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Teratogenicity of Australian Simbu Serogroup and Some Other Bunyaviridae Viruses: The Embryonated Chicken Egg as a Model

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The use of embryonated chicken eggs as a model for assessing the teratogenic potential of animal viruses was investigated with 12 members of the Bunyaviridae family. Infection of 4-day-old embryonated chicken eggs via the yolk sac with 10 of the viruses resulted in deaths or congenital deformities that were similar to those observed in Akabane virus infections of fetal ruminants and included arthrogryposis, scoliosis, mandible defects, and retarded development. Statistical analysis showed that the viruses fell into three main groupings, namely, those that caused both death and deformities (Akabane, Aino, Tinaroo, and Belmont viruses), those that mainly caused death (Peaton, Thimiri, and Facey's Paddock viruses), and those that required very high doses to cause either death or deformities (Douglas and CSIR0296 viruses). In addition, two viruses (Kowanyama and Mapputta viruses) caused neither death nor deformities. A difference in the pathogenic potential between two Akabane isolates (B8935 and CSIR016) in the embryonated chicken egg model was found to correlate with differences previously observed in experimentally infected sheep; Akabane CSIR016 was the more pathogenic. It is concluded that the embryonated chicken egg model should also be of value in assessing the teratogenic potential of other Bunyaviridae and attenuated vaccine viruses, although it does not assess the ability of the virus to cross the placenta.

Akabane (AKA) virus (Simbu serogroup), Rift Valley fever virus (phlebotomus fever serogroup), and Nairobi sheep disease virus (Nairobi sheep disease serogroup) are three Bunyaviridae members known to be associated with fetal death, abortions, and developmental defects in domestic animals (20). In Australia, several Bunyaviridae members have been isolated, some of which are Simbu serogroup members. Several of these are known to infect cattle, sheep, humans, and other species (Table 1). Apart from AKA virus, little is known of the potential of these viruses to cause congenital deformities. Aino virus has been implicated on serological evidence as the cause of congenital deformities in calves in Japan (17) and Australia (4).

Chicken embryos have been used extensively as an animal model for the study of teratogenic mechanisms of a number of animal viruses (3). AKA virus is known to cause death and deformities when inoculated into the yolk sac of 4- or 6-day-old embryonated chicken eggs (ECE) (14, 16). We have explored the potential of the ECE as a possible model for assessing the teratogenic potential of selected Australian Bunyaviridae members (Table 1), particularly those in the Simbu serogroup. Such a model would be of economic value by reducing the number of large animals needed to assess viruses.

This study shows that ECE are susceptible to infection with the majority of Bunyaviridae viruses examined, the resulting developmental defects being similar to those observed in natural AKA virus infections in cattle and sheep.

MATERIALS AND METHODS

Viruses. The original source and major vertebrate hosts of all the virus isolates used in this study are listed in Table 1, together with abbreviations of names used in this paper. All of the Simbu serogroup viruses except FP Ch16129 were provided by Long Pocket Laboratories, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Queensland, Australia. The two strains of AKA virus (B8935 and CSIR016) used differ in their pathogenicity for sheep (6, 19). FP Ch16129 was provided by the Queensland Institute of Medical Research, Queensland. The other Bunyaviridae members tested, BEL, KOW, and MAP viruses, were provided by the Department of Microbiology, Monash University, Victoria, Australia. Infectious virus was assayed by a plaque assay with Vero cells (15).

Inoculation of ECE. Four-day-old ECE were inoculated via the yolk sac (1) with 0.2 ml of virus suspension or with virus diluent only (controls). The shell was then sealed with an acetate-based cement (Aeroflyte, Sydney, Australia), and the eggs were incubated at 37°C. Deaths occurring up to 24 h post-inoculation were considered nonspecific and are not included in the results.

Virus replication in ECE. To demonstrate that virus replication occurring in ECE was the possible cause of death and deformities, virus replication of AKA virus was assessed. A total of 160 embryos were inoculated with 1.1×10^3 PFU of AKA CSIRO16 virus; 40 controls were included. From day 0 to day 14, two infected and two control live embryos were harvested. Each group was pooled, ground, and clarified, and the supernatant was stored at −80°C until assayed (14a). Infected embryos that died after day 1 were treated similarly.

