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AFRICAN SWINE FEVER. I. MORPHOLOGICAL CHANGES AND VIRUS REPLICATION IN BLOOD PLATELETS OF PIGS INFECTED WITH VIRULENT HAEMADSORBING AND NON-HAEMADSORBING ISOLATES

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ABSTRACT

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Replicating and mature viral particles were detected with the transmission electron microscope in blood platelets of pigs infected with virulent haemadsorbing and non-haemadsorbing African swine fever virus isolates. Although platelet numbers decreased terminally in infected pigs, the most noticeable morphological damage to these cells apparent in the last 2 days of the disease included cytoplasmic swelling, vacuolation, fragmentation and loss of dense granules.

INTRODUCTION

African swine fever (ASF) is a disease of domestic and some wild suids caused by a virus of uncertain classification (Carrascosa, Carazo, Carrascosa, Garcia, Santisteban & Vinuela, 1984). The course of the disease in pigs varies, but classically it is responsible for acute death, widespread haemorrhage being the most prominent change (De Kock, Robinson & Keppel, 1940; Maurer, Griesemer & Jones, 1958; Moulton & Coggins, 1968; Edwards, 1983).

The cause of death in acute ASF is not clear. Although the virus replicates and causes cytolysis, primarily in cells of the reticuloendothelial system as well as in many other cell types, there is a body of evidence which suggests that the indirect effects of virus multiplication on the immune system and blood platelets may be more important (Wardley, Andrade, Black, De Castro Portugal, Enjuanes, Hess, Mebus, Ordas, Rutili, Sanchez Vizcaino, Vigario, Wilkinson, Moura Nunes & Thomson, 1983).

Haemorrhage in acute ASF may follow vascular damage as a result of replication of the virus in endothelial cells (Wilkinson & Wardley, 1978), thrombocytopenia (Edwards, 1983), defective fibrin clot formation (Edwards, 1983), or a combination of these. Edwards (1983) considered thrombocytopenia and defective fibrin clot formation the most important.

Thrombocytopenia in ASF occurred only terminally in pigs infected with an isolate that caused death within 8 days (Edwards, 1983; Edwards, Dodds & Slauson, 1985b). Furthermore, Edwards (1983) found that thrombocytopenia developed 6–7 days after infection, but counts usually returned to normal within 10–12 days in pigs infected with a less virulent isolate. The majority of pigs recovered. Basing their conclusion on the failure to detect virus antigen in platelets from infected pigs and the ability of soluble virus antigen to induce aggregation of thrombocytes obtained from recovered pigs, Edwards (1983) and Edwards *et al.* (1985b) proposed that thrombocytopenia in ASF is immune-mediated. No evidence for decreased thrombocytopoiesis during the disease could be found (Edwards, 1983; Edwards & Dodds, 1985).

African swine fever virus isolates can be grouped into haemadsorbing (HD), non-haemadsorbing (NHD) and possibly atypical subgroups, according to their ability to produce haemadsorption of erythrocytes in porcine bone marrow and leucocyte cultures (Malmquist & Hay, 1960; Coggins 1968; Pini & Wagenaar, 1974; Vigario, Terrinha & Moura Nunes, 1974; Pini 1977; Pan & Hess, 1985). It is not clear to what extent these differences reflect pathological variations of importance *in vivo*, since HD-ASF or NHD-ASF viruses may be both virulent or relatively avirulent (Coggins, Moulton & Colgrove, 1968; Thomson, Gainaru & Van Dellen, 1979; Pan & Hess, 1985).

In this paper we present transmission electron microscopical evidence for the replication of ASF virus in blood platelets of experimentally infected pigs. Cytopathic changes of affected platelets are also described. A subsequent paper will deal with the functional changes induced in blood platelets by this virus. This study was also an attempt to determine possible differences in the pathogenesis and pathology induced in platelets by virulent HD-ASF and NHD-ASF virus isolates.

MATERIALS AND METHODS

Twenty-five white, cross-bred pigs, 4–6 months old, were housed separately in raised cages to prevent cross infection.

Viruses: Four different virulent HD-ASF virus isolates, namely, Control (CV), 951, Malawi (MWI), Dominican Republic (DR) and a virulent NHD-ASF virus, namely, the Lillie-148 isolate (L-148), were used. The CV (HD) and L-148 (NHD) isolates were described in a previous report (Thomson *et al.*, 1979) while the 951 and Malawi isolates were obtained from outbreaks of acute ASF in Malawi. The DR isolate was received as a donation (Dr P. Wilkinson, Animal Virus Research Institute, Pirbright, England). Thirteen pigs were infected by intramuscular injection with the 4 different HD-ASF isolates and 12 with the Lillie-148 (NHD-ASF) virus (Table 1). The passage levels and virus titres inoculated are indicated in Table 1.

Specimen collections: Wherever possible, blood specimens for platelet counts and transmission electron microscopy were collected daily or every 2nd day from 2–6 days before infection to the time of death (Tables 2a and 2b). Bleeding was performed from the venae cava anterior by the procedure of Schulze (1981) under light anaesthesia performed with azaparone[1] and trichloroethylene[2]. The anaesthetic method ensured prompt and efficient collection of blood specimens with minimal discomfort and uneventful recovery in every animal. The

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TABLE 1 The isolate, HD characteristic, passage level and inoculum titre of ASF virus injected

Haemadsorbing (HD)				Non-haemadsorbing (NHD)			
Pig No.	Virus isolate	Passage level	Inoculum titre*	Pig No.	Virus isolate	Passage level	Inoculum titre*
1	CV	BC 5	6,1	14	L-148	BC 7	7,9
2	CV	BC 5	6,1	15	L-148	BC 7	7,9
3	CV	BC 5	6,1	16	L-148	BC 7	7,9
4	CV	BC 5	6,1	17	L-148	BC 6	6,3
5	951	BC 2	6,3	18	L-148	BC 7	7,9
6	951	BC 2	6,3	19	L-148	BC 7	7,9
7	MWI	BC 2	5,9	20	L-148	BC 6	6,3
8	MWI	BC 2	5,9	21	L-148	BC 9	4,9
9	DR	BC 2	6,7	22	L-148	BC 9	4,9
10	DR	BC 2	6,7	23	L-148	BC 14	7,7
11	CV	LLC-MK2/BC 6	5,9	24	L-148	BC 14	7,7
12	CV	LLC-MK2/BC 6	5,9	25	L-148	BC 14	7,7
13	CV	LLC-MK2/BC 6	5,9	—	—	—	—

* = $\log_{10}$ TCD_{50} or HD_{50}/mℓ
BC = blood leucocyte culture
CV = Control Virus
MWI = Malawi Virus
DR = Dominican Republic Virus
L-148 = Lillie-148 Virus
LLC-MK2 = "American type culture collection", Rockville, Maryland, USA

vena cava anterior and adnexal tissues tolerated the repeated blood collections well, even in the NHD-ASF isolate-infected pigs where the course of the disease was longer.

