiments were performed on 10% tonsil tissue emulsions and the results are shown in Table I. In infected tonsil tissues, amounts as small as 0.6 infectious unit per 100 mg of tissue could be detected by RT-PCR, which represented a thousand-fold dilution, whereas, in VI-IPA, sensitivity dropped ten-fold to 6 infectious units per 100 mg of tissue.

Diagnostic sensitivity of the assay

The internal coded panels (shown in Tables III and IV) were tested by both VI-IPA and RT-PCR. The first blind panel tested (Table III) consisted of eleven positive samples and one negative sample. The samples were from five animals, each of which was experimentally infected with one of three different strains of CSFV, namely: the UK, Standard and BAI Nervous isolates. All infected samples returned positive results on the first round of RT-PCR. Nine of eleven samples gave positive results by VI-IPA without any amplification in PK15 cells. The remaining two samples, tissues from a mesenteric lymph node and a kidney, returned positive results after passage in PK15 cells. All the samples in the second panel, twelve positive and one negative, were correctly identified by RT-PCR in the first round of testing, and all but one by VI-IPA upon initial testing. The remaining sample, an infected ileum, tested positive after a passage in PK15 cells. For the external panel, both assays correctly identified 18 out of the 20 samples, giving complete agreement between the tests (Table V). However, both RT-PCR and VI-IPA missed two samples, tissues from the intestine and ileum, that originally gave
### Table III
Comparative evaluation of reverse transcription-polymerase chain reaction and immunoperoxidase assays for the detection of classical swine fever virus

The first internal blind panel was tested by RT-PCR and IPA, as described in 'Materials and methods', above.

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Sample</th>
<th>Strain</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RT-PCR IPA</td>
</tr>
<tr>
<td>1</td>
<td>Tonsil</td>
<td>UK isolate</td>
<td>+ +</td>
</tr>
<tr>
<td>1</td>
<td>SMLN</td>
<td>UK isolate</td>
<td>+ +</td>
</tr>
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<td>Spleen</td>
<td>BAI[18]</td>
<td>+ +</td>
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<tr>
<td>2</td>
<td>Kidney</td>
<td>BAI[19]</td>
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</tr>
<tr>
<td>3</td>
<td>MLN</td>
<td>Standard</td>
<td>+ +</td>
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<td>Blood</td>
<td>Standard</td>
<td>+ +</td>
</tr>
<tr>
<td>4</td>
<td>MLN</td>
<td>Standard</td>
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<td>Standard</td>
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<td>+ +</td>
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<td>5</td>
<td>MLN</td>
<td>Standard</td>
<td>+ +</td>
</tr>
<tr>
<td>5</td>
<td>SMLN</td>
<td>Standard</td>
<td>+ +</td>
</tr>
<tr>
<td>6</td>
<td>Blood</td>
<td>Uninfected</td>
<td>- -</td>
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</table>

RT-PCR: reverse transcription-polymerase chain reaction
IPA: immunoperoxidase assay
UK: United Kingdom
SMLN: submandibular lymph node
MLN: mesenteric lymph node

- Samples were processed to mimic field conditions
- IPA returned positive test results after one round of amplification in porcine kidney 15 cells

### Table IV
Comparative evaluation of reverse transcription-polymerase chain reaction and immunoperoxidase assays for the detection of classical swine fever virus

The second internal blind panel was tested by RT-PCR and IPA, as described in 'Materials and methods', above.

<table>
<thead>
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<th>Detection method</th>
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<td></td>
<td></td>
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<td>UK isolate</td>
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<td>+ +</td>
</tr>
<tr>
<td>1</td>
<td>Liver</td>
<td>Standard</td>
<td>+ +</td>
</tr>
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<td>Standard</td>
<td>+ +</td>
</tr>
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<td>Standard</td>
<td>+ +</td>
</tr>
<tr>
<td>2</td>
<td>Lung</td>
<td>Standard</td>
<td>+ +</td>
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<td>Lung</td>
<td>Standard</td>
<td>+ +</td>
</tr>
<tr>
<td>2</td>
<td>Ileum</td>
<td>Standard</td>
<td>+ +</td>
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<td>3</td>
<td>Brain</td>
<td>Standard</td>
<td>+ +</td>
</tr>
<tr>
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</tr>
<tr>
<td>7</td>
<td>Lung</td>
<td>Standard</td>
<td>+ +</td>
</tr>
</tbody>
</table>

RT-PCR: reverse transcription-polymerase chain reaction
IPA: immunoperoxidase assay

- IPA returned positive test results after one round of amplification in porcine kidney 15 cells
- Tissues were processed to mimic field conditions

To assess the degree of agreement between the two tests, data obtained from testing the three coded panels were analysed by the kappa statistic. The results showed that the two tests were in total accord.