Examination of embryos for developmental defects. Eggs were inoculated with a range of viruses and inoculum levels (Tables 2 and 3) and candled daily for 14 days to check viability. The surviving embryos were then examined for gross abnormalities, fixed in neutral buffered Formalin, and stained with alizarin red to demonstrate skeletal defects (23).

Statistical methods—ED_{50} estimation. Four or five dilutions of each virus were prepared and 20 ECE were inoculated per dilution (Table 3). Attempts were made to use dilutions that would cover the range of 20 to 80% deaths. The data collected for each virus were divided into three

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TABLE 1. Bunyaviridae members used in ECE inoculation

Virus	Serogroup	Abbreviation	Original source	Major vertebrate host(s)	Reference
Akabane B8935	Simbu	AKA B8935	*Culicoides brevitarsis*	Cattle, sheep, goats	10
Akabane CSIRO16	Simbu	AKA CSIRO16	*C. brevitarsis*	Cattle, sheep, goats	25
Aino B7974[a]	Simbu	AIN	*C. brevitarsis*	Cattle, sheep, buffalo	5, 10
Peaton CSIRO110	Simbu	PEA	*C. brevitarsis*	Cattle, sheep, horses, buffalo	26
Tinaroo CSIRO153	Simbu	TIN	*C. brevitarsis*	Cattle, sheep	24
Douglas CSIRO150	Simbu	DOU	Cattle blood	Cattle, buffalo	24
Unnamed (CSIRO296)[b]	Simbu	CSIRO296	Cattle blood	Cattle, buffalo, sheep	St. George and Cybinski, personal communication
Facey's Paddock Ch16129	Simbu	FP	*Culex annulirostris*	Not known	8
Thimiri CSIRO1	Simbu	THI	*Culicoides histrio*	Birds	27
Belmont R8659	Ungrouped	BEL	*Culex annulirostris*	Wallabies, kangaroos, cattle	7, 10, 18
Kowanyama MRM1178	Ungrouped	KOW	*Anopheles annulipes* and *Anopheles amictus amictus*	Kangaroos, wallabies, humans	7, 11
Mapputta MRM186	Mapputta	MAP	*Anopheles meraukensis*	Kangaroos, wallabies	7, 9

[a] Originally called Samford virus.
[b] This virus isolate has not yet been fully compared with other Simbu serogroup members and has not yet been named.

