VIRULENCE ASSAY OF *COCCIDIOIDES IMMITIS* IN EMBRYONATED EGGS

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During work on *Coccidioides immitis* the need arose for a relatively rapid method for the determination of the virulence of a number of strains of this microorganism. A satisfactory method has been described by Friedman, Smith, and Gordon (1955) in which a standard dose, usually 100 arthrospores, is inoculated intraperitoneally into mice. The virulence was determined from (i) the percentage of animals dead after a fixed interval of time or (ii) the number of days after inoculation when a specified percentage of the animals died. Karrer (1953) suggested that intracerebral injections in mice could be used for virulence assays. This method may be too sensitive, however, because the LD₅₀ for the two strains tested was less than 10 arthrospores. Characteristic of such virulence assays, considerable time and numbers of animals are required.

Several workers have shown that *C. immitis* could be subcultured in the developing chick egg. Moore (1941) used the chorioallantoic membrane for cultivation and histopathological study of pathogenic fungi. Newcomer, Wright, and Tambllyn (1952) studied the growth and development of the spherule phase following injections into the allantoic cavity or the yolk sac. Brueck and Buddingh (1951) used the yolk sac of embryonated eggs for the isolation and growth of pathogenic fungi and Burke, Salvin, and Gerloff (1952) and Vogel and Conant (1952) studied the growth and morphology of *C. immitis* in embryonated eggs using various sites of inoculation. Goodman, Fountaine, and Vincent (1953) reported that LD₅₀ values for chick embryos were not obtained even with injections of 10⁶ organisms unless the eggs were cooled immediately prior to and following injection. The site of inoculation, however, was not specified in Goodman's paper. Vogel, Peace, and Koger (1957) studied the histopathological reactions of the yolk sac tissue to *C. immitis*. On the basis of these reports, a

virulence assay method employing the embryonated chicken egg has been devised and is described in this report.

MATERIALS AND METHODS

Preparation of inoculum. The material injected into eggs was a suspension of *C. immitis* arthrospores from a culture grown for 28 days at 34 C on a reciprocal shaker in a glucose, ammonium acetate, inorganic salts medium (Roessler et al., 1946). To provide aeration for maximal growth, the shaker was operated at 90 excursions per min with a 6-in. stroke.

The arthrospores were washed twice in 0.85 per cent saline solution and chains broken up, if necessary, by shaking for 10 min with glass beads on a Kahn shaker. Viable particle counts of all inocula were made by plating serial dilutions on agar containing 2 per cent glucose, 1 per cent peptone, 0.1 per cent yeast autolysate, and 2 per cent agar (GPY agar) adjusted to pH 7.0 to 7.2.

Inoculation of eggs. Five-day-old embryonated chicken eggs were used in all except the preliminary experiments. Ten-fold dilutions of the arthrospore suspension were made in 0.85 per cent saline; appropriate dilutions (0.25 ml per egg) were inoculated by the selected route. Before inoculation, the area over the air sac was cleansed with 4 per cent tincture of iodine. Injections were made into the yolk sac with a 2-ml tuberculin syringe using a 1-in. 21-gauge hypodermic needle. Injections in the allantoic fluids of 8-day-old embryonated eggs also were made with a 3/4-in., 24-gauge needle. Ten eggs per dilution plus at least 10 controls were used in all virulence assays.

Incubation. The inoculated eggs were incubated at 38.5 C for 14 days in a standard egg incubator with controlled humidity. Embryo deaths were determined daily by candling. Values for LD₅₀ were calculated by the method of Reed and Muench (1938) from the number of cumulative deaths for the second through the tenth day after inoculation. Deaths occurring in the first

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48 hr were considered to have resulted from traumatic injury; these accounted for 8 per cent or less of the eggs injected.

Microscopic and cultural examination of eggs. Yolk material from eggs in which embryo death occurred was cultured in liquid defined medium (Roessler et al., 1946) for growth of *C. immitis*. The yolk was examined microscopically for the presence of spherule-like structures after clearing with 10 per cent potassium hydroxide. Stained sections of embryonic tissues were examined for the presence of specific pathological changes due to coccidioidomycosis.

The amount of growth of the organism in the egg was determined by viable plate counts of whole eggs ground for 2 min in a Waring Blender. It was determined in an experiment that the grinding process was not detrimental to the viability of arthrospores injected into eggs; grinding could increase viable counts if spherules

were disrupted or mycelia or spore chains were fragmented. Before grinding, the surface of the egg was cleansed with 70 per cent ethanol. Dilutions of the eggs were plated on GPY agar.

RESULTS

Site of inoculation. The first experiment compared the yolk sac and the allantoic fluid as sites of inoculation in 8-day-old embryonated eggs. The majority of embryo deaths in eggs inoculated by the yolk sac route occurred between the second and sixth day following inoculation (table 1). The large number of deaths occurring on the 11th and 12th days was considered to be the normal prehatching mortality rise which occurs in incubated eggs immediately before the hatching period. Cultures made from these eggs were negative.

