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Tyzzer's Disease of Rabbits: Isolation and Propagation of *Bacillus piliformis* (Tyzzer) in Embryonated Eggs

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Bacillus piliformis (Tyzzer) was isolated from the liver of rabbits with Tyzzer's disease and serially passaged in embryonated hens' eggs. Weanling rabbits given the 32nd egg passage developed lesions typical of Tyzzer's disease and died. *B. piliformis* was reisolated from the liver of these rabbits in embryonated eggs. Outside the host cell, the motile vegetative phase appeared to be unstable, and no means was found to preserve its viability; the results of titrations were believed to be dependent upon the resistant stage or spore. The spore withstood repeated freeze and thaw and was resistant to heat treatment of 56 C for 1 hr but not 80 C for 0.5 hr. None of several antibacterial substances tested in embryonated eggs was completely inhibitory; *B. piliformis* was resistant to sulfamethazine and chloramphenicol. The taxonomic position of this pleomorphic, gram-negative, sporeforming, pathogenic bacterium which appears to grow only in certain cells of several species remains unresolved.

"There is no analogy to this phenomenon anywhere in the field of bacteriology" (8). This statement by Gard concerning the sporeforming bacterium (*Bacillus piliformis*) which multiplies only within certain cells seems as appropriate today as 26 yr ago. Numerous reports (4, 9, 15, 19) have confirmed Tyzzer's original description (20) of a fatal enteric disease of mice, since known as Tyzzer's disease. The diagnostic feature of Tyzzer's disease is the demonstration of bundles of gram-negative, pleomorphic bacilli (*B. piliformis*) randomly arranged in the cytoplasm of apparently viable liver cells which border small areas of coagulation necrosis. Though unable to culture *B. piliformis* outside the mouse, Tyzzer demonstrated the transmissability of the disease by injecting mice with tissue suspensions prepared from liver of diseased mice and also by exposing mice to contaminated bedding which had been stored for a year at room temperature.

Naturally occurring Tyzzer's disease in a species other than the laboratory mouse was first reported by Allen, Ganaway, Moore, and Kinard (1) who described an epizootic associated with high mortality in the rabbit production colony at the National Institutes of Health. The disease has since been reported in the rhesus monkey (*Macaca mulatta*, reference 12), gerbil (*Meriones unguiculatus*, references 2, 13, 21), and laboratory rat (*Rattus norvegicus*, references 10, 17).

Cultivation of *B. piliformis* in cell-free media has not been successful (1, 4, 8, 15, 19, 20). The single report of successful culture by Kanazawa and Imai (11) remains unconfirmed. Isolation in mouse embryo cell cultures was reported by Rights, Jackson, and Smadel (15), but pathogenicity for mice was lost with the second serial passage. Craigie (4), using the yolk sac route in embryonated eggs, isolated two strains of *B. piliformis* from the liver of spontaneous cases of Tyzzer's disease of laboratory mice. Typical lesions were observed in mice after parenteral inoculation with serially passaged yolk sac suspensions. The present study deals with the isolation, passage, and character of *B. piliformis* associated with Tyzzer's disease of rabbits (1).

MATERIALS AND METHODS

Rabbits. A source of New Zealand White rabbits infected with *B. piliformis* was maintained using contaminated bedding as previously described (1). Transmission of the disease was thus accomplished by placing 4- to 6-week-old rabbits on bedding previously soiled by rabbits that died of Tyzzer's disease. Cortone acetate (Cortisone Acetate, Merck Sharp and Dohme Research Laboratories, Div. of Merck & Co., Inc., Rahway, N.J.), 0.05 mg/g of body weight, was administered intramuscularly to rabbits on the first day of exposure. Pelleted food and water were available ad lib. In certain experiments to be described, sulfaquinoxaline-treated water (1.5 g/gal) was given

in lieu of tap water. The rabbits were housed in Horsfall-type units supplied with filtered air under slight negative pressure. Necropsy was performed on all rabbits used in this study. Histopathology procedures were described previously (1).