TABLE 2. Death and deformity rates in ECE infected with Bunyaviridae viruses

Virus	Isolate	Inoculum titer (PFU/egg)	Deaths[a]	Deformities[b]	Virus	Isolate	Inoculum titer (PFU/egg)	Deaths[a]	Deformities[b]
Akabane	B8935	3.0×10^5	4/5	1/1			9.0×10^3	5/5	0/0
		3.0×10^4	5/5	0/0			9.0×10^2	5/5	0/0
		3.0×10^3	4/6	0/2			9.0×10^1	3/6	0/3
		3.0×10^2	3/6	2/3			9.0×10^0	2/6	0/4
		3.0×10^1	0/6	0/6			9.0×10^{-1}	1/5	0/4
		3.0×10^0	0/6	0/6					
					Thimiri	CSIRO1	5.8×10^4	4/4	0/0
Akabane	CSIRO16	8.0×10^4	5/6	1/1			5.8×10^3	5/5	0/0
		8.0×10^3	6/6	2/2			5.8×10^2	4/4	0/0
		8.0×10^2	4/6	2/2			5.8×10^1	4/4	0/0
		8.0×10^1	4/6	2/2			5.8×10^0	4/6	0/2
		8.0×10^0	2/6	1/4			5.8×10^{-1}	3/3	0/0
		8.0×10^{-1}	1/6	0/5					
					Facey's Paddock	Ch16129	2.7×10^6	6/6	0/0
Aino	B7974	1.6×10^5	5/6	1/1			2.7×10^5	6/6	0/0
		1.6×10^4	4/6	1/2			2.7×10^4	6/6	0/0
		1.6×10^3	3/6	2/3			2.7×10^3	6/6	0/0
		1.6×10^2	1/6	1/5			2.7×10^2	5/6	1/1
		1.6×10^1	0/6	0/6			2.7×10^1	4/6	2/2
		1.6×10^0	1/6	0/5					
Tinaroo	CSIRO153	1.3×10^5	6/6	0/0	Belmont	R8659	6.8×10^4	5/6	1/1
		1.3×10^4	5/6	1/1			6.8×10^3	2/6	3/4
		1.3×10^3	3/6	3/3			6.8×10^2	3/6	2/3
		1.3×10^2	1/6	3/5			6.8×10^1	2/6	1/4
		1.3×10^1	0/6	0/6			6.8×10^0	1/6	0/5
		1.3×10^0	1/6	0/5			6.8×10^{-1}	2/6	0/4
Douglas	CSIRO150	1.4×10^6	3/5	2/2	Kowanyama	MRM1178	8.0×10^5	1/6	0/5
		1.4×10^5	4/6	1/2			8.0×10^4	0/6	0/6
		1.4×10^4	2/5	0/3			8.0×10^3	0/5	0/5
		1.4×10^3	1/5	0/4			8.0×10^2	0/6	0/6
		1.4×10^2	1/6	0/5			8.0×10^1	0/5	0/5
		1.4×10^1	0/6	0/6			8.0×10^0	0/6	0/6
Unnamed	CSIRO296	4.0×10^5	5/6	1/1	Mapputta	MRM186	2.3×10^5	0/5	0/5
		4.0×10^4	4/6	1/2			2.3×10^4	1/6	0/5
		4.0×10^3	4/6	1/2			2.3×10^3	0/6	0/6
		4.0×10^2	1/6	1/5			2.3×10^2	1/6	0/5
		4.0×10^1	1/6	0/5			2.3×10^1	0/5	0/5
		4.0×10^0	1/6	0/5			2.3×10^0	0/6	0/6
Peaton	CSIRO110	9.0×10^4	6/6	0/0	Control			11/103	0/92

[a] Number affected/number inoculated on day 4.
[b] Number affected/number alive at day 18. The deformities do not include those seen in both infected and control groups (see text).

TABLE 3. Pathogenicity of Australian Bunyaviridae viruses for chicken embryos infected on day 4

Virus	Isolate	Inoculum titer (PFU/egg)	No. of embryos:				Death		Death plus deformities[a]	
			Tested	Dead	Alive					
					Deformed	Normal	ED_{50}[b]	95% confidence limits	ED_{50}[b]	95% confidence limits
Akabane	CSIRO16	4.5	19	4	0	15	26,800	4,410, 280,000	249	28.6, 1,470
		190	18	2	5	11				
		10,000	18	9	7	2				
		1,180,000	20	14	6	0				
Akabane	B8935	100	19	11	4	4	74.0	0.6, 278	3.2	0, 37.3
		320	15	11	2	2				
		1,597	19	12	6	1				
		8,500	18	17	1	0				
		4,000,000	16	16	0	0				
Aino	B7974	18.8	16	4	1	11	723	206, 2,590	30.5	3.5, 113
		232.5	19	8	8	3				
		3,468	17	10	6	1				
		51,680	19	17	2	0				
Peaton	CSIRO110	16.5	17	6	0	11	23.5	14, 31.6	22.5	12.9, 30.3
		32	20	14	0	6				
		52	17	12	1	4				
		110	17	17	0	0				
Tinaroo	CSIRO153	3	19	1	0	18	41,000	10,200, 244,000	465	116, 1,918
		20	19	2	0	17				
		2,400	15	4	9	2				
		88,000	19	9	10	0				
Douglas	CSIRO150	21,000	19	2	1	16	286×10^4	66×10^4, $8{,}894 \times 10^4$	28.8×10^4	7.9×10^4, 165×10^4
		30,715	20	3	3	14				
		400,713	19	3	3	13				
		1,700,000	20	11	6	3				
Unnamed	CSIRO296	30	17	2	0	15	39.6×10^4	5.7×10^4, $41{,}190 \times 10^4$	24.2×10^4	3.8×10^4, $16{,}020 \times 10^4$
		300	18	2	0	16				
		3,000	16	0	2	14				
		30,000	17	9	1	7				
		650,000	19	18	0	1				
Facey's Paddock	Ch16129	3	17	3	1	13	32.7	18.7, 65.5	22.8	11.9, 40.8
		18	19	4	1	14				
		30	19	8	2	9				
		90	20	17	1	2				
Thimiri	CSIRO1	0.5	16	2	0	14	118	40.4, 918	101	34.4, 682
		17	18	2	0	16				
		49	17	8	0	9				
		349	18	12	1	5				
Belmont	R8659	24	16	1	3	12	577	287, 1,504	108	37.3, 227
		375	18	4	8	6				
		636	16	11	3	0				
		100,000	18	18	0	0				
Control			130	10	0	120				

