Application of a blocking enzyme-linked immunosorbent assay for serological monitoring of hog cholera (classical swine fever) in Poland

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Summary: Between 1990 and 1992, serum samples from 55,478 domestic swine were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of hog cholera virus (HCV) antibodies. The amount of antibody in the sera was expressed as the mean percentage inhibition (PI). For diagnosis, the tested sera were diluted 1:2 and considered positive if the PI was less than 25%. Sera giving PI values in the range of 25-50% were retested against HCV and bovine virus diarrhoea virus (BVDV), by neutralising peroxidase-linked assay. Comparison of the serum titres obtained was used for serological diagnosis of hog cholera; the tested sera were considered negative for hog cholera if the titre for BVDV was higher than that obtained for HCV. All sera with a PI higher than 50% were considered negative for HCV and BVDV. All sera were found to be free of antibodies to HCV. BVDV antibodies were demonstrated in 0.40% of the sera tested in 1990, in 1.80% in 1991 and 1.06% in 1992.


INTRODUCTION

Hog cholera (HC) – also known as “classical swine fever” (3) – is an infectious viral disease of domestic and wild pigs caused by a pestivirus (hog cholera virus: HCV) (4). Recently, based on new insights into the replication strategy and genome organisation of pestiviruses, these were allocated to the family Flaviviridae (earlier, pestiviruses were considered as belonging to the family Togaviridae) (7). HC remains an important disease economically, particularly in countries with a high pig population density (1). The spread of HCV strains of low and medium virulence, which cause atypical and subclinical forms of HC, underlines the significance of serological monitoring in the eradication of this disease (14).

In accordance with the OIE International Animal Health Code and European Community (EC) regulations (10), a country exporting swine or swine products is obliged to demonstrate freedom from HC on the basis of serological investigations.

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In 1990, Poland implemented a system of obligatory serological screening for HCV antibodies. The primary objective of the present three-year study was to obtain further data on the seroprevalence of HC in Poland.

MATERIALS AND METHODS

Sera

In accordance with instructions issued by the Polish Ministry of Agriculture (Department of Veterinary Services), blood samples were collected from sows and boars kept on private farms. In 1990, tests were conducted on 17,545 field sera from all except one of the 49 provinces in Poland; in 1991, 21,378 sera from 47 provinces were tested and in 1992, 16,555 sera from 46 provinces were tested. The sera were heat-inactivated at 56°C for 30 min, and kept frozen at -20°C until testing. The quality of the serum samples varied, with regard to haemolysis and bacterial contamination.

Serology

Blocking enzyme-linked immunosorbent assay (ELISA) (5) was used for serological screening for HCV antibody. In view of the fact that antibodies against bovine virus diarrhoea virus (BVDV) had been identified in the Polish swine population (11), the neutralisation peroxidase-linked assay (NPLA) (6, 8) was used for the differentiation of BVDV and HCV antibodies.

Enzyme-linked immunosorbent assay

HCV antigen for coating microplates was produced using the method described by Have (5), with the modifications proposed by Dalsgaard and Overby (2). The Alfort strain of HCV was used for production of antigen in PK15 cell cultures propagated in roller bottles. Cells were scraped with a rubber policeman and pelleted by centrifugation. The cell pellet was extracted in two volumes of 20 mM tris (pH 7.2), 1% Triton X-100, 0.5 mM ethylene diamine tetra-acetic acid (EDTA) by sonication. The extract was clarified at 5,000 g for 20 min, precipitated by ammonium sulphate at 40% saturation and resuspended in 20 mM tris (pH 7.2), 0.1% Triton X-100 and 0.5 mM EDTA.

Depending on the titre, the antigen was diluted from 1:800 to 1:2,000 in carbonate buffer (pH 9.6) and placed on microplates. Plates were coated for 18 h at room temperature. After washing the plates three times with washing buffer (phosphate-buffered saline: PBS) containing 0.05% Tween 20 (PBST), test sera (diluted 1:2 in PBST) were placed in appropriate wells and incubated for 18 h at 4°C. Subsequently, microplates were washed three times with PBST. Microplates were then incubated for 1 h at 37°C with rabbit anti-HCV serum diluted 1:100-1:200. The microplates were then washed three times with PBST.

