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AFRICAN SWINE FEVER: PATHOGENICITY AND IMMUNOGENICITY OF TWO NON-HAEMADSORBING VIRUSES

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ABSTRACT

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The virulence of 2 non-haemadsorbing African swine fever virus isolates were compared with 2 haemadsorbing viruses. While 3 of these isolates usually produced acute death in pigs, 1 non-haemadsorbing virus caused either a fatal infection with an extended course, or few or no obvious signs of infection. Pigs that survived infection with the latter virus were resistant to the lethal effects of the other 3 strains as well as to a pool of 7 isolates made from *Ornithodorus porcinus porcinus* (*sensu* Walton, 1964) and warthog obtained in the Northern Transvaal.

Résumé

PESTE PORCINE AFRICAINE: PATHOGÉNICITÉ ET POUVOIR IMMUNOGÈNE DE DEUX VIRUS NON-HÉMADSORBANTS

*On a comparé la virulence de 2 isolats de virus non-hémadsorbants de la peste porcine africaine avec celle de 2 virus hémadsorbants. Tandis que 3 de ces isolats ont déterminé habituellement une mort rapide chez le porc, 1 virus non-hémadsorbant a causé soit une infection fatale à longue échéance, soit peu ou pas de signes manifestes d'infection. Les porcs qui avaient survécu à l'infection par le dernier virus ont résisté aux effets léthaux des 3 autres souches ainsi qu'à un pool de 7 isolats tirés d'*Ornithodorus porcinus porcinus (sensu *Walton,* 1964) *et de phacochères provenant du nord du Transvaal.*

INTRODUCTION

The observation by Malmquist & Hay (1960) that African swine fever (ASF) virus produces changes in porcine blood leucocyte (BC) and bone marrow (BM) cell cultures which render adherent cells capable of adsorbing erythrocytes provided an important technique for the *in vitro* study of this virus. However, Coggins (1968) observed that at least some ASF viruses comprised both haemadsorbing (HD) and non-haemadsorbing (NHD) subpopulations and, although NHD viruses segregated *in vitro* sometimes re-acquire the HD characteristic, they appeared to have reduced virulence for pigs. Later, Pini & Wagenaar (1974) and Vigario, Terrinha & Moura-Nunes (1974) isolated NHD viruses from diseased pigs and demonstrated that the isolates did not induce haemadsorption of erythrocytes even after extensive passaging (Pini, 1977).

The Commission of the European Communities (Anon., 1976) reported that 1% of 22 406 positive specimens examined contained NHD virus. These isolates were made from animals suffering from subacute, chronic or clinically inapparent forms of the disease. Although these authors did not present detailed information, Vigario *et al.* (1974) felt that the virulence of their NHD isolates was reduced for pigs, while the isolates made in South Africa varied in this respect (Pini, 1977).

Even though little is known of the mechanism of haemadsorption in ASF other than that it is not associated with a haemagglutinin (Malmquist, 1963), the haemadsorption inhibition (HAI) test is capable of distinguishing antigenic types or subtypes of HD viruses (Malmquist, 1963; Vigario, Bastos, Moura-Nunes, Marques & Silva, 1970) of which there would appear to be at least 3, with the NHD viruses constituting a further group (Vigario *et al.*, 1974). However, it is not known whether these serological differences reflect immunological and pathological differences of importance *in vivo*.

The investigations reported in this paper were aimed both at comparing the responses of pigs following inoculation with 2 NHD viruses and those resulting from inoculation with HD isolates known to produce acute lethal infections in domestic swine, and at evaluating the ability of NHD viruses to confer protection against heterologous isolates.

MATERIALS AND METHODS

Viruses

Four different ASF isolates were used, 2 of which (CV and TS237) produced haemadsorption (HD) in blood leucocyte cultures and 2 (Lillie-148 and Zaire) which did not (NHD). The CV strain was isolated from a pig which died during an ASF outbreak in 1962 and was known to produce fatal infection in pigs. The Lillie-148 and TS237 strains have been described elsewhere (Pini, 1977), and the Zaire virus strain was isolated from an outbreak of pig mortality in Zaire (A. Pini, personal communication, 1978).