[a] Deformities in live embryos on day 18.
[b] The ED_{50} was determined by using maximum likelihood.

levels of response—dead (these may or may not be deformed, but it is assumed they died due to virus infection), alive but deformed, and alive but not deformed. Such a response is termed polytomous (12) and was analyzed with a logit dose-response curve in which 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 β are equal in the two cases ensures that the response curves never cross. The data were then analyzed by both minimum logit χ^2 (13) and maximum likelihood (12). 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. Confidence intervals for these were obtained by using Fieller's theorem (12).

RESULTS

Virus replication in ECE. AKA CSIRO16 virus (Fig. 1A) replicated extensively in the host; high titers were recovered from live embryos on days 3, 4, and 6, with the peak occurring at day 6. Virus was also recovered from pooled dead embryos on 6 of the 8 days (Fig. 1B). On days 4 and 13, only one infected embryo died, and virus was not recovered. The highest titers from dead embryos occurred on days 6, 8, 9, and 11 post-inoculation, with the peak at day 8.

Death due to virus infection. Deaths that occurred from day 2 to day 14 after inoculation with the 12 viruses studied are shown in Table 2. Increasing the virus inoculum from approximately 10^1 PFU to 10^5 to 10^6 PFU per egg resulted in an increase in deaths with all viruses except MAP and KOW. The deaths with these last two appeared to be nonspecific, and no more than a single death (17%) occurred at any dilution that was comparable to the control group mortality rate (11%); further comments of deaths and deformities exclude these viruses. At inoculation levels of $\geq 10^4$ PFU per egg all viruses, except DOU, caused 67 to 100% mortality; any embryos that survived were usually abnormal (congenitally deformed), except for those infected with AIN, DOU, and CSIRO296 viruses (Table 2).

Deformities due to virus infection. Many of the infected embryos that survived to day 14, particularly those inoculated with 10^4 to 10^6 PFU of virus, showed congenital defects. The most obvious were arthrogryposis and severely retarded development (Fig. 2 and 3). The arthrogryposis varied from relatively mild to very severe and was confined to the legs and feet. Other deformities seen in infected embryos were scoliosis, beak and wing deformities, reduced feathering, lack of abdominal wall, and abdominal swelling (Fig. 2). Some deformities, seen in both control and infected embryos, are not listed in Tables 2 and 3. Of 1,842 embryos examined, in these and other experiments (data not shown), 15 (1 control, 14 infected) were observed with hydranencephalocoele, 6 (2 control, 4 infected) had a misaligned beak and only one eye, and 13 (5 controls, 8 infected) had a protruding eye. In the majority of infected embryos, the yolk sac and abdominal organs and tissues were stained green with biliverdin; this was not seen in any control embryo.

Comparative pathogenicity of viruses tested. A direct comparison of the pathogenicity of the 10 viruses that caused deaths and deformities is shown in Table 3. Nonspecific deaths occurred in the controls at a level of 7.7%, 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 were made in the analyses.

