THE PROPAGATION OF A VIRULENT GOAT PLEUROPNEUMONIA-LIKE ORGANISM IN THE CHICK EMBRYO

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Although the organisms of the pleuropneumonia group isolated from man (Paine *et al.*, 1950; Keller and Morton, 1954) and from several species of animals (Tang *et al.*, 1936; Delaplane, 1948; Hoyt *et al.*, 1951; Sheriff and Piercy, 1952) have been cultivated in the chick embryo, the goat pleuropneumonia-like organism (PPLO) recently isolated from goats and reported by us, to our knowledge, has not been studied in this medium.

It is the purpose of this paper to describe the chick embryo propagation of a virulent goat PPLO isolated for the first time on this continent (Cordy *et al.*, 1955, *in press*).

MATERIALS AND METHODS

Chick embryos. Fertile eggs from an apparently disease free white leghorn flock managed by this department were used throughout the experiment. All embryo inoculations (chorioallantois, allantoic cavity, and yolk sac) were made according to the method of Cunningham (1952). Six to seven day old embryos were used for the yolk sac route of inoculation. For the chorioallantois (CA) and allantoic cavity (AC) routes, embryos that had received a preliminary incubation of 10, 13, and 15 days were used.

PPLO culture. A 48 hour, 5th passage culture of PPLO originally isolated from the joint fluid of a goat was the inoculum for the first serial passage. Inocula for the subsequent embryo passages consisted of infected yolk material from previous passages.

Counting procedures. The titer of the infected embryo material and the numbers for the *in vitro* growth study were determined according to the method of Adler (unpublished data). Briefly, the material to be titrated was diluted in veal infusion broth, pH 7.2 in tenfold dilutions. To determine whether growth occurred in each of the dilutions, 0.1 ml quantities were transferred into each of five tubes of PPLO broth (Difco) containing 20 per cent horse serum and 250 units of penicillin per ml of medium. Usually, four tenfold dilutions were found to be adequate. After incubating at 37 C for 3 days, a loopful from each broth tube was streaked to PPLO agar (Difco) containing 20 per cent horse serum. The plates were incubated for three days and then examined with a dissecting microscope at a magnification of 36 diameters for the presence or absence of growth for the respective dilutions tested. The most significant number was then obtained by referring to the table of significant numbers as given by Buchanan and Fulmer (1928).

Infected yolk from the third serial (g-3) passage was used to calculate the LD_{50}. Five embryos were inoculated per dilution via the yolk sac route. From the death pattern obtained, the LD_{50} was calculated by the method of Reed and Muench (1938).

RESULTS

Preliminary embryo death pattern. All embryos were dead within 72 hours with the first embryo passage when inoculated via the CA and yolk sac routes. Direct culture of fluids from individual embryos yielded confluent growth of PPLO and no contaminants. All embryos showed cutaneous hemorrhages. Smears of yolk sac tissue from embryos inoculated by the yolk sac route, when stained by the acid-hydrolysis Giemsa method as described by Cordy *et al.* (1955, *in press*), showed numerous tiny coccoid bodies (figure 1). The embryos inoculated via the CA route exhibited a slight thickening of the membranes. The allantoic and amniotic fluids of dead embryos were clear. Similar observations were made with subsequent passages.

Although a majority of the embryos of the g-3 passage were dead at 72 hours, there were 2 early deaths at 24 hours. A loopful of a tenfold dilution of the composited yolks from these embryos streaked on 20 per cent serum agar yielded heavy growth of PPLO. The pooled yolk of the

