

1Pos copy
9602782-6

- 1 -

64130J.63

THIS SHEET HAS BEEN
REPLACED

The invention relates to the isolation of a viral agent associated with "mystery swine disease" and methods of growing it, methods of attenuating the virus and producing a vaccine.

BACKGROUND

Recently, an apparently new swine disease causing heavy losses in breeding herds has appeared in the United States and in certain regions of Canada (Keffaber, K.K., "Reproductive Failure of Unknown Etiology", American Association of Swine Practitioners Newsletter, 1:109 (1989)). A similar disease has also been reported in certain regions of Germany, in the Netherlands and also in the United Kingdom and Spain (Wensvoort, G. et al., "Blue Ear Disease of Pigs", Vet. Rec., 128:574 (1991)). The most prominent clinical symptom of the disease is the farrowing of dead or sickly piglets, with some healthy-appearing piglets subsequently faring poorly or developing impaired breathing, CNS symptoms and dying. In some affected herds, up to seventy-five percent of all piglets may be lost. The economic consequences of the disease, accordingly, are devastating. It has been called "mystery swine disease", Swine Infertility and Respiratory Syndrome (SIRS), "blue ear disease", and "Porcine Reproductive and Respiratory Syndrome".

SIRS is an apparently infectious disease, characterized by reproductive failure, respiratory disease and variable clinical signs including anorexia, fever, dyspnea and mild neurological signs. The disease affects all types of swine production facilities, and may be among the most costly of diseases affecting the swine industry today (Polson, D.P. et al "Financial Implications of Mystery Swine Disease", Proceedings,

This Sheet Has Been Replaced



Livestock Conservation Institute, Denver, CO. Oct. 6, 1990).

The infection of sows may go unnoticed, or may manifest itself by impaired general condition lasting one or a few days. For example, the sows may go off feed and show body temperatures above or below normal. In the farrowing phase, the signs of disease include depression, lethargy, pyrexia, occasional vomiting, abortions, stillbirths and/or delivery of mummified fetuses. The most frequent clinical sign is the dramatic increase in the number of stillborn pigs. The number of stillbirths can be as high as twenty percent to thirty percent of all births in an affected herd. Many of the fetuses delivered as stillbirths will be macerated, appearing to have been dead for 24 to 48 hours.

As explained above, the main component of SIRS is reproductive failure, which manifests itself in premature births, later abortions, pigs born weak, increased stillbirths, mummified fetuses, increased farrowing rates and delayed return to oestrus. Such clinical symptoms will typically be observed in a herd from 4 to 16 weeks, or even longer. Stillborn fetuses in affected litters are often in the early stages of mummification, as evidenced by yellowish-brown discoloration of the skin and post-mortem autolysis. Dome-shaped malformation of foetal skulls is sometimes seen.

Clinical signs of respiratory disease are most pronounced in piglets under 3 weeks of age, but are reported to occur in pigs of all ages in affected herds. The diseased piglets grow slowly, have roughened hair coats, respiratory distress and increased mortality (up to about eighty percent pre-weaning mortality).

Findings in preliminary studies of gross and microscopic lesions of SIRS diseased swine suggest that microscopic lung lesions are an important feature of

this disease. Despite pronounced respiratory signs, lungs uncomplicated by secondary bacterial infection are either grossly normal or have a mild, diffuse brownish-grey discoloration of lung surface. Microscopic examination of lung tissue of SIRS diseased piglets, however, reveals a characteristic pattern of interstitial pneumonitis (Collins, J.E. et al.

"Respiratory Disease in a Swine Herd Experiencing a Reproductive Failure Syndrome", Proceedings, Minnesota Swine Conference for Veterinarians, p. 254, St. Paul, MIN. (Sept. 10-18, 1990)).

The incidence of SIRS or "mystery swine disease" is widespread in the United States, reported in at least eleven states. The primary causative agents being studied, as reported in the published literature, include encephalomyocarditis virus (EMC), swine influenza virus (SIV), mycotoxins and chlamydia.

