Epidemiol. Infect. (1991), **106**, 179–188
Printed in Great Britain

Teratogenicity of the Palyam serogroup orbiviruses in the embryonated chicken egg model

T. WHISTLER AND R. SWANEPOEL

National Institute for Virology and Department of Virology, University of the Witwatersrand, Sandringham 2131, Republic of South Africa

(*Accepted 4 July 1990*)

SUMMARY

Embryonated chicken eggs were used as a model for assessing the teratogenic potential of several Palyam serogroup orbiviruses. Infection of 4-day-old embryonated chicken eggs via the yolk sac with eight of the viruses resulted in deaths or congenital deformities which included retarded development, arthrogryposis and reduced feathering. Statistical analysis showed that the viruses could be divided into three groups: those that caused death (Gweru virus isolates 866/77 and 1726/76; and Apies River virus), those that caused deaths only when large amounts of virus were inoculated (Gweru isolate AR11869 and Marondera virus) and those that caused death and deformities (Abadina, Kasba, Nyabira, Petevo and Vellore viruses). Differences in pathogenic potential were noted between isolates identified as the same serotype by serological tests.

INTRODUCTION

The Palyam serogroup of orbiviruses (family Reoviridae) comprises at least 15 viruses in six antigenic complexes [1–5]. They are widely distributed in Africa, Asia and Australia and are presumed to be vector-borne since they have commonly been isolated from haematophagous arthropods such as mosquitoes, midges and ticks. One hundred and twelve isolations of Palyam serogroup viruses made from cattle in Australia, were obtained from blood samples of sentinel animals in the absence of evidence of disease [6]. In contrast 14 isolations in Zimbabwe were obtained from aborted cattle fetuses and one from visceral organs of a cow which died during a Rift Valley fever epizootic [3, 7], while one isolation was made from a cow with mild fever in South Africa [5]. The apparent differences in findings on pathogenicity, may stem from differences in the specimens tested in the various studies. The viruses may produce relatively mild infection in cattle, but may be abortigenic in pregnant cattle. Chuzan virus has been implicated as the causal agent of the hydranencephaly-cerebellar hypoplasia (HCH) syndrome of calves in Japan on the basis of serological and epidemiological evidence [4].

Clearly, there is a need for pathogenicity trials with African and other Palyam serogroup viruses in cattle and other species of livestock. However, the use of a large number of suitably susceptible farm animals would be expensive and a more convenient laboratory animal model is required for the initial assessment of the teratogenic potential of the viruses. The chick embryo model has been used to

study the relative pathogenicity and teratogenicity of a wide range of viruses [8–10] and accordingly the use of this system for assessing Palyam serogroup viruses was investigated here.

MATERIALS AND METHODS

Viruses

Reference strains of known Palyam serogroup viruses were obtained from Dr R. B. Tesh of the Yale Arbovirus Research Unit, New Haven, Conn., USA. Southern African viruses were as described previously [3, 5], all with identical passage histories: viruses were passaged three times in suckling mice then passed thrice in Vero cells. Inocula were prepared by freeze-thawing Vero cells twice and clarifying the supernatant by centrifugation. Ten-fold serial dilutions of virus were prepared in PBS for titration and each virus dilution tested in Vero cells using a fluorescent focus assay [3].

Inoculation and observation of embryonated chicken eggs (ECE)

Four or five tenfold dilutions of each virus were prepared and 10 ECE inoculated per dilution. Attempts were made to use dilutions that would cover the range of 20–80% deaths of chick embryos. Four-day-old ECE were inoculated with 0·1 ml virus into the yolk sac and the control ECE were inoculated with PBS. The shells were sealed with molten wax and the ECE incubated at 37 °C in a humidified incubator. The ECE were examined by transillumination daily for 14 days and deaths recorded. Deaths which occurred up to 24 h post infection (p.i.) were considered non-specific and were not included in the results. The surviving ECE were killed by refrigeration on day 18 i.e. 14 days after inoculation, and the embryos examined for gross abnormalities.

Statistical methods

The data collected for each virus were divided into three levels of response: (*a*) dead (these may or may not have been deformed, but it is assumed they died due to virus infection), (*b*) alive but deformed (*c*) alive but not deformed. This response is termed polychotomous [11] and was analyzed with a logit dose–response curve using the computer program of Raymond [12]. In essence, the probability P_1 of death at dose x and the probability P_2 of being either dead or deformed at dose x are $1/(1+e^{-\alpha_1-\beta \log x})$ and $1/(1+e^{-\alpha_2-\beta \log x})$ respectively. The fact that the slopes are equal in the two cases ensures that the response curves never cross. The data were then analyzed by minimum logit χ^2 [13]. The 50% effective dose (ED_{50}) values for both death and death plus deformities (these are deformities seen in live embryos at day 14 post-inoculation only) are given by $-a_1/b$ and $-a_2/b$ respectively, where a_1, a_2 and b are the estimated values of α_1, α_2 and β, respectively. Ninety-five per cent confidence limits for these values were obtained by using Fieller's theorem [11].