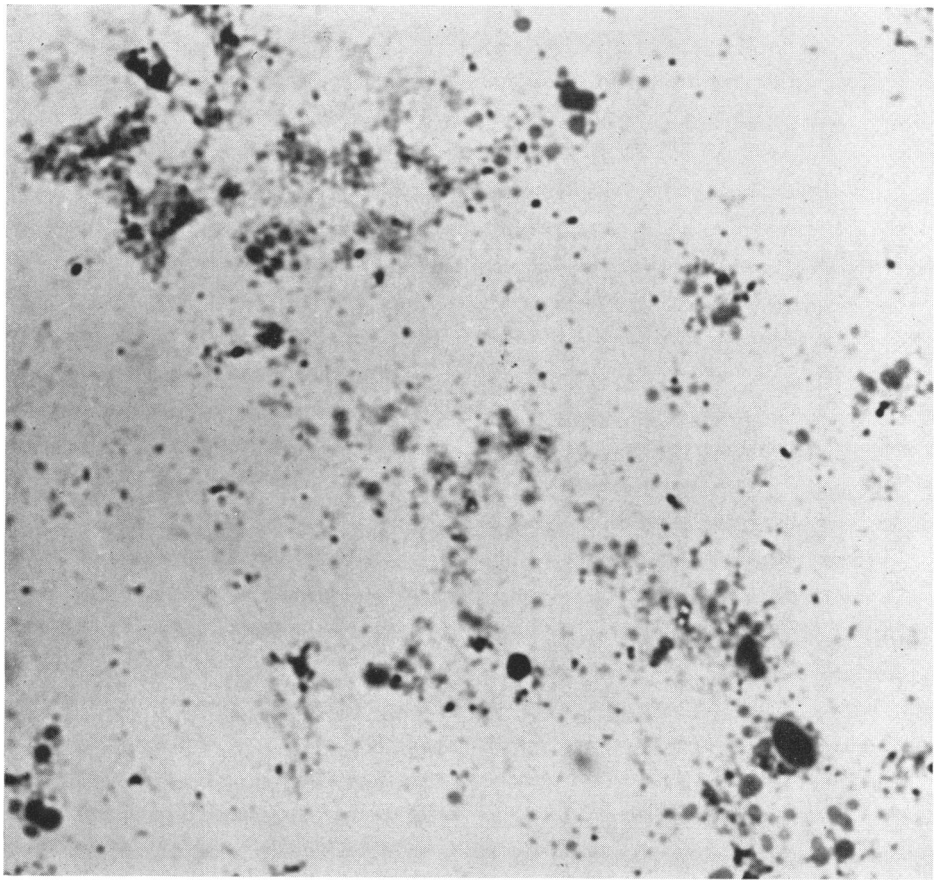

Figure 1. Yolk sac smear of a pleuropneumonia-like organism stained by acid hydrolysis Giemsa. Photomicrograph × 2,250.

g-3 passage was dispensed into screw cap vials, shell frozen, and placed in the dry ice chamber at −60 C, and used for the subsequent LD_{50} calculation and other studies.

Calculation of the LD_{50}. Table 1 gives the death pattern obtained for the calculation of the LD_{50}, which was found to be $10^{8.31}$. Yolk from all embryos which died during this trial yielded positive cultures on PPLO agar.

Inoculation of 7 and 10 day old embryos. Table 2 shows the death pattern obtained when yolk of g-3 was inoculated by the CA and AC routes of 10 day old embryos. The death patterns and embryo lesions were similar to the 13 day old embryo inoculations and will be described later. The chorioallantois of embryos inoculated by this route were pooled, gently washed in saline, and ground in alundum. The yolk and allantoic fluids of embryos which were inoculated by the AC routes were pooled, and counts were made of the respective material (table 3). Four 7 day old embryos were inoculated via the yolk sac route with 10^6 LD_{50} of the g-3 yolk. All embryos were dead within 72 hours. The yolk, allantoic fluid,

TABLE 1

Death pattern obtained for the LD_{50} calculation

	Dilution of Infected Yolk*						
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
No. of embryos dead out of five.....	5	5	5	5	3	1	0
Average day of death.	2.6	3.4	3.6	4.0	8.6	12.1	14

* Seven day old embryos were inoculated via the yolk sac route with infected yolk of the g-3 passage. The average day of death: the number of deaths occurring on each day was multiplied by that day. The sum total of these values divided by the total number of embryos used was calculated as the value for the average day of death.

TABLE 2

Death patterns of embryos of various age group inoculated by various routes with 10^6 LD_{50} of infected yolk

Day of Death Postinoculation	Route of Inoculation			
	Chorioallantois		Allantoic cavity	
	Trial 1	Trial 2	Trial 1	Trial 2
10 day old embryos				
1	1	ND	0	2
2	0		1	0
3	8		1	4
4	1		2	1
5	—		—	2
Add.....	2.9		3.3	3.1
13 day old embryos				
1	0	0	2	2
2	0	0	0	0
3	7	13	1	3
4	5	2	3	7
5	1	—	1	2
6	—	—	1	2
7	—	—	2, 1s	2
Add.....	3.5	3.1	4.2	4.7
15 day old embryos				
1–4	0	ND	0	ND
5	4		1	
6	1		5s, 4h	
Add.....	5.1		—	

ND: Not done; Add: average day of death; s: sacrificed; h: hatched.

and the embryos of these dead embryos were pooled individually, and dilution counts were made from each source. The results are also tabulated in table 3.

The in vitro (growth) count. In order to correlate the *in vitro* growth of the PPLO with the growth in the chick embryo, the numbers of PPLO for a 96 hour, 14th passage broth culture were determined by the dilution method. As can be seen in table 3, maximum growth occurred in 24 hours (10^{11} organisms per ml) and remained at this level for several days.

Inoculation of 13 day old embryos. Various routes of inoculations were employed, and the results are summarized in table 2. All of the infected fluids yielded positive cultures on artificial medium. Cutaneous hemorrhages, especially around the head, wings, and feet, were observed in embryos inoculated by the three routes used. The chorioallantois of the embryos inoculated by this route showed plaques 0.75 cm in diameter at the site of inoculation, appearing necrotic at the center with a raised edematous periphery. Diffuse hemorrhages surrounding the plaques were also observed. Control embryos inoculated with an equal volume of sterile veal infusion broth for each group remained unaffected.