Few embryo deaths occurred in eggs inoculated in the allantoic fluid. Cultures and microscopic

TABLE 1
Death of 8-day-old chick embryos inoculated with Coccidioides immitis strain 6212

Dilution Injected	Days after Inoculation												Ratio Embryos Dead/Eggs Injected
	1	2	3	4	5	6	7	8	9	10	11*	12*	
Undiluted 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴ 10 ⁻⁵ 10 ⁻⁶ 10 ⁻⁷	Yolk sac inoculation†												
	1‡	1‡	7 6	2 3 3									9/9 9/9 10/10 2/10 3/10 2/10 2/9 0/9
					2 1 1	3 1		1 1	1 1			3 1 1	
Undiluted 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴ 10 ⁻⁵ 10 ⁻⁶ 10 ⁻⁷	Allantoic fluid inoculation†												
						2		1			2	4	0/10 3/10 1/10 0/10 0/10 1/8 0/10 0/8

* Normal prehatching mortality occurring between the 19th and 21st day of incubation. These deaths were not considered in the calculations.

† Ten eggs were inoculated for each dilution. The undiluted cultures contained 5.5×10^6 cells per ml; 0.25 ml was injected per egg.

‡ Traumatic deaths considered to be due to mechanical injury.

TABLE 2

Effect of embryo age on susceptibility to infection with Coccidioides immitis strain 6212

Age of Embryo	Dilution Injected*	Ratio Embryos Dead/Eggs Injected†	LD ₅₀
5 Days	10 ⁻¹	10/10	794
	10 ⁻²	10/10	
	10 ⁻³	7/9	
	10 ⁻⁴	0/4	
6 Days	10 ⁻¹	10/10	676
	10 ⁻²	9/9	
	10 ⁻³	7/10	
	10 ⁻⁴	2/9	
7 Days	10 ⁻¹	8/8	7,762
	10 ⁻²	5/9	
	10 ⁻³	3/10	
	10 ⁻⁴	0/9	
8 Days	10 ⁻¹	9/9	5,495
	10 ⁻²	5/8	
	10 ⁻³	2/10	
	10 ⁻⁴	2/10	
9 Days	10 ⁻¹	5/8	98,680
	10 ⁻²	1/10	
	10 ⁻³	0/10	

* The undiluted culture contained 7.3×10^6 cells per ml; 0.25 ml was injected per egg.

† Traumatic deaths subtracted.

examinations of embryo tissues, yolk material, and allantoic fluid were all negative. Consequently this route of inoculation was discontinued.

Age of embryo. In a preliminary experiment which compared the response of 8-, 9-, and 10-day-old embryos to yolk sac inoculation with *C. immitis*, it was demonstrated that the age of the embryo markedly influenced the LD₅₀ value. These results were confirmed in an experiment which measured the effect of equivalent inocula on 5-, 6-, 7-, 8-, and 9-day-old chick embryos. The marked difference in susceptibility of embryos of different ages is shown in table 2.

There were approximately 10-fold increases in the number of viable particles necessary to kill 50 per cent of the embryos from the 5- and 6-day to the 7- and 8-day groups and from the latter to the 9-day age group. In the experiments which followed, 5-day-old embryos were used because of their greater susceptibility and because the titrations could be completed before the embryo was 15 days old. This enabled a sharper separation of deaths due to infection

from deaths which normally occur just prior to hatching. A graded time response was obtained which depended on the dose injected.

Growth of C. immitis in eggs. Table 3 shows the results of an experiment to determine the viable counts reached in embryonated or unfertile eggs after injections of various strains of *C. immitis*. The viable counts were made on eggs selected at random from various experiments. In nearly all cases the viable count was 1×10^6 particles per egg or greater at the time of death of the embryo although the number of arthrospores injected varied considerably. The Mouser strain did not kill the embryo even after 14 days of incubation. However, this strain, which was one of the least virulent in the collection, reached the same viable count in the embryonated egg as strains which were much more virulent.

Several nonfertile eggs were injected with strain Cash and incubated for 7 days before viable cell numbers were determined. During this interval the count was increased from 1.0×10^4 to 1.9×10^7 particles per egg indicating that this strain of *C. immitis* grows as well in nonfertile eggs as it does in embryonated eggs.

Comparative virulence. The virulence of 37 strains of *C. immitis* as determined by egg inoculation is shown in table 4. All strains were grown in the liquid defined medium under the conditions described above. The LD₅₀ values ranged, with one exception, between 4 and 2,800 viable particles.

