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Summary: Since there has been no evidence of swine vesicular disease (SVD) in Switzerland since 1973, and because the disease can circulate in an inapparent form, a sero-epidemiological survey was undertaken. For this purpose, an ELISA was developed, using a cytoplasmic extract from infected and uninfected cell cultures. This extract was later analysed by PAGE and immunoblotting, and was shown to contain the structural viral proteins VP₀, VP₁, VP₂, VP₃ and VP₄.

Having shown a satisfactory correlation between ELISA and the serum neutralisation test, the former was employed to test 9,529 serum samples from breeding pigs. Of these, 9,358 (98.2%) were negative, 8 (0.09%) were positive and the remaining 163 (1.71%) could not be interpreted.

Positive samples and those impossible to interpret were retested by the serum neutralisation test against SVD virus and porcine enterovirus serotypes 1, 2, 3, 6 and 8. These sera neutralised the other enterovirus serotypes to a greater extent than the SVD virus. Thus, it would appear that the positive reactions in the anti-SVD virus ELISA were due to antibodies against other enterovirus serotypes.

Therefore SVD is not present among breeding pigs of the Swiss pig population.

KEYWORDS: Enterovirus - Epidemiological survey - Freedom from disease - Swine diseases - Swine vesicular disease - Switzerland.

INTRODUCTION

Swine vesicular disease (SVD) is characterised by the formation of vesicles on the coronary band, on distal parts of the limbs, and occasionally on the snout, lips, tongue and teats. Suidae are the only natural hosts (14, 15).

Since the symptoms of SVD are similar to those of foot and mouth disease, vesicular stomatitis and vesicular exanthema (14, 15), these four infections can be distinguished only by virological or serological tests.

SVD virus (SVDV) is an enterovirus of the family Picornaviridae, called serotype 9 of porcine enterovirus. It is closely related to the human Coxsackie B5 enterovirus.

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The capsids of porcine enteroviruses are formed from analogous viral structural proteins, namely: VP\(_0\), VP\(_1\), VP\(_2\), VP\(_3\) and VP\(_4\). Protein VP\(_0\) is associated with incomplete virions; it is cleaved into VP\(_2\) and VP\(_4\) for incorporation into complete virions (that is, the virus particles which contain the RNA genome). All of these proteins can elicit the formation of antibodies which may give cross-reactions in various serological tests (6, 20). Techniques employed for detecting SVD antibodies are counter-immunoelectrophoresis, double immunodiffusion in gels, serum neutralisation and enzyme immunoassay (ELISA) (6, 8, 14, 15, 17, 20). An inapparent infection can be detected solely by the presence of antibodies in infected animals, and occurs only in pigs exposed to small doses of virus (15). The virus has also been isolated from clinically healthy individuals (11).

SVD was reported for the first time in Italy in 1966 (16). It reappeared in 1971 in both Hong Kong and Italy. In 1972 it reached Britain and other European countries (15). The following year it was diagnosed in Switzerland, in the Zurich Canton (19). Since then there have been sporadic outbreaks in Hong Kong, Europe, Malta and Japan (15).

Since SVD has appeared only once in Switzerland, and the disease can exist in an inapparent form, it was considered necessary to perform a serological examination of a representative number of the Swiss pig population. For this purpose, a serum bank was prepared using samples collected from boars and sows used for breeding (18). The samples were tested by an ELISA which used a cytoplasmic extract from SVD virus-infected cells (1). Polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting, were used to identify the virion proteins present in this extract. In order to establish a correlation between ELISA and the serum neutralisation test, a representative percentage of the samples was analysed, using both methods. The possible occurrence of the cross-reactions described by Golding (6) and Sörensen (20) was determined by testing the anti-SVDV antibody positive sera for their capacity to neutralise porcine enterovirus serotypes 1, 2, 3, 6 and 8.

ELISA proved to be a simple method for rapid testing of a large number of samples. Moreover, plates sensitised and stored at \(-20^\circ\text{C}\) remained stable for six months.

The results obtained in this survey suggest that SVD does not occur in Switzerland, even in an inapparent form.

**MATERIALS AND METHODS**

**Serum samples**

Serum samples were collected from breeding sows and boars from the following sources: various abattoirs in Switzerland, certain Pig Health Centres and a number of AI centres. The serum bank totalled 9,529 samples collected between December 1984 and September 1987 (18).