Both statistical methods (minimum logit χ^2 and maximum likelihood) produced similar results. However, for subsequent analyses, we concentrated on maximum likelihood results since the confidence intervals for the parameters were narrower. The ED_{50} values for $-a_1/b$ (dead) and $-a_2/b$ (dead plus live deformed) are given for each virus in Table 3. The goodness of fit was checked for each virus by using a χ^2 goodness-of-fit test for maximum likelihood and the χ^2 value was obtained from the minimum logit χ^2 method. The data are presented for these viruses based on their ED_{50} values in a format that indicates relationships based on pathogenicities (Fig. 4). The horizontal axis ($-a_1/b$) is the log of the amount of infectious virus needed to cause 50% embryo deaths. The vertical axis ($a_2 - a_1/b$ is the difference between the logarithms of the two ED_{50} values and is the horizontal shift between the two dose-response curves when dose is plotted

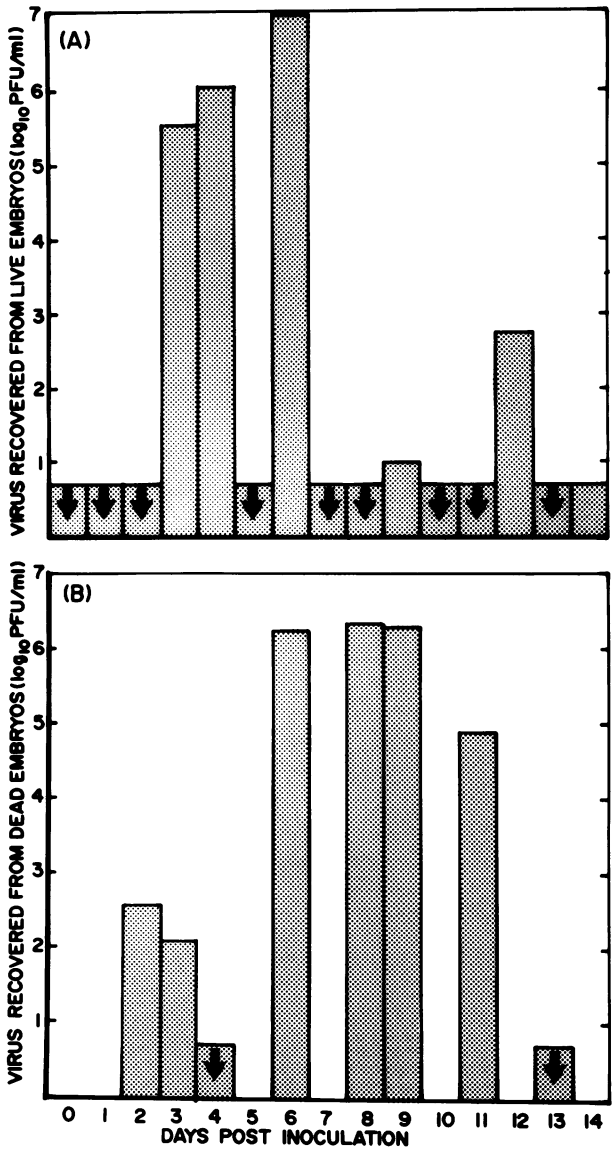

FIG. 1. Virus replication in 4-day-old ECEs inoculated with 1.1×10^3 PFU of AKA CSIRO16 virus. Samples were assayed daily from (A) two live embryos and (B) embryos that died on the given day. Days marked by a downward pointing arrow indicate that titers were indeterminable by the plaque assay technique, i.e., <5 PFU/ml.

