Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1970, 42, 767-770

Experimental Infection of Weanling Pigs with A/Swine Influenza Virus *

2. The Shedding of Virus by Infected Animals

D. BLAŠKOVIČ,[1] O. JAMRICHOVÁ,[1] V. RATHOVÁ, D. KOČIŠKOVÁ [1] & M. M. KAPLAN [2]

As part of a study described in a previous paper observations were made to determine whether and for how long experimentally infected young pigs would transmit their infection to new groups of weanlings maintained in contact with them. When groups of 4 or 5 susceptible weanlings 2–3 months old were placed in contact for a month with infected pigs 42 days or 3, 6, 9 or 12 months after experimental infection, no antibody rises were observed in the contact pigs. However, a strain of virus identical with the infecting strain was isolated from lung suspensions from 2 of the 5 contact pigs exposed to pigs infected 3 months previously. Possible sources of technical error such as laboratory contamination could be almost certainly excluded. It is considered that a shedder state of virus had occurred some time during the fourth month following experimental infection. There was suggestive serological evidence that the shed virus acted as a booster dose to previously infected pigs.

The aim of this study was to determine whether and for how long young pigs, after an acute experimental infection with A/swine influenza virus, are infective for susceptible animals in contact with them.

MATERIAL AND METHODS

Infected animals

Twenty-two weanling pigs, 42, 52, 54 or 77 days old, were infected as described in a previous paper (Blaškovič et al., 1970). All animals recovered from the infection.

Contact animals

Groups of 4 or 5 new weanlings each were placed in pens together with the recovered pigs 42 days or 3, 6, 9 or 12 months after infection. The weanlings, approximately 2–3 months old, came from an outside herd, where no influenza infection had been recognized, and all animals were free of swine influenza antibodies (haemagglutination-inhibition test). The new animals were maintained in contact for one month and then killed and their organs examined for the presence of virus.

Virus isolation

Attempts to isolate virus were made in either of the following ways.

Egg inoculation. Suspensions (10%) were made in broth (containing penicillin and streptomycin) from tonsils, nasal mucosa, trachea, bronchi, lung, submandibular salivary gland, cervical lymph nodes, mediastinal lymph nodes and pericardial fluid and then inoculated into embryonating eggs.

Organ explants.[3] The organs or tissues (upper, middle and lower parts of trachea, lungs, and lymph nodes) were cut by scissors into pieces roughly 3 mm × 3 mm. Ten explants from each sample were placed in a Petri dish (4 cm diameter). The explants were overlaid with 3 ml of growth medium 199 (containing 2% inactivated calf serum with Earle's solution, and 250 IU of penicillin and 250 μg streptomycin) and kept in the incubator at 34°C in an atmosphere of 5% carbon dioxide. After 24 hours, and then every 3 days for 15 days, the original medium was removed

* This work was partially supported by financial assistance from the World Health Organization.

[1] Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia.

[2] Chief, Veterinary Public Health, Division of Communicable Diseases, World Health Organization, Geneva.

[3] This method was kindly suggested by Professor B. C. Easterday, University of Wisconsin, Madison, Wis., USA.

and new medium added. The removed fluid was tested for haemagglutination activity with chicken and guinea-pig erythrocytes. The fluid was also inoculated intra-amnionically into 11–12-day-old embryonating eggs. The method of organ explants was used in all but the first contact group. The inoculation of material was usually made immediately the fluid was drawn; exceptionally, the material was stored at −60°C.

All attempts at virus isolation were carried out in a laboratory where no work with influenza viruses had previously been done.

Serology

For the haemagglutination-inhibition (HI) test, serial twofold dilutions of serum (0.25 ml) were mixed with an equal volume of purified virus containing 4 HA units, and the mixture was incubated at 4°C for 30 minutes. Then 0.5 ml of a 0.5% suspension of chicken erythrocytes was added to each test-tube, and the mixture kept at 4°C for 60 minutes.

The HI titre is expressed as the reciprocal of the highest dilution of serum (i.e., per 0.25 ml) showing complete inhibition of haemagglutination.

The virus neutralization test was performed as described in the previous paper (Blaškovič et al., 1970).

Immunization of hamsters. Ten Syrian hamsters (weight about 70 g) were immunized intranasally with 4 drops of partially purified [1] virus of the strain A/Swine/Mĕrotin/57 after 96 egg, 10 swine and a further 4 egg passages and having an HA titre of 1:12 800 (per ml) and, simultaneously, with a dose of 0.5 ml intraperitoneally. One week later a second dose of 0.5 ml was administered intraperitoneally.