The Zaire isolate was twice plaque-purified, using LLC-MK_2 cells under an agarose overlay, as described by Pini (1977).

The passage histories of the viruses used are given in Tables 1–4.

Animals

Thirty-three cross-bred Landrace pigs between 4 and 6 months of age were used, except where otherwise stated. They were maintained inside an isolation block, singly or in pairs, in pens raised above the floor.

Cell cultures

Blood leucocytes (BC) were prepared from heparinized blood as described by the Commission of the European Communities (Anon., 1976). Stationary culture tubes were inoculated with 1,5 mℓ of cell suspension and incubated at 37 °C for 2 days prior to use.

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The propagation and maintenance of LLC-MK_2 cells which were used for the plaquing of adapted strains has been described by Pini (1977).

Virus titrations

The concentration of ASF virus in porcine tissues was determined by preparing ten-fold dilutions of a 10% suspension of the tissue in modified Eagle's medium (Glasgow) containing penicillin (0,6 mg/mℓ), streptomycin (1 mg/mℓ), neomycin (0,5 mg/mℓ) and fungizone (8 μg/mℓ), and inoculating 0,2 mℓ of each dilution into 4 or 5 BC tube cultures. The method of Kärber (1931) was used to calculate the log_{10} reciprocal of the 50% culture infective dose (CID_{50}) and/or the 50% haemadsorbing dose (HD_{50}). Titres were expressed per gram of tissue.

Titres of cell culture-derived stocks of the Zaire strain were obtained by a plaque method (Pini, 1977) and expressed as pfu/mℓ.

Inoculation of pigs

Virus was administered to pigs parenterally by intramuscular injection into the rump. Intranasal infection was effected by holding the pig in dorsal recumbency and squirting the inoculum into both nostrils with a 5 mℓ syringe.

Serological tests

All the sera were tested using the immunoelectro-osmophoresis (IEOP) technique of Pan, De Boer & Hess (1972), and some of them by haemadsorption inhibition (HAI) as well (Anon., 1976).

Pathology

Tissues for histopathological examination were collected in 10% buffered (pH 7,4) formalin, while the uncut brain was fixed in 25% unbuffered formalin. Paraffin-embedded blocks of tissue were cut at 6 μm and stained with hematoxylin and eosin (HE). The special staining methods (Luna, 1968) of Gram (Humberstone modification) and Wilder were applied to selected sections for demonstration of bacteria and reticulin, respectively.

The classification of pathological changes in individual cases into acute, subacute and chronic ASF was made on the basis of previously published macroscopic and histopathological features of each stage (Moulton & Coggins, 1968; Konno, Taylor & Dardiri, 1971; Maurer, 1975). Overlapping of pathological features occurred, especially between the acute and subacute stage. Two cases did not fit readily into a category and were therefore indicated as "p", which identified the principal feature of lymphoreticular proliferation.

Statistical analysis

An analysis of variance was calculated for a one-way classification from which significance at the 1% or 5% levels could be detected. The least significant differences were calculated by Benferroni's multiple comparison method.

RESULTS

Stability of the NHD characteristic

The original Lillie-148 and Zaire isolates were each passaged 10 times in BC cultures which were examined daily for the appearance of haemadsorption. In no instance did this occur. Furthermore, the virus titres in tissues from 11 pigs inoculated with these 2 NHD strains were determined in BC cultures; no haemadsorption was observed (Tables 2 & 3).

Comparison between the virulence of 2 NHD and 2 HD viruses

With one exception, all 14 pigs inoculated with the 2 HD viruses died of acute ASF within 11 days of infection (Table 1). All 6 pigs infected with Lillie-148 (NHD) also died of acute or subacute ASF, although 1 pig lived for 16 days after inoculation (Table 2). However, only 4 of the 12 animals inoculated with Zaire (NHD) virus died (Table 3). The difference between the Zaire group on the one hand and the HD and Lillie-148 (NHD) groups on the other was significant ($P<0{,}01$). In the 4 pigs that died following inoculation with the Zaire strain, the time between inoculation and death and the duration of pyrexia were also significantly longer ($P<0{,}01$ and $P<0{,}05$, respectively) than that for the HD group. Hoewever, the titres of virus in the lungs and spleens of pigs that died of Zaire virus infection did not differ significantly from those found in pigs which died following inoculation of HD strains or Lillie-148 (cf., Tables 1, 2 & 3).