Microscopic and cultural studies. The yolk material from representative eggs containing dead embryos was examined microscopically after clearing with 10 per cent potassium hydroxide. In every instance, spherule-like structures were present and in some instances mycelial growth was noted. Although the spherule-like bodies were not positively identified, these structures showed a strong resemblance to the type of growth in vivo of *C. immitis*. It was only after considerable experience that these spherule-like structures could be differentiated from other globular bodies that normally exist in the yolk material. Staining of unwashed yolk material to demonstrate these structures was difficult.

In early experiments, yolk material from eggs containing dead embryos was cultured in the liquid medium to demonstrate the presence of *C. immitis*. In later experiments, only representative samples were cultured. Growth of the organism occurred in every instance.

TABLE 3
Growth of Coccidioides immitis in eggs

Strain	Days of Incubation	Condition of Eggs	Organisms per Egg	
			In inoculum	After incubation*
Cash.....	3	Embryo dead	5.5×10^2	6.9×10^6
M-11.....	3	Embryo dead	1.2×10^2	1.2×10^6
Hutchinson.....	3	Embryo dead	3.0×10^2	1.1×10^6
Hutchinson.....	3	Embryo dead	3.0×10^2	1.4×10^6
M-11.....	3	Embryo dead	2.0×10^1	2.7×10^6
M-11.....	3	Embryo dead	1.2×10^2	1.3×10^6
Dog A.....	3	Embryo dead	3.9×10^2	2.5×10^6
Cash.....	6	Embryo dead	3.5×10^2	2.1×10^6
Cash.....	3	Embryo dead	6.0×10^2	4.6×10^6
Cash.....	5	Embryo dead	6.0×10^2	1.5×10^6
Cash.....	7	Embryo dead	6.0×10^1	0.5×10^6
Mouser.....	14	Embryos alive	2.6×10^4	3.6×10^6
Cash.....	7	Nonfertile	1.0×10^4	1.9×10^7

* Two or three eggs were ground together and assayed for each value given.

TABLE 4
*Calculated LD₅₀ values of different strains of Coccidioides immitis**

Strain	LD ₅₀	Strain	LD ₅₀	Strain	LD ₅₀
Stencio.....	32	Knaggs.....	13	Smith Arizona..	28
Bates.....	7	Loustalot....	98	Soil 3a.....	2,793
Brisco.....	12	Mouser.....	24,060	SOM 1.....	48
Cash.....	88	Maxwell.....	1,000	SOM 2.....	8
Davis.....	236	M-11.....	4	Stacey.....	464
Dog A.....	192	Nelson.....	4	Steinbiss.....	589
Dog B.....	30	Norman.....	130	Thomas.....	8
Edralin.....	27	Perry.....	57	Tidwell.....	225
Euphrat.....	397	P.W.....	23	Venezuela.....	2,748
Herreth.....	141	Scarborough..	1,205	Warren.....	173
Hutchinson.....	65	Seigrist.....	26	Weible.....	97
Jones.....	17	Silveira.....	40		
King.....	10	6212.....	794		

* Mean values of 2 to 13 determinations.

The possibility of death due to some adjuvant used in the assay was investigated. The injection of sterile defined medium, sterile physiological saline, sterile culture filtrate, heat-killed *C. immitis* culture, or sterile filtrate of ground eggs (in which the embryos had died after inoculation with 5.9×10^6 viable fragments of *C. immitis*) failed to cause an appreciable number of deaths in uninfected 5-day-old embryonated eggs. Realizing that death of the embryos may have been due to the competition for or depletion of the nutrients in the yolk, autolyzed yeast and

glucose were added periodically to the yolk. This procedure tended to decrease rather than increase the LD₅₀ values without affecting uninoculated controls.

Replicate titrations. A large experiment, using the Cash strain of *C. immitis* and approximately 280 five-day-old embryonated eggs, was performed. The mean and standard deviation of this virulence titration was computed so that an estimate of the true experimental error was afforded when replicate groups of eggs injected at the same time with the same stock suspension

TABLE 5

Results of replicate titrations in embryonated eggs of one culture of *Coccidioides immitis* strain Cash

Group	Ratio of Embryos Dead/Eggs Injected*				LD ₅₀
	No. of particles injected:				
	59,000	5,900	590	59	
A	9/9	9/9	8/9	3/7	100
B	10/10	8/8	10/10	4/8	59
C	9/9	6/6	9/9	4/8	59
D	7/7	10/10	9/9	2/9	133
E	7/7	10/10	10/10	4/10	86
F	9/9	9/9	8/9	3/7	100
G	10/10	8/8	10/10	4/10	86

* Traumatic deaths were subtracted.

were used. The results of the experiment are shown in table 5. The mean LD₅₀ of these titrations was 89.0 viable particles; the standard deviation was 25.8. Separate cultures of the Cash strain grown under the same conditions were assayed in subsequent virulence titration experiments with other strains. Statistical analysis of the results for the Cash strain in these titrations (table 6, experiments 52 to 69) showed that the mean LD₅₀ changed only slightly to 85.7 and the standard deviation changed to 39.1.