Blood platelet counts and light microscopical examinations: Five mℓ of blood was collected in EDTA anticoagulant[3] from which the blood platelet count was determined with the electronic Coulter Counter[4] (Models S plus). In addition, the simple indirect microscopical platelet counting method (Schalm, Jain & Carroll, 1975) was performed on EDTA-preserved blood films, fixed in methanol and stained with 10 % Giemsa[5] solution for 45 minutes. Two blood films and 50 fields per film were examined under oil immersion (× 1200) magnification to determine the mean number of platelets per field. At the same time platelet morphology and size were studied. Their approximate size was assessed by comparing platelets with red blood cells.

Extrapolation of the indirect microscopical count to platelets × 10^9/ℓ was made on the basis of Schalm's *et al.* (1975) statement that 3 platelets per oil immersion field is roughly equal to 50 × 10^9 platelets per litre. A count of 200 × 10^9/ℓ was accepted as the low normal level for swine platelet counts (Christoph & Meyer 1965; Bowie, Owen, Zollman, Thomson & Fass, 1973). By extrapolation the low normal indirect microscopical platelet count for swine would therefore be 12 platelets per 1200 × oil immersion field.

Electron microscopical examinations: Seven pigs infected with HD-ASF isolates (pigs 1, 2, 9–13) and 7 infected with NHD/ASF isolate (Pigs 17, 20–25) were studied (Tables 2a and 2b).

A modification of the method of Zwierzina, Schmalzl, Kunz, Dworzak, Linker & Geissler (1983) to ensure immediate fixation and minimal activation of platelets before processing was applied. Briefly, 3 mℓ of blood was drawn directly into a 10 mℓ syringe containing 7 mℓ of a 2,5 % glutaraldehyde solution, using a 19G needle. A platelet-rich plasma (PRP) supernatant was then prepared by centrifugation for 10 min at 200 *g*. The PRP was recentrifuged for 10 min at 1500 *g* to produce platelet-rich pellets. These were post-fixed in 1 % osmium tetroxide[6] and cacodylate buffer[6], dehydrated in graded ethanols, cleared in 1,2 propylene oxide[7] and embedded in Polaron 812[6].

Virological examinations: Viraemia was determined in blood collected terminally from 3 pigs infected with a HD-ASF isolate and from 3 infected with a NHD-ASF isolate (Table 3). In addition, the concentration of ASF virus in selected tissues and lymph nodes of the remaining pigs was also determined. Specimens were titrated in blood leucocyte cultures as described by Thomson *et al.* (1979).

RESULTS

Course of infection

While all the pigs infected with the HD-ASF viruses died within 4–6 days (mean 4,6 ± 0,5) of inoculation (Table 2a), those exposed to the L-148 (NHD-ASF) virus lived for 6–15 days (mean 9,8 ± 2,8) (Table 2b).

Blood platelet counts

Sequential platelet counts and mean platelet volume determinations by the electronic method, as well as the indirect microscopical counting method, are recorded in Tables 2a and 2b, except where terminal specimens were not suitable for examination or unobtainable owing to unexpected deaths. Indirect microscopical counting proved more useful in this investigation, since the electronic Coulter Counter rejected the platelet counts as the disease progressed, especially in the 1st group (Table 2a), hence the former method was discontinued in the last 3 pigs of each group. Post-inoculation electronic platelet counts varied over a wide range in both groups but did not decrease during the course of the disease in most animals (Tables 2a and 2b).

Furthermore, in only 1/9 (11 %) of pigs infected with HD and NHD isolates (Tables 2a and 2b), where electronic counts were possible during the last 3 days before death, were they below 200 × 10^9/ℓ. Conversely, microscopical counts were below normal (12 platelets per 1200 × oil immersion field) in 9/12 (75 %) of pigs infected with HD-ASF isolates (Table 2a) and in 6/11 (56 %) of pigs infected with NHD/ASF isolate (Table 2b).

[1] Stressnil, Ethnor (Pty) Ltd, Halfway House, RSA 1685

[2] Trilene, Imperial Chemical Industries Limited, England, P.O. Box 11270, Johannesburg, RSA 2000

[3] Vacutainer, Raydem Laboratories, P.O. Box 391606, Bramley, RSA 2018

[4] Coulter Electronics Inc., Hialeah Florida, USA

[5] Giemsa-Lösung, Merck Chemicals, Darmstadt

[6] Polaron Equipment Ltd., 21 Greenhill Crescent, Watford, Hertfordshire, England

[7] BDH Chemicals Ltd., Poole, England

TABLE 2a Sequential mean platelet volume and blood platelet counts determined by 2 methods before and after infection with 4 different HD/ASF virus isolates