RESULTS

Deaths due to virus infection

Deaths that occurred from day 2–14 after inoculation with the viruses studied are shown in Table 1. Increasing the virus inoculum from approximately 10^1 to 10^4

https://doi.org/10.1017/S0950268800056545 Published online by Cambridge University Press

fluorescent focus units (ffu)/egg resulted in an increase in deaths with all viruses. At inoculation levels of 10^3 ffu/egg all viruses, except Abadina 2582/78, Gweru isolate AR11869, Marondera and Vellore, caused 70–100% mortality; the majority of embryos that survived were abnormal (congenitally deformed), except for some infected with Gweru virus isolate 690/80 (Table 1).

Deformities due to virus infection

Of the 253 infected embryos that survived to day 14 post inoculation at dilutions where virus-induced pathology (either death or deformity) occurred, 60 (23·7%) showed congenital defects. Photographs of the defects observed are shown in Fig. 1. In all cases embryos showed severely retarded development and 16/60 had obvious arthrogryposis. The arthrogryposis varied from relatively mild to severe and was confined to legs and feet. Other deformities in infected embryos were reduced feathering, 'hairy' feathers and in some cases a large umbilical hernia containing abdominal viscera was evident. No deformities were seen in control embryos which were inoculated with diluent only.

Comparative pathogenicity of viruses tested

Non-specific deaths up to 14 days after inoculation occurred in the controls at a level of 7·1% (Table I) and a similar rate was assumed for the infected embryos. Because of the low level of these deaths and the comparative nature of the tests, no adjustments for non-specific deaths between days 2 and 14 p.i. were made in the analysis.

The data derived from a direct comparison of the pathogenicities of several Palyam serogroup viruses using the minimum logit χ^2 statistical method are shown in Fig. 2 in a format that indicates the relationships between pathogenicities. The horizontal axis $(-a_1/b)$ is the natural logarithm of the amount of infectious virus needed to cause 50% embryo deaths. The vertical axis $(a_2-a_1)/b$ is the natural logarithm of the difference between the two ED_{50} values and is the horizontal shift between the two dose response curves when dose is plotted on a logarithmic scale and represents the ED_{50} for live deformities. It has been demonstrated previously that viruses that cause few deformities have a very small value for $(a_2-a_1)/b$, whereas those that cause a large number of deformities have a larger value [8]. Fig. 2 shows that the larger the amount of infectious virus required for 50% deaths, the greater the number of deformities. Two exceptions to this were evident. Very large amounts of Marondera virus and the Gweru isolate AR11869 were required to cause 50% deaths and only 1/37 and 0/25 surviving embryos respectively, showed any abnormalities (Table 1). Vellore virus proved to be the most teratogenic of all the viruses examined and Gweru virus (866/77) the most lethal. The remainder of the viruses had intermediate values.

Comparison of the pathogenicity of six Gweru virus isolates with other Palyam serogroup viruses shows that mortality, rather than deformity, was the predominant effect of these viruses on the chick embryo (Fig. 2). Isolate 866/77 from Zimbabwe caused a large number of deaths with small doses of virus, for example, inoculation of approximately 3 ffu/egg resulted in 56% deaths (Table 1). Embryos infected with larger amounts of virus did not survive long enough to develop any defects. Isolates AR18422, AR11022 and 690/80 appeared to be the

Table 1. *Pathogenicity of Palyam serogroup virus isolates for chicken embryos infected on day 4*

