Lethality of Pathogenic and Non-Pathogenic Staphylococci in Embryonated Eggs. (27547)

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Satisfactory experimental staphylococcal infections have been difficult to produce in laboratory animals. Localized infections have been produced by injection of staphylococci into areas subjected to various types of trauma(1-3) or by injection of extremely large numbers of staphylococci into normal skin (4,5). Even larger numbers of bacteria have been required for production of lethal systemic infections(6,7). At least one strain of *Staphylococcus aureus* is capable of producing a lethal systemic infection in mice with a relatively small inoculum ($\pm$ 10^4 bacteria) but its virulence appears to be related to factors which have not been demonstrated to be important in human infections(8). The study of a more suitable experimental infection might allow more thorough understanding of the pathogenesis of staphylococcal infections in humans and clarification of the importance of various host factors. This report describes differences in the lethality of pathogenic and non-pathogenic staphylococci for embryonated eggs observed during evaluation of experimental staphylococcal infections in chick embryos.

Materials and methods. Coagulase positive mannitol positive strains of *Staphylococcus aureus* were isolated from purulent exudates from patients with clinical infections. Strains of coagulase negative, mannitol negative *Staphylococcus epidermidis* were obtained from nose, throat and urine specimens of patients without evidence of clinical infection. These isolates were selected since they clearly represented strains of documented pathogenicity and strains which were non-pathogenic.

Serial 10-fold dilutions of overnight tryptose phosphate broth cultures were made in saline. One-tenth milliliter of 10^{-3} and 10^{-7} dilutions were injected into the chorioallantoic cavity of commercial fertilized eggs which had been incubated for 10 days. Ten eggs were injected with 0.1 ml of each of these 2 dilutions of one strain of coagulase positive and one strain of coagulase negative staphylococci in each experiment. Ten other eggs were treated similarly and injected with 0.1 ml of sterile saline to serve as viability controls. The number of bacteria injected was determined by serial 10-fold dilutions of the inocula used for egg injection. The eggs were examined and survival rate was determined daily for 9 days.

Overnight broth cultures were thoroughly mixed and divided into three 5-ml aliquots to study the effect of preformed exotoxin. One aliquot was centrifuged at 5000 rpm for 15 minutes, the sedimented bacteria were resuspended in sterile saline to the original volume and recentrifuged. Three washings were made in this manner. The second specimen was heated at 60°C for 30 minutes while no manipulations were performed with the third aliquot. The specimens were serially diluted and injected into embryonated eggs as described above and their lethality was determined.

Results. The number of embryos which survived for 9 days after injection of each dilution of each strain of coagulase positive and coagulase negative staphylococci is shown in Table I. The 2 upper groups show the number of survivors after challenge with 10^4 coagulase positive or coagulase negative staphylococci while the next 2 groups demonstrate the survival after injection of a smaller inoculum. Growth was relatively constant with coagulase positive strains and allowed the selection of dilutions which gave an inoculum of 10^4 and less than 10 bacteria. Growth was less constant with coagulase negative strains and it was not always possible to select the desired number of bacteria for injection.

Five of the 90 eggs injected with 10^4 coagulase positive staphylococci and 15 of the 90

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TABLE I. Lethality of Coagulase Positive and Coagulase Negative Staphylococci in Embryonated Eggs.

Strain of staphylococcus	No. of bacteria inj.	No. of eggs — Injected	No. of eggs — Alive after 9 days
Coagulase positive			
101	2×10^4	10	0
102	2 "	"	1
103	2 "	"	0
104	1 "	"	0
17	2 "	"	2
105h	4 "	"	0
106nh	2 "	"	2
107	1 "	"	0
108	2 "	"	0
Coagulase negative			
131	1×10^4	10	7
132	1 "	"	8
133	1 "	"	7
1131	1 "	"	7
630	3 "	"	6
134	5 "	"	5
Coagulase positive			
101	2	10	0
102	3	"	0
103	2	"	2
104	1	"	2
17	2	"	3
105h	4	"	1
106nh	2	"	3
107	1	"	2
108	2	"	2
Coagulase negative			
130	300	10	6
131	1	"	7
132	1	"	8
133	1	"	9
1131	1	"	8
630	3	"	7
135	20	"	8
136	3	"	7
134	3	"	8
Saline controls	0	47	43