Positive control serum was prepared in a pig which was infected on four occasions with the 1973 Swiss isolate of SVDV. Twelve days after the final infection, the animal was slaughtered and its blood collected to prepare hyperimmune serum (neutralisation titre 1:5012).
Serum from a sow which had had no contact with SVD virus was used as the negative control.

Positive control sera were prepared in guinea-pigs immunised with strains UKG 73, Italy 66, Italy 72 and UKG 72, as well as in pigs immunised with strains France 73 (7), Italy 72 and UKG 72.

A total of 24 positive serum samples from infected British herds were provided by the Institute for Animal Health at Pirbright.

Twelve monospecific sera were produced against porcine enterovirus serotypes 1, 2, 3, 6 and 8 by infecting specific pathogen free (SPF) piglets.

**Virus strains**

In order to prepare a stock of virus with which to produce antigen for ELISA and PAGE, strain UKG 27/72 of SVD virus (isolated in Staffordshire in December 1972) (6) was propagated three times in PK15 cells. Virus from the third passage was used for antigen production. The infectivity titre of the different passages of virus ranged from $10^{7.7}$ to $10^{8.4}$ TCID$_{50}$ per ml.

A stock of virus for the serum neutralisation test was produced from the same strain by two passages on IBRS$_{II}$ cells (6), resulting in titres ranging from $10^{7.3}$ to $10^{7.9}$ TCID$_{50}$ per ml.

The other strains of porcine enteroviruses (PEV) used in this study were: PEV$_1$ (Talfan virus from the Virology Institute at the Bern Veterinary Faculty), PEV$_2$, PEV$_3$, PEV$_6$ and PEV$_8$ (reference strains from the National Veterinary Services Laboratories at Ames, Iowa, USA) (23).

**Antigen production**

Antigen for ELISA was produced in PK$_{15}$ cells from the third passage of the stock virus. After 20-24 h of incubation, a cytopathic effect was present in 90-100% of the infected cell monolayer. A negative control antigen was prepared from uninfected PK$_{15}$ cells. The cells (infected or control) were collected and diluted with double distilled water and allowed to stand for 5 min. After adding 0.5% (v/v) N-octyl-beta-D-glucopyranoside for 5 min, the mixture was centrifuged. The cytoplasmic extract present in the supernatant fluid constituted the antigen (1). In order to study the stability of this antigen, microplates were sensitised with some of the antigen and frozen at $-20^\circ$C, whilst other portions of antigen were stored at $-70^\circ$C.

Antigen for PAGE was produced from cells and cytoplasmic extract diluted in a buffer solution containing 55% (w/v) sucrose, 2% (w/v) sodium dodecyl sulphate and 10% (v/v) beta-mercaptoethanol. The antigen was subjected to ultrasonication for 2 min and then immersed in a water bath at 100°C for 4 min.

**ELISA**

An ELISA was developed similar to that described by Bommeli for infectious bovine rhinotracheitis (2). The antigen was diluted with sodium carbonate/bicarbonate buffer (0.1M, pH 9.6). Each washing and all dilutions were performed with PBS containing 0.01% (v/v) Tween 20. Volumes of 200 µl per well were used throughout the ELISA. Incubation took place in a moist chamber at room temperature for one hour.
Sensitisation of plates

Antigen diluted with sodium carbonate/bicarbonate buffer was placed in the wells of microplates (Dynatech M129A or Petra Plastic), incubated at 4°C overnight, and then frozen at -20°C. Negative and positive antigens were placed in the wells of adjacent rows of the plates (2, 4).

Test technique

After thawing, the microplates were rinsed three times. A 1% solution of skimmed milk powder was added to the wells to reduce the occurrence of non-specific reactions. After incubation and three washings, serum diluted 1:80 was placed in four wells (two with negative and two with positive antigen). Both positive and negative control sera were also included in each ELISA. After additional incubation and washing, the conjugate was added to each well; this was either peroxidase conjugated anti-pig IgG diluted 1:200 (Dr W. Bommeli, Ltd., Bern, Switzerland), or peroxidase-conjugated protein A diluted 1:40000 (Kirkegaard & Perry, Maryland, USA). A final incubation and washing was followed by addition of substrate (ABTS and H2O2). After a reaction time of 30 min, the result was read photometrically at 404 nm. Optical densities (A404) were recorded on a "SORD M23" computer, which calculated the difference between the A404 obtained with positive antigen and that with negative antigen (this gave the ΔA404 value), in addition to the percentage ΔA404 in relation to that of the positive control serum. A serum was judged positive when the percentage ΔA404 was at least 30%. A serum could not be classified as positive or negative when it reacted equally with both positive and negative control antigens, or more strongly with the negative antigen.