Pig No.	Virus inoculated	Platelet count	Days before and after virus inoculation										Surivial time (days)
			−3	−2	−1	0	1	2	3	4	5	6	
1	CV	MPV	—	—	—	—	—	—	R	—			
		EPC	—	—	—	—	—	—	R	—	NA	NA	4
		MIMC	—	—	—	9	—	—	12	—			
2	CV	MPV	—	—	—	11,3	—	—	14	R			
		EPC	—	—	—	243	—	—	249	R	NA	NA	4
		MIMC	—	—	—	9	—	—	10	6			
3	CV	MPV	8,6	—	—	8,9	—	—	7,7	R	—		
		EPC	499	—	—	563	—	—	838	R	—	NA	5
		MIMC	—	—	—	—	—	—	—	—	—		
4	CV	MPV	—	—	—	—	—	—	9,4	R			
		EPC	—	—	—	—	—	—	245	R	R	NA	5
		MIMC	—	—	—	—	—	—	8	12	11		
5	951	MPV	—	—	—	8,6	—	—	9,1	R			
		EPC	—	—	—	439	—	—	415	R	NA	NA	4
		MIMC	—	—	—	17	—	—	13	11			
6	951	MPV	8,5	—	—	8,4	—	—	8,7	R			
		EPC	409	—	—	404	—	—	196	R	NA	NA	4
		MIMC	15	—	—	33	—	—	11	12			
7	MWI	MPV	8,5	—	—	8,3	—	—	7,9	R			
		EPC	269	—	—	262	—	—	325	R	NA	NA	4
		MIMC	15	—	—		—	—	10	13			
8	MWI	MPV	9,2	—	—	9,1	—	—	8,6	10,1	R		
		EPC	307	—	—	312	—	—	253	319	R	NA	5
		MIMC	18	—	—	—	—	—	12	10	9		
9	DR	MPV	8,0	—	—	8,0	—	—	9,1	10,4	—		
		EPC	500	—	—	513	—	—	337	505	—	NA	5
		MIMC	14	—	—	—	—	—	14	15	—		
10	DR	MPV	—	—	—	8,9	—	—	8,8	10,1	R		
		EPC	—	—	—	331	—	—	289	658	R	NA	5
		MIMC	—	—	—	17	—	—	—	16	14		
11	CV	MPV	—	—	—	—	—	—	—	—	—		
		EPC	—	—	—	—	—	—	—	—	—	NA	5
		MIMC	—	—	28	24	—	—	6	6	—		
12	CV	MPV	—	—	—	—	—	—	—	—	—		
		EPC	—	—	—	—	—	—	—	—	—	NA	5
		MIMC	—	—	24	27	—	—	—	3	—		
13	CV	MPV	—	—	—	—	—	—	—	—	—		
		EPC	—	—	—	—	—	—	—	—	—	NA	5
		MIMC	—	—	16	19	—	—	8	3	—		
TOTAL:	13												Mean ± SD 4,6 ± 0,5

MPV = mean platelet volume × fl as determined by the electronic Coulter Counter
EPC = electronic platelet count $\times 10^9/\ell$ as determined by the electronic Coulter Counter
MIMC = mean indirect microscopic platelet count × platelets/oil immersion field
R = rejection of platelet counts by Coulter Counter
NA = not applicable, i.e. animal dead
SD = standard deviation
— = not done

The discrepancy between the counts obtained by the 2 methods was greater in pigs infected with HD-ASF isolates than in those infected with the NHD-ASF isolate (Table 2a and 2b). There was no obvious decrease in microscopical count in either group until 2–3 days before death. Furthermore, it would appear that the most marked decrease in microscopical platelet counts took place in pigs where the survival period was 9 days or less, irrespective of the haemadsorption characteristic of the virus inoculated (Tables 2a and 2b).

Electronic mean platelet volume

Although there was considerable variation in pre-inoculation values, there was a slight but consistent increase in platelet volume of *c.* 0,1–3,0 femtolitre (1–20 %) in 6/8 (75 %) pigs infected with HD-ASF isolates and in 3/8 (38 %) animals infected with NHD-ASF isolates (Tables 2a and 2 b).

Light microscopical examinations

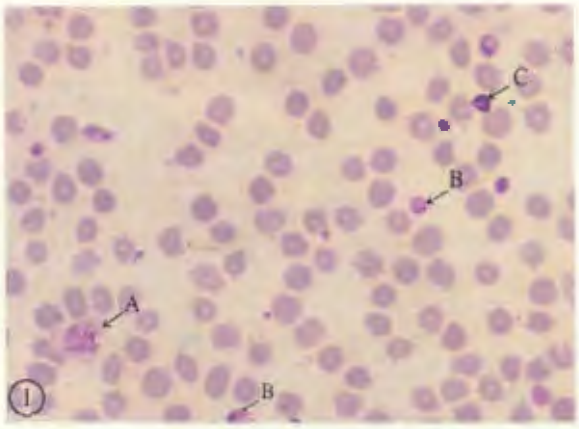
Degeneration and fragmentation of thrombocytes and leucocytes were conspicuous changes in blood films prepared from both groups on the last 2 days of life. These changes were more pronounced in pigs from both groups with survival periods of 9 days and less (Tables 2a and 2b).

Scattered cytoplasmic and nuclear fragments, presumably derived from leucocytes, were sometimes difficult to distinguish from degenerative platelets (Fig. 1).

TABLE 2b Sequential mean platelet volume and blood platelet counts determined by 2 methods before and after infection with the Lillie NHD-ASF virus isolate

Pig No.	Platelet volume and counts	Days before and after virus inoculation																							Survival time (days)
		−7	−6	−5	−4	−3	−2	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
14	MPV	—	—	—	—	—	—	—	—	—	8,1	—	—	—	—	7,9	—	8,1	—	7,4	—	8,3	—	—	15
	EPC	—	—	—	—	—	—	—	—	—	286	—	—	—	—	275	—	348	—	320	—	431	—	—	
	MIMC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
15	MPV	—	—	8,7	—	7,7	—	—	7,7	—	7,4	—	7,8	—	—	8,3	—	8,5	—	8,2	—				12
	EPC	—	—	331	—	485	—	—	376	—	376	—	346	—	—	396	—	354	—	319	—	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	34	—	18	—				
16	MPV	—	—	7,7	—	7,0	—	—	7,0	—	7,8	—	8,3	—	—										6
	EPC	—	—	401	—	441	—	—	419	—	336	—	308	—	—	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	—	—	11	—	11	—	—										
17	MPV	—	—	—	—	—	—	—	—	—	—	—	—	—	9,7	—	—								8
	EPC	—	—	—	—	—	—	—	—	—	—	376	—	—	278	—	—	NA	NA	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	—	—	—	16	—	—	8	—	7								
18	MPV	8,9	—	8,6	—	—	8,4	—	8,7	—	8,0	—	—	8,3	—	8,7	—	8,6	—	—	9,4	—	9,8		14
	EPC	360	—	345	—	—	261	—	334	—	449	—	—	354	—	397	—	371	—	—	441	—	426	NA	
	MIMC	22	—	17	—	—	—	—	22	—	16	—	—	18	—	25	—	27	—	—	24	—	13		
19	MPV	8,0	—	8,0	—	—	7,8	—	8,4	—	7,7	—	—	8,7	—	—	—	8,2	—	—					11
	EPC	458	—	373	—	—	308	—	401	—	411	—	—	339	—	—	—	287	—	—	NA	NA	NA	NA	
	MIMC	29	—	16	—	—	17	—	12	—	11	—	—	16	—	—	—	12	—	—	—				
20	MPV	—	—	—	—	—	—	—	9,5	—	9,4	—	—	—	—	—									7
	EPC	—	—	—	—	—	297	—	287	—	290	—	—	220	—	R	NA	NA	NA	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	11	—	13	—	—	11	—	4									
21	MPV	—	—	—	—	—	7,5	—	7,0	—	—	—	7,5	—	—	9,1	8,6	8,3	—						10
	EPC	—	—	—	—	—	600	—	713	—	—	—	375	—	—	165	160	165	—	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	35	—	32	—	—	—	13	—	—	15	—	14	—						
22	MPV	—	—	—	—	—	7,9	—	7,8	—	—	—	8,0	—	—	9,1	8,3	9,4	—						10
	EPC	—	—	—	—	—	570	—	664	—	—	—	481	—	—	373	298	—	—	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	28	—	32	—	—	—	16	—	—	29	—	12	—						
23	MPV	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—								8
	EPC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	NA	NA	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	—	—	—	—	48	—	—	18	5								
24	MPV	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—								8
	EPC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	NA	NA	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	—	—	—	—	66	—	—	14	4								
25	MPV	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—								8
	EPC	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	NA	NA	NA	NA	NA	NA	NA	
	MIMC	—	—	—	—	—	—	—	—	—	—	—	38	—	—	12	8								
Total	12																								Mean ± SD 9,8 ±2,81