The majority of the embryos in the virulence titration died between the third and sixth day after injection, the larger doses giving the more rapid response. A typical death curve plotted from these results of experiment 50 is shown in figure 1. Normally no deaths occurred between the 10th and 14th day after injection when 5-day-old embryos were used.

DISCUSSION

Vogel, Peace, and Koger (1957) reported that a direct invasion of tissues of the yolk sac by the fungus was observed when 13-day-old embryos were studied. Although no systematic study was made by us of tissues of the embryonated eggs, it was shown that (i) onset of embryo death was influenced by the amount of agent injected into the yolk sac, (ii) large differences in the calculated LD₅₀ resulted when different strains of *C. immitis* were compared, and (iii) 5-day-old embryos were more susceptible than older embryos. Even though these results were

TABLE 6

Titration of replicate cultures of *Coccidioides immitis* strain Cash

Expt No.	No. of Particles Injected	Ratio of Embryos Dead/Eggs Injected*	LD ₅₀
52	66,000	10/10	99
	6,600	10/10	
	660	7/7	
	66	4/10	
53	5,000	7/7	63
	500	7/8	
	50	4/8	
	5	0/8	
60	5,400	9/10	103
	540	7/9	
	54	4/9	
	5	1/10	
65	280	9/10	87
	28	1/9	
	2.8	0/8	
	0.28	0/8	
69	100,000	8/8	<100†
	10,000	9/9	
	1,000	10/10	
	100	7/10	
84	4,400	20/20	24
	440	18/20	
	44	11/19	
	4.4	5/20	
85	4,300	17/18	140
	430	10/18	
	43	5/19	
	4.3	5/19	

* Traumatic deaths were subtracted.

† Dose range too high to permit calculation of LD₅₀.

not correlated with specific pathological changes in the embryo, the above evidence suggests a relationship between death of the embryo and virulence of the organism.

The fact that certain strains of the organism failed to cause death of the embryo even after growth in the egg reached 10⁶ particles would seem to eliminate the possibility that this test is a growth index rather than a virulence index. Since the injection of 10⁶ viable fragments of *C. immitis* generally caused death of 5-day-old embryos within 48 hr, it is very unlikely that the nutrients of the yolk were depleted to such an extent that the embryo could no longer survive, particularly since yeast extract and glucose

Figure 1. Death of 5-day-old chick embryos after injection of *Coccidioides immitis* strain Cash. Experiment 50. Seventy eggs were injected for each dilution.

added to the yolk tended to decrease rather than increase the LD_{50} .

No physical or chemical cause of death of the embryo was demonstrated. Death did not result from the mechanical process of injection, nor from the chemical or nonviable materials of biological origin present in the inoculum as was shown by the lack of activity of heat-killed culture, spent medium, or sterile filtrates of infected eggs.

The statistical analysis of the data indicates that the estimation of the virulence of *C. immitis* in the embryonated egg is as reproducible as other assays in the embryonated egg and nearly as good as that obtained with mice (Friedman et al., 1956). The data show that there was little variability in the virulence of strain Cash among experiments performed under identical conditions at different times. A comparison of these data with those reported by Friedman et al. (1956) shows that, in general, the results of virulence assays in eggs correlate with those in mice.

The results reported here are somewhat in variance with those reported by Newcomer et al. (1952). These workers reported that they found no correlation between the survivability of 8-day-old embryos and the number of organisms in the inoculum. Further, they found that *C. immitis* developed more rapidly in nonembryonated eggs than in embryonated eggs. These differences possibly can be explained by considering the age of the chick embryo used, the plating

medium employed to evaluate growth in the egg and to quantitate the inoculum, and the medium used to produce material for inoculation. Practically all the work reported here involved 5-day-old embryos which were shown to be more sensitive to injections of *C. immitis* than 8-day-old embryos. In experiments which employed the proper dose range, graded responses were always obtained; such experiments involved several thousands of eggs. Sabouraud's agar at the pH commonly used in clinical laboratories has been shown to be rather poor for the cultivation of *C. immitis*. The use of this medium may also have contributed to the differences observed.

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SUMMARY

A method of virulence assay of *Coccidioides immitis* in embryonated chicken eggs is described. Embryo deaths were proportional to the amount of the fungus injected, the majority occurring between the third and sixth day following yolk sac inoculation of 5-day-old embryonated eggs. Growth of the fungus in the egg reached 10^6 particles whether or not death of the embryo occurred.

Death of the embryo was not due to mechanics

of injection or to the effects of chemical or non-viable biological material injected with the fungus. It also was concluded that death probably was not the result of depletion of nutrients in the yolk.

The virulence of 37 strains of *C. immitis* was determined by this technique.

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