THE INVENTION

A tissue homogenate obtained from piglets in SIRS-affected herds consistently reproduced the respiratory and reproductive forms of SIRS when intranasally inoculated in gnotobiotic piglets and pregnant sows. Gnotobiotic piglets so inoculated with either unfiltered or filtered (0.45, 0.22, or 0.1 μ m) inoculum became anorectic and developed microscopic lung lesions similar to lesions seen in SIRS-affected herds. The same inoculum also caused reproductive effects identical to those seen in SIRS-affected herds. A viral agent has been recovered from the tissue homogenate. The viral agent will cause a disease that mimics SIRS in piglets and pregnant sows. A deposit of the viral agent has been made on July 18, 1991, with the American Type Culture Collection in Rockville, Maryland under the accession number ATTC-VR2332. The viral agent is a demanding, non-haemagglutinating, coated RNA virus.

A. ISOLATION

Lung tissue and combined brain-spleen-liver-kidney tissues obtained from an infected piglet in a SIRS-infected herd were homogenized separately. The individual homogenates were mixed with "Modified Eagle's Medium" (MEM) containing gentamycin at about 100 μ g per ml. Both samples were centrifuged at about 4000 x g for about twenty-five minutes. The supernatant was then removed and filtered through a 0.45 micron filter. The tissue and lung homogenates were then combined, and the combined material was used to infect various tissue culture bottles.

Two tests were conducted using 75cm² plastic bottles. In test no. 1, the combined material was inoculated into two bottles of full cell sheet of each of the cell lines listed below. Additionally, to one bottle of each cell line about 2.5 mg of trypsin was added. All other remaining conditions were the same for each bottle of cell line. Serum was not added to the culture medium. The inoculum was about 1 ml. All bottles were held for about seven days at approximately 4°C. The results were recorded at the end of seven days. After freezing and thawing, a sample was taken for a second passage in the same cell line. The remaining material was frozen and stored at about -60°C.

In test no. 2, the combined material was inoculated into bottles of the same cells as were used in test no. 1. However, the cell sheets were only 20-40% confluent at the time of inoculation. The media contained about 10% foetal calf serum. Again the inoculum was 1 ml, and the cultures were incubated at about 34°C for approximately seven days. The results of both test no. 1 and test no. 2 are summarized below:

CELL LINE USED	TEST NO. 1	TEST NO. 2
BOVINE TURBINATE (BT)	-	-
FELINE KIDNEY (CRFK)	-	-
MONKEY KIDNEY (VERO)	-	-
MONKEY LUNG (VERO)	-	-
CANINE KIDNEY (MDCK)	-	-
PORCINE KIDNEY (PK2a)	-	-
MINK LUNG	-	-
FERRET LUNG	-	-
BOVINE LUNG	-	-
BUFFALO LUNG	-	-
BOVINE KIDNEY (MDBK)	-	-
SWINE TESTICLE (ST)	-	-
MONKEY KIDNEY (MA-104)	-	+
HUMAN RECTAL TUMOR (HRT-18)	-	NT
HUMAN LUNG	NT	-
+ = cytopathic effect (CPE)		
- = no cytopathic effect		
NT = not tested		

There was no cytopathic effect observed in test no. 1 in any of the cell lines evaluated. In test no. 2, however, small clumps of MA-104 cells began to swell and form "weak holes" in the monolayer around the edges of the bottle. Fluid was separated from the bottle, and passed into a new bottle of MA-104 cells (again 20-40% cell sheet), and then subsequently passed a third time. The cytopathic effect became stronger with each passage. The above-described procedures were repeated for the MA-104 cell line employing a full cell sheet. A cytopathic effect (CPE) was also observed.

Further testing also demonstrated that the viral agent will also grow at 37°C. The presence of serum may be helpful for the initial isolation of the viral agent. Subsequent passages of the viral agent in the MA-104 cell line will produce the CPE without the presence of

serum. However, more pronounced CPE is observed with the use of serum in the growth medium for the MA-104 cell line.