The test was considered invalid if the positive control serum gave an ΔA404 of less than 0.2.

Serum neutralisation test

The technique used was that of Golding et al. (6). A serum was judged positive for anti-SVDV antibody if its titre was greater than 1:32, negative if it was less than 1:16, and doubtful if between 1:16 and 1:32 (17).

Neutralisation tests against other serotypes of porcine enterovirus were performed in primary pig kidney cell cultures, according to the method of Zindel (23). A serum was judged positive if it had a titre greater than 1:80, negative if less than 1:40, and doubtful between 1:40 and 1:80 (23).

Each neutralisation test included positive and negative control sera.

Correlation between ELISA and the serum neutralisation test

In order to calculate the sensitivity and specificity of ELISA, serum samples were tested by both ELISA and serum neutralisation. These samples comprised 8 positive control sera, 24 sera from animals of infected herds and 421 from pigs apparently free from SVD.

In addition, the actual correlation between the two tests was determined from the 'kappa' value calculated according to the method of Landis and Koch (13).
PAGE and immunoblotting

Polyacrylamide gel electrophoresis (PAGE) was performed using 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, and both infected cells and extracted cytoplasmic antigen. This was followed by electrophoretic transfer to nitrocellulose sheets (3, 9, 10, 22).

For immunoblotting, the nitrocellulose was first blocked with a 3% (w/v) solution of skimmed milk powder in PBS for an hour at 37°C, then incubated for another hour at 37°C in PBS containing 1% (w/v) skimmed milk powder and the serum under test (diluted 1:25). The conjugate (peroxidase-conjugated protein A from Kirkegaard & Perry, Maryland, USA), was diluted 1:500 with PBS and incubated on the nitrocellulose sheets for one hour at 37°C. Chloronaphthol was used as substrate to reveal the presence of antibodies. Washing with PBS was done between each stage of immunoblotting (10, 21).

RESULTS

Evaluation of the ELISA

For this study, an ELISA was developed to detect anti-SVDV antibody, in which cytoplasmic extracts from both infected and uninfected cell cultures were used.

In order to determine the optimum dilution of antigen for sensitising the ELISA plates, each batch of antigen was titrated. A dilution of 1:80 and an incubation time of 30 min gave an $\Delta A_{404}$ of 0.2 with the positive control serum, and this reaction was visible to the naked eye. The $\Delta A_{404}$ of negative control serum was nil (Fig. 1).

The anti-SVDV antibody positive control sera were tested by ELISA against different strains of the virus in order to verify their reaction. All gave $\Delta A_{404}$ greater than 0.2 after a reaction time of 30 min (Fig. 2). Positive serum of Swiss origin gave an $\Delta A_{404}$ of 0.5, while the negative serum had no $\Delta A_{404}$. The reactivity of different positive sera was related to their immunoglobulin content.

The twelve serum samples from SPF piglets which were monospecific for a given serotype of porcine enterovirus were all negative. Hence the ELISA was apparently specific for detecting anti-SVDV antibody.

In order to establish the correlation between ELISA and serum neutralisation, 453 serum samples (8 positive control sera, 24 from infected herds and 421 from the serum bank) were analysed by both assays. Of 421 samples found to be negative by serum neutralisation, 415 were also negative by ELISA, giving a specificity of 98.6%. From the 32 samples positive for serum neutralisation, 26 were positive also in the ELISA, giving a sensitivity of 81.3%. Comparison of the two tests gave a satisfactory correlation, the 'kappa' value being 0.8 (Table I).

In order to discover which structural viral proteins were present in the cytoplasmic extract, the SVD-positive control sera and six sera from infected herds were examined by immunoblotting. The results showed that VP₀, VP₁, VP₂, VP₃ and VP₄ were present in the infected cytoplasmic extract. SVD virus infected cell lysates served as positive control antigens. Some of the samples from infected herds tested by immunoblotting reacted solely with VP₀ (data not shown).
Antigen dilution

- reaction between positive control serum and positive antigen
- reaction between positive control serum and negative antigen
+ reaction between negative control serum and positive antigen
- reaction between negative control serum and negative antigen

The arrow marks the 1:80 antigen dilution chosen for sensitising the plates. ΔA₄₀₄ of the positive control serum was greater than 0.2. This reaction was visible to the naked eye.