Of the 8 pigs that survived inoculation with Zaire virus, 2 only showed clinical evidence of infection which consisted of pyrexia of 4 days duration in one pig and a single day in the other.

Surprisingly, a piglet (811, Table 3), which was killed 7 days after inoculation with Zaire and had been clinically normal prior to sacrifice, had high virus concentrations in both its lung and spleen together with proliferative changes in the lumph nodes. An older pig (770, Table 3), which was also killed 7 days after infection with Zaire virus, failed to show any virus in its tissues, although it too had proliferative lesions in its lymph nodes.

Challenge with virulent ASF virus after prior inoculation with Zaire virus

Seven of the pigs that survived infection with Zaire were challenged between 23 and 35 days later with virulent CV, Lillie-148 and TS 237 viruses (Table 4). Both intramuscular and intranasal inoculation were used as routes for challenge virus administration. The results, summarized in Table 4, demonstrated that prior inoculation with Zaire virus protected 7/7 pigs from lethal infection during the observation period. Furthermore, no clinical evidence of illness, apart from pyrexia of a single days duration in 2 pigs was observed (Table 4). On 3 out of the 9 occasions when pigs were challenged there was a four-fold or greater increase in IEOP antibody titre, while two-fold increases followed the other challenge inoculations (Table 4). This suggests that, even though the challenge virus had little or no clinical effect, it did provide an antigenic stimulus, even following intranasal administration. This was confirmed in the cases of pigs 771, 772 and 798 by the animals developing HAI antibody after challenge with CV where none was present before.

Of the 4 pigs (Nos. 792, 794, 807 and 808; Table 4) that were killed after challenge to determine the titre and type (HD or NHD) of virus in lungs and spleens, 3 were found to be infected, while 2 of them contained virus too low in concentration to titrate. Pig 794 was the only one in which HD virus was detected, indicating that the challenge strain had established itself.

TABLE 1 The response of pigs to inoculation with 2 haemadsorbing viruses (CV and TS237)

Pig No.	Virus strain and passage level	Inoculum[2] +route	Lethal (L) or non-lethal (N) infection	Time until death[1]	Observation period[1]	Duration of pyrexia[1]	Antibody titre 2–4 weeks after infection	Virus titre in		Pathology
								Spleen[2]	Lung[2]	
788....	CV BC5	8,1 i/n	L	8	8	4	NA	8,6	7,4	A
666....	CV BC10	9,2 i/n	L	6	6	3	NA	7,5	6,5	A
667....	CV BC10	9,2 i/n	L	6	6	3	NA	7,3	6,4	A
668....	CV BC10	9,2 i/n	L	7	7	2	NA	6,8	ND	A
692....	CV BC10/P1	2,0 i/m	L	9	9	4	NA	9,2	8,5	A
693....	CV BC10/P1	2,0 i/m	N	NA	380	34	1/400	ND	ND	CR
694....	CV BC10/P1	2,0 i/m	L	6	6	3	NA	8,2	9,0	A
695....	CV BC10/P1	2,0 i/m	L	7	7	3	NA	6,4	8,7	A
696....	CV BC10/P1	2,0 i/m	L	7	7	1	NA	9,0	8,1	A
697....	CV BC10/P1	2,0 i/m	L	6	6	3	NA	8,5	8,0	A
707....	CV BC10/P1	2,0 i/m	L	11	11	5	NA	7,7	7,8	A
708....	CV BC10/P1	2,0 i/m	L	9	9	3	NA	ND	ND	A
768....	TS 237 BC26	7,9 i/m	L	7	7	4	NA	8,8	7,8	A
796....	TS 237 BC26	6,1 i/m	L	8	8	4	NA	8,6	8,4	A
Totals + means	NA	NA	13/14	7,5±1,5*	NA	3,2±10*	NA	8,1±0,9*	7,9±0,8*	13A 1 CR