Nine adult Syrian hamsters (weight about 70 g) were immunized with virus (partially purified) isolated from pig No. 145 (a contact animal) in its 5th egg passage and with an HA titre of 1:6400 per ml. The immunization procedure was the same as that just described.

Absorption of sera with the virus. To 1 ml of serum, 2 ml of purified virus with an HA titre of 1/8200 and 1 ml of phosphate-buffered saline were added. Sera were diluted 1:4. The mixture was kept at 4°C overnight. It was then centrifuged for 1 hour at 20 000 rev/min. All sera were heated at 56°C for 30 minutes.

Electron microscopy

Swine influenza virus, Mĕrotin strain, after 96 egg, 10 swine and a further 4 egg passages, and the new isolate from contact animal No. 145 in the 6th egg passage were adsorbed and eluted several times on chicken erythrocytes. Specimens for negative staining of the purified viral material in a dilution of 1:10 were treated with phosphotungstic acid and observed in the electron microscope (JEM 6c at 80 kV). The electron micrography was kindly performed by Dr M. Nermut, Institute of Virology, Bratislava.

RESULTS

No virus was isolated from the first contacts (4 pigs), that were introduced among the recovered weanlings 42 days after experimental infection of the latter.

The second group of 5 contact animals was introduced among the recovered weanlings on 12 January 1968 (3 months after infection) and killed on 12 February 1968 (pigs No. 141 to 145). In the first intra-amniotic egg passage of lung suspension from animals No. 144 and 145, slight haemagglutination was observed. In the second and third passages, the amniotic fluid gave haemagglutination titres of 1:8 to 1:16, higher titres being obtained with guinea-pig erythrocytes than with chicken erythrocytes. In further egg passages the haemagglutination titres increased, and the virus recovered was compared antigenically (see accompanying table) and under the electron microscope. Both strains, Mĕrotin and the isolate, gave identical HI titres with immune hamster sera.

Under the electron microscope, the Mĕrotin strain was seen to contain elongated virions, including very large particles. The configuration of the nucleocapsid and the spikes were characteristic of influenza viruses. The isolate from pig No. 145, in its 6th egg passage, gave similar results, and contained filamentous forms. There were no differences in shape between the two viral preparations.

No virus was isolated from the additional groups of 5 contact animals introduced 6, 9 and 12 months after infection.

Negative results were obtained in serological tests (HI and VN) of the live contact animals during the entire period of observation.

No lungworms were found in the autopsied animals.

DISCUSSION

The results obtained in this experiment show that pigs intranasally infected with swine influenza virus may maintain the virus in their lung tissue without

[1] By adsorption on to and elution from erythrocytes.

HAEMAGGLUTINATION-INHIBITION WITH IMMUNE HAMSTER SERA AGAINST MĚROTIN STRAIN AND VIRUS ISOLATE FROM CONTACT ANIMAL No. 145 [a]

Immune serum	Antigen		
	Purified Měrotin strain	Purified swine 145 isolate	Serum control
Hamster anti-Měrotin strain	128	256	<8
Hamster anti-Měrotin absorbed with virus isolate 145	8	8-16	<8
Hamster anti-isolate 145	128	256	<8
Hamster anti-isolate 145 absorbed with Měrotin strain	<8	<8	<8

[a] Results expressed as reciprocals of end-point serum dilutions.

clinical signs for at least 3 months after being infected, even in the presence of humoral antibody (Blaškovič et al., 1970). The virus can be spread to susceptible animals, and the contacts can acquire the infection without showing any clinical signs.

The possible objection that the virus was already present in the newly introduced contacts is not tenable because these contact animals originated from a herd where no swine influenza had been observed in the past, were antibody-negative, and did not develop antibodies during the entire period of contact with the experimental herd.

These observations suggest that infection occurred in one group of animals during their last week of contact, since infection was detected at autopsy by virus isolation, and before antibody response had occurred.

There is no indication as to which tissues or cells maintained the virus in the experimentally infected animals, because they were kept for a further 9 months in the herd and found not to be infectious for newly introduced contacts after the third month.

There is also no indication that the virus was brought into the herd by the persons taking care of the animals (see Blaškovič et al., 1970). The opposite, in fact, seems to have occurred; i.e., swine influenza virus was acquired by these persons from pigs or from laboratory sources during the first months of the experiment.