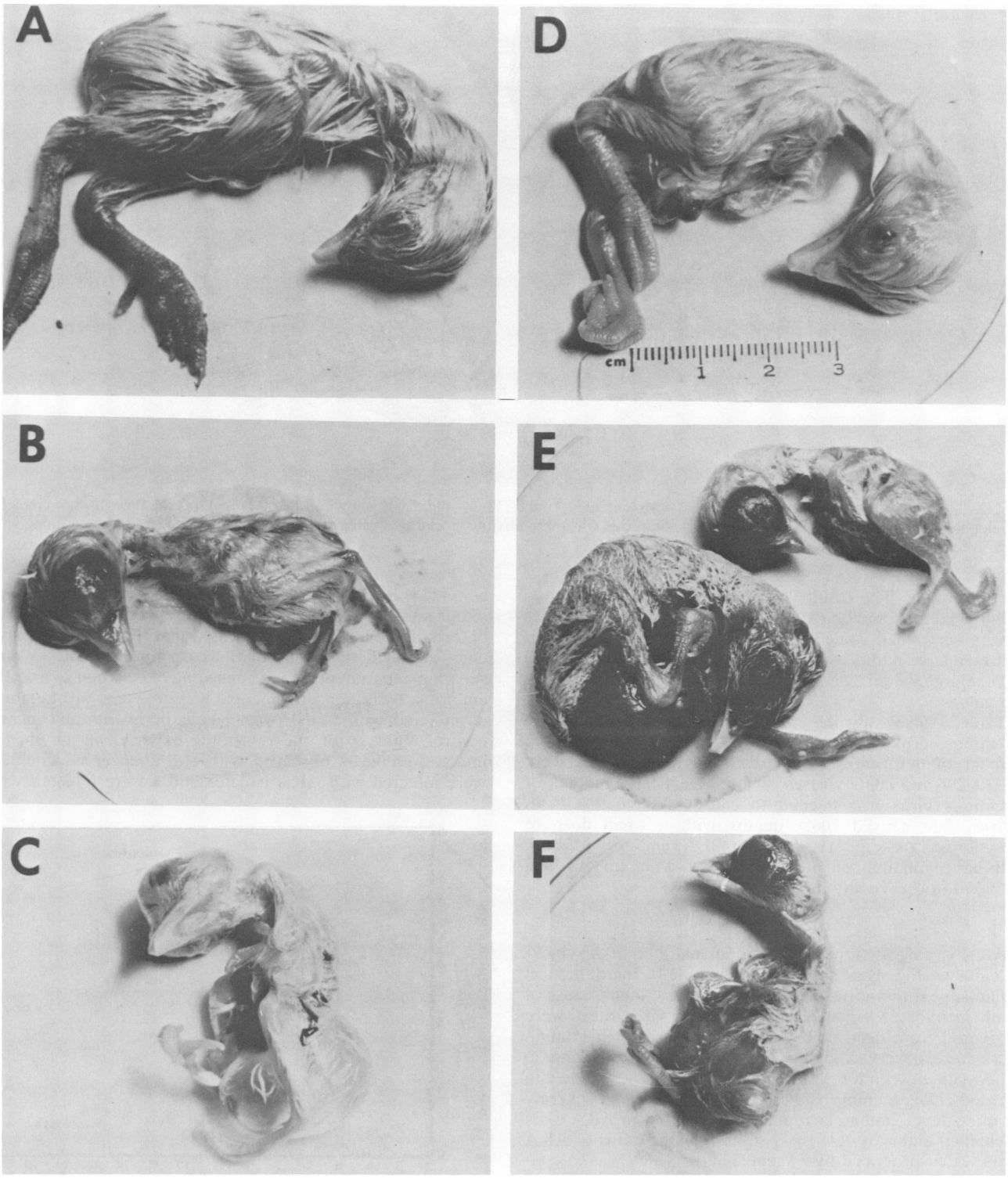

FIG. 2. Gross morphology of 18-day-old SPF chicken embryos inoculated with virus on day 4. (A) Uninfected control; (B,C) infected with AKA CSIRO16; (D) infected with AIN; (E) infected with TIN; (F) infected with DOU. Deformities include arthrogryposis of legs or feet (B, C, D, E, and F), grossly retarded development (B, C, E, and F), abdominal swelling (C, E, and F), and mandible defects (E and F). All photos are at the same magnification.

