A SEROLOGICAL SURVEY AND THE DIAGNOSIS OF PSEUDORABIES VIRUS AMONG PIGS IN ARGENTINA

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Abstract

A SEROLOGICAL SURVEY AND THE DIAGNOSIS OF PSEUDORABIES VIRUS AMONG PIGS IN ARGENTINA.

An enzyme linked immunosorbent assay (blocking ELISA) was used to detect antibodies to pseudorabies virus (PRV) in serum samples from 5955 pigs. The results of this test indicated that 10.5% of the samples were positive to pseudorabies. Virus neutralization (VN) test was used as a confirmatory test on 207 positive and 191 negative sera respectively. Using the VN test as standard, the ELISA showed a relative specificity and sensitivity of 88.8% and 98.9%, respectively. An outbreak of pseudorabies was also studied by virological, immuno-histochemical and in situ nucleic acid hybridization methods.

1. INTRODUCTION

Pseudorabies (Aujeszky's disease) has a worldwide distribution in pigs, causing heavy losses for the swine industry. The disease is caused by suid herpesvirus type 1 (pseudorabies virus, PRV), a member of the alphaherpesvirinae subfamily. Pseudorabies is an acute and often fatal neurological disease in neonatal pigs, whilst in adult pigs PRV induces sporadic disease with relatively low morbidity and mortality.

PRV is harbored by recovered pigs in a latent stage, from where it can be reactivated causing recrudescence and spread to susceptible animals [1]. Pseudorabies has been known to cause disease in pigs in South America since 1912 [2]. However, Argentina remained apparently free until 1979 [3]. Thereafter, several outbreaks have been described and the disease confirmed by virus isolation [4-6].

There are approximately 3.5 million pigs in Argentina. The production system is mainly extensive (40%) and semi-extensive (40%), being intensive only in 20% of the farms. No surveys investigating the prevalence of PRV infection have been carried out in Argentina, and this was the main purpose of the present study.

2. MATERIALS AND METHODS

2.1. Collection of serum samples

Serum samples from 5955 pigs on 265 different farms were collected at random. The farms were classified according to the production system i.e. extensive, semi-extensive and intensive farming.

2.2. Virus and cells

The rabbit kidney (RK-13) cell line was used for virus isolation and for the virus neutralization (VN) test. Cells were grown in Eagle's minimal essential medium containing 10% foetal calf serum and antibiotics. The virus strain used in the VN test was the reference strain PRV/66/Sweden, kindly provided by Dr. Moreno-Lopez, Uppsala, Sweden. The virus, with a titer of $10^{5.5}$TCID 50, was stored at -70°C until use.

2.3. Virus isolation

Samples from the brain, tonsils, lung and lymph nodes were collected during an outbreak of the disease which occurred in the southern part of the Province of Santa Fe. The samples collected from August 1987 to December 1988, were from 114 piglets showing disorders of the central nervous system. The virus isolates were identified by VN, indirect immunofluorescence (IIF) and peroxidase-antiperoxidase (PAP) techniques.

2.4. Virus neutralization (VN) test

The micro-neutralization test in 96-well plates was carried out as described by Banks and Cartwright [7]. The titres were reported as maximum protective end point dilution of serum [8]. The VN test was conducted on 207 positive and 161 negative sera as determined by ELISA.

Chi square (x^2) method was used to determine the correlation between the results obtained by ELISA and VN test.

2.5. Enzyme linked immunosorbent assay (ELISA)

For the serological survey, a blocking ELISA kit was provided by the Joint FAO/IAEA Division, Vienna [9]. The test was carried out according to the procedure described in the ELISA kit manual. Briefly, undiluted test sera, reference positive serum (diluted 1:80) and reference negative serum (diluted 1:100) were added to the antigen coated wells in duplicate. The plates were covered with a sealing tape, incubated overnight at room temperature and then after three washes, rabbit anti-PRV diluted 1:1000 was added. After incubation for 30 minutes at room temperature, the plates were washed three times and swine anti-rabbit IgG-HRP conjugate diluted 1:2000 was added. A further incubation for 30 minutes was carried out at room temperature, the plates were washed three times and then substrate was added. The reaction was stopped

by adding 50 μl of 1M H_2SO_4 to all wells. The results were interpreted by reference to the controls, and expressed as a percentage of optical density (OD). Sera with OD% values lower than 40% were considered as negative.

2.6. Indirect immunofluorescence (IIF) test

The IIF was performed in a conventional way on virus infected cell cultures using rabbit antiserum as primary antibody. Fluorescein isothiocyanate (FITC) labelled swine anti-IgG (Cappel, USA) was employed as second antibody.

2.7. Histopathology

Cerebrum, cerebellum, brain stem, Gasserian ganglion spinal cord, tonsils and lung samples were taken from 22 piglets with disorders of the central nervous system. The specimens were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin (HE).