It has also been determined that the viral agent is partially resistant to heating at about 56°C for approximately 50 minutes. As a result of such heat treatments, the virus titer was reduced by 3 logs. The viral agent has been passed eight times in the MA-104 cell line with good CPE developing in three days at passage five and greater. The titer obtained is approximately $10^{5.5}$. The viral agent will grow also in other simian cell lines. Consequently, it is within the capabilities of an expert in this field to grow the SIRS virus ATCC-VR2332 or mutants derived therefrom in other cell lines by modifying the growth conditions, growth media, etc.

B. IN VIVO TESTING

A third passage harvest was used to inoculate two three-day old gnotobiotic piglets. Both piglets were exposed intranasally, one with 1 ml and the other with 2 ml. The piglets were observed for seven days, and were then killed.

Tissue samples were collected for histopathology and for recovery of the viral agent. The histopathology report confirmed that lung lesions in the infected piglets were identical to lung lesions from piglets known to have SIRS.

The tissue samples were processed as before, and then cultured on 20-40% and 100% monolayers of the MA-104 cell line with foetal calf serum. The viral agent was again recovered.

A third passage harvest was also used to inoculate sows in order to verify that the reproductive effects of the disease can be duplicated and confirmed. Two multiparous sows were inoculated intranasally at 93 days of gestation. The sows delivered litters with fifty

percent stillborn piglets (8/13 and 6/14 stillborn/live) on days 112 and 114 of gestation, respectively. Seven of the stillborn piglets were partial mummies and the liveborn piglets were weak and failed to suckle vigorously. The viral agent was recovered from tissues of the stillborn piglets.

The viral agent has been recovered from three herds known to have SIRS. Antibody titers to the ATCC-VR2332 agent have been identified in these same herds.

Although there are some differences in clinical signs, i.e. cutaneous cyanosis of the ears, tail and udder in European swine, the prevailing opinion is that the North American and European disease are caused by the same virus.

C. VIRAL CHARACTERISTICS

ATCC-VR2332 leads to clinical signs and pulmonary lesions in gnotobiotic pigs and negative ImF results, indicating that the virus does not have any group antigens which are antigenically similar to those of the viruses from the present genera of Togaviridae. ATCC-2332 might therefore be an as yet unidentified genus of Togaviridae. ATCC-2332 is not a known swine pathogen either since specific antisera against numerous known viral swine pathogens were unable to neutralise the virus or detect antigens in infected cells.

ATCC-VR2332 is a demanding, non-haemagglutinating, coated RNA virus. The ATCC-VR2332 virus grows in a continuous cell line, especially in MA-104 and other monkey cell lines. The ATCC-VR2332 virus grows to a high titre (10^7 TCID₅₀/1 ml) in the commercially available cell line MA-104.

The ATCC-VR2332 virus has a lipid coat, which was detected by a loss of infectivity after chloroform treatment. A lipid coat may also be detected as a translucent ring around the empty particles. The morphology of the SIRS virus is not marked in direct

electron microscopy (DEM) and can only be identified with considerable difficulty in preparations containing cell residues. The ATCC-VR2332 virus can be identified after purification by means of CsCl gradients, followed by immunogold labelling of the virion with anti-ATCC-VR2332 hyperimmune sera and gold conjugate. The suspension density of the ATCC-VR2332 virions in CsCl gradients is 1.18 to 1.19 g/ml. Sucrose gradients have a considerable virus titre loss and were eliminated as suitable gradients for purification. The morphology of the gradient-purified ATCC-VR2332 particles and the average diameter of 62 nm are similar to those of the virions of equine arteritis and those of the lactate dehydrogenase viruses. For equine arteritis viruses a size range of 50-73 nm is given, similar to the 48-84 nm size of the ATCC-VR2332 virus. 30-35 nm nuclei were observed in numerous ATCC-VR2332 particles, these nuclei being reminiscent of the nucleocapsid nuclei which have been described for the equine arteritis virus. Morphologically, the ATCC-VR2332 virus is closest to the arteritis virus group.