### Discussion

Previous work on the RT-PCR of CSFV developed in the laboratory of the authors (5, 6) indicated that the test is highly specific. Testing was conducted for viruses other than CSFV and all samples gave negative results. A limited number of negative tissues were also tested, producing similar results (6).

The work described in this report is a comparative validation of RT-PCR and VI-IP assays using a larger number of tissues, positive results. Furthermore, two infected liver tissue samples, which returned positive results by RT-PCR, tested positive by VI-IPA only after passage in PK15 cells. To confirm the RT-PCR results, the generated fragments were isolated and digested with Ava I. The size and number of fragments generated (data not shown) were as expected (6), confirming the identity of the products.
both positive and negative. The results clearly reinforce previous conclusions and support the use of the assay as a routine diagnostic test.

The importance of the RT-PCR assay is underlined in the comparison of its analytical sensitivity with that of VI-IPA. In infected tissue culture fluids, VI-IPA is able to detect one infectious unit; in infected tonsil tissue, sensitivity decreases to approximately six infectious units. This could be due to deterioration of the sample following storage for several months or the effect of freezing and thawing on virus infectivity. A ten-fold increase in sensitivity is observed when RT-PCR is performed. The high analytical sensitivity achieved by RT-PCR (less than 1 infectious unit per ml) has also been noted in the case of other viruses (2, 7, 15).

In terms of diagnostic sensitivity, the VI-IPA and RT-PCR tests were in total agreement. All the samples were detected by both assays when the internal panels were used, and both assays missed two positive samples when the external panel was tested. Two passages of the two false negatives did not improve the results in either test. That would suggest that the virus, present at one point in those samples, had been destroyed. The samples – infected intestine and ileum tissues – may have been improperly stored or handled, leading to degradation of the infectious particles and the genome. The results of the three coded panels together gave an overall sensitivity of RT-PCR to CSFV of 95%, the same sensitivity as VI-IPA. However, in the latter, five samples required amplification in PK15 cells before giving positive test results.

The five samples were from four different tissues, harvested from three different pigs which had been infected with three strains of the virus. This would suggest that the failure to detect CSFV was not due to an CSFV strain or tissue type. One explanation could be that there were too few infectious particles for detection by VI-IPA, and an amplification cycle brings the amount up to detectable levels. Another possibility is that the virus has to adapt to the cell line, provided by a passage in PK15 cells, before antigen production reaches detectable levels. Although a few positive tissues were missed, all infected animals were correctly identified as most of their tissues gave positive test results.

It is well documented that RT-PCR assays require less time than most other tests (18, 21), an important feature in diagnostic virology. Similarly, in the work described in this paper, a minimum of 48 hours is required for a VI-IPA to be completed, whereas the result for an RT-PCR is known within 24 hours. Moreover, if an amplification round is required, VI-IPA results will be known only after four days. Taking into consideration the sensitivity of the assay and the time required for completion of the test, RT-PCR would be a significant addition to the tools available for the diagnosis of CSFV.

Acknowledgements

The authors are grateful to Y. Robinson of the Health of Animals Laboratory, Canadian Food Inspection Agency, St Hyacinthe, Quebec, for kindly providing the tissue samples from abattoirs, and J.E. Pearson of the Diagnostic Virology Laboratory, National Veterinary Services Laboratories, United States Department of Agriculture, Ames, Iowa, for providing CSFV-infected tissues.

Comparaison entre la technique de transcription inverse-amplification en chaîne par polymérase et l’isolement du virus pour la recherche du virus de la peste porcine classique

S. Vydelingum, T. Tao, K. Balazsi & R. Hecker

Résumé

Les auteurs évaluent la capacité d’une épreuve de transcription inverse-amplification en chaîne par polymérase (RT-PCR) à déceler le virus de la peste porcine classique, par comparaison avec l’isolement et la détection du virus selon la méthode d’immunoperoxidase indirecte (VI-IPA). Pour déterminer la spécificité de l’épreuve, des prélèvements ont été effectués sur 60 rates, 45 amygdales, 10 ganglions lymphatiques sous-mandibulaires, 8 ganglions lymphatiques mésentériques et 4 reins, provenant de porcins d’âges divers abattus au Canada (où la population porcine est indemne du virus de la peste porcine classique). Tous les prélèvements ont donné des résultats négatifs par les deux techniques,
VI-IPA et RT-PCR. En tout, 20 échantillons ont été passés dans des cultures cellulaires de rein de porc 15 (PK15) et vérifiés à nouveau par les deux méthodes. Ils ont tous donné des résultats négatifs, confirmant la spécificité (100%) des épreuves.

