Pseudorabies (Aujeszky’s disease) in Argentina *

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Summary: Various methods have been employed for the diagnosis of pseudorabies in Argentina. A large serological survey was carried out by means of enzyme-linked immunosorbent assay (blocking ELISA) and virus neutralisation (VN). An outbreak was studied by virological and immunohistochemical methods and in situ nucleic acid hybridisation.

KEYWORDS: Argentina - Aujeszky’s disease Epidemiological surveys Histology Immunochemistry - In situ hybridisation.

INTRODUCTION

Pseudorabies (Aujeszky’s disease) has a world-wide distribution in pigs, resulting in heavy losses in the swine industry. The disease is caused by porcine herpesvirus type 1 (pseudorabies virus: PRV), a member of the Alphaherpesvirinae subfamily. Pseudorabies is an acute and often fatal neurological disease in neonatal and young pigs; however, in adult pigs, PRV induces sporadic disease with relatively low mortality and, in most cases, animals recover from the illness. PRV establishes latent infection from which the virus can be reactivated (11).

Pseudorabies has been described in South America since 1912 (4). However, Argentina remained apparently free until 1979 (1); since then, a number of outbreaks have been described and confirmed by virus isolation (6, 13, 16).

There are approximately 3.5 million pigs in Argentina. The production system is mainly extensive (40%) and semi-extensive (40%), being intensive on only 20% of farms. No surveys of this disease have been carried out in Argentina.

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The main purpose of the work conducted by the authors was to determine the possibility of exposure of large numbers of pigs to PRV in Argentina. Serum samples were tested by enzyme linked immunosorbent assay (blocking ELISA) and the virus neutralisation (VN) test. Results of both methods were compared. In addition, an outbreak in 1988 was studied by virological, histopathological and immunohistochemical methods and in situ nucleic acid hybridisation.

**MATERIALS AND METHODS**

**Serum sample collection**

A total of 5,955 serum samples were collected at random on 265 farms. The farms were classified according to whether the production system was extensive, semi extensive or intensive.

**Virus and cells**

The RK-13 cell line was employed for virus isolation and the VN test. Cells were grown in Eagle's minimal essential medium containing 10% foetal calf serum and antibiotics. The virus used in the VN test was strain PRV 66 Sweden, kindly provided by Dr J. Moreno López of the Department of Veterinary Microbiology at the Swedish University of Agricultural Sciences, Biomedicum, Uppsala, Sweden. The virus, with a titre of $10^{5.5}$ TCID$_{50}$, was stored at 70°C until use.

**Virus isolation**

Samples were collected during an outbreak which had occurred in the southern part of the Province of Santa Fe. From August 1987 to December 1988, samples were collected from 114 piglets which showed nervous clinical signs. The virus isolates were identified by VN, indirect immunofluorescence (IIF) and peroxidase-antiperoxidase (PAP) techniques.

**Virus neutralisation test**

The conventional method was used in 96-well plates (12). The titres were reported as maximum protective endpoint dilution of serum (15). VN tests were conducted on 207 ELISA positive and 161 ELISA-negative sera.

The chi-square method was used to determine the correlation between the results obtained by ELISA and VN.

**Enzyme-linked immunosorbent assay**

An ELISA kit was used for the serological survey, kindly provided by the International Atomic Energy Agency (IAEA) in Vienna.

Undiluted pig sera were used. Rabbit anti-PRV was employed as second antibody and goat anti-rabbit IgG labelled with horseradish peroxidase as third antibody.

Standard negative and positive pig anti-PRV sera were employed as controls. The results were interpreted by reference to the controls and expressed as a percentage of optical density (OD). Sera with an OD value lower than 40% were considered as negative.
Indirect immunofluorescence test

An IIF was performed on virus-infected cell cultures using rabbit antiserum as primary antibody. Fluorescein isothiocyanate, labelled anti swine IgG (Cappel, United States of America: USA) was employed as second antibody.

Histopathology

Samples of cerebrum, cerebellum, brain stem, gasserian ganglion, spinal cord, tonsil and lung were taken from 22 piglets showing signs of nervousness. The specimens were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin (HE).

Immunohistochemistry

The PAP technique, essentially described by Sternberger (19), was performed on tissue sections and cell cultures, with a rabbit anti-PRV serum diluted 1:2,000 (the serum was kindly provided by Dr R. Ducatelle of the Department of Veterinary Pathology, Faculty of Veterinary Medicine at the University of Ghent, Belgium). Swine anti-rabbit IgG and rabbit PAP complex were commercially purchased (Dakopatts, Denmark). Controls were treated with normal rabbit antiserum as primary antibody.