Virus	Isolate	Inoculum titre (ffu/egg)	Number of embryos: Tested	Dead	Alive*: Deformed	Alive*: Normal	Death†: ED_{50}	Death†: 95% Confidence limits	Death plus deformities*†: ED_{50}	Death plus deformities*†: 95% Confidence limits
Abadina	IbAR 22388§	0·8	9	2	0	7	12	1, 88	6	1, 30
		8·0	9	6	0	3				
		80·0	7	3	2	2				
		800·0	9	7	1	1				
		8000·0	8	8	0	0				
	Ark 58§	0·3	6	1	0	5	747	23, 11×10^3	115	2, 1640
		2·7	10	4	0	6				
		27·0	9	2	3	4				
		270·0	10	6	0	4				
		2700·0	10	7	1	2				
		27000·0	9	8	0	1				
	2041/76	0·4	9	1	0	8	198	11, 7309	33	5, 186
		4·1	9	3	0	6				
		41·0	9	5	0	4				
		410·0	9	3	2	4				
		4100·0	10	8	2	0				
	2582/78	2·5	9	1	0	8	2214	78, 15×10^6	293	19, 1675
		25·0	9	3	0	6				
		250·0	8	4	1	3				
		2500·0	9	4	2	3				
		25000·0	9	7	2	0				
Gweru	1726/76	0·3	9	0	0	9	91	20, 500	51	13, 208
		3·0	8	1	0	7				
		30·0	8	4	1	3				
		300·0	9	5	0	4				
		3000·0	9	8	1	0				

866/77	0·3	10	0	0	10	7	1, 31	5	1, 22	
	2·8	9	5	0	4					
	28·0	8	6	1	1					
	280·0	9	8	0	1					
	2800·0	6	6	0	0					
690/80	0·4	10	0	0	10	1317	$99, 34 \times 10^4$	279	22, 6479	
	3·9	9	3	1	5					
	39·0	9	3	1	5					
	390·0	10	5	0	5					
	3900·0	10	7	0	3					
	39000·0	10	5	2	3					
AR 11022	0·2	6	1	0	5	178	$1, 38 \times 10^3$	49	7, 188	
	1·8	9	1	1	7					
	18·0	10	3	1	6					
	180·0	8	4	2	2					
	1800·0	9	8	1	0					
Gweru	AR11869	1·5	9	0	0	9	1547	$80, 12 \times 10^6$	1547	$80, 12 \times 10^6$
	15·0	9	4	0	5					
	150·0	10	5	0	5					
	1500·0	9	3	0	6					
AR18422	0·2	8	1	0	7	6	2, 22	2	1, 7	
	1·7	10	4	2	4					
	17·0	9	6	3	0					
	170·0	10	8	0	2					
	1700·0	10	10	0	0					
Apies River	0/4518	0·6	9	0	0	9	29	8, 102	18	5, 63
	6·3	9	4	1	4					
	63·0	10	4	1	5					
	630·0	10	10	0	0					
Kasba	G 15534§‖	1·5	9	2	3	4	58	7, 312	5	81×10^{-3}, 48
	15·09	4	1	4						
	150·010	4	1	5						
	1500·09	7	1	1						
	15000·010	10	0	0						

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Table 1. (*cont.*)

Virus	Isolate	Inoculum titre (ffu/egg)	Number of embryos				Death†		Death plus deformities*†	
			Tested	Dead	Alive* Deformed	Alive* Normal	ED_{50}	95% Confidence limits	ED_{50}	95% Confidence limits
Marondera	1070/78	0·8	9	0	0	9	6847	538, 10×10^6	6284	370, 16×10^7
		8·0	10	3	1	6				
		80·0	10	3	0	7				
		800·0	9	3	0	6				
		8000·0	9	4	0	5				
		80000·0	9	6	0	3				
Nyabira	4646/76	0·3	9	1	0	8	73	12, 912	26	6,129
		2·6	8	3	0	5				
		26·0	9	4	0	5				
		260·0	10	5	1	4				
		2600·0	10	8	2	0				
Petevo	ArTB 2032§	0·2	8	1	0	7	14	3, 105	5	1, 16
		2·0	10	3	1	6				
		20·0	10	5	1	4				
		200·0	10	8	2	0				
Vellore	68886§‖	7·6	8	0	0	8	3440	695, 28×10^3	140	17, 627
		76·0	7	1	4	2				
		760·0	9	4	3	2				
		7600·0	9	4	3	2				
		76000·0	8	7	1	0				
Control	—	—	154	11	0	143	—	—	—	—

* Deaths occurring from day 2 to 14 post inoculation.
† Embryos alive at day 18.
‡ Deformities in live embryos on day 18.
§ Reference strains of Palyam group viruses obtained from Dr Tesh.
‖ Viruses isolated in India, all others isolated in Africa.