Embryonated eggs. Portions of liver were collected aseptically from rabbits which died of Tyzzer's disease and from rabbits killed (chloroform) at daily intervals after exposure to contaminated bedding. Glass tissue grinders were used to prepare 10% (w/v) liver suspensions in sterile phosphate-buffered saline (PBS) (0.85% NaCl, *p*H 7.2). White Leghorn hens' eggs (Duckworth Hatchery, Hanover, Md.), 7-day embryonated, were inoculated with the rabbit liver suspensions via the yolk sac route, 0.5 ml per egg. Eggs were incubated at 37 C in the upright stationary position. Viable embryos were determined prior to inoculation and daily thereafter by candling. Embryonic deaths which occurred within 24 hr of inoculation were considered to be due to trauma (<5%) and were not included in the data. Cause of embryo death was verified by the routine examination of representative yolk sac smear preparations. These were air dried, fixed in methanol, and stained with Giemsa. In addition, infective yolk sac suspensions were examined by conventional aerobic and anaerobic cultural methods as previously described (1). Yolk sac suspension (20% w/v in PBS) for serial passage was prepared by brief but vigorous shaking of freshly harvested, unwashed yolk sacs and PBS in a prescription bottle containing glass beads. Normal yolk sacs were harvested and passaged similarly to detect endogenous viruses (3). None was detected. Yolk sac suspensions and individually harvested yolk sacs were frozen and stored at −70 C for reference.

Quantitation. Titrations were performed in 7-day embryonated eggs via the yolk sac route, eight eggs per dilution, by using decimal dilutions in PBS. End points, expressed as ELD_{50}, were determined by the method of Reed and Muench (14).

Antibacterial testing. Sensitivity testing was performed by adding varying concentrations of antibacterial agents in a constant volume to equal samples of a freshly harvested pool of 20% yolk-sac suspension (15th and 16th passages) in PBS. A sample of untreated suspension, similarly diluted, served as control. After brief mixing, groups of 8 or more eggs were inoculated with each suspension via the yolk sac route.

Transmission studies. A litter of 7-week-old rabbits was divided into two groups of four. Each rabbit of one group received by mouth 5.0 ml (via stomach tube) of 20% yolk sac suspension in PBS of the 32nd passage of *B. piliformis* (4×10^3 ELD_{50} per rabbit) and a single intramuscular injection of cortisone (0.05 mg/g of body weight). Each rabbit of the other group received by mouth 5.0 ml of a 20% normal yolk sac suspension in PBS and a comparable single injection of cortisone. Each group was housed in separate Horsfall-type units. The rabbits were examined daily for evidence of disease. Necropsy was performed on all rabbits soon after death or when killed (chloroform) while moribund. Giemsa-stained tissue sections of liver and ileocecal junction were examined histologically. Embryonated eggs (7-day) were inoculated via the yolk sac route with rabbit liver suspensions (20% in PBS) containing 0.5 mg of chloramphenicol per ml for reisolation of *B. piliformis*.

RESULTS

Initial attempts to isolate *B. piliformis* in embryonated eggs failed repeatedly due to an overgrowth of various enteric bacteria found in the liver suspensions prepared from rabbits dead of Tyzzer's disease or from rabbits killed when clinically ill.

Successful isolation in embryonated eggs was achieved from the liver of four of seven rabbits killed on the 3rd, 4th, or 5th postexposure day when sulfaquinoxaline was added to the drinking water. (A preliminary experiment indicated that sulfaquinoxaline did not prevent the infection; when 6-week-old rabbits were given a single injection of cortisone, placed on contaminated bedding, and offered sulfaquinoxaline-treated water, they usually died of Tyzzer's disease on the 5th or 6th day postexposure). Though the rabbits appeared normal clinically, lesions of a typical but mild character were seen at necropsy. Petechial hemorrhages and edema were present in the small intestine at the ileocecal junction. Histological examination of the tissues revealed lesions of a mild enteritis, with atrophy of the villous epithelial cells, edematous ballooning of the tips of the villi, and, in one instance, patchy areas of necrosis in the glandular portion of the mucosa. Bacilli typical of *B. piliformis* were seen in the cells of the mucosal epithelium of four rabbits and in the muscularis mucosa of two. Neutrophils were slightly increased in number in the submucosa of the intestines having the most bacilli. Lesions of Tyzzer's disease were not seen in the livers. However, the liver cells were severely vacuolated (laden with glycogen) and had a moth-eaten appearance.