Inoculation of 15 day old embryos. Using 15 day old embryos the CA and AC routes were inoculated with the same PPLO concentration as in the previous trials (table 2). The membranes of embryos inoculated by this route were thickened with occasional whitish plaques 1 cm in diameter.

TABLE 3

Dilution count of infected embryo material and of cultural growth taken at various time intervals

Route of Embryo Inoculation	Pooled Material after Death	Count per Ml	Broth Culture Time of Count	Count per Ml
Allantoic cavity	Yolk	8.2×10^9	0 time*	2.3×10^8
	Allantoic fluid	2.5×10^{11}		
Allantoic cavity	Yolk	4.5×10^9	24 hours	4.0×10^{11}
	Allantoic fluid	2.5×10^{10}	48 hours	3.5×10^{10}
Chorioallantois	Chorioallantois	2.5×10^{11}	72 hours	4.0×10^{10}
Yolk sac	Yolk	3.0×10^{10}	96 hours	5.0×10^9
	Allantoic fluid	6.0×10^{10}		
	Embryo	5.0×10^{10}	144 hours	1.3×10^8

* Original four day old culture from which fresh medium was inoculated and subsequent counts made.

DISCUSSION

A virulent PPLO of goat origin was inoculated into chicken embryos by various routes. In every instance, PPLO were recovered from the infected embryo material upon cultivation on artificial medium. Giemsa stained smears of infected yolk sacs showed tiny coccoid bodies approximately 0.2 μ in diameter. They appeared tinctorially and morphologically indistinguishable from the agents of the psittacosis-lymphogranuloma group. Others who have observed these organisms in tissue have described morphological forms ranging from coccoid, cocco-bacillary, to ring forms (Edward, 1954). It would seem wise to inoculate artificial medium to rule out the possible presence of PPLO whenever one sees such suspected tissues. Special enrichment techniques such as the one described by Adler *et al.* (1954) may facilitate isolation of the more fastidious PPLO, for it has been pointed out by Sabin (1941) that some strains fail to adapt readily to artificial media.

By the dilution counting method it was found that regardless of the route of inoculation the PPLO titer appeared to be high in all of the tissues tested. There also appeared to be a close correlation between the numbers of organisms obtained in artificial culture and in the chick embryo, similar to the results of Keller and Morton (1954). Using 13 day old embryos the PPLO was lethal by both the CA and AC routes of inoculation, but much more irregular death patterns were observed with the latter route of inoculation. When 15 day old embryos were used, the mortality rate was very poor. No macroscopic lesions were observed in the sacrificed embryos nor in the chicks hatched.

The areas of edema and plaques on the chorioallantois as described with the bovine pleuropneumonia organism (Tang *et al.*, 1936; Sheriff and Piercy, 1952) were also demonstrated with this goat isolate.

It appears that the death pattern of PPLO in the chick embryo depends on the virulence of the strain used, the route of inoculation, and the age of the embryo at the time of inoculation. Early in the course of the study of PPLO associated with chronic respiratory disease of chickens, a very irregular death pattern resulted when the embryos were inoculated via the allantoic route (Delaplane, 1948). Subsequent studies by Hoyt *et al.* (1951) with PPLO from turkey origin indicated that a more uniform death pattern resulted when the yolk sac route of inoculation was used. With strains of the organisms isolated from poultry in this laboratory, we have found usually that the yolk sac route of inoculation gave uniform and high death rates (all embryos dead within 5–7 days). However, a few strains have been encountered which multiplied profusely in the yolk sac but did not cause embryo mortality (unpublished data). Sheriff and Piercy (1952) have found that with bovine pleuropneumonia, the yolk sac route of inoculation was the most lethal although deaths were also obtained when the agent was inoculated on the chorioallantois. Keller and Morton (1954) working with several human strains of PPLO found that no embryo mortality occurred when the PPLO strains were inoculated by the allantoic route. From this investigation, using a strain isolated from goats, comparable mortality rates were observed when the embryos were inoculated via the yolk sac, chorioallantois, and the allantoic routes. For the latter two routes, embryos of 10 days' preliminary incubation gave the most uniform results.

SUMMARY

The death pattern and lesions produced in chick embryos by a strain of a pleuropneumonia-like organism isolated from a highly fatal disease of goats are described. Inoculation of suspensions of yolk in a chorioallantois, allantoic cavity, and yolk sac gave comparable mortality rates in the chick embryo. The *in vitro* growth number correlated closely with the numbers obtained in infected embryo tissues, the titer of the latter being comparably high in all of the tissues tested.

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