In situ hybridisation

In situ hybridisation (ISH) was conducted in sections made from paraffin blocks, as described in a previous communication (3). The clone TM 16 (kindly provided by Dr T. Linne of the Department of Veterinary Microbiology, Virology Section, at the Biomedical Centre in Uppsala, Sweden) was biotinylated and used as a probe. Specific hybridisation was detected using the avidin-biotin peroxidase complex (ABC-kit, Vector Laboratories, USA), followed by diaminobenzidin silver enhancement (Amersham, United Kingdom) (3).

RESULTS

Virus isolation

Suspensions of tissues from the central nervous system and lung of clinically-ill piglets were inoculated onto RK-13 cell monolayers. Cytopathic effect (CPE) was observed after two passages. The CPE was characteristic of herpesvirus, with round cell and syncytia formation and intranuclear inclusion bodies clearly detected by HE staining. Six isolates were identified as PRV by VN, IIF and PAP.

Serological survey

A total of 5,955 serum samples belonging to 265 farms were tested by ELISA; 624 of these sera (10.5%) taken from 68 farms (25.7%) were seropositive. The highest percentage was detected in the Province of Santa Fe, with 35.8% infected herds (Table I).

There was significant agreement between ELISA and the VN test (Table II). The proportion of positive sera as measured by ELISA and VN was 56% and 51%,
TABLE I

Pseudorabies seroprevalence among pigs in Argentina determined by blocking ELISA

<table>
<thead>
<tr>
<th>Province</th>
<th>Serum samples Tested</th>
<th>Positive</th>
<th>Herds Tested</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Santa Fe</td>
<td>3,036</td>
<td>439</td>
<td>106</td>
<td>38</td>
</tr>
<tr>
<td>Córdoba</td>
<td>493</td>
<td>36</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>Buenos Aires</td>
<td>2,105</td>
<td>146</td>
<td>122</td>
<td>23</td>
</tr>
<tr>
<td>Chaco</td>
<td>58</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>La Pampa</td>
<td>158</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Entre Ríos</td>
<td>35</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Salta</td>
<td>44</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Tucumán</td>
<td>26</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5,955</td>
<td>624</td>
<td>265</td>
<td>68</td>
</tr>
</tbody>
</table>

(10.5%) (25.7%) respectively. Fifty per cent of the sera were positive in both tests and 43% were negative. Using the chi square ($\chi^2$) method, the proportion with both serological techniques was: $\chi^2 = 287$, where $P<0.001$ (Table II).

TABLE II

Comparison of results of ELISA and virus neutralisation test for pseudorabies virus obtained with 368 field serum samples

<table>
<thead>
<tr>
<th>ELISA</th>
<th>VN positive</th>
<th>VN negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA positive</td>
<td>187</td>
<td>20</td>
<td>207</td>
</tr>
<tr>
<td>ELISA negative</td>
<td>2</td>
<td>159</td>
<td>161</td>
</tr>
<tr>
<td>Total</td>
<td>189</td>
<td>179</td>
<td>368</td>
</tr>
</tbody>
</table>

VN virus neutralisation

With regard to pig production systems, the prevalence of seropositive animals was higher in extensive (32.2%) than in semi extensive (23.7%) or intensive (6.9%) farming conditions (Table III).

TABLE III

Distribution according to breeding system of herds infected with pseudorabies virus

<table>
<thead>
<tr>
<th>Production system</th>
<th>Tested</th>
<th>Farms</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extensive</td>
<td>118</td>
<td>38</td>
<td>32.2%</td>
<td></td>
</tr>
<tr>
<td>Semi extensive</td>
<td>118</td>
<td>28</td>
<td>23.7%</td>
<td></td>
</tr>
<tr>
<td>Intensive</td>
<td>29</td>
<td>2</td>
<td>6.9%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>265</td>
<td>68</td>
<td>25.7%</td>
<td></td>
</tr>
</tbody>
</table>

$P<0.001$
Histopathology and immunohistochemistry

The principal lesions were found in the central nervous system. These consisted of diffuse, non-suppurative meningoencephalomyelitis and ganglioneuritis. Perivascular cuffings, neuronal necrosis and gliosis were also prominent. These changes were frequently observed in the cerebrum and brain stem, occasionally in the gasserian ganglion and rarely in the cerebellum and spinal cord.

Inflammatory-necrotic foci were seen in the tonsils, affecting lymphoreticular tissue and crypt epithelium. Intranuclear inclusion bodies were occasionally present.

Lungs frequently showed pneumonic lesions of variable magnitude. Necrotic foci were not detected.

The PAP-stained sections revealed PRV antigen in the cerebrum, brain stem and tonsils. In the nervous system tissues, the positive reaction usually involves both the nucleus and the cytoplasm of the neurons. Immunoreactive cells were distributed in areas in which histological changes were found.