Pour déterminer la sensibilité analytique des épreuves, une autre étude comparée a été réalisée, utilisant le virus de la peste porcine classique obtenu en culture cellulaire et à partir de tissus d’amygdales provenant d’un porc infecté. Pour les tissus infectés comme pour les milieux de culture cellulaire liquides, la sensibilité de la méthode RT-PCR était dix fois supérieure à celle de la technique VI-IPA. La méthode RT-PCR a ainsi permis de déceler 0,6 unité infectieuse pour 100 mg de tissus, un titre particulièrement faible, contre 6 unités infectieuses détectés par la technique VI-IPA. De même, la méthode RT-PCR décelait jusqu’à 0,1 unité infectieuse par ml dans les milieux de culture liquides, contre 1 unité infectieuse par ml détectée par l’épreuve VI-IPA.

Pour déterminer la sensibilité diagnostique, trois séries codées (deux internes et une externe) de 45 échantillons provenant de 14 porcs ont été soumises à des tests. La sensibilité diagnostique des deux techniques, VI-IPA et RT-PCR, a été de 100 % pour les deux séries internes. Les résultats de la série externe coïncidaient également, à l’exception de deux prélèvements qui ont échappé à la RT-PCR comme à la VI-IPA. Ces deux prélèvements donnaient toujours des résultats négatifs après amplification en cellules PK15. Tous les résultats de la méthode RT-PCR ont été obtenus lors d’une épreuve unique, alors qu’avec la technique VI-IPA, des résultats positifs n’ont été obtenus pour cinq prélèvements qu’après amplification dans des cellules PK15. L’application de l’épreuve RT-PCR au diagnostic de la peste porcine classique permettrait d’améliorer la détection du virus et de réduire les délais d’obtention des résultats.

Mots-clés

Comparación entre una prueba de transcripción inversa-reacción en cadena de la polimerasa y una de aislamiento vírico aplicadas a la detección del virus de la peste porcina clásica

S. Vydelingum, T. Tao, K. Balazsi & R. Hecker

Resumen
Los autores evaluaron la capacidad de una prueba de transcripción inversa-reacción en cadena de la polimerasa (reverse transcription-polymerase chain reaction, RT-PCR) para detectar el virus de la peste porcina clásica, comparándola con el aislamiento y detección del virus por el método de inmunoperoxidasa indirecta (VI-IPA). Para determinar la especificidad del ensayo se analizaron muestras de 60 bazos, 45 amígdalas, 10 ganglios linfáticos submandibulares, 8 ganglios linfáticos mesentéricos y 4 riñones procedentes de cerdos de edades diversas sacrificados en mataderos canadienses (una población libre del virus de la peste porcina clásica). Todas esas muestras arrojaron respuesta negativa tanto a la prueba de aislamiento como a la prueba de detección por VI-IPA como a la de RT-PCR. Tras pasar un total de 20 muestras por un cultivo de células de riñón de cerdo 15 (PK15), se sometieron nuevamente esas muestras a ambos ensayos, con resultados nuevamente negativos. De ahí se deduce una especificidad del 100%.
Para determinar la sensibilidad analítica del ensayo se llevó a cabo un estudio comparativo similar, utilizando virus de la peste porcina clásica reproducidos en cultivo celular y tejido amigdalar de un cerdo infectado por el virus. Tanto en el caso del tejido infectado como en el del líquido del cultivo celular, la prueba de RT-PCR resultó diez veces más sensible que la del aislamiento con detección por VI-IPA. Mientras que la primera era capaz de detectar dosis ínfimas, de hasta 0,6 unidad infecciosa por 100 mg de tejido, el método VI-IPA alcanzaba sólo a discriminar 6 unidades infecciosas. Análogamente, la prueba de RT-PCR podía detectar 0,1 unidad infecciosa por ml de líquido de cultivo celular contra 1 unidad infecciosa por ml en el caso del método VI-IPA.

Para determinar la sensibilidad de diagnóstico se analizaron tres paneles codificados (dos internos y uno externo), que comprendían 45 muestras procedentes de 14 cerdos. La sensibilidad de diagnóstico de ambas pruebas, VI-IPA y RT-PCR, resultó ser de un 100% para los dos paneles internos. Los resultados del panel externo también coincidían, con la salvedad de dos muestras que ambos ensayos pasaron por alto. Tras amplificarlas en células PK15, esas dos muestras siguieron dando resultado negativo. Mientras que todos los resultados del método RT-PCR se obtuvieron aplicando una prueba única, en el caso del método VI-IPA hizo falta una ronda de amplificación en células PK15 para obtener resultados positivos para cinco de las muestras. La aplicación del ensayo de RT-PCR al diagnóstico del virus de la peste porcina clásica haría posible una mejor detección en un lapso menor de tiempo.

**Palabras clave**

Detección – Ensayo de inmunoperoxidasa indirecta – Transcripción inversa-reacción en cadena de la polimerasa – Virus de la peste porcina clásica.

**References**