**FIG. 1**

Determination of the optimum antigen dilution for sensitising microplates

**TABLE I**

*Correlation between ELISA and serum neutralisation*

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Serum neutralisation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>positive (a)</td>
<td>negative (b)</td>
</tr>
<tr>
<td>positive (c)</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>negative (d)</td>
<td>6</td>
<td>415</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>421</td>
</tr>
</tbody>
</table>

(a) neutralisation titre greater than 1:16
(b) neutralisation titre of 1:16 or less
(c) The ΔA₄₀₄ of the test sample was at least 30% of that obtained with the positive control serum
(d) The ΔA₄₀₄ of the test sample was less than 30% of that obtained with the positive control serum
The arrow marks the point when, after 30 min, all positive sera had given a ΔA₄₀₄ greater than 0.2. The different reactions correlated with the titre measured by serum neutralisation. The negative serum had a negative ΔA₄₀₄.

**Fig. 2**

Reaction between positive control sera from pigs immunised with different isolates of SVD virus in comparison with a negative control serum

Plates sensitised with freshly prepared antigen and kept frozen at −20°C remained stable for five to six months (Fig. 3). These plates gave higher ΔA₄₀₄ for positive control serum than plates sensitised with antigen which had been stored at −70°C (data not shown). Antigen frozen at −70°C had degraded within five months (data not shown).

**ELISA as a screening test**

In order to determine whether or not anti-SVDV antibody was present in the Swiss pig population, 9,529 samples from the serum bank were tested by ELISA. Of these, 9,358 were judged to be negative (having an ΔA₄₀₄ which was less than 30% of that obtained with the positive serum). Eight of the samples were positive, one of which gave a similar ΔA₄₀₄ to that of the control positive serum. In 163 cases the result was inconclusive (Table II).
Each point is the mean of the $\Delta A_{404}$ of the positive control serum when tested against five batches of antigen. The vertical lines show the standard deviation.

**FIG. 3**

**Stability of microplates sensitised and then stored at $-20^\circ C$**

**TABLE II**

*Application of ELISA to the serum bank*

<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>9,358</td>
<td>98.20</td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>0.09</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>163</td>
<td>1.71</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9,529</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

**Serum neutralisation**

A total of 171 serum samples, comprising the 8 positive and 163 which gave inconclusive results by ELISA, were tested for their capacity to neutralise SVD virus (Table III).

Two of the eight ELISA-positive samples had serum neutralisation titres between 1:16 and 1:32, whilst the other six sera had titres less than 1:16.
TABLE III

Application of the serum neutralisation test to samples positive or inconclusive in the ELISA

<table>
<thead>
<tr>
<th>Serum neutralisation</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Titre</td>
<td></td>
</tr>
<tr>
<td>less than 1:16</td>
<td>6</td>
</tr>
<tr>
<td>between 1:16 and 1:32</td>
<td>2</td>
</tr>
<tr>
<td>greater than 1:32</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
</tr>
</tbody>
</table>

In 151 of the 163 inconclusive samples, serum neutralisation titres were less than 1:16; six had suspect titres between 1:16 and 1:32; and six had positive titres greater than 1:32 (Table III).

The 38 samples which were SVD-positive by ELISA and/or serum neutralisation were tested for their capacity to neutralise serotypes 1, 2, 3, 6 and 8 of porcine enterovirus. Higher titres of serum neutralisation were obtained against these serotypes than against SVD virus. Moreover, all sera neutralised one or more enterovirus serotypes (Table IV).

TABLE IV

Reaction of 38 “SVD-positive” sera against other porcine enteroviruses (PEV)

<table>
<thead>
<tr>
<th>No. of sera(^{(a)})</th>
<th>Serotype</th>
<th>Antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>PEV(_1)</td>
<td>1:56 - &gt; 1:320</td>
</tr>
<tr>
<td>11</td>
<td>PEV(_2)</td>
<td>1:40 - &gt; 1:320</td>
</tr>
<tr>
<td>4</td>
<td>PEV(_3)</td>
<td>1:40 - 1:95</td>
</tr>
<tr>
<td>12</td>
<td>PEV(_6)</td>
<td>1:67 - &gt; 1:320</td>
</tr>
<tr>
<td>3</td>
<td>PEV(_8)</td>
<td>1:40 - 1:190</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Samples which were SVD-positive by the ELISA and/or serum neutralisation test and which gave a higher antibody titre against one particular PEV serotype than against the others. Each serum could neutralise one or more of the other four PEV serotypes.