In tonsils, the immunoperoxidase reaction product was mostly confined to the small necrotic foci and to the margin of the large foci. Groups of infected cells were also observed in the tonsillar crypts, surface epithelium, peripheral nerve fibre and salivary glands (Fig. 1).

**FIG. 1**

Photomicrograph (250×) of tonsil section from a pig naturally infected with pseudorabies virus (PRV) (peroxidase-antiperoxidase technique using rabbit anti-PRV serum on formalin-fixed and paraffin-embedded tissue; positive immunoreactivity demonstrated by dark brown colour produced by p-dimethylaminoazobenzene)
**In situ hybridisation**

Viral DNA was detected only in the tonsil sections. Positively stained cells were mainly located in the surface and crypt epithelium. Few cells were shown to contain virus DNA in necrotising areas and salivary glands (Fig. 2).

**FIG. 2**

Photomicrograph (400 × ) of formalin-fixed paraffin-embedded tonsil section from a pig infected with pseudorabies virus showing positively stained cells (section hybridised with a biotin-labelled probe with silver enhancement)

**DISCUSSION**

Indirect and blocking ELISA tests have been widely used for detection of PRV antibodies (2, 9, 10, 18). Differences in the results of these tests have been reported; for example, the number of false positive sera was lower when using the blocking ELISA than in indirect ELISA (18). In contrast to the indirect ELISA, test samples need not be diluted before use in the blocking ELISA. Some sera could not be assayed in VN due to their cytotoxic effect. The differences observed in the comparative results of ELISA and VN (Table II) could be due to the occurrence of contaminated or cytotoxic sera. In the survey conducted by the authors, approximately 10% of the samples were either contaminated or cytotoxic. In addition, serum samples were tested by ELISA immediately after arrival, while samples for VN were frozen at −20°C until use. Repeated freezing and thawing could have been responsible for a decrease in the titre of antibodies.

In the present study, serum sampling was performed at random; information about the clinical status of each animal was thus lacking and it was not possible to evaluate the sensitivity and specificity of the two techniques employed.
The chi-square method showed a larger proportion of positive sera with ELISA. However, ELISA detects not only neutralising antibodies but also antibodies against antigenic determinants not involved in neutralisation (10).

With ELISA it was possible to test 400 samples every two days, whereas only 250 sera per week could be tested by VN. ELISA was therefore more suitable than VN for the detection of PRV antibodies in large numbers of samples. These results agree with the results obtained by other authors (8, 17, 20).

The results of this study clearly indicate the importance of pseudorabies in Argentina. The prevalence of seropositive pigs is higher in the central part of the country (Santa Fe and Córdoba Provinces) (Table I) where the disease was first reported in 1979 (1) and all the following outbreaks were localised (6, 13, 16). Santa Fe and Córdoba Provinces form the main swine producing region in the country and the best farms are located there.

An interesting observation was the much lower prevalence of pseudorabies in intensive breeding conditions (6.9%) despite the close contact between animals. In extensive production systems, the prevalence increased to 32.2% (Table III). This difference could be attributed to the higher sanitary standards maintained on the intensive farms.

In one outbreak, six strains of PRV were isolated from the central nervous system tissues of sick animals. The isolated strains were identified as PRV by serological and immunohistochemical methods. Further studies will be necessary for better characterisation of these strains.

The histopathological picture agreed with the results obtained by other authors (5, 14). PRV antigen demonstrated by the PAP technique appeared in direct association with degenerated or necrotic cells, similar to previous descriptions (7). PAP-positive cerebrum and brain stem sections were negative by ISH. The hybridisation method has viral DNA as the detection target, while the PAP technique detects viral antigenic proteins. In the immunohistochemical method, a polyclonal PRV antiserum was employed, which probably reacted even with the proteins of early viral replication (3).

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Résumé : Différentes méthodes ont été employées pour le diagnostic de la maladie d’Aujeszky en Argentine. Une importante étude sérologique a été effectuée à l’aide de la méthode ELISA (anticorps bloquants) et de la neutralisation virale. Un foyer a été étudié par des méthodes virologiques et immunohistochimiques, et par hybridation in situ des acides nucléiques.


Resumen: Se han empleado diversos métodos para el diagnóstico de la enfermedad de Aujeszky en Argentina. Se llevó a cabo un extenso estudio serológico utilizando las pruebas de dosificación inmuno-enzimática (ELISA bloqueante) y de neutralización del virus. Se estudió un foco mediante métodos virológicos e immunohistoquímicos y la hibridación del ácido nucleico in situ.

PALABRAS CLAVE: Argentina Enfermedad de Aujeszky - Estudios epidemiológicos Hibridación in situ Histología - Inmunocuímica.

REFERENCES