injected with from one to 10 coagulase positive staphylococci survived for 9 days. In contrast, 40 of the 60 eggs injected with 10^4 coagulase negative staphylococci and 68 of the 90 injected with a smaller number of coagulase negative staphylococci survived for 9 days. The fatality rate ranged from 80 to 100% in strains injected with 10^4 coagulase positive staphylococci and from 70 to 100% in those injected with less than 10 pathogenic staphylococci. The fatality rate with coagulase negative strains ranged from 20 to 50% with a large inoculum and from 10 to 40% with a small inoculum. There was no overlap of the fatality rates between coagulase positive and coagulase negative strains at either dilution and the fatality rate was significantly greater in eggs challenged with less than 10 coagulase positive staphylococci than in eggs injected with 10^4 coagulase negative staphylococci ($X^2 = 5.6$; $P<.02$). Deaths were significantly more frequent in eggs challenged with a large inoculum of coagulase positive staphylococci than with a small inoculum ($X^2 = 5.6$; $P<.02$) but with coagulase negative strains, fatalities were not significantly related to number of bacteria injected ($X^2 = 1.2$; $P>.2$). Some strains of coagulase positive staphylococci (102, 17, 106 nh) produced a significant number of fatalities with an inoculum of less than 10 bacteria but were not uniformly lethal even when an inoculum 10,000 times this size was injected. A large inoculum of coagulase positive staphylococci was also more rapidly fatal than a small inoculum. Eighty-three % of the fatalities in eggs injected with 10^4 coagulase positive staphylococci occurred within 3 days while more than one-half of the fatalities with an inoculum of less than 10 bacteria occurred during the latter half of the experimental period.

The lethality of 2 strains of coagulase positive staphylococci was retested at 4-week intervals as a check of the reproducibility of these results. Table II shows that the fatality rates with each dilution of these 2 strains were almost identical in each experiment.

Table III illustrates the lethality of both washed and unwashed coagulase positive staphylococci. Washing to remove preformed

TABLE II. Lethality of 2 Strains of Coagulase Negative Staphylococci in Separate Experiments Done at 4-Week Intervals.

Strain of staphylococcus	Exp. No.	No. of bacteria inj.	No. of eggs — Inj.	No. of eggs — Alive after 9 days
105h	4	4×10^4	10	0
105h	8	5×10^4	10	0
106nh	5	2×10^4	10	2
106nh	9	5×10^4	10	2
105h	4	4	10	1
105h	8	5	10	1
106nh	5	2	10	3
106nh	9	5	10	4

TABLE III. Lethality of Washed, Unwashed, and Heat-Killed Staphylococci.

Strain of staphylococcus	Treatment	No. of bacteria inj.	No. of eggs Inj.	No. of eggs Alive after 9 days
104	None	2×10^4	10	0
104	Saline washed	1×10^4	10	0
104	Heated (60°C × 30 min.)	0	10	9
104	None	2	10	4
104	Saline washed	1	10	3
104	Heated (60°C × 30 min.)	0	10	9

exotoxin did not diminish the lethality while heat killed cultures diluted 10^{-4} and 10^{-8} were essentially avirulent. Similar results were obtained with a second strain of coagulase positive staphylococcus treated similarly.

Discussion. More thorough understanding of specific virulence factors, variations in the virulence of individual strains, and factors associated with host resistance might be obtained by the study of a sensitive and reproducible experimental staphylococcal infection. Many of the previously described lethal experimental infections have required such a large bacterial challenge that their usefulness has been hindered. Embryonated eggs have proven to be extremely valuable for propagation of viruses and study of viral infections. Some bacterial infections have been studied in the chick embryo, but usually more satisfactory experimental animals have been found. Knothe(9) studied a variety of bacterial infections in embryonated eggs and noted that staphylococci were quite lethal in this system. Frappier and Sonea(10) also found that some strains of staphylococci were quite lethal when injected directly into 18- and 19-day-old chick embryos but other strains were relatively avirulent. Because of the extreme difficulty in characterizing the pathogenicity of coagulase positive staphylococci, only coagulase positive strains which had produced infections in humans and nonpathogenic coagulase negative strains were studied in the present report. These data confirm the sensitivity of chick embryos to pathogenic staphylococci and demonstrate that a reproducible experimental staphyloccal infection can be produced in chick embryos by the chorioallantoic route. Coagulase positive staphylococci of proven human pathogenicity were quite lethal while coagulase negative strains were relatively avirulent. The LD_{50} of these coagulase positive strains could not be precisely defined but was less than 10 bacteria while 10,000 or more coagulase negative staphylococci were significantly less lethal and produced an overall fatality rate of only 33%. A large inoculum (10^4) of coagulase positive strains was not only more lethal but also was more rapidly lethal than a small inoculum (1-10 bacteria). An occasional embryo (6%) survived even the larger challenge dose. Whether this represented innate resistance or other undefined factors remains to be determined.