All conventions as for previous table



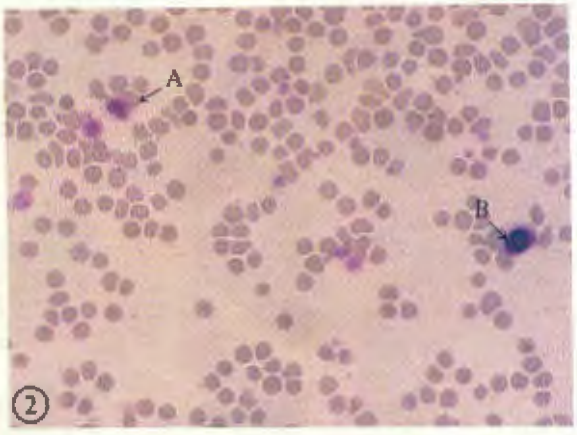


FIG. 1 Enlarged vacuolated and hypogranular degenerated platelet (A), hypogranular and vacuolated platelets (B), and suspected leucocyte fragments (C) 1–2 days before death of the pig. 10 % Giemsa: × 1250

FIG. 2 Enlarged pale staining hypogranular platelets (A) and mononuclear leucocyte (B) 1–2 days before death of the pig. 10 % Giemsa: × 1200

An estimated 1–20 % of identifiable platelets showed various changes, consisting of cytoplasmic swelling and vacuolation, loss of dense granules and fragmentation (Fig. 1). A distinct population of *c.* 4 % of platelets with a size larger than red blood cells could also be identified (Fig. 2). These were considered to be immature, a conclusion based on their non-vacuolated hypogranular cytoplasm (Paulus & Aster, 1983). A considerable number of immature neutrophils was also present.

Electron microscopical examination

Blood platelets in PRP fractions of 7 pigs infected with HD-ASF isolates (Table 2a) and 7 infected with the NHD-ASF isolate (Table 2b) showed obvious morphological disturbances (Fig. 3 and 4) at 2–3 days before death, when they became larger and more irregular in shape. Approximately 5–10 % of these platelets became fragmented (Fig. 5) or displayed degenerative changes, such as cytoplasmic swelling and vacuolation, dilatation of the canalicular system and loss of organellar detail (Fig. 5–9).

In 2 pigs infected with the NHD-ASF isolate (Pigs 21, 22) (Table 2b) where the survival was longer than 9 days, no obvious increase in the degree of platelet damage became apparent on the day before death. Platelets were not examined on the last day of life. However, in the other 5 pigs (Table 2b) and in the pigs infected with the HD-ASF isolates (Table 2a), where the survival period was shorter, the proportion of severely damaged platelets increased to at least 50–75 % on the day of death. A precise assessment under the electron microscope was difficult, however.

Typical, unenveloped, hexagonal or polygonal ASF virus particles *c.* 200 nm in diameter, as well as numerous curved to roughly circular open or closed double layered membranous structures *c.* 10 nm wide, were present in *c.* 0,1–1 % of damaged platelets or fragments thereof (Fig. 6–8) in 5/7 pigs (Pigs 10, 11, 18, 19 and 28) infected with the HD-ASF isolates and in 4/7 (Pigs 4, 7, 23 and 25) which received the NHD-ASF isolate (Fig. 10). Mature, enveloped virions were occasionally found extracellularly (Fig. 10). Both extra and intracellular hexagonal particles displayed an electron-dense nucleoid core, while it was either absent or ill-defined within less angular virions arranged in characteristic array (Fig. 7 and 8). Structures which were suspected as being virions budding from platelet membranes were also seen (Fig. 9 and 10).

Virus titrations

The titres of ASF virus in EDTA preserved blood specimens collected on the day of death are presented in Table 3. Lymph node and organ specimens titrated from the remaining pigs were positive for ASF virus (Neser, Thomson & Gainaru, 1985, unpublished results).

TABLE 3 Virus titres in EDTA preserved blood specimens collected on the day of death

Haemadsorbing (HD) isolate			Non-haemadsorbing (NHD) isolate		
Pig No.	Virus isolate	Virus titre*	Pig No.	Virus isolate	Virus titre*
11	CV	−7,4	23	L-148	−7,8
12	CV	−7,8	24	L-148	−8,0
13	CV	−7,8	25	L-148	−8,2

* = $\log_{10}$ HD_{50}/mℓ blood

All other conventions as for previous tables

DISCUSSION

Pigs infected with HD-ASF isolates died more acutely (4–5 days) than those infected with the NHD-ASF isolate (6–15 days). Judged by the length of the survival period, all the isolates used in this study were virulent (Pan & Hess, 1984). The Dominican Republic (HD-ASF) isolate, however, was more virulent in our experiments than in those reported previously by Edwards (1983). The reason for this is not clear, but the difference may be explained by the postulation that ASF virus isolates can be composed of mixed clones of heterogenous virulence (Pan & Hess, 1985). At necropsy, varying degrees of haemorrhage and oedema of superficial and deep lymph nodes and viscera, as well as effusions of body cavities, were present in both groups of pigs (Neser, Thomson & Gainaru, 1985, unpublished results). Although these changes were more pronounced in the pigs infected with the HD-ASF viruses than in animals infected with the NHD-ASF isolate, they were less obvious than those described by Edwards (1983) and Edwards *et al.* (1985b).