The presence of an RNA genome of ATCC-VR2332 was confirmed by the ability of the virus to continue replication in the presence of 5-bromo-2-deoxyuridine and mitomycin C, which are known to inhibit the replication of DNA and an RNA virus family (Retroviridae), but not other RNA viruses. Moreover, the preliminary classification of the ATCC-VR2332 virus as an RNA virus conforms to the observation that the virus replicates in the cytoplasm of a cell, as indicated by the presence of virus antigens which were detected by ImF. Actinomycin D, which interferes with the DNA-dependent RNA transcription, has no effect on the replication of the ATCC-VR2332 virus. These results show that the ATCC-VR2332 virus needs no nuclear functions in order to replicate.

The ATCC-VR2332 virus is thermally unstable at 37°C

and 56°C but is stable for long periods at 4°C and -70°C. The thermal instability of the virus at 37°C shows that the virus is relatively unstable in a warm environment. This also has a practical significance in the replication of the virus, in that growth temperatures below 37°C produce higher yields of ATCC-VR2332 virus. Cooling should be sufficient for short term storage of diagnostic samples for isolation of the virus, otherwise the sample should be frozen.

To sum up, the size, morphology, presence of an RNA genome and other biological properties of the ATCC-VR2332 virus seem to indicate that it belongs to the family of the Togaviridae. ATCC-VR2332 cannot, however, be definitely classified in a genus of this family. Although the ATCC-VR2332 appears to be closely related to the arteritis viruses, from a morphological point of view, the virus should not be classified in a specific genus until additional information is available on its RNA and protein structure.

D. MODIFIED LIVE VACCINE PRODUCTION

The production of a vaccine was formulated by the inclusion of a modified or attenuated live ATCC-VR2332, the administration of which successfully immunised pigs against an infection. The SIRS virus ATCC-VR2332 was propagated in the continuous MA-104 cell line. The cell line was cultured in bottles containing MEM to which 10% foetal calf serum had been added. The pH of the medium was adjusted to 7.2 and the medium was incubated at about 37°C. The virus was inoculated into the cells by the addition of about 1 ml of a frozen inoculum to the liquid medium. The virus was then allowed to be absorbed into the cells for 24 hours.

The growth medium was then replaced by a maintenance medium consisting of MEM to which 4% foetal calf serum pH 7.6, had been added. The ambient temperature was 35-37°C. The virus was cultured until

50% of the MA-104 cell sheet had been destroyed by the virus. The sample was frozen and used for a passage in another flask with MA-104 cells. This process was carried out for 25 passage of the virus in the cell line. The virus was then propagated another 12 times at about 31°C, by contrast to the 35-37°C, but using the same techniques as described above. The 12th passage was frozen in the form of small aliquots and termed the "Master Seed Virus".

Specifically, the invention thus comprises the following objects:

- a biologically pure culture of the SIRS (swine infertility and respiratory syndrome) virus, ATCC-VR2332 and all zoopathogenic mutants, the said culture being capable of mimicking swine infertility and respiratory disease in pigs,
- a vaccine composition containing a modified live SIRS virus, ATCC-VR2332, and a pharmaceutically acceptable carrier, the virus being modified into a virus which is non-pathogenic in pigs, a vaccine composition containing a killed or inactivated SIRS virus, ATCC-VR2332, and a pharmaceutically acceptable carrier,
- a method of immunising pigs against SIRS, characterised in that it comprises giving one of the above-mentioned vaccine compositions to pigs,
- a method of cultivating and isolating the SIRS virus, ATCC-VR2332, characterised in that the virus is inoculated on a complete or partial sheet of monkey cells in the presence of a serum in a suitable growth medium and the inoculated cell sheet is incubated at about 34-37°C until a cytopathic effect is observed, the process described above, characterised in that the monkey cell line MA-104 is used,
- a method of attenuating the SIRS virus, ATCC-VR2332, characterised in that it comprises

passage of the virus in a monkey cell line at about 35-37°C and then passage of the resulting virus in a monkey cell line at about 31°C,

- a process for attenuating as described above, characterised in that the passages are carried out in a monkey cell line in maintenance medium in the presence of serum at a pH of about 7.6,

- a process for attenuating as described above, characterised in that the passage at about 34-37°C is carried out 25 times and the passage at 31°C is carried out 12 times,

- a process for attenuating as described above, characterised in that the monkey cell line is MA-104,

- a process for attenuating as described above, characterised in that the passages are carried out without CO₂,

- use of the vaccine composition for preparing a pharmaceutical composition for immunising pigs against SIRS.

EXAMPLE

Example of a preparation of attenuated ATCC-VR233

I. Media:

- a. "Eagles Minimum Essential Medium (MEM) made by JRH Biosciences, #200-2041
- b. Foetal calf serum (FCS) obtained from JRH Biosciences
- c. Growth medium for cell cultivation, MEM and 10% foetal calf serum
- d. Maintenance medium: MEM, 4% foetal calf serum
- e. Trypsin-Versene IX
- f. Sodium bicarbonate, 5% or saturated

II. Tissue culture:

Cell line used: MA-104 ("African Green Monkey Kidney" cells maintained within a limit of 20 cell passage (cell passage 58-78)).

III. Equipment:

75 cm tissue culture flasks

Incubator at 35-37°C

Incubator at 31°C

Centrifuge

IV. Method of producing attenuated SIRS VR-2332 virus cultivated at 35-37°C

A. Preparation of a tissue seed culture:

1. 75 cm² seed flasks containing 5 to 7 day old MA-104 were divided up 1:4 as follows:
 - a. Pouring off all the medium (50 ml per flask)
 - b. Removing the cell sheet by means of 10 ml of trypsin-versene by incubating at 37°C for 5 to 10 minutes
 - c. Removing the cells from the flask and centrifuging at 270 x g for 5 to 10 minutes
 - d. Decanting the supernatant and resuspending the cells in 5-10 ml of growth medium (MEM, 10% FCS).
 - e. Taking up all the cells in 200 ml of MEM, 10% FCS, which is then suspended and divided up 1:4 between four 75 cm flasks, 50 ml per flask. The flasks are then stored at 35-37°C until ready for use (may also be carried out without CO₂).
 - f. Flasks 3 or 4 days old which have formed a total cell sheet are now ready for use.
 - g. Adjustment of the pH of 50 ml of medium in flasks to pH 7.2 and subsequently adding 1 ml of SIRS virus to the medium and incubating the flasks

at 35-37°C (may be carried out without CO₂).

h) After 24 hours the medium is discarded and the flasks are filled with 50 ml of MEM, 4% FCS, pH 7.6, and incubated again at 35-37°C.

i) 24 hours after the change of liquid a cytopathic effect should be apparent; if 50-60% of the cell sheet is perforated, the flasks are frozen.

j) After the above flasks have been thawed, 1 ml of this liquid may be taken and transferred into a new 75 cm flask as described above, in order to carry out a new cell passage of the SIRS VR-2332 virus.

The procedure described above was carried out a total of 25 times or the SIRS VR-2332 was subjected to cell passage at 35-37°C 25 times.

V. Process for preparing attenuated SIRS VR-2332 virus, cultured at 31°C

(It is important to establish that the VR-2332 virus subjected to 25 cell passages at 35-37°C was used to carry out the first cell passage for the SIRS VR-2332 virus cultivation at 31°C)

A. Preparation of the tissue seed culture:

1. 75 cm² seed flasks containing 5 to 7 day old MA-104 were divided up 1:4 as follows:
 - a. Pouring off all the medium (50 ml per flask)
 - b. Removing the cell sheet by means of 10 ml of trypsin-versene by incubating at 37°C for 5 to 10 minutes
 - c. Removing the cells from the flask and centrifuging at 270 x g for 5 to 10 minutes
 - d. Decanting the supernatant and resuspending the cells in 5-10 ml of growth medium (MEM, 10% FCS).

- e. Taking up all the cells in 200 ml of MEM, 10% FCS, which is then suspended and divided up 1:4 between four 75 cm flasks, 50 ml per flask. The flasks are then stored at 35-37°C until ready for use (may also be carried out without CO₂).
- f. Flasks 3 or 4 days old which have formed a total cell sheet are now ready for use.
- g. Adjustment of the pH of 50 ml of medium in flasks to pH 7.2 and subsequently adding 1 ml of SIRS virus to the medium and incubating the flasks at 31°C (may be carried out without CO₂).
- h) After 24 hours the medium is discarded and the flasks are filled with 50 ml of MEM, 4% FCS, pH 7.6, and incubated again at 31°C.
- i) 24 hours after the change of liquid a cytopathic effect should be apparent; if 50-60% of the cell sheet is perforated, the flasks are frozen.
- j) After the above flasks have been thawed, 1 ml of this liquid may be taken and transferred into a new 75 cm flask as described above, in order to carry out a new cell passage of the SIRS VR-2332 virus.

The above procedure was carried out 12 times in all at 31°C. The 12th passage of the virus cultivated at 31°C was termed the "Master Seed Virus" for the vaccine production.

The cold adapted virus ("Master Seed Virus") with a titre of about 10^{4.5} to about 10^{5.0} TCID₅₀ per millilitre was administered intranasally and intramuscularly to ordinary pigs. A pharmaceutical carrier was administered by intranasal and intramuscular route to ordinary pigs as a control. The pigs inoculated with the attenuated virus developed no SIRS symptoms after being exposed to the ATCC-VR2332 virus (titre: 10^{4.2} TCID₅₀ per millilitre). The cold-adapted virus can be combined with any compatible, conventional

pharmaceutical carrier material. Although the vaccine preparation was administered by intranasal and intramuscular route, other preparations may of course be envisaged.

For the person skilled in the art it is naturally obvious that various modifications may be made to the growth conditions and various methods may be used for attenuating the SIRS virus ATCC-VR2332 and preparing the vaccine formulations. All these modifications are included in the invention.



THIS SHEET HAS BEEN
REPLACED

Patent Claims

1. A biologically pure culture of SIRS (swine infertility and respiratory syndrome) virus, ATCC-VR2332 and all zoopathogenic mutants, the said culture being capable of mimicking swine infertility and respiratory disease in pigs.
2. Vaccine composition containing a modified live SIRS virus, ATCC-VR2332, and a pharmaceutically acceptable carrier, the virus being modified into a virus which is non-pathogenic in pigs.
3. Vaccine composition containing a killed or inactivated SIRS virus, ATCC-VR2332, and a pharmaceutically acceptable carrier.
4. Process for immunising pigs against SIRS, characterised in that it comprises administering a vaccine composition according to claim 2 to pigs.
5. Process for immunising pigs against SIRS, characterised in that it comprises a vaccine composition according to claim 3.
6. Process for culturing and isolating the SIRS virus, ATCC-VR2332, characterised in that the virus is inoculated on a complete or partial sheet of monkey cells in the presence of a serum in a suitable growth medium and the inoculated cell sheet is incubated at about 34 to 37°C until a cytopathic effect is observed.
7. Process according to claim 6, characterised in that the monkey cell line is MA-104.
8. Process for attenuating the SIRS virus, ATCC-VR2332, characterised in that it comprises passing

This Sheet Has Been Replaced





THIS SHEET HAS BEEN
REPLACED

the virus in a monkey cell line at about 35 to 37°C and then passing the resulting virus in a monkey cell line at about 31°C.

9. Process according to claim 9, characterised in that the passages are carried out in a monkey cell line in maintenance medium in the presence of serum at a pH of about 7.6.

10. Process according to claim 9, characterised in that the passage at about 34-37°C is carried out 25 times and the passage at 31°C is carried out twelve times.

11. Process according to one of claims 8 to 10, characterised in that the monkey cell line is MA-104.

12. Process according to claim 11, characterised in that the passages are carried out without CO₂.

13. Use of the vaccine composition according to one of claims 2 or 3 for preparing a pharmaceutical composition for immunising pigs against SIRS.