DISCUSSION

There has been no evidence of clinical SVD in Switzerland since 1973. In order to ascertain if the infection was present in an inapparent form, an ELISA developed for detecting anti-SVDV antibody was applied to a serum bank (obtained from breeding sows and boars). The samples tested in this survey represented 5% of the Swiss pig breeding stock of about 200,000 head.
Since breeding boars and sows live longer than fattening pigs, they provide a more accurate reflection of the epidemiological situation in a population. As their average life span is 2.5 years, they are more at risk of being infected by a given pathogen. Moreover, a sow may produce litters of 10-12 piglets 2-3 times a year, and could act as a reservoir. If antibodies were present, they would be detected more readily in breeding stock (18).

An infection may remain localised among fattening pigs because their movement is limited to transfer to fattening premises. By contrast, breeding animals are moved around more often (to markets, change of ownership, etc.) and could spread disease over a wide area.

In the case of enteroviruses, the structural viral proteins and their precursors are formed and accumulated within the cytoplasm of infected cells. For this reason cytoplasmic extracts of infected and uninfected cell cultures were used as antigens in ELISA. Cytoplasmic extraction is relatively easy, and ELISA is a rapid method, suitable for testing large numbers of samples. In comparison with the serum neutralisation test, ELISA has proved to be very specific for detecting anti-SVDV antibody. It is true that the sensitivity of ELISA is average, but it seemed more important to have a highly specific test, rather than a very sensitive one, in order to examine a large pig population. The ratio of positive to negative samples identified by the serum neutralisation test was about 1:13 (Table I). Thus, the correlation between ELISA and serum neutralisation could be calculated only for a limited number of serum samples; nevertheless, a satisfactory correlation ('kappa' value of 0.8) was obtained between the two tests.

PAGE and immunoblotting techniques showed that all of the structural viral proteins (VP0, VP1, VP2, VP3 and VP4) were present in the cytoplasmic extract. The SVD-positive control sera recognised VP0, VP1 and VP3. Some samples from infected herds recognised only VP0, but they were negative by ELISA. In immunoblotting, the antibodies evidently reacted with epitopes which were inaccessible in the ELISA, probably due to the SDS treatment of the antigen which would have altered the structure of the viral proteins.

Serum samples positive for anti-SVDV antibody by ELISA and/or serum neutralisation were tested for neutralisation of porcine enterovirus serotypes 1, 2, 3, 6 and 8; all gave a positive reaction against at least one serotype. Thus, cross-reactions could be detected between these five PEV serotypes and SVD virus. Such cross-reactions were probably responsible for the false-positive results in ELISA and the serum neutralisation test using SVD virus, which is in agreement with the reports by Golding (6), Sörensen (20) and Zindel (23). The twelve monospecific sera against particular serotypes of PEV gave high neutralising titres solely against the inducing serotype. Such a relationship was not observed by Zindel who found strong cross-reactions between these same monospecific sera (23). An explanation might be that successive freezing and thawing had destroyed some of the IgM, thereby diminishing the cross-reactions between these monospecific sera. Since the SVD-positive samples from the serum bank had been thawed only once before testing, their IgM content should have been intact at the time of ELISA testing, which could explain their positive reactions. Other serum samples from the same locality as these positive sera were free from anti-SVDV antibody.

Most of the samples of Swiss origin were negative for anti-SVDV antibody, and those that were positive neutralised more strongly porcine enteroviruses of serotypes
1, 2, 3, 6 and 8. Moreover, the ELISA method used was specific for anti-SVDV antibody, and no clinical case has been reported since 1973. From these results and observations, it can be stated that SVD is not present, even in an inapparent form, among sows and boars of the Swiss pig population.

It would be interesting to find out if the SVD-positive sera possess antibodies directed against the human Coxsackie B5 enterovirus. This virus is antigenically similar to SVD virus, and has been recovered from nasal and faecal swabs of experimentally-infected pigs. Coxsackievirus B5 does not produce clinical signs in infected pigs, but the induced antibodies are capable of neutralising SVD virus (5, 12).

Since 75% of human beings in contact with this virus fail to develop clinical symptoms, it is highly probable that pigs which show SVD-positive serum reactions may have been infected with Coxsackievirus B5. The antibodies which form in such pigs would recognise SVD virus, and produce false-positive results in serological tests for SVD.

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