The ability of the test sera to inhibit the reaction between antigen and rabbit anti-HCV serum was examined by reaction with peroxidase-labelled anti-rabbit immunoglobulin G (IgG). Microplates were incubated with this conjugate (diluted according to the recommendations of the manufacturer) at 37°C for 30 min. After washing three times, the reaction was visualised by incubating the microplates for 15 min at room temperature in the dark with a substrate solution containing 4.6 mM ortho-phenylene diamine, 9 mM H$_2$O$_2$, in 38 mM citric acid and sodium phosphate buffer (pH 5). After 15 min, the reaction was stopped with 0.5 N sulphuric acid. Optical densities (OD) were then recorded at 492 nm using an ELISA reader. On each microplate, two wells with negative reference sera and two wells with strong positive reference sera were included as controls.
Any uncoated sites on microplates were blocked using several blocking agents (horse serum, 1% bovine serum albumin, and 2%, 5% and 10% solutions of defatted dry bovine milk) in preliminary trials. The authors have previously shown that to be highly sensitive and specific, ELISA requires no blocking agents for the serodiagnosis of HC (12), and therefore such agents were not used.

**Interpretation of the results**

In accordance with the method described by Have (5), the antibody reactivity of the test sera was calculated as percentage inhibition (PI), where:

\[
PI = \frac{(OD \text{ negative reference} - OD \text{ test serum}) \times 100}{OD \text{ negative reference} - OD \text{ strong positive reference}}.
\]

In accordance with the recommendation of Have (5) and the results presented by Leforban and colleagues (9), the cut-off value for inhibition was established at 25%. Therefore, samples diluted 1:2 were considered positive if the PI was less than 25%.

(Since the completion of the present investigations, a slightly different method of calculating PI values, where:

\[
PI = \frac{(OD \text{ negative reference} - OD \text{ test serum}) \times 100}{OD \text{ negative reference}}
\]

has been adopted by the Standards Commission of the OIE.)

BVDV reactions may reach as high as 50% inhibition in the ELISA (5, 9). Therefore, sera giving PI values in the range of 25-50% were retested against HCV and BVDV. Testing was conducted by neutralisation assay in microplates, using a 1:25 dilution of the tested serum as the initial dilution, and results were read by NPLA (6). Results were determined by comparison of the serum titres obtained. The tested sera were considered negative for HC if the titre with BVDV was higher than that obtained with HCV. Sera with PI higher than 50% were considered HCV- and BVDV-negative.

**RESULTS**

The results demonstrated that, in 1990, 71 serum samples (0.40%) had BVDV antibodies; all other samples (17,464 sera), were free from HCV and BVDV antibodies (Table I).

In 1991, 21,378 serum samples were tested. All sera were found to be free from HCV antibodies, but the prevalence of BVDV-positive sera increased to 385 (1.8%). In 1992, 16,555 serum samples were tested and again all sera were free from HCV antibodies, and 176 serum samples (1.06%) were BVDV-positive. Differences were found in BVDV seroprevalence between the different provinces. In some provinces, 5.0% of pigs tested were BVDV-seropositive, whereas in other provinces all tested samples were negative for BVDV. The comparative serology by NPLA of the sera inhibiting 25-50% of the signal usually provided a clear-cut result, showing either the absence of antibody against HCV or titres distinctly lower than the corresponding antibody titre against BVDV.
### Table I

**Seroprevalence of antibodies to hog cholera virus (HCV) and bovine virus diarrhoea virus (BVDV) in pigs in Poland between 1990 and 1992**

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of swine tested</th>
<th>No. of swine with antibodies in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HCV</td>
</tr>
<tr>
<td>1990</td>
<td>17,535</td>
<td>-</td>
</tr>
<tr>
<td>1991</td>
<td>21,378</td>
<td>-</td>
</tr>
<tr>
<td>1992</td>
<td>16,555</td>
<td>-</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The choice of cut-off value is essential with regard to the sensitivity and specificity of blocking ELISA. A high cut-off value will eliminate false-positive results, but will also decrease the sensitivity of the test, leading to a risk of obtaining false-negative results (9). The cut-off value of 25% applied in these studies has been previously determined experimentally (5, 9). Corresponding standards (positive and negative sera) prepared by the authors were used. The specificity and sensitivity of the ELISA technique used in the present study was also controlled by using a set of HCV- and BVDV-positive and HCV- and BVDV-negative sera obtained from the EC Liaison Laboratory for Classical Swine Fever in Hanover (Germany), as well as a similar set of sera supplied by the *Station de Pathologie Porcine* in Ploufragan (France). Preliminary investigations demonstrated the balance between the required sensitivity and the number of heterologous reactions which could be tolerated. Simultaneously, it was demonstrated that, because of frequent occurrence of antibodies to BVDV in swine populations, it is necessary, in addition, to use NPLA to test sera with a PI ranging from 25-50%, for antibodies against this heterologous pestiviruses. This confirms the findings of a previous report (5).