The cause of death in the embryo is uncertain. Preformed free exotoxin does not appear to be important since washed staphylococci were as lethal as unwashed bacteria and heat killed cultures were relatively avirulent. These data do not exclude the importance of exotoxin formed after infection. Destruction of various staphylococcal exotoxins by methods used in obtaining bacterial free toxins, however, has prompted the use of toxigenic and nontoxigenic bacterial mutants for studies of the effect of individual staphylococcal exotoxins now in progress.

The extreme sensitivity of embryonated eggs to pathogenic staphylococci, their relative resistance to coagulase negative staphylococci, reproducibility of the infection, and ease of handling suggest that this model may prove quite satisfactory for investigation of factors important in human staphylococcal infections.

Summary. Ten-day-old chick embryos are extremely susceptible to infection with pathogenic coagulase positive staphylococci and the LD_{50} of the strains studied was less than 10 bacteria. More than 10^4 coagulase negative staphylococci were necessary to produce a 50% fatality rate in 10-day-old chick embryos.

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1. James, R. C., Mac Leod, C. J., *Brit. J. Exp.*

Path., 1961, v42, 266.

2. Goshi, K., Cluff, L. E., Johnson, J. E., III, Conti, C. R., *J. Exp. Med.*, 1961, v113, 249.

3. ———, *ibid.*, 1961, v113, 259.

4. Dold, H., *Zbl. Bakt., I.* Abt. Orig., 1927, v102, 1.

5. Panton, P. N., Valentine, F. C. O., *Brit. J. Exp. Path.*, 1929, v10, 257.

6. Forssman, J., *Acta path. microbiol. scand.*, 1935, v12, 536.

7. Bass, J. A., Higginbotham, R. D., *Tex. Rep. Biol. Med.*, 1960, v18, 364.

8. Hunt, G. A., Moses, A. J., *Science*, 1958, v128, 1574.

9. Knothe, H., *Zbl. Bakt., I.* Abt. Orig., 1952, v158, 453.

10. Frappier, A., Sonea, S., *Un. med. Can.*, 1954, v83, 382.

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Effect of a *Clostridium* Species Upon Cecal Size of Gnotobiotic* Mice.† (27548)

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One of the frequent morphological anomalies of the axenic(1) rat and mouse is enlargement of the cecum(2) which may occupy 30-90% of the abdominal cavity. The wall of the cecum is thin(2) and the contents are fluid. Such enlargement simulates the appearance of pregnancy, and may lead to a fatal volvulus(3). Similar distention of the cecum has been reported for the conventional rat maintained either on a diet of raw unboiled potato starch(4), or lactose(5), or one deficient in potassium(6). Evidence that the cecal anomaly may be unrelated to diet has been reported(7). Addition of antibiotics to the diet of conventional rats also caused occasional enlargement of the cecum (8,9). This report describes the effect of a *Clostridium* species on the restoration of the distended cecum of axenic mice to normal size.

Materials and methods. Axenic and conventional ("normal") albino ND-2 mice§ (male and female, 21-365 days old) were housed in flexible film isolators which were provided with sterile supplies of San-i-cel bedding,‖ Purina chow 5010C,¶ and water. Animals and materials were introduced into the closed system through a "lock" sterilized with 2% peracetic acid spray(10). Preliminary experiments were designed to determine if any organisms in the aerobic microflora of conventional mice would restore the enlarged cecum of axenic mice to normal size. Dilutions of cecal contents of conventional mice were incubated aerobically on Desoxycholate agar, Endo agar, S. S. agar, *Staphylococcus* Medium No. 110, MacConkey agar, L.B.S. medium, and trypticase soy agar at 26, 37, and 55°C. Cultures of aerobic bacteria were then isolated by routine bacteriological technics. Selected isolates were propagated for 24 hours in thioglycollate broth to which a small chip of calcium carbonate had been added. Axenic mice which had been starved for 6 hours were fed a diet soaked in the broth culture. Both axenic and conventional control animals were maintained on similar but untreated diets. Animals were sacrificed at various time intervals by cervical fracture. Ceca were excised and weights were expressed as percentage of total body weight. Bacteriological tests were made to

* The term "gnotobiote" refers to an animal of known microbial content.

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§ Originally derived from the Rabstein colony, Fort Detrick, Md.

‖ Paxton Processing Co., Paxton, Ill.

¶ Ralston Purina Co., St. Louis, Mo.