[1]=Days; [2]=LOG_{10} TCD_{50} or HD_{50}/mℓ; *=mean (excluding 693)±SD; A=acute; CR=chronic; S/A=subacute; NA=not applicable; BC=blood leucocyte culture; P=pig passage; ND=not done

TABLE 2 The response of pigs to infection with Lillie-148 (non-haemadsorbing) virus

Pig No.	Virus strain and passage level	Inoculum[2] +route	Lethal (L) or non-lethal (N) infection	Time until death[1]	Observation period[1]	Duration of pyrexia[1]	Antibody titre 2–4 weeks after infection	Virus titre in		Pathology
								Spleen[2]	Lung[2]	
783....	Lillie-148 BC_1	$10^{6,1}$ i/m	L	7	7	4	NA	8,5	8,5	A
784....	Lillie-148 BC_1	$10^{6,1}$ i/m	L	16	16	13	1/32	6,9	8,5	S/A
785....	Lillie-148 BC_1	$10^{6,1}$ i/m	L	8	8	4	NA	8,9	6,5	A
782....	Lillie-148 BC_1/P_1	$10^{4,9}$ i/m	L	7	7	3	NA	9,1	8,1	A
789....	Lillie-148 BC_1/P_1	$10^{4,9}$ i/m	L	7	7	4	NA	7,9	8,3	A
787....	Lillie-148 BC_1/P_2	D	L	Unknown	17	6	ND	8,1	7,9	A
Totals + Means	NA	NA	6/6	9,0±3,9*	NA	5,7±3,7*	NA	8,2±0,8*	8,0±0,8*	5A 1 S/A

D=infection by direct contact. Other conventions as for Table 1
* =mean (excluding 693)±SD

By contrast, 2 pigs inoculated initially with attenuated CV, which had undergone 44 passages *in vitro*, were not protected from lethal infection with virulent homologous virus (Nos. 664 and 665, Table 4), although they survived longer than the controls. It must be pointed out, however, that pig No. 665 received an extremely large challenge dose.

The pig (No. 693, Table 4) that survived infection with virulent CV virus was completely protected from challenge with both homologous virus and TS237 administered 181 and 211 days respectively after first infection. Its antibody titre continued to decline despite the administration of large amounts of live virus.

Pigs (Nos. 693, 772 and 798) were subsequently challenged intramuscularly with a pool of 7 ASF isolates obtained from tampans (*Ornithodorus porcinus porcinus*) (3 isolates) and warthog (*Phacochoerus aethiopicus*) sampled in the Northern Transvaal. None of these pigs developed any evidence of infection and no virus was isolated from lung, spleen, gastro-hepatic or cervical lymph nodes when the pigs were killed a month later.

Discussion

This investigation demonstrated that the reduced virulence of NHD isolates reported by Coggins (1968) and Vigario *et al.* (1974) is not a constant phenomenon, since Lillie-148, a stable NHD isolate, produced an acute and lethal infection indistinguishable from that following inoculation with virulent HD viruses (Tables 1 & 2). Virus titres in tissues (the figures for lung and spleen only are shown in Table 2) were similar to those of pigs that succumbed to HD virus infection (Table 1 and Plowright, Parker & Staple, 1968).