Besides the morphological changes in platelets, prolonged bleeding time, decreased clot retraction and impaired platelet aggregation occurred in all the pigs in this study (Neser, 1985, unpublished observations), which also indicated functional disturbances of thrombocytes (Schalm *et al.*, 1975).

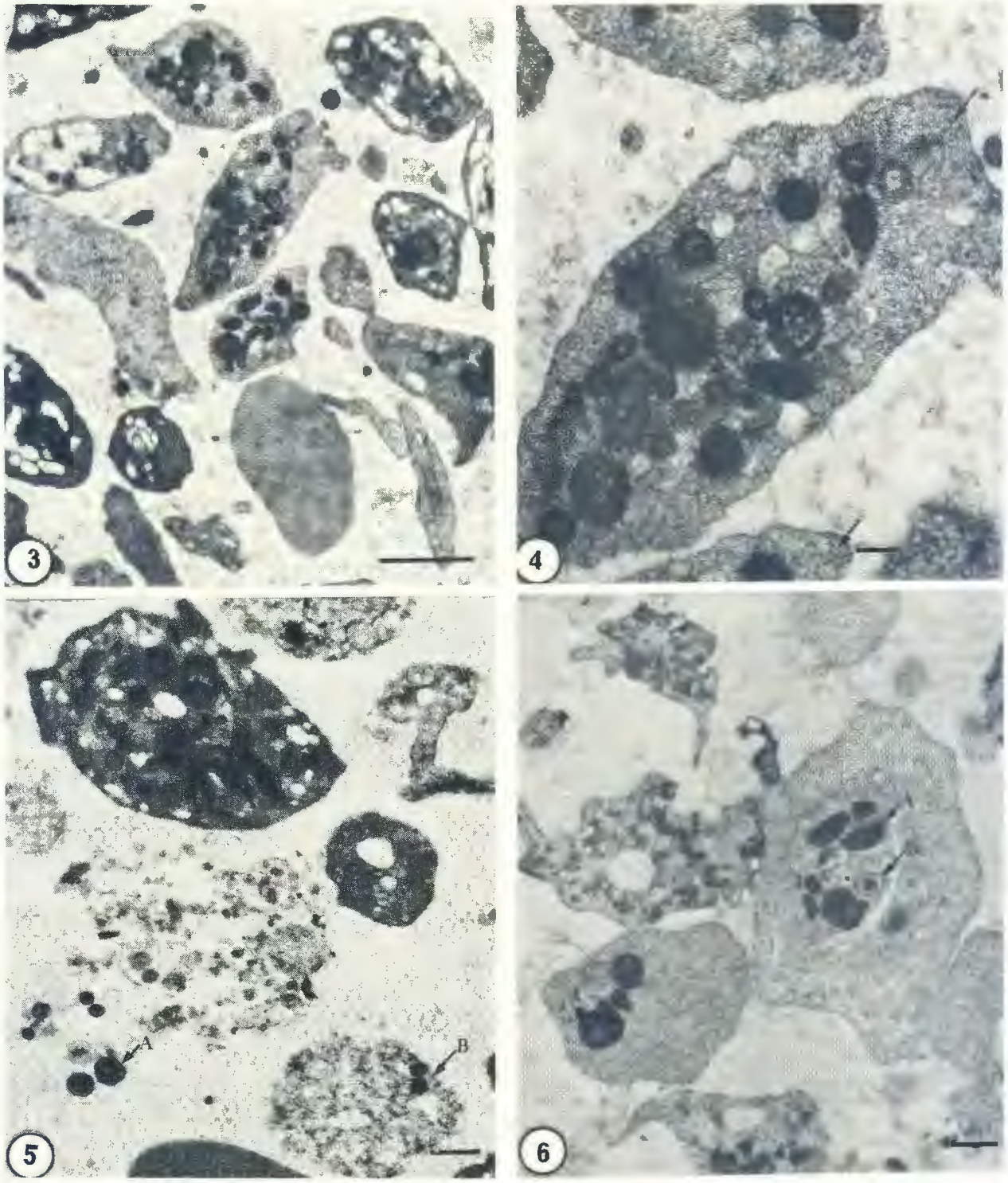


FIG. 3 & 4 Ultrastructure of normal platelet (control): Note electron-dense granules, canalicular system and peripheral microtubules (arrow) × 11,00 (Bar = 1000 nm) and × 36,000 (Bar × 200 nm) respectively

FIG. 5 Electron-dense granules from fragmented platelet (A) and ASF virus particles (B) in degenerate platelet 1–2 days before death of the pig: × 17000 (Bar = 500 nm)

FIG. 6 Electron-dense granules (A) and immature ASF virus particle (B) in platelet 1–2 days before death of the pig: × 17000 (Bar = 500 nm)

The rejection of the platelet counts by the Coulter Counter Model S plus in specimens collected during the terminal stages of the disease in both groups casts some doubt on the suitability of this instrument for these studies in acute ASF. Electronic counting of platelets in acute ASF is rendered potentially spurious by the presence of leucocyte fragments (De Kock *et al.*, 1940) and the increase in platelet size. This emphasizes the assertion of Paulus & Aster (1983) that electronic platelet counts may be artifactually elevated by leucocyte fragments and ought to be routinely checked microscopically on blood films. It would therefore be reasonable to suspect these fragments and the fragmented, degenerated or large immature platelets of being the cause of disturbed and persisting high electronic platelet counts in most pigs infected with the HD-ASF isolates. Although