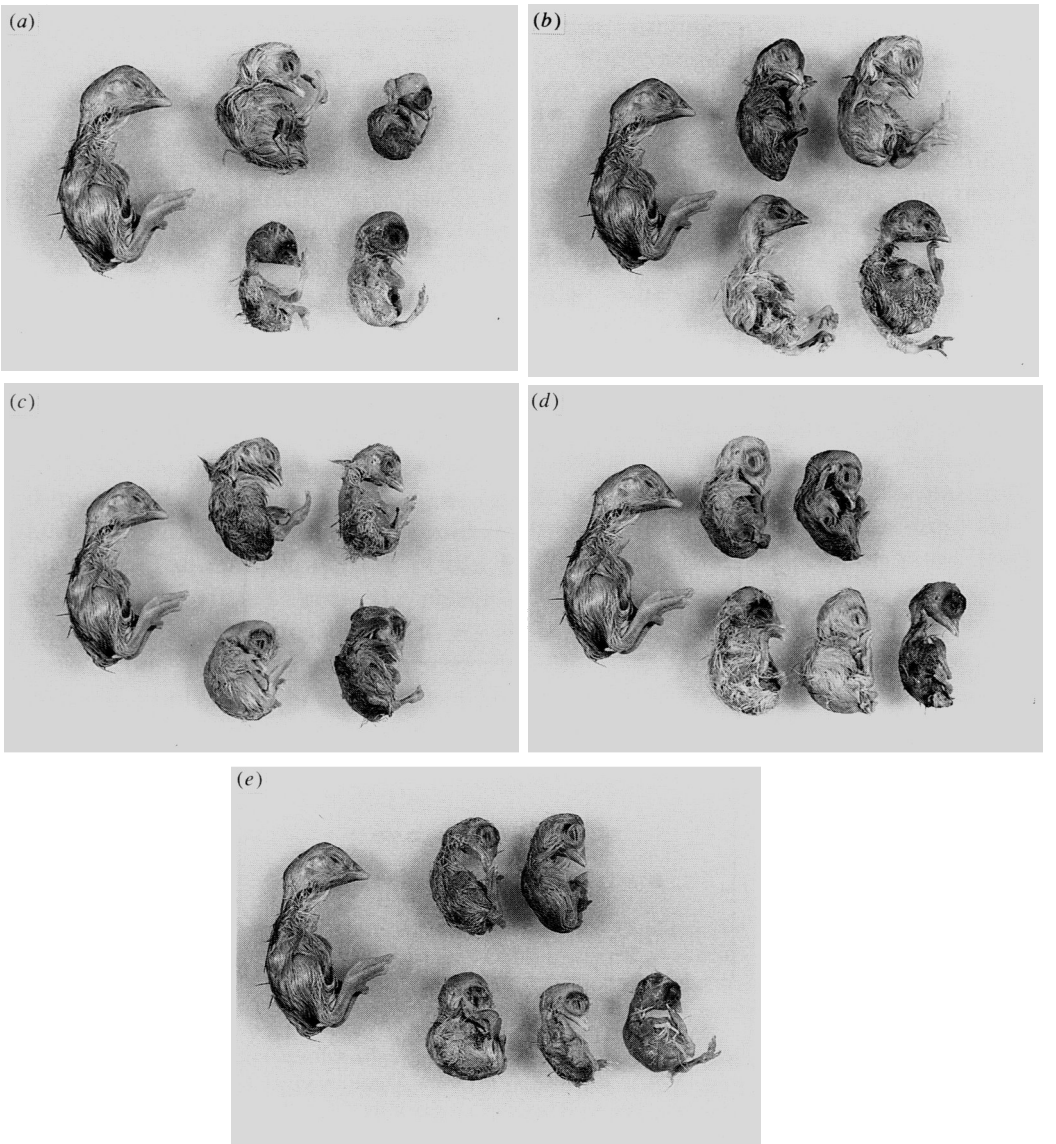

Fig. 1. Gross morphology of 18-day-old chicken embryos inoculated on day 4 with (*a*) Kasba virus, (*b*) Vellore virus, (*c*) Abadina virus – isolate 2041/76 (top row) and 2582/78 (bottom row), (*d*) Gweru virus – isolate 1726/76 (top row) and 690/80 (bottom row), (*e*) Apies River virus (top row) and Nyabira virus (bottom row). Control embryos on the left-hand side of each photograph are the same age. Deformities include retarded development, varying degrees of arthrogryposis and reduced feathering. Photographs all of the same magnification.

most teratogenic of the Gweru viruses, with AR18422 requiring the smallest dose of virus to produce deformities (Fig. 2). Isolate AR11869 was the least pathogenic/teratogenic of the Gweru isolates.