TABLE 3 The response of pigs to infection with Zaire (non-haemadsorbing) virus

Pig No.	Age (months)	Virus strain and passage level	Inoculum[2]	Lethal (L) or non-lethal (N) infection	Time until death[1]	Observation period[1]	Duration of pyrexia[1]	Antibody titre 1–4 weeks after infection	Virus titre in		Pathology
									Spleen[2]	Lung[2]	
795	4–6	Zaire BC_2	$10^{6,5}$	L	25	25	12	$^1/_{64}$**	6,9	7,1	CR
798	4–6	Zaire BC_2	$10^{6,5}$	N	NA	35	0	$^1/_2$**	ND	ND	NA
770	4–6	Zaire BC_2/MK_5	$10^{6,4}$	X	NA	7	0	—ve**	—ve	—ve	P
771	4–6	Zaire BC_2/MK_5	$10^{6,4}$	N	NA	27	0	$^1/_8$**	ND	ND	NA
772	4–6	Zaire BC_2/MK_5	$10^{6,4}$	N	NA	27	0	$^1/_8$**	ND	ND	NA
792	4–6	Zaire BC_2/MK_6	$10^{5,7}$	N	NA	23	0	—ve	ND	ND	NA
794	4–6	Zaire BC_2/MK_6	$10^{5,7}$	N	NA	23	4	$^1/_{32}$**	ND	ND	NA
807	2	Zaire BC_2/MK_6	$10^{5,7}$	N	NA	23	0	$^1/_{16}$**	ND	ND	NA
809	2	Zaire BC_2/MK_6	$10^{5,7}$	L	14	14	3	$^1/_4$**	8,3	7,7	A[3]
810	2	Zaire BC_2/MK_6	$10^{5,7}$	L	20	20	11	$^1/_{64}$**	9,5	9,7	A[3]
811	2	Zaire BC_2/MK_6	$10^{5,7}$	X	NA	7	0	$^1/_2$	8,7	6,7	P
793	4–6	Zaire $BC_2/MK_6/P_1$	D	L	Unknown	26	2	$^1/_2$	9,5	9,1	A
797	4–6	Zaire $BC_2/MK_6/P_1$	D	N	Unknown	26	0	$^1/_{64}$**	ND	ND	NA
808	2	Zaire $BC_2/MK_6/P_1$	D	N	Unknown	23	1	$^1/_8$**	ND	ND	NA
Totals & Means	NA	NA	NA	$^4/_{12}$	NA	NA	NA	NA	NA	NA	NA

[3]=suppurative lesions in lymph nodes; p=proliferative lesions in lymph nodes; **=sera tested for HAI antibody and found negative; D=infection by direct contact; X=pig killed one week after inoculation. Other conventions as for Table 1.

TABLE 4 The response of pigs that survived inoculation with haemadsorbing and non-haemadsorbing isolates to challenge with virulent virus

Pig No.	Primary inoculation			Challenge inoculation									
	Virus dose and route[1]	Duration of pyrexia	Antibody titre before challenge	Virus, dose and route[2]	Period after 1° inoculation[1]	Observation period[1]	Death + or — (time until death)[1]	Duration of pyrexia	Virus titre in		Antibody titre after challenge		Pathology
									Spleen[2]	Lung[2]	IEOP	HAI	
664......	CV BC_{43}/BM_1 $10^{7,5}$ i/n	0	$^1/_{64}$	CV BC_{10} $10^{8,2}$ i/n	28	45	+(17)	10	3,0	3,8	$^1/_{256}$	ND	S/A[3]
665......	CV BC_{43}/BM_1 $10^{7,5}$ i/n	0	$^1/_4$	CV BC_{10} $10^{8,2}$ i/n	28	NA	—	0	ND	ND	$^1/_{32}$	ND	NA
			$^1/_{32}$	CV BC_{10} $10^{9,6}$ i/p	46	66	+(20)	9	1,4	4,6	$^1/_{64}$	ND	CR
693......	CV BC_{10}/P $10^{2,0}$ i/m	34	$^1/_{128}$	CV BC_{10} $10^{9,0}$ i/p	181	NA	—	0	ND	ND	$^1/_{64}$	$^1/_{3125}$	NA
			$^1/_{64}$	TS237 BC26 $10^{6,8}$ i/m	211	380	—	0	ND	ND	$^1/_{16}$	ND	CR
798......	Zaire BC_2 $10^{6,5}$ i/m	0	$^1/_2$	TS237 BC 26 $10^{6,8}$ i/m	35	140	—	0	ND	ND	$^1/_4$	$^1/_{125}$	CR
771......	Zaire BC_2/MK_5 $10^{6,4}$ i/m	0	$^1/_8$	Lillie-148 BC_1 $10^{5,1}$ i/m	27	NA	—	1	ND	ND	$^1/_{32}$	$<^1/_5$	NA
			$^1/_{16}$	CV BC_{10} $10^{6,2}$ i/m	54	103	—	0	ND	ND	$^1/_{32}$	$^1/_{625}$	ND
772......	Zaire BC_2/MK_5 $10^{6,4}$ i/m	0	$^1/_8$	Lillie-148 BC_1 $10^{5,1}$ i/m	27	NA	—	1	ND	ND	$^1/_{16}$	$<^1/_5$	NA
			$^1/_8$	CV BC_{10} $10^{6,2}$ i/m	54	174	—	0	ND	ND	$^1/_{64}$	$^1/_{125}$	CR
792......	Zaire BC_2/MK_6 $10^{5,7}$ i/m	0	—ve	CV BC_5 $10^{7,1}$ i/n	23	40	N	0	—ve	+ve	$^1/_{64}$	$<^1/_5$	CR
794......	Zaire BC_2/MK_6 $10^{5,7}$ i/m	4	$^1/_{32}$	CV BC_5 $10^{7,1}$ i/n	23	31	N	0	4,1	4,1	$^1/_{64}$	ND	CR
807......	Zaire BC_2/MK_6 $10^{5,7}$ i/m	0	$^1/_{16}$	CV BC_5 $10^{7,1}$ i/n	23	40	N	0	—ve	—ve	$^1/_{32}$	$<^1/_5$	CR
808......	Zaire $BC_2/MK_6/P_1$	1	$^1/_8$	CV BC_5 $10^{7,1}$ i/n	NA	40	N	0	+ve[6]	—ve	$^1/_{16}$	$<^1/_5$	CR