It has several times been suggested that the reactivation of swine influenza infection is provoked by unfavourable climatic conditions when " masked " virus is present in the lungworms of swine (Shope, 1941, 1943). Whether the experimentally infected weanlings had lungworms prior to infection is not known, but precautions were taken to prevent lungworm infection during the experiment (Blaškovič et al., 1970) and no lungworms were found on autopsy. Also, the animals were selected from a herd in which no influenza infection had been observed in the past, and the infection was not recognized in neighbouring herds either clinically or serologically (see Blaškovič et al., 1969).

The climatic conditions of the period in question do not support the weather component in Shope's hypothesis (see above) if we assume that the shedder state had occurred at the end of January or at the beginning of February 1968.

From 12 to 14 January 1968, the lowest average outside daily temperature was −9.3°C to −9.9°C, followed by an abrupt rise until 20 January. The temperature dropped again on 27 January (average between −1°C and +0.8°C). From then until 12 February 1968 the daily temperature fluctuated between +4.2°C (the highest) and +0.1°C (the lowest). It is difficult to believe that the fluctuations in temperature outside the piggery would be reflected in the indoor microclimate and would be responsible for any reactivation of virus in the infected animals. The only evidence suggesting that the virus might have been circulating among the animals in the experiment is the slight increase of average antibody titres of 52–54 and 77-day-old weanlings during the period in question between the third and fourth months after infection of these animals. This slight rise could be interpreted as a booster effect of shed virus. And some slight support for this suggestion is afforded by the interpretation of the minor rise in

antibody titre of the persons handling the animals that has been advanced in the previous paper (Blaškovič et al., 1970).

However, when the curves representing antibody titres during the 1-year observation period are considered, the minor rises just referred to are not convincing evidence that the virus had been spread widely in the environment.

The possibility of a technical error is small because the primary isolation of the virus from the contact animal resembled in all respects a new isolation of virus—a low initial HA titre that increased after passage, and a higher titre with guinea-pig than with chicken erythrocytes. Finally, the virus isolation procedure was carried out in a laboratory that had never previously dealt with influenza viruses.

The only reasonable explanation, therefore, is that the virus was shed from animals which had been infected 3 months earlier with swine influenza virus and had become carriers of the virus.

ACKNOWLEDGEMENTS

The technical help of Dr T. Sadlon, Mrs K. Kovalčíková, Mrs B. Záhumenská and Mr K. Vančo is greatly appreciated.

RÉSUMÉ

INFECTION EXPÉRIMENTALE DE PORCS RÉCEMMENT SEVRÉS PAR UN VIRUS GRIPPAL PORCIN DE TYPE A: 2. EXCRÉTION DU VIRUS PAR LES ANIMAUX INFECTÉS

Dans le cadre des recherches sur l'infection expérimentale du porc par un virus de la grippe porcine (souche Měrotin) décrites dans l'article précédent, on a étudié les modalités de la propagation de l'infection ainsi provoquée à des animaux neufs. Des groupes de 4-5 porcs récemment sevrés et non immuns, âgés de 2-3 mois, ont été placés en contact étroit, pendant un mois, avec les animaux infectés, respectivement 42 jours, 3, 6, 9 et 12 mois après la date de l'inoculation expérimentale.

Toutes les épreuves (inhibition de l'hémagglutination, neutralisation du virus) pratiquées sur le sérum des animaux contacts sont restées négatives. Une souche de virus identique à la souche Měrotin a été isolée à partir d'une suspension de tissu pulmonaire chez 2 des 5 porcs mis en contact avec leurs congénères infectés 3 mois auparavant. Aucun virus n'a été isolé chez les autres animaux contacts.

Les auteurs excluent toute éventualité d'une contamination accidentelle et estiment qu'un processus d'excrétion et de propagation du virus est intervenu chez les animaux infectés expérimentalement au cours des 3e-4e mois suivant l'inoculation. Les observations sérologiques donnent à penser que le virus ainsi éliminé a agi comme une dose de rappel chez les porcs déjà infectés.

REFERENCES

Blaškovič, D., Rathová, V., Skoda, R., Kočišková, D. & Kaplan, M. M. (1970) *Bull. Wld Hlth Org.*, **42**, 757-765

Blaškovič, D., Skoda, R., Rathová, V., & Kočišková, D. (1969) *Cš. Epidem.*, **18**, 278-283

Shope, R. E. (1941) *J. exp. Med.*, **74**, 49-68

Shope, R. E. (1943) *J. exp. Med.*, **77**, 127-138