2.8. Immunohistochemistry

The peroxidase-antiperoxidase (PAP) technique was performed on tissue sections and cell cultures basically as described by Sternberger [10]. A rabbit anti-PRV serum (kindly provided by Dr. Ducatelle, Ghent, Belgium), diluted 1:2000 was used. Swine anti-rabbit IgG and rabbit PAP complex were commercially purchased (Dakopatts, Denmark). Controls were treated with normal rabbit antiserum as the primary antibody.

2.9. In situ hybridization (ISH) test

This technique was conducted in tissue sections prepared in paraffin blocks as previously described [11]. The biotinylated PRV-DNA clone TM 16 (kindly provided by Dr. Linne, Uppsala, Sweden) was used as a probe. The specific nucleic acid hybridization was detected using the avidin-biotin peroxidase complex (ABC-kit, Vector Laboratories, USA), followed by diaminobenzidin (DAB) silver enhancement (Amersham, UK) [11].

3. RESULTS

3.1. Virus isolation and serological survey

Suspensions of tissues from the central nervous system and lungs of 25 clinically ill piglets were inoculated onto RK-13 cell monolayers. A cytopathic effect (CPE) was observed after two passages. The CPE was characteristic of herpesvirus, with rounded cells, syncytia formation, and intranuclear inclusion bodies clearly detected with HE staining. Six isolates were identified as PRV by VN, IIF and PAP tests.

TABLE I. PSEUDORABIES SEROPREVALENCE AMONGST PIGS IN ARGENTINA DETERMINED BY THE BLOCKING ELISA

Province	Serum Samples		Herds	
	Tested	*Positives*	*Tested*	*Positive*
Santa Fe	3036	439	106	38
Cordoba	493	36	23	5
Buenos Aires	2105	146	122	23
Chaco	58	2	2	1
La Pamba	158	1	3	1
Entre Rios	35	-	2	-
Salta	44	-	4	-
Tucuman	26	-	3	-
Total	5955	624 (10.5%)	265	68 (25.7%)

TABLE II. COMPARISON OF ELISA AND VN RESULTS OBTAINED WITH 368 FIELD SERUM SAMPLES

ELISA	VN positive	VN negative	Total
ELISA positive	187	20	207
ELISA negative	2	159	161
Total	189	179	368

For the serological survey a total of 5955 sera collected from 265 farms were tested by blocking ELISA and the results of this assay indicated that 624 sera (10.5%) from 68 farms (25.7%) were positive. The highest percentage of positive sera was detected in the Province of Santa Fe with 35.8% of the herds infected (Table I).

There was a good correlation between ELISA and the VN test (Table II). Using the Chi square method the proportion of positive sera in both tests was $x^2=287$ ($p<0.001$). The relative sensitivity and specificity of the ELISA in comparison with the VN test was 98.9% and 88.8%, respectively.

With respect to the 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 OF PRV INFECTED HERDS ACCORDING TO THE BREEDING SYSTEM

Production system	Farms		
	Tested	Positive	Percentage
Extensive farming	118	38	32.2
Semi-extensive farming	118	28	23.7
Intensive farming	29	2	6.9
Total	265	68	25.7 p= <0.001

3.2. Histopathology and immunohistochemistry analysis

The principal lesions were found in the central nervous system (CNS). These consisted of diffuse, non-suppurative meningoencephalomyelitis and ganglioneuritis. Perivascular cuffings, neuronal necrosis and gliosis were also prominent. The changes were frequently observed in the cerebrum and brain stem, occasionally in the Gasserian ganglion, and rarely in the cerebellum and the 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 lesion of variable magnitude. Necrotic foci were not detected.

The PAP-stained sections revealed PRV antigens in the cerebrum, brain stem and tonsils. In the nervous tissues, the positive reaction usually involved both the nucleus and the cytoplasm of the neurones. Immuno-reactive cells were distributed in areas in which histological changes were found.

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

3.3. <u>In situ</u> hybridization

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

4. DISCUSSION

Indirect and blocking ELISA tests have been widely used for detection of PRV antibodies [7, 12-14]. Differences in the results of these tests have been reported. A number of reports have claimed a higher