[4] =cultures contaminated; pig died of bacterial peritonitis;
[5] =haemadsorbing virus observed; BM—bone marrow culture;
[6] =positive culture but titre too low to titrate.
Other conventions as for Table 3.

That pigs which recover from or are chronically infected with ASF virus are sometimes resistant to challenge with homologous virus although susceptible to lethal infection with heterologous isolates, points to the conculsion that immunologically distinct types may occur naturally (Malmquist, 1963; De Boer, Pan & Hess, 1972). Both Malmquist (1963) and Vigario *et al.* (1970) demonstrated that HAI was capable of distinguishing some isolates from others and suggested that this *in vitro* technique might be useful in classifying immunologically distinct viruses. There is, however, no conclusive information as to whether this *in vitro* differentiation reflects differences of importance *in vivo.* Nevertheless, Vigario *et al.* (1974) have proposed at least 4 subgroups on the basis of cross HAI tests.

The observation that an NHD virus (Zaire) was capable of conferring protection against heterologous HD and NHD viruses indicated that the determinants associated with the HD effect are not fundamental to the development of immunological resistance to ASF. Therefore differences in HAI activity are unlikely to reflect differences of significance in terms of immunity. This finding is in agreement with that of Coggins (1968), who observed that NHD segregates were capable of providing protection against homologous HD virus. However, it is clear from his results that, although he was working with what was essentially a NHD virus population, it was not pure in this respect.

Malmquist (1963) found that the capacity to induce resistance in swine was related to the virulence of the virus inducing the resistance. Our findings, on the contrary, indicate that animals (Table 4) which were clinically unaffected by infection with Zaire virus were protected from the lethal effects of virulent virus (CV). Thus we were unable to confirm Malmquist's (1963) assertion.

Even when there were no clinical signs of infection following inoculation with Zaire virus and subsequent challenge, microscopic and sometimes macroscopic lesions consistent with chronic ASF were observed at a post-mortem examination.

Although prior inoculation of pigs with Zaire virus protected against death following challenge, this was not due to protection from infection, since 3 pigs (Nos. 771, 772 and 792; Table 4) developed significantly increased antibody titres after challenge and, in 3 cases (Nos. 771, 772 and 798; Table 4), the animals developed HAI antibody, indicating that they had been infected with an HD virus. Furthermore, the spleen of pig No. 794 (Table 4) was found to contain HD virus after intranasal challenge.

It was concluded, therefore, that, although haemadsorption is a useful laboratory phenomenon, it cannot be used as a marker for virulence, since HD virus may be relatively avirulent (Coggins, Moulton & Colgrove, 1968) and *vice versa* (Table 2). Furthermore, because the determinants of haemadsorption are not required for the induction of immunological resistance (Table 4), the HAI test cannot be used to determine immunological differences of significance *in vivo.*

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