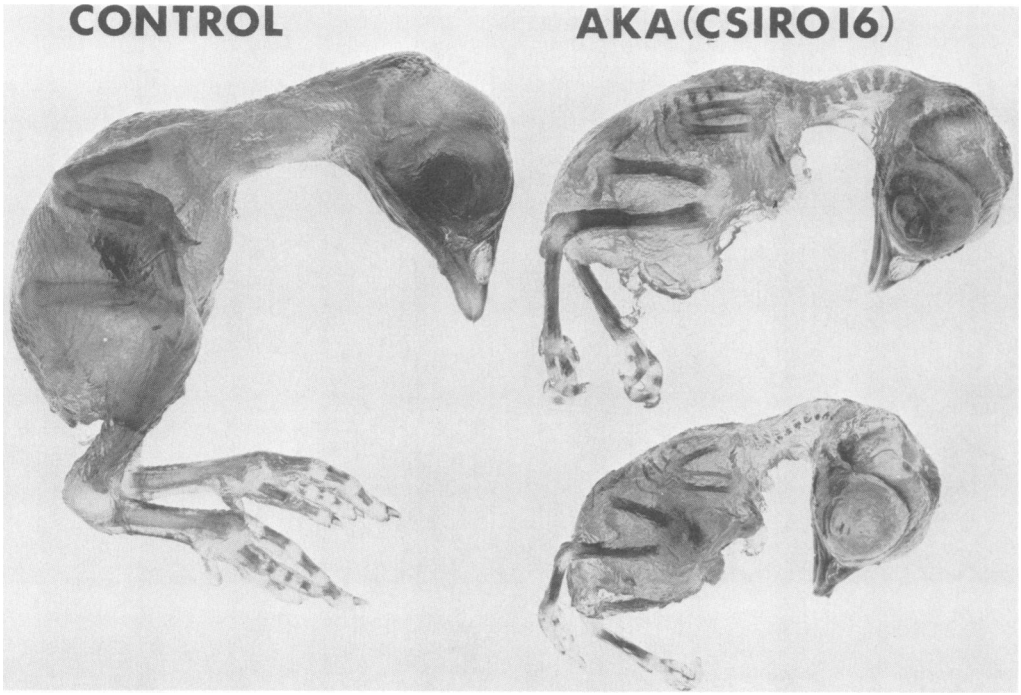

FIG. 3. Control (left) and AKA CSIRO16 virus-infected (right) 18-day-old ECE showing development of calcified tissue. Skeletal development was retarded in infected embryos. Scoliosis was more obvious in clarified embryos. Specimens were cleared after staining with alizarin red.

on a log scale. The results clearly show that those viruses (PEA, THI, FP, and CSIRO296) that caused few deformities have a very small value for $(a_2 - a_1)/b$, whereas those that caused a large number of deformities (AKA CSIRO16, TIN) have a large value. The remainder of viruses had intermediate values.

Table 3 also indicates that, the larger the amount of infectious virus required for 50% deaths, the greater the number of deformities. The two exceptions to this were CSIRO296 and DOU viruses, where a very large amount of infectious virus was needed to cause 50% deaths. With viruses that caused 100% deaths with a small dose of infectious virus, e.g., 110 PFU for PEA, embryos infected with larger amounts of infectious virus did not survive long enough to observe any defects.

DISCUSSION

AKA virus growth was clearly demonstrated in the chicken embryo (Fig. 1) as shown previously (14). In addition, it would appear that virus replication was the primary cause of death in infected embryos, as maximum virus titers were recovered from dead embryos a few days after maximum titers were observed in live embryos. The most common abnormalities seen for AKA and the other viruses studied in affected chicken embryos were arthrogryposis and severely retarded development, as reported previously for AKA virus in chicken embryos (14, 16). Neither of the strains of AKA virus tested produced hydranencephaly, which was in contrast to results reported with the OBE-1 strain inoculated into 6-day-old embryos via the yolk sac (14). This difference may have been due to the stage of embryonic development at inoculation or to strain differences between viruses. However, other deformities noted previously with AKA virus strains OBE-1 and B8935 (hydranencephalocoele, defective cranium, protruding eye, and abnormal occlusion of the beak) (14, 16) were seen in both infected and control groups in our studies. The relative incident rates of the latter three defects (data not shown) suggested that these were nonspecific, although this was not the case for the first. In nine separate experiments, each consisting of between 26 and 236 embryos, hydranencephalocoele was observed in 14 embryos infected with virus from eight experiments and in one control embryo for the remaining experiment. Of the 14 infected embryos observed with hydranencephalocoele, 5 were infected with AKA isolates and 4 were infected with