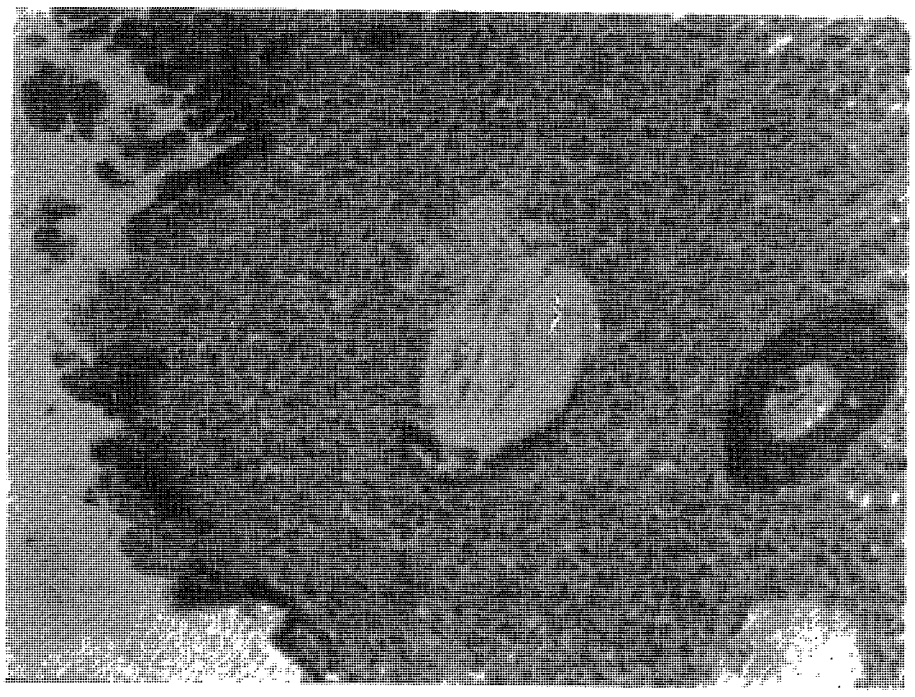

Fig. 1. Photomicrograph of tonsil section from a pig naturally infected with PRV. PAP technique using rabbit anti-PRV serum on formalin-fixed and paraffin-embedded tissue. Positive immunoreactivity is demonstrated by the darker coloured areas (250 x).

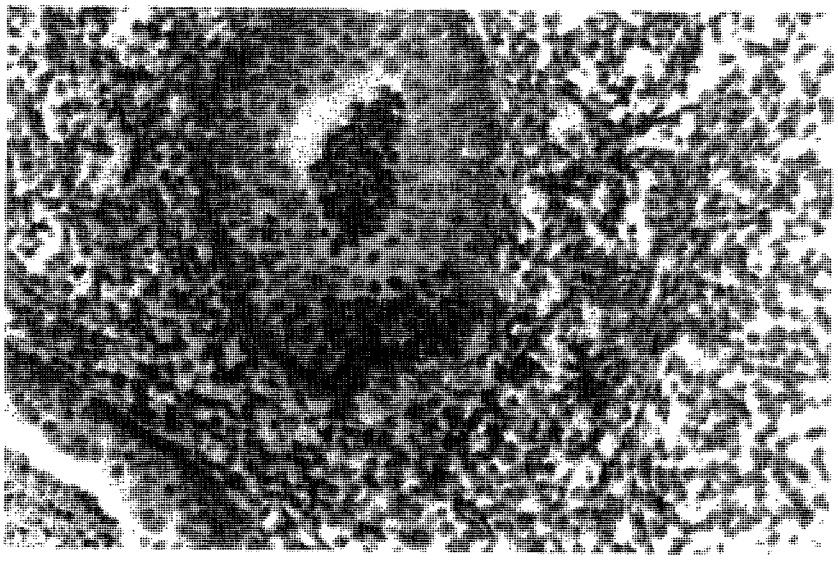

Fig. 2. Photomicrograph of formalin-fixed paraffin-embedded tonsil section from PRV-infected pig showing positively stained cells. Section hybridized with a biotin-labelled probe with silver enhancement (X 400).

sensitivity for the PVR indirect ELISA when compared with the VN test and argued substantial practical advantages over the VN for routine use (for references see [15]). This was also evident from our work. Using ELISA more than 400 serum samples were processed in 2 days whereas only 250 sera could be tested by VN in a week. Moreover, serum samples could be tested by ELISA immediately after arrival, while samples for VN test for practical reasons were frozen at -20 C until tested. The repeated freezing and thawing of the sera may affect the results of the test.

A good correlation between the results obtained with the blocking ELISA and the VN test was reported by Soerensen and Lei [14]. The specificity of the ELISA in our hands was lower than that reported by these workers and this may be caused by technical factors but also by the nature of the individual immunological response to infection.

The results in this study clearly indicate the importance of pseudorabies in Argentina. The prevalence of seropositive pigs was higher in the central part of the country, (Santa Fe and Cordoba Provinces) (Table I). The Santa Fe and Cordoba Provinces are the main swine producing regions in the country and the farms with the highest production are located there.

It was of interest to observe that the prevalence of pseudorabies was much lower in intensive breeding conditions (6.9%) in spite of the close contact of the animals. In extensive production system the prevalence increased to 32.2% (Table III). This difference may be attributed to the higher sanitary conditions in the intensive production system.

Six strains of PRV were isolated from the central nervous system of diseased animals in one outbreak. The isolated strains were identified as PRV by serological and immunohistochemical methods. Further studies will be necessary for better characterization of these strains.

The histopathological picture agreed with the results obtained by others [17, 1]. PRV antigen demonstrated by the PAP technique appeared in direct association with degenerated or necrotic cells, similarly to previous descriptions [16]. PAP positive cerebrum and brain stem sections were negative with the ISH method. The hybridization method has viral DNA as detection target, while the PAP technique detects viral proteins. In the immuno-histochemical method, a polyclonal PRV antiserum was employed, which probably cross-reacted even with the proteins of early viral replication [11]. This could be one of the reasons for its higher sensitivity compared to ISH. However, it can not be excluded that ISH is a less sensitive than the PAP. This has to be further evaluated.

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