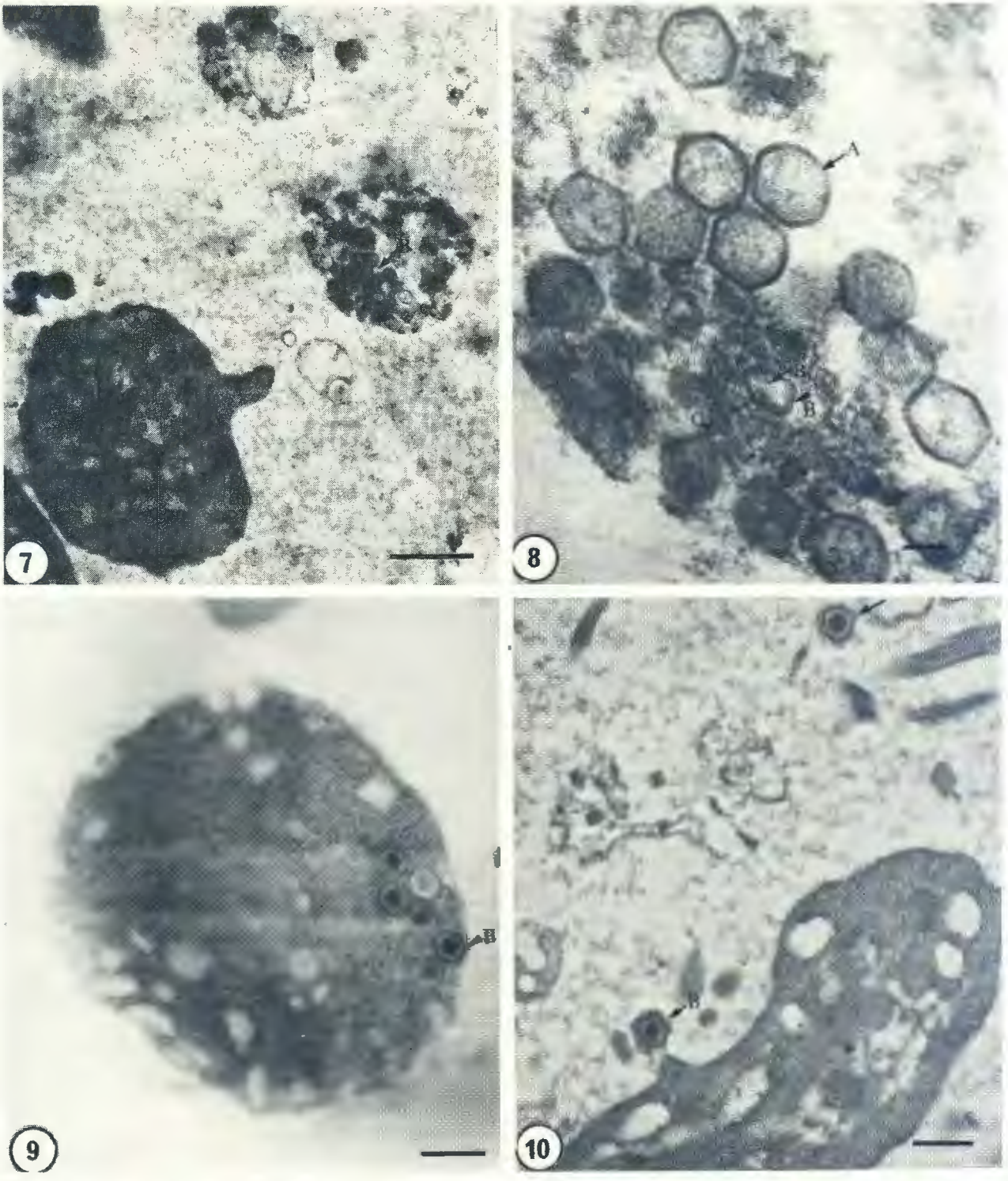


FIG. 7 & 8 Suspected platelet from specimen collected 1–2 days before death. Note immature replicating polygonal ASF virus particles (A) arranged in characteristic array and circular (B) to curved (C) membranous viral structures: × 1500 (Bar = 1000 nm) and: × 70 000 (Bar = 100 nm) respectively

FIG. 9 Blood platelet from specimen collected 1–2 days before death. Immature replicating ASF virus particles and suspected early budding particle from platelet membrane (B) × 30 000 (Bar = 550 nm)

FIG. 10 Blood platelet from specimen collected 1–2 days before death. Note ASF virus particle suspected to have budded from platelet membrane: × 20 000 (Bar = 500 nm)

the indirect microscopical counting method is not generally accepted as being as accurate as the electronic method, it provided the additional advantage that blood platelet and leucocyte changes could be evaluated and that platelets could be counted and classified according to their relative size to red blood cells. For these reasons, more reliance was placed on the indirect microscopical method than on the electronic counting method.

The terminal onset of platelet reduction observed in both groups by the indirect microscopical method is in agreement with the findings of Edwards (1983) and Edwards *et al.* (1985b). However, the marked transient thrombocytopenia described by him in less acute cases infected with the DR 79 isolate from the Dominican Republic was never seen where the course of the disease was more extended. It should be emphasized, however,

that none of our pigs recovered from the infection. Thrombocytopenia (fewer than 12 platelets per — × 1200 field) was more frequently observed in the pigs infected with the HD-ASF virus isolates than in animals infected with the NHD-ASF isolate. In addition, thrombocytopenia was more pronounced in pigs that died within the first 8 days after being infected with the NHD-ASF isolate. The degree of thrombocytopenia, therefore, also appears to depend on the duration of the disease. Since the indirect microscopical platelet counting method employed in this study is generally considered to be less accurate (Schalm *et al.*, 1975), this aspect needs to be investigated further. However, the presence of the blood cell fragments described earlier remains a complicating factor in the determination of platelet counts by conventional methods in the acute form of this disease. Platelets may be extremely difficult to distinguish microscopically from blood cell fragments by the haemocytometer method described by Schalm *et al.* (1975).

The presence of fragmented, degenerated and large immature platelets in the majority of pigs infected with the HD-ASF and NHD-ASF viruses is indicative of peripheral platelet destruction rather than deficient thrombocytopoiesis (Paulus & Aster, 1983). This is in agreement with the conclusion reached by Edwards (1983), Edwards *et al.* (1985a) and Edwards *et al.* (1985b). Although ASF virus could be found ultrastructurally in a small percentage of bone marrow megakaryocytes (Neser & Phillips, 1985, unpublished results) as well as by positive immunofluorescence (Edwards, 1983; Edwards, Dodds & Slauson, 1985a) the increase in numbers of immature platelets and leucocytes in our study indicates stimulated haematopoiesis rather than deficient thrombopoiesis.

Despite the decrease in platelet numbers in most pigs during the progression of the disease, in no animal did the indirect microscopical count fall below 3 platelets per oil immersion field or $50 \times 10^9/\ell$ (Schalm *et al.*, 1975). It is unlikely that thrombocytopenia alone could have resulted in the degree of haemorrhage seen in some pigs at necropsy. Lie (1968) found that in piglets suffering from iso-immune thrombocytopenia, haemorrhage was only evident when pletelets fell below $40 \times 10^9/\ell$.