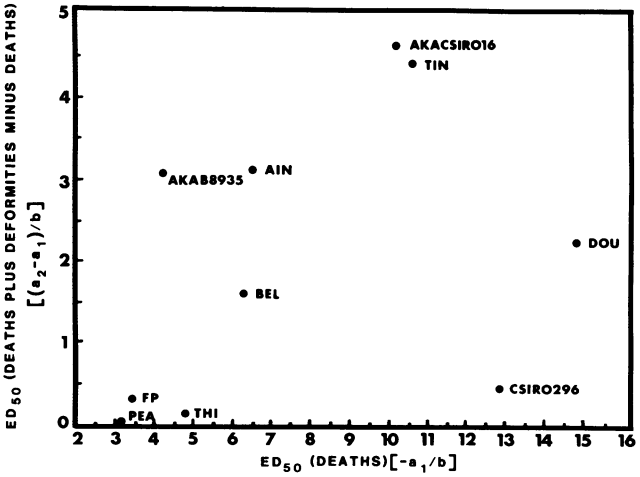

FIG. 4. Graphical representation of determined ED_{50} values for the 10 Bunyaviridae members. The horizontal axis $(-a_1/b)$ is the logarithm of infectious virus needed to cause 50% embryonic deaths. The vertical axis $(a_2 - a_1)/b$ is the difference between the logarithms of infectious virus causing 50% total deaths plus deformities and that causing 50% deaths.

DOU. These incidence rates suggest that infection with certain viruses may increase the occurrence of this defect.

As with AKA virus infection of sheep (21) and ECE, other viruses in this study are also able to replicate in ECE, causing limited cellular damage at replication sites such that the host survives despite the developmental deformities. Considerable variability in virulence among viruses was also demonstrated by the fact that some caused mainly deaths with a low titer of virus (e.g., PEA virus caused 100% deaths with 110 PFU per egg), whereas others required a high virus titer to cause either death or deformities (e.g., AKA CSIRO16 virus caused 70% deaths and 30% deformities after inoculation of 1,180,000 PFU per egg). From these results, there is a need to investigate the effects that these viruses may have in their natural hosts, which include cattle and sheep.

Results from this study indicate that AKA (CSIRO16 and B8935) virus strains cause a similar range of congenital deformities in ECE. However AKA B8935 virus has a lower ED_{50} than has AKA CSIRO16, whereas the latter is the more teratogenic. This is consistent with the finding that AKA CSIRO16 is more teratogenic than is AKA B8935 in experimentally infected pregnant sheep (6, 19).

For the Bunyaviridae members studied in the ECE model, viruses could be divided into four distinct groupings: those that cause death only (PEA, FP, and THI viruses), those that cause death and deformities (AKA CSIRO16, AKA B8935, TIN, AIN, and BEL viruses), those that cause death or deformities only when very large amounts of virus are inoculated (CSIRO296 and DOU viruses), and those that do not cause either death or deformities (KOW and MAP viruses). With the last group it is possible that replication may not have occurred in ECE, whereas the possibility of the first group being teratogenic under different experimental conditions also cannot be excluded.

In assessing the ECE model system for determination of the pathogenicity of Bunyaviridae viruses, it must not be overlooked that before infection of the fetus can occur in its natural host (ruminants for the majority of viruses tested), the virus must first infect and replicate in the dam and subsequently cross the placenta (21). In the ECE we can only define relative pathogenicities once the fetus becomes infected. To define which viruses are able to cross the placenta and infect the fetus we are presently experimentally infecting pregnant sheep with the same virus inocula used in these ECE experiments.

An in vitro technique for determining the factors involved in the replication of potential teratogenic viruses in placentomal tissues would greatly aid in reducing requirements for in vivo animal studies, and this proposition is currently under examination. The combined evidence of the ability of a virus to replicate in placentomal tissue, plus that of its teratogenicity in the ECE model, will then perhaps provide an excellent and cheap alternative to the use of natural ruminant hosts for assessing the pathogenic potential of Bunyaviridae viruses. It could be expected that this approach to the assessment of teratogenicity might also be applicable to other important Bunyaviridae viruses, such as Rift Valley fever and other viruses infecting humans, and to attenuated vaccines (2, 20, 22).

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