Four Abadina virus isolates were compared: the prototype strain (IbAR 22388), isolate Ark 58 from *Amblyomma variegatum* ticks collected in the Republic of Guinea [1] and two Zimbabwean isolations from aborted cattle fetuses (2041/76

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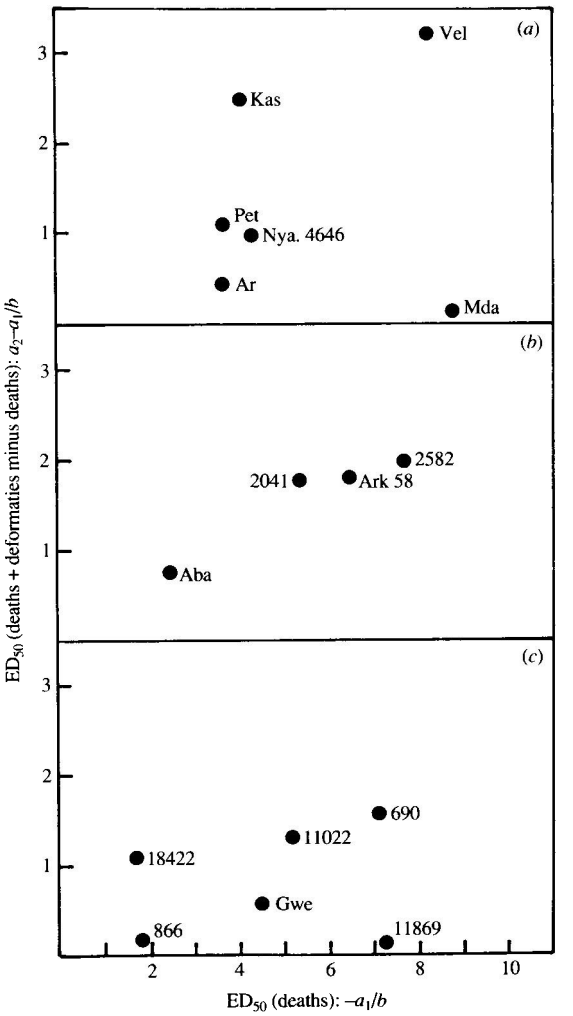

Fig. 2 Graphical representation of determined ED_{50} values for several Palyam group viruses: (*a*) isolates from Africa and India, (*b*) four Abadina virus isolates. (*c*) Gweru viruses isolated in southern Africa. The horizontal axis $(-a_1/b)$ is the logarithm of infectious virus needed to cause 50% embryonic deaths. The vertical axis (a_2-a_1/b) is the logarithm of the difference between infectious virus causing 50% total deaths plus deformities and that causing 50% deaths.

and 2582/78). Comparisons of ED_{50} values shows (Table 1) the prototype strain to be the most lethal and the remaining three isolates more teratogenic (Fig. 2).

DISCUSSION

The results of this preliminary investigation showed that the Palyam serogroup viruses studied in the chick embryo model, could be divided into three groups: those that caused death (Gweru isolates 866/77, 1726/76 and Apies River virus), those that caused deaths only when large doses of virus were inoculated (Gweru AR11869 and Marondera viruses) and viruses that caused deaths and deformities

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(Fig. 2). Considerable variation in virulence occurs among viruses of the serogroup, even between isolations of a single serotype. For example, Gweru isolate 866/77 has a lower ED_{50} than have isolates AR11022 and 690/80, whereas the latter two are possibly more teratogenic.

The viruses within the serogroup appear to cause a similar range of congenital deformities in the ECE, with severely retarded development being the most pronounced defect, followed by reduced feathering and arthrogryposis.

The chick embryo is considered to be a good experimental model to examine the teratogenicity of viruses and the mechanism whereby they cause congenital defects in livestock, because similarities in gross pathological findings between chick embryos and ruminants has been demonstrated for Akabane virus [10] and bluetongue virus [14]. The results of the present study indicate that the ECE can be used to define the relative pathogenicities of the Palyam serogroup viruses. The ECE has the advantage of being economical and shows a rapid response. However, it must not be overlooked that before infection of the fetus can occur in the natural host, the virus must first infect and replicate in the mother and then cross the placenta. The main drawback of the ECE model is that it does not assess the ability of the virus to cross the placenta.

Nevertheless, it is reasonable to conclude that the teratogenic effects observed were a consequence of infection with Palyam serogroup viruses as they were not seen in control embryos. It remains necessary to perform pathogenicity experiments in pregnant cattle and it would be interesting to see if the differences in lethal and teratogenic effects for the embryo observed between different viruses in the ECE, is reflected in cattle.

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