The direct fixation method of blood platelets before differential centrifugation (Zwierzina *et al.*, 1983) provided a useful method for studying intact and damaged platelets. Processing of affected platelets by centrifugation before fixation failed to provide suitable results. No significant improvement of specimen quality could be achieved by using a wide-bore needle as recommended by Zwierzina *et al.* (1983).

Damaged and fragmented platelets were identified on the grounds of their approximate size, lack of a nucleus and the presence of alpha granules and a canalicular system. However, peripheral concentric microtubules, a hallmark of platelet ultrastructure (Bloom & Fawcett, 1975), could not be demonstrated in most fragments or damaged platelets. Considering the degree of cytoplasmic damage, their dissolution is nevertheless conceivable. Cytoplasmic leucocyte fragments were difficult to distinguish from damaged platelets when devoid of nuclear remnants. A distinction could only be attempted on the lack of alpha granules and a canalicular system.

Less than *c.* 2 % of damaged platelets and fragments studied in the terminal stages of the disease contained regularly arrayed hexagonal and polygonal, capsid-bound, viral particles *c.* 200 nm in diameter. However, the rate of platelet infection may be significantly higher, considering that only a single plane of platelet section is viewed under the transmission electron microscope. These particles were similar to mature and immature virions and structures associated with viral replication (Moura Nunes, Vigario & Terrinha, 1975; Els & Pini, 1977; Carrascosa *et al.*, 1984). As far as could be ascertained, this is the first morphological evidence of the presence and replication of ASF virus in blood platelets contrary to the findings of Edwards (1983) and Edwards *et al.*, (1985b). How much virus is produced by thrombocytes was not determined in this investigation because of the difficulty in purifying platelets adequately, the high infectivity levels associated with other blood components, particularly erythrocytes, which contaminated thrombocyte rich fractions, and the relatively inaccurate titration techniques available. Unadapted ASF isolates do not usually produce plaques in conventional cell cultures.

Daily titrations of ASF virus in blood were not performed in this study. However, viraemic levels determined at death were high in both groups and were comparable with maximal levels reached 72–96 hours post-inoculation in other studies (Plowright, Parker & Staple, 1968; Wardley & Wilkinson, 1977a; Edwards, 1983).

Most virus in the blood of pigs acutely affected with ASF virus are located in and on the surface of erythrocytes (Plowright *et al.*, 1986; Wardley & Wilkinson, 1977a; Rodriques, Andrade, Da Silva & Baptista, 1983). Apart from virus free in the plasma (Plowright *et al.*, 1968), ASF virus has been shown to replicate in monocytes (Wardley & Wilkinson, 1977b) and in a small proportion of polymorphonuclear leucocytes (Casal, Enjuanes & Vinuela, 1984). Conflicting results have been reported with respect to the possible replication of ASF virus in lymphocytes (Wardley & Wilkinson, 1977c; Wardley & Wilkinson, 1980; Sanchez-Vizcaino, Slauson, Ruiz-Gonzalvo & Valero, 1981; Casal *et al.*, 1984).

Taking together the presence of replicating virus in putative blood platelets and the generally cytolytic nature of ASF virus infection, it is tempting to speculate that platelet damage is a direct consequence of virus multiplication in these cells. However, the fact that viral structures were visible in only a minority of damaged platelets, and, in the case of Lillie-148 infected pigs, platelet abnormalities may only become apparent up to 5 days after the onset of viraemia (Neser, Phillips, Thomson & Gainaru, 1985, unpublished results) suggests that the rate of infection of platelets is low or, alternatively, that the virus growth cycle in these cells is extended. It is conceivable that the fact that platelets are not nucleated could have such an effect on ASF virus replication (Ortin & Vinuela, 1977). *In vitro* studies are required to elucidate this point.

Immunological destruction of platelets in ASF was proposed by Edwards (1983) and Edwards *et al.*, (1985b). However, our studies showed that platelet damage was more frequent and more severe in pigs that died within 5 days of infection with the HD-ASF viruses than in those animals inoculated with the Lillie-148 isolate that survived longer. It is improbable, therefore, that immune mechanisms could have played a part in platelet destruction where pigs died acutely.

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REFERENCES

BLOOM, W. & FAWCETT, D. W., 1975. Blood platelets. *In:* A textbook of histology. (10th edn) 139 pp. Philadelphia, USA: W. B. Saunders Company.

BOWIE, E. J. W., OWEN, C. A., ZOLLMAN, P. E., THOMPSON, J. H. & FASS, D. N., 1973. Tests of hemostasis in swine: Normal values and values in pigs affected with Von Willebrand's disease. *American Journal of Veterinary Research,* 34, 1 405–1 407.

CARRASCOSA, J. L., CARAZO, J. M., CARRASCOSA, A. L., GARCIA, N., SANTISTEBAN, A. & VINUELA, E., 1984. General morphology and capsid fine structure of African swine fever virus particles. *Virology,* 132, 160–172.

CASAL, I., ENJUANES, L. & VINUELA, E., 1984. Porcine leucocyte cellular subsets sensitive to African swine fever virus *in vitro*. *Journal of Virology,* 52, 37–45.

CHRISTOPH, H. J. & MEYER, H., 1965. Klinisches Laborotorium Arbeits Methoden des Laboratoriums in der Veterinarmedizin. *In:* LEMAN, A. D., GLOCK, R. D., MENGELING, W. L., PENNY, R. H., SCHOLL, E. & STRAW, B. (eds). Diseases of swine. (5th edn), 31 pp. 1981. Ames, Iowa, USA: Iowa State University Press.

COGGINS, L., 1968. Segregation of a non-haemadsorbing African swine fever virus in tissue culture. *Cornell Veterinarian,* 58, 12–20.

COGGINS, L., MOULTON, J. E. & COLGROVE, G. S., 1968. Studies with Hinde attenuated African swine fever virus. *Cornell Veterinarian,* 58, 525–540.

DE KOCK, G., ROBINSON, E. M. & KEPPEL, J. J., 1940. Swine fever in South Africa. *Onderstepoort Journal of Veterinary Science and Animal Industry,* 14, 31–93.

EDWARDS, J. F., 1983. The pathogenesis of thrombocytopaenia and hemorhage in African swine fever. Ph. D. thesis, Cornell University, Ithaca, New York.