In each of the four instances when the bacillus was isolated, the chick embryos were found dead between the 6th and 9th post-inoculation day (PID). Bacilli were readily demonstrated within the cytoplasm of epithelial cells of Giemsa-stained yolk sac smears. The bacilli were similar in all respects to those described in spontaneous Tyzzer's disease of mice (20), rabbits (1), monkeys (12), gerbils (2, 13, 21), and rats (11, 17), and to those seen in yolk sac smears of embryonated eggs inoculated with diseased-mouse liver suspension (4). The predominant forms seen were the evenly stained filamentous rods (Fig. 1) and spores (Fig. 2). Monilial, beaded, banded, club-shaped, and short rod forms were also seen (Fig. 3). No growth was obtained in cell-free media when infective yolk sac suspensions were ex-

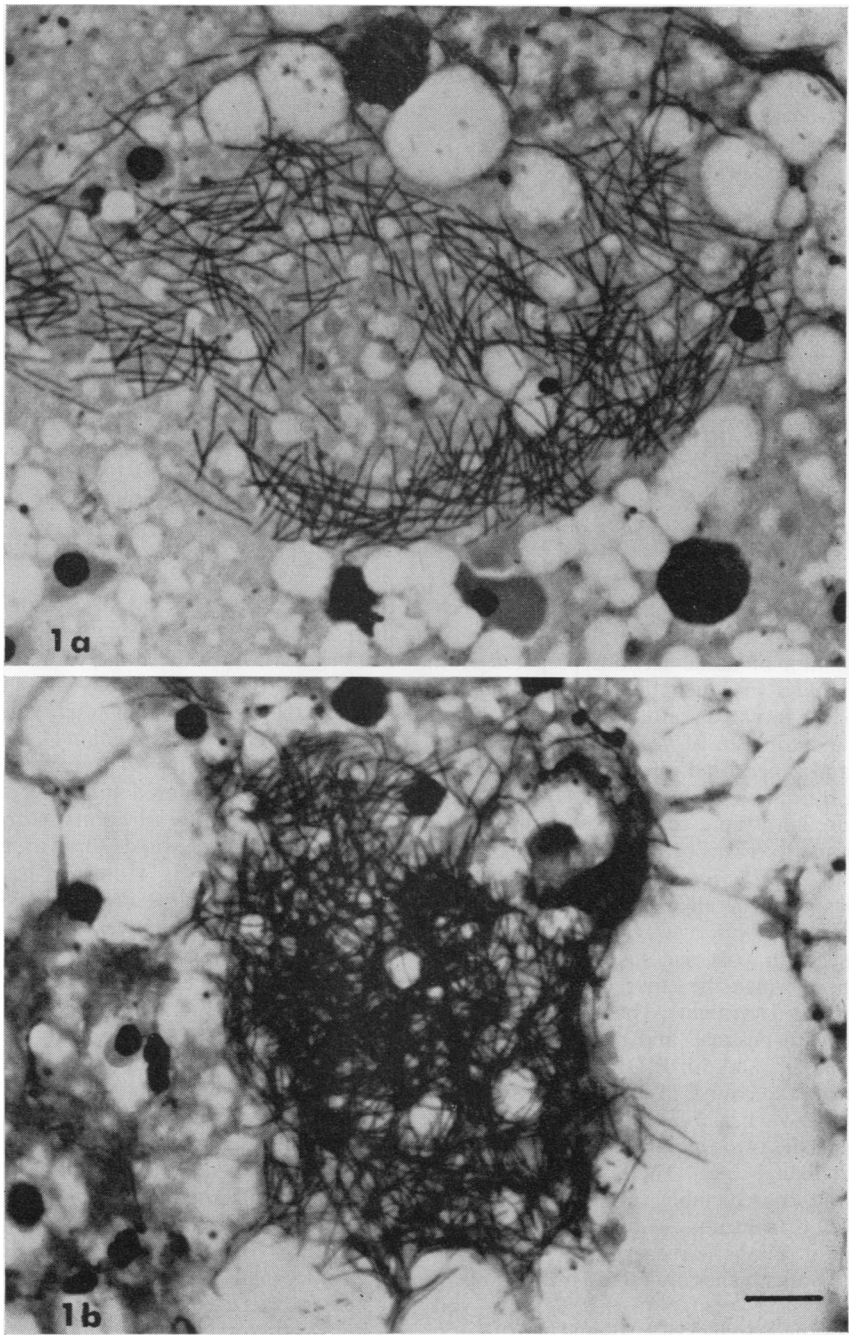

FIG. 1. *Predominant form of Bacillus piliformis seen in epithelial cells of a yolk sac smear. (a) Numerous single bacilli are evenly stained and randomly arranged within the host cell. (b) A heavily parasitized cell. Giemsa stain. Bar = 10 μm.*