Prior to 1991, approximately two million doses of hog cholera vaccine were used annually for vaccination against this disease in Poland. A programme of vaccination was followed only in State farms and in farms where garbage feed was used, while it was forbidden to vaccinate pigs in private enterprises. Therefore, according to Ministry of Agriculture regulations, only swine serum samples from private farms were tested. This excluded the possibility of obtaining false-positive reactions due to the detection of any residual vaccine.

The breeding pig population on private farms in Poland totals approximately 15 million animals. Of these, approximately 20,000 pigs are tested annually for HC seroprevalence. This represents a far greater proportion than in other European countries such as Denmark where, from a total of approximately 16 million pigs, only 4,500 are tested for HCV antibodies each year (P. Have, personal communication, 1993).

BVDV seroprevalence in Poland may be compared with that in other HCV-free countries. For example, Holm-Jensen (6) demonstrated BVDV antibodies in 6.4% of 3,000 serum samples tested in Denmark. Surveys revealed that the prevalence of naturally-occurring antibodies to BVDV in breeding pigs in Australia, Germany, the
Netherlands and Ireland ranged from 3% to 40% (6, 13). The frequent occurrence of antibodies to BVDV in swine populations, observed by other authors and in this study, justifies the need for precise determination of the cut-off value of the immunoenzymatic method applied. These findings also lend support to the use of NPLA to clarify doubtful ELISA results.

The methods used in this study may also be used for indirect (serological) differential diagnosis of infection with BVDV in pigs which have signs resembling HC (13).

CONCLUSIONS

The blocking ELISA, applied together with NPLA, enables accurate surveillance of large numbers of samples for HCV antibodies.

All swine sera tested during the study between 1990 and 1992 were found to be free from HCV antibodies.

The study showed a non-significant increase of BVDV seroprevalence in pigs in Poland.


Résumé : Entre les années 1990 et 1992, des prélèvements de sérum effectués sur 55 478 porcs domestiques ont fait l'objet de recherches d'anticorps dirigés contre le virus de la peste porcine classique (hog cholera virus : HCV) à l'aide d'une épreuve enzyme-linked immunosorbent assay (ELISA). Le titrage des anticorps était exprimé en pourcentage moyen d'inhibition (mean percentage inhibition : PI). Aux fins de diagnostic, les sérums testés ont été dilués au demi et considérés comme positifs lorsque PI était inférieur à 25 %. Les sérums dont le pourcentage d'inhibition se situait entre 25 % et 50 % ont été soumis à de nouvelles recherches d'anticorps contre le virus HCV et celui de la diarrhée virale bovine (bovine virus diarrhoea virus : BVDV) à l'aide de l'épreuve peroxydasique de neutralisation. La comparaison des titres obtenus a ensuite été appliquée au diagnostic sérologique de la peste porcine : les sérums testés étaient considérés comme négatifs vis-à-vis de la peste porcine lorsque le titre du virus BVDV était supérieur à celui du virus HCV. Tous les sérums dont le pourcentage d'inhibition était supérieur à 50 % étaient considérés comme négatifs vis-à-vis des virus HCV et BVDV. Aucun anticorps du virus HCV n'a été décelé dans les sérums. Les anticorps du virus BVDV ont été mis en évidence dans 0,40 % des sérums testés en 1990, 1,80 % en 1991 et 1,06 % en 1992.


Resumen: Entre los años 1990 y 1992, se investigó la presencia de anticuerpos contra el virus de la peste porcina clásica en muestras de suero recogidas en 55,478 cerdos domésticos mediante una prueba inmunoenzimática (ELISA). La titulación de los anticuerpos estaba expresada en porcentaje medio de inhibición (PI). Se observaron los sueros con fines diagnósticos, diluidos en su mitad y considerados positivos cuando el PI era inferior a 25%. Los sueros cuyo PI oscilaba entre 25% y 50% fueron sometidos a nuevas búsquedas de anticuerpos contra los virus de la peste porcina clásica y de la diarrea viral bovina, por medio de la prueba peroxidásica de neutralización. La comparación de los títulos obtenidos se aplicó a continuación para establecer el diagnóstico serológico de la peste porcina clásica: los sueros investigados se consideraron negativos en relación con la peste porcina clásica cuando el título del virus de la diarrea viral bovina era superior al obtenido del virus de la peste porcina clásica. Todos los sueros cuyo PI era superior a 50% se consideraron negativos en relación con los virus de la peste porcina clásica y de la diarrea viral bovina. No se identificó ningún anticuerpo del virus de la peste porcina clásica en los sueros. Los anticuerpos del virus de la diarrea viral bovina se manifestaron en 0,40% de los sueros investigados en 1990, en 1,80% de los investigados en 1991 y en 1,06% de los investigados en 1992.


REFERENCES