EDWARDS, J. F. & DODDS, W. J., 1985. Platelet and fibrinogen kinetics in healthy and African swine fever affected swine: [^{75}Se] Selenomethionine-labeling study. *American Jounal of Veterinary Research,* 46, 181–184.

EDWARDS, J. F., DODDS, W. J. & SLAUSON, D. O., 1985a. Megakaryocytic infection and thrombocytopaenia in African swine fever. *Veterinary Pathology,* 22, 95–196.

EDWARDS, J. F., DODDS, W. J. & SLAUSON, D. O., 1985b. Mechanism of thrombocytopenia in African swine fever. *American Journal of Veterinary Research,* 46, 2 058–2 063.

ELS, H. & PINI, J., 1977. Negative staining of a non-haemadsorbing strain of African swine fever virus. *Onderstepoort Journal of Veterinary Research,* 44, 39–55.

LIE, H., 1968. Thrombocytopenic purpura in baby pigs—clinical studies. *Acta Veterinaria Scandinavia,* 9, 285–301.

MALMQUIST, W. A. & HAY, D., 1960. Hemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures. *American Journal of Veterinary Research,* 21, 104–108.

MAURER, F. D., GRIESEMER, R. A. & JONES, T. C., 1958. The pathology of African swine fever—a comparison with hog cholera. *American Journal of Veterinary Research,* 19, 517–539.

MOULTON, J. & COGGINS, L., 1968. Comparison of lesions in acute and chronic African swine fever. *Cornell Veterinarian,* 58, 364–388.

MOURA NUNES, J. F., VIGARIO, J. D. & TERRINHA, A. M., 1975. Ultrastructural study of African swine fever virus replication in cultures of swine bone marrow cells. *Archives of Virology,* 49, 59–66.

ORTIN, J. & VINUELA, E., 1977. Requirement of cell nucleus for African swine fever virus replication in Vero cells. *Journal of Virology,* 21, 902–905.

PAN, I. C. & HESS, W. R., 1984. Virulence in African swine fever: its measurement and implications. *American Journal of Veterinary Research,* 45 (2), 361–366.

PAN, I. C. & HESS, W. R., 1985. Diversity of African swine fever virus. *American Journal of Veterinary Research,* 46, 314–320.

PAULUS, J. M. & ASTER, R. H., 1983. Clinical evaluation of thrombokinetics. *In:* WILLIAMS, W. J., BEUTLER, E., ERSLEV, A. J. & LICHTMAN, M. A. (eds). Hematology. (3rd edn) 1196 pp. 1983. New York McGraw-Hill Book Co.

PINI, A. & WAGENAAR, G., 1974. Isolation of a non-haemadsorbing strain of African swine fever virus from a natural outbreak of the disease. *Veterinary Record,* 94, 2.

PINI, A., 1977. Strains of African swine fever virus isolated from domestic pigs and from *Ornithodorus moubata* in South Africa. D.V.Sc. thesis, University of Pretoria.

PLOWRIGHT, W., PARKER, J. & STAPLE, R. F., 1968. The growth of a virulent strain of African swine fever virus in domestic pigs. *Journal of Hygiene,* Cambridge, 66, 117–134.

RODRIQUES, L. C., ANDRADE, C. DE M., DA SILVA, A. G. & BAPTISTA, M. DE F. D., 1981. Ultramicroscopic observations of African swine fever virus in pig erythrocytes. *Proceedings CEC/FAO (EUR 8466) of Research Seminar on African Swine Fever,* Sassari, Sardinia, 181. pp. 161–164.

SANCHEZ-VIZCAINO, J. M., SLAUSON, D. O., RUIZ-GONZALVO, F. & VALERO, M. S. 1981. Lymphocyte function and cell-mediated immunity in pigs with experimentally induced African swine fever. *American Journal of Veterinary Research,* 42, 1 335–1 341.

SCHALM, O. W., JAIN, N. C. & CARROLL, B. S., 1975. Materials and methods for the study of the blood, including brief comments on factors to be considered in interpretation. *In:* Veterinary Hematology, 3rd. edn. Philadelphia, USA: Lea & Febiger Publ. Co.

SCHULZE, W., 1981. Physical examination. *In:* LEMAN, A. D., GLOCK, R. D., MENGELING, W. L., PENNY, R. H., SCHOLLE, E. & STRAW, B. (eds). Diseases of swine. (5th edn) 690 pp. 1981. Ames, Iowa: Iowa State University Press.

THOMSON, G. R., GAINARU, M. D. & VAN DELLEN, A. F., 1979. African swine fever: pathogenicity and immunogenicity of two non-haemadsorbing viruses. *Onderstepoort Journal of Veterinary Research,* 46, 149–154.

VIGARIO, J. D., TERRINHA, A. M. & MOURA-NUNES, J. F., 1974. Antigenic relationships among strains of African swine fever virus. *Archiv für die gesamte Virusforschung,* 45, 272–277.

WARDLEY, R. C. & WILKINSON, P. J., 1977a. The association of African swine fever virus with blood components of infected pigs. *Archives of Virology,* 55, 327–334.

WARDLEY, R. C. & WILKINSON, P. J., 1977b. The growth of virulent African swine fever virus in pigs monocytes and macrophages. *Journal of General Virology,* 38, 183–186.

WARDLEY, R. C. & WILKINSON, P. J., 1977c. African swine fever replication in porcine lymphocytes. *Journal of General Virology,* 37, 425–427.

WARDLEY, R. C. & WILKINSON, P. J., 1980. Lymphocyte responses to African swine fever virus infection. *Research in Veterinary Science,* 28, 185–189.

WARDLEY, R. C., ANDRADE, C. DE M., BLACK, D. N., DE CASTRO PORTUGAL, F. L., ENJUANES, W. R., HESS, C., MEBUS, A., ORDAS, D., RUTILI, J., SANCHEZ VIZCAINO, J. D., VIRGARIO, J. D., WILKINSON, P. J., MOURA NUNES, J. F. & THOMSON, G. R., 1983. African swine fever virus—brief review. *Archives of Virology,* 76, 73–90.

WILKINSON, P. J. & WARDLEY, R. C., 1978. The replication of African swine fever virus in pig endothelial cells. *British Veterinary Journal,* 134, 280–282.

ZWIERZINA, W. D., SCHMALZL, F., KUNZ, F., DWORZAK, H., LINKER, D. & GEISSLER, 1983. Studies in a case of Bernard-Soulier Syndrome. *Acta Haematologica,* 69, 195–203.