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In vivo and *in vitro* studies on temperature-sensitive mutants of swine vesicular disease virus

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SUMMARY

Two temperature-sensitive mutants of the Ukg 27/72 strain of swine vesicular disease virus were isolated in tissue culture and a third was derived following adaptation in mice. All three were found to have similar growth restrictive temperatures, but varied considerably in their virulence when administered to pigs. The route of inoculation appeared to exert a considerable influence on the apparent degree of attenuation, the antibody titre engendered and the transmission of disease to pigs held in contact with inoculated animals. One strain appeared almost totally attenuated when inoculated into pigs but spread to animals in contact causing severe disease. Virus re-isolated from one such animal was found to have retained its temperature sensitive phenotype, suggesting that virulence in this case was not directly related to temperature sensitivity. Pigs with high antibody titres were found to be susceptible when placed in contact with challenge animals, although the lesions which developed were mild.

INTRODUCTION

Fenner (1968*a*) suggested that temperature sensitive (*ts*) mutants might be specifically selected for their degree of attenuation, thus enabling them to be used in the formulation of live virus vaccines.

Such mutants arise as a result of conditional lethal mutations in parent (ts^+) or wild type virus populations. The mutant progeny loses its capacity to function at supra optimal temperatures (i.e. under non-permissive conditions). *ts* mutants arising spontaneously from virus populations have been reported, e.g. for vesicular stomatitis virus by Flamand (1970), but the frequency of isolation is always very low. It has now become common practice to use a chemical mutagen to increase the mutation frequency. For example, the work on polio virus by Cooper (1964) and on foot-and-mouth disease virus by Pringle (1968) and Lake (1975), has indicated the usefulness of 5-fluorouracil (5-FU) as a mutagen increasing the frequency of *ts* mutations in virus populations.

Since 1968 *ts* mutants from a number of different viruses have been shown to be less virulent than their respective parent strains, e.g. rabbit pox virus (Fenner, 1968*b*), influenza virus (Mackenzie, 1969), respiratory syncytial virus (Wright, Woodend & Chanock, 1970), reovirus (Fields & Raine, 1972), parainfluenza virus (Zygraich, Lobmann & Huygelen, 1972) and herpes virus (Zygraich & Huygelen,

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1973). It is with this apparent relation between temperature sensitivity in growth, loss of virulence and its application to the development of an attenuated vaccine against swine vesicular disease (SVD) that the present paper is concerned.

The study describes the production, isolation, characterization, degree of attenuation and immunogenicity of two *ts* tissue culture mutants and a *ts* mouse adapted strain of the SVD virus Ukg 27/72. This virus, used as the wild type parent in this work, is said to typify the causal agent of the recent outbreaks of SVD in the United Kingdom.

MATERIALS AND METHODS

Cells

For the production of virus and its subsequent assay, monolayers of the continuous pig kidney cell line IB-RS-2 (de Castro, 1964) were used. Monolayers were grown in lactalbumin yeast hydrolysate (LYH) medium containing 10% bovine serum, although the latter was omitted for virus production.

Virus infectivity assays

Monolayer cultures in 6 cm plastic Petri dishes were infected with 0·2 ml of virus diluted in phosphate buffered saline (PBS). The infected plates were maintained at 37 °C for 30 min allowing adsorption of the virus to take place. Excess fluid containing unadsorbed virus was then removed with a pipette, and the plates overlaid with 5 ml of LYH medium containing 1% of agarose. Assay plates were incubated for 72 h at 38 °C, after which plaques could be counted without staining.

Incubation temperatures

The plaquing efficiency of the parent SVD virus was found to be essentially similar at both 38 and 41 °C. These temperatures were chosen as permissive (38 °C) and restrictive (41 °C) conditions of incubation for the selection of *ts* mutants.

Isolation of temperature sensitive mutants

Temperature sensitive mutants were obtained by treating the wild type SVD virus Ukg 27/72 (ts^+) with 5-fluorouracil (5-FU). Stock 5-FU treated virus was prepared by infecting IB-RS-2 monolayers in 1 oz. bottles and overlaying with LYH containing 500 μg/ml of 5-FU. The bottles were incubated at 38 °C until the cells showed approximately 100% CPE. The harvest from these cultures was immediately repassaged under identical conditions.

The isolation of *ts* mutants from limiting dilutions of 5-FU treated stock virus was done using a modified form of the agar-cell suspension system described by Cooper (1961).

The plates were prepared in 6 cm plastic Petri dishes and consisted of: (i) a basal layer of 5 ml of 0·6% agarose in LYH medium supplemented with 10% ox serum; (ii) a second layer consisting of 1 ml of freshly harvested IB-RS-2 cells ($1{\cdot}2 \times 10^7$

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viable cells/ml) in LYH medium to which 0·2 ml dilutions of stock 5-FU treated virus had been added.

The cell–virus suspension was incubated at 38 °C for 30 min allowing the virus to adsorb onto the cells. After virus adsorption 1·2 ml of 1·2% agarose in LYH medium supplemented with 10% ox serum was mixed with the virus–cell suspension and evenly distributed over the basal layer.

The plates were incubated for 48 h in sealed plastic boxes submerged in a 38 °C water bath (permissive conditions).

After this incubation, the plates were stained with 2·5 ml of an overlay containing 3 vols of 0·15% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and 1 vol. 3·6% agarose, both solutions prepared in physiological saline. After 3 h further incubation at 38 °C well isolated plaques were given an identification number and their diameter measured. The plates were resealed in their boxes and immersed in a 41 °C water bath for 48 h (restrictive conditions).

After incubation at the restrictive temperature, the plaques were again measured. Those showing no increase in diameter were picked into 1 ml of 10% ox serum in PBS. Possible *ts* mutants isolated in this way were plaque purified twice and examined for their plaquing efficiency over the temperature range 34–41 °C.

Preparation of mouse-adapted SVD virus (M1)

The sixth IB-RS-2 passage of Ukg 27/72 was passaged in 5-day-old P strain mice by the intracerebral inoculation of 0·03 ml of a clarified tissue culture harvest. Animals were observed for 7 days and brain material was collected from mice which either died or developed paralysis. The sample was ground up in 0·04 M phosphate buffer to give an approximate ten-fold dilution, clarified by centrifugation and used to initiate the second mouse passage. This procedure was repeated until the virus had received 15 intracerebral passages.

Pig skin organ culture of SVD virus

The growth of SVD virus in fragments of hairy pig skin isolated in organ culture is a technique developed at AVRI by Mr J. Mann (personal communication).

Each culture consisted of three pieces of tissue approximately 4–6 mm square, from a freshly killed animal, cultured in a ½ oz. screwtop bottle containing 10 ml of Eagle's complete medium with 50 units of mycostatin and 70 units of neomycin per ml (maintenance medium).

To infect the culture the maintenance medium was replaced with 10 ml of virus suspension containing approximately $10^{5\cdot0}$ p.f.u./ml. The virus was adsorbed for 1 h at 38 °C and the tissue washed five times with PBS containing antibiotics as in the maintenance medium. The final wash was replaced by 10 ml maintenance medium and the cultures incubated at 38 °C.

Samples were collected over a period of 9 days, the old medium being replaced daily with fresh.

Animal experiments

Large White cross-bred pigs weighing 12–14 kg were used. Details of experimental procedures are given in the text. Serum samples were obtained before and at intervals after inoculation and pigs were examined clinically each day for up to 21 days after inoculation or exposure. Lesions developing during the course of the experiment were point scored according to the method of Burrows, Mann & Goodridge (1974) which allows a theoretical maximum score of 100 points per animal. In practice, scores of 40–60 are typically observed in severely diseased cases. Only the score on the tenth day after inoculation or exposure has been quoted since this conveniently represents the peak of lesion severity.

Antibody assays

Serum neutralizing antibody titres were determined against the Ukg 27/72 strain of SVD by a microtitre method in IB-RS-2 cells as described by Golding *et al.* (1976).

RESULTS

Preparation of stock mutagenized virus and ts *mutant isolation*

Two successive passages of parental (*ts*$^+$) virus on IB-RS-2 cells in the presence of 500 μg/ml of 5-FU gave a tissue culture harvest in which virus infectivity had been reduced by approximately $10^{3\cdot0}$ p.f.u./ml. This reduced harvest provided a stock virus from which *ts* mutants were isolated.

Using the 5-FU treated stock virus in agar-cell suspension plates, several thousand plaques were examined for their inability to grow under restrictive incubation conditions. This approach yielded 31 potential *ts* mutants of which two (*ts*9 and *ts*16) were confirmed to be true *ts* mutants by assay under permissive and restrictive incubation conditions. These two mutants were cloned twice and their phenotype determined by plaque assay over the temperature range 34–41 °C. The growth profile of *ts*9, *ts*16, M1 and also *ts*$^+$ parental virus over this range is shown in figure 1.

Growth of ts$^+$, ts9 *and* ts16 *in pig tissue*

Figure 2 shows the growth of *ts*$^+$, *ts*9 and *ts*16 in pig skin organ cultures during the first 7 days after infection of the isolated tissue. The graph shows that *ts*16 gave a reduced virus yield over the first 4 days compared with that given by the wild type parent virus. *ts*9 showed a similar trend although the difference in virus yield was smaller. After day 4 the results from all three viruses converged and significant differences in their pattern of growth could not be detected.

Animal experiments

After the laboratory production of the two *ts* mutants and a mouse adapted strain of Ukg 27/72, these viruses were examined for their ability to produce clinical disease in the natural host. Briefly, this part of the work was conducted

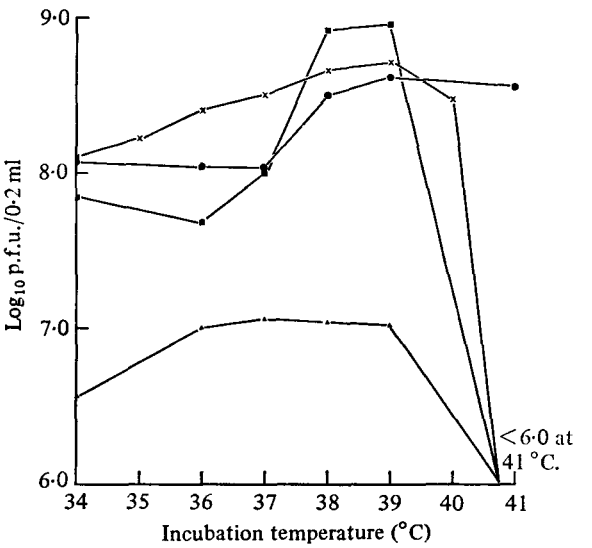

Fig. 1. Production of infective virus by the M1, *ts*9, *ts*16 and *ts*$^+$ isolates of Ukg 27/72 over the temperature range 34–41 °C. ●, *ts*$^+$; ×, *ts*9; ■, *ts*16; ▲, M1A/2.

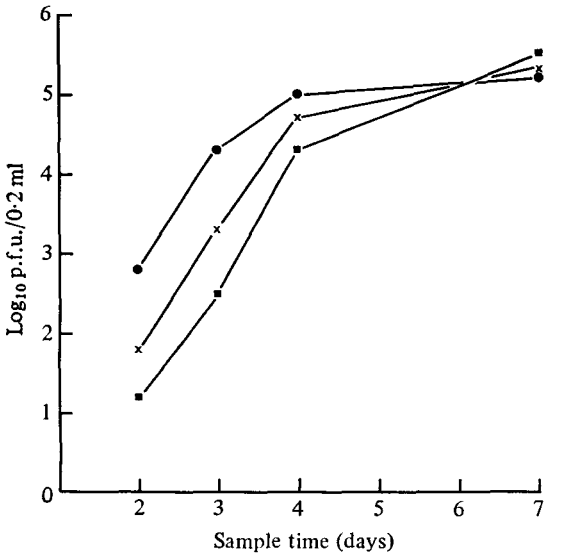

Fig. 2. Growth of *ts*$^+$, *ts*9 and *ts*16 in pig tissue. Each point on the graph is a mean result of four separate experiments. ●, *ts*$^+$; ×, *ts*9; ■, *ts*16.

in two phases: (1) Inoculation of animals with the three viruses to investigate the degree of attenuation. These animals were subsequently challenged with wild type virus. (2) Further testing of the virulence and immunogenicity of the *ts*16 mutant administered by different routes.

Phase 1

Groups of five pigs housed in separate loose-boxes were used to test for virus attenuation. A further box containing two animals was used as a control to check

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disinfection procedures between boxes of potentially infected animals. These pigs were always examined last.

Each group of five animals received 0·1 ml of virus by intradermal injection into the coronary band as follows:

Left forefoot	10^8 p.f.u./ml
Right forefoot	10^6 p.f.u./ml
Left hind foot	10^4 p.f.u./ml
Right hind foot	Not inoculated

The serum-neutralizing antibody responses of the four groups of animals, and the corresponding day 10 lesion scores, are shown in Table 1. Since the animals used as disinfectant controls remained free of clinical disease or detectable antibody it may be assumed that decontamination procedures between adjacent boxes were satisfactory.

All three groups of animals receiving virus gave a good antibody response over a 20 day period, but in those animals receiving the M1 and *ts*9 viruses the antibody response was also accompanied by clinical disease as shown by the lesion scores in Table 1. Animals receiving *ts*16, however, gave a good antibody response and only one animal of the group developed a single minor lesion.

In order to effect challenge by contact with an infected animal the vaccinated animals were rearranged into four groups: Group 1 comprised eight new animals

Table 1. *Neutralizing antibody titres (reciprocal) and lesion scores in pigs inoculated with M*1, ts9 *and* ts16 *mutants of Ukg* 27/72 *SVD virus*

Virus given to each group of six pigs	Group serum neutralization titres: geometric mean (Day 0)	Group serum neutralization titres: range (Day 20)	Lesion scores, mean and range (Day 10)
M1	⩽ 0·80	2·64 (2·55–2·70)	29·8 (14–51)
*ts*9	⩽ 0·80	2·61 (2·25–3·00)	33·2 (18–46)
*ts*16	⩽ 0·80	2·37 (2·10–3·00)	One animal showed one small lesion scoring 1
Disinfection controls (uninoculated)	⩽ 0·80	⩽ 0·80	0·00

of which one received 0·1 ml (approximately $10^{8·0}$ p.f.u./ml) of wild type Ukg 27/72 by inoculation of the coronary band of four feet. This control group was used to check that the challenge was effective. Group 2. Five animals convalescent from M1 and *ts*9 virus. Group 3. Five animals inoculated with *ts*16. Group 4. The two disinfection control animals.

Groups 2, 3 and 4 were challenged by the addition of a further three fresh pigs, one of which was inoculated with 0·1 ml of the Ukg 27/72 wild type virus via the coronary band.

From Table 2 it may be seen that whereas all the inoculated donors developed generalized disease, uninoculated animals in contact with them showed considerable variation in their response to challenge (Lesion scores 0–51). The contact control

Table 2. *Post challenge lesion scores and antibody titres persisting in animals 'vaccinated' with M*1, ts9 *and* ts16 *mutants of Ukg* 27/72

Group	Status	Individual lesion scores, day 10 post vaccination	Individual and group mean geometric serum neutralization titres and ranges – post challenge	
			Day 0	Day 21
1	New Animals [1 inoculated, 7 in contact]*	70	0·0	2·55
		0, 0, Sn, 2, 4, 49, 51	0·0	⩾ 2·68 (1·95–⩾ 3·15)
2	'Vaccinated' Animals [1 inoculated, 2 in contact]	60	0·0	2·85
		0, Sn	0·0	0·0 and 2·25
	*ts*9 and M1 5 'vaccinated'	0, 0, 0, 1, 1	2·43 (2·40–2·55)	2·58 (2·55–2·70)
3	'Vaccinated' Animals [1 inoculated, 2 in contact]	82	0·0	2·85
		0, 21	0·0	2·25 and 2·85
	*ts*16 5 'vaccinated'	0, 0, 0, 2, 3	2·10 (1·95–2·55)	⩾ 2·79 (2·25–⩾ 3·15)
4	Disinfection Controls [1 inoculated, 2 in contact]	60	0·0	2·70
		28, 45	0·0	3·00 and 3·00
	2 Disinfection control	63, 72	0·00	⩾ 3·15
	Collected challenge and challenge control animals from all groups.			
	Inoculated donor pigs (4)		0·00	2·74 (2·55–2·85)
	New in contact pigs (13)		0·00	⩾ 2·47 (0·0–⩾ 3·15)

sn, Snout lesion.

* Animals grouped in [ ] are those comprising the inoculated and in contact challenge pigs.

pig (Group 2) developed neither lesions nor measurable antibody following exposure.

Some animals in Groups 2 and 3 developed signs of mild clinical disease following challenge despite the presence of high antibody titres at that time.

Phase 2

In this part of the work four groups of animals were inoculated with *ts*16 as shown in Table 3.

Table 3. *Details of the inoculation of groups of pigs with* ts16 *virus*

Group	Dose		Route	Site
	Volume	$\log_{10}$ p.f.u.		
5	6 × 0·05 ml	8·5	Intradermal	Coronary band of three feet, two sites per foot
6	1 × 0·1 ml	8·0	Intradermal	Ear pinna
7	1 × 1·0 ml of 1:10 dilution	8·0	Subcutaneous	Behind ear
8	1 × 1·0 ml of 1:10 dilution	8·0	Intramuscular	Gluteal muscle

Each group consisted of six inoculated animals with a further two uninoculated animals kept in contact. On day 28 all animals were challenged by the introduction into each group of two pigs inoculated with wild type Ukg 27/72 SVD virus and a further two fresh pigs were placed in contact. Lesion scores and serum antibody titres are given in Table 4.

The lesion scores indicate that the site of inoculation influenced the apparent virulence of the *ts*16 mutant as seen in the inoculated and contact animals. Thus mild disease followed the use of the intradermal coronary band route (Group 5, lesion scores 0–8), but one of two in-contact animals developed severe lesions (score 57), suggesting that reversion towards virulence may have occurred during contact transmission. A similar but less marked effect emerged in Group 8 where the intramuscular route was used. However, in Groups 6 and 7 inoculated intradermally on the pinna or subcutaneously behind the ear, mild lesions (scores 0–4) occurred only in Group 7 and all in-contact animals remained free of clinical disease.

The effect of inoculation site on the development of homologous antibodies is also shown in Table 4. The highest 21 day antibody response followed intradermal inoculation into the coronary band.

In this phase of the experiment control animals in contact with pigs inoculated with wild type virus all displayed symptoms of disease (scores 14–64) and in this respect the challenge of vaccinated animals was more effective than in Phase 1.

The group found to be most resistant to challenge was that which had received *ts*16 virus intradermally into the coronary band and which had also developed the highest 21 day antibody titres. The severity of disease within the groups after challenge reflected the 21 day antibody titres shown in Table 4.

Table 4. *Lesion scores and antibody titres developed by animals 'vaccinated' at different sites with the* ts16 *mutant of Ukg* 27/72 *SVD virus*

Group	Site of inoculation and group composition	Group mean lesion scores and range†		Group geometric mean serum neutralization titres and range (post 'vaccination')	
		Post 'vaccination'	Post challenge	Day 0	Day 21
5	i/d coronary band				
	6 'vaccinated'	3·66 (0–8)	0·33 (0–2)	0	2·25 (2·10–2·85)
	2 contact	28·5 (0–57)	0	0	⩾ 3·15
	[2 inoculated]*	—	55·5 (43–68)	—	—
	[2 contact]	—	22 (17–27)	—	—
6	i/d ear				
	6 'vaccinated'	0	2·16 (0–9)	0	1·72 (1·34–2·10)
	2 contact	0	28·5 (0–47)	0	0
	[2 inoculated]	—	65·5 (62–69)	—	—
	[2 contact]	—	51·5 (39–64)	—	—
7	s/c behind ear				
	6 'vaccinated'	0·66 (0–4)	13·83 (2–50)	0·15 (0·00–0·90)	1·37 (0·90–1·65)
	2 contact	0	68 (64–72)	0	0·90 (0·00)
	[2 inoculated]	—	55·5 (46–68)	—	—
	[2 contact]	—	19·5 (14–25)	—	—
8	i/m gluteal muscle				
	6 'vaccinated'	2·33 (0–10)	2·5 (0–12)	0	2·15 (1·65–3·00)
	2 contact	6 (0–12)	20·3 (0–4)	0·60 (0·00–1·20)	1·65 (1·34–2·85)
	[2 inoculated]	—	56·5 (60–53)	—	—
	[2 contact]	—	39·5 (37–42)	—	—

* Animals grouped in [ ] are those comprising the inoculated and in contact challenge pigs.
† Lesion score 10 days after 'vaccination' or challenge.

DISCUSSION

Successful attenuated vaccines rely on sufficient *in vivo* replication of the virus to stimulate host defences, without the accompaniment of clinical disease. The data presented here give some support to the concept of *ts* lesions being associated with such attenuation.

Initial experiments with Ukg 27/72 parent virus grown over the temperature range 34–41 °C indicated an optimal growth temperature in the range 38–41 °C and 38 °C was chosen as the permissive incubation temperature used throughout this work.

The *ts* mutants isolated had a reduced ability to replicate at 41 °C. It is probable that they were deficient in one or more gene products necessary for the production of infectious virus at the restrictive temperature. During mutagen treatment a number of other lesions must have been induced in the parent Ukg 27/72 virus population. Although these lesions were not selected for during mutant isolation, they may have accompanied the *ts* lesion and have been present in the genome of *ts*9 and *ts*16.

The growth curves of *ts*9, *ts*16, M1 and parental ts^+ virus (Fig. 1) were interesting in that the mouse-adapted strain showed the same cut-off temperature (39 °C) as the *ts*9 and *ts*16 mutants.

In Phase 1 of the animal work all pigs given M1 and *ts*9 virus developed typical SVD lesions. Recipients of *ts*16, however, displayed virtually no signs of disease but developed antibody titres similar to those of the other groups. A single animal from this group developed a very minor lesion, scoring one on the point system used.

The differing degree of attenuation between *ts*9 and *ts*16 was reflected in their ability to grow in isolated pig tissue. This technique might be of considerable use in any future work for screening viruses before testing in the natural host.

The challenge of the Phase 1 animals was unsatisfactory. Four of thirteen control pigs, with no previous experience of SVD, failed to develop disease when placed in contact with infected animals. The challenge, however questionable, did provide some surprising results. Animals in Group 2 which developed severe lesions when inoculated with *ts*9 and M1 and which had high antibody titres at the time of challenge, developed mild SVD. Similarly, animals in Group 3 inoculated with *ts*16, with high antibody titres at the time of challenge, also developed mild SVD.

Intradermal inoculation into the coronary band was used initially since this tissue is a site of predilection in the natural disease but, as it was considered impracticable for routine use in the field, an attempt was made in Phase 2 to find an alternative, more accessible site and also to determine the effect of inoculation site on the expression of virulence. In this phase, work was restricted to the *ts*16 mutant as the only isolate exhibiting signs of significant attenuation in Phase 1.

Administration of *ts*16 either intradermally into the coronary band or intramuscularly, gave rise to similar antibody titres. In both of these groups, however, one of the two in-contact animals developed clinical disease, suggesting that reversion of the *ts*16 mutant had occurred on contact transmission. Similarly

some pigs from each group, except those inoculated intradermally into the ear, showed signs of disease following inoculation with *ts* 16.

In Phase 2, all pigs in contact with the inoculated challenge animals succumbed, indicating that animals in contact were being effectively challenged. Response to the challenge varied, but a number of animals from each group developed SVD regardless of vaccination site.

Throughout this work the results obtained in the natural host inidcated that complete attenuation of *ts*16 had not been achieved. It was evident, however, that animals vaccinated by whatever route with *ts*16 contracted only a mild form of disease on challenge. Although *ts*9 and *ts*16 appeared quite stable *in vitro* it was quite possible that reversion to the wild type occurred *in vivo*. For example, one animal in contact with animals vaccinated with *ts*16 (Phase 2, Group 5) developed severe disease with a total lesion score of 57. However, virus isolated from vesicular fluid taken from this animal was found to display the *ts* phenotype, suggesting that virulence in this case was not directly associated with the *ts* lesion.

The data obtained in the present study have highlighted two main problems in using SVD vaccines in pigs. First, it was found that animals with high antibody titres were not always completely protected and even convalescent animals may develop mild lesions on re-exposure to infection. It therefore appears unlikely that vaccine, either attenuated or inactivated, can be expected to provide complete clinical immunity against disease. Secondly, considerable variation exists in the susceptibility of pigs to SVD virus; a phenomenon already reported by Burrows *et al.* (1974).

Vaccination with the *ts*16 mutant into the coronary band or on the pinna of the ear was found to give levels of protection similar to those achieved by Mowat *et al.* (1974) using conventional inactivated vaccines. However, the demonstration of possible reversion of *ts*16 to the wild type, and the success of the control measures adopted in this country, has led to the natural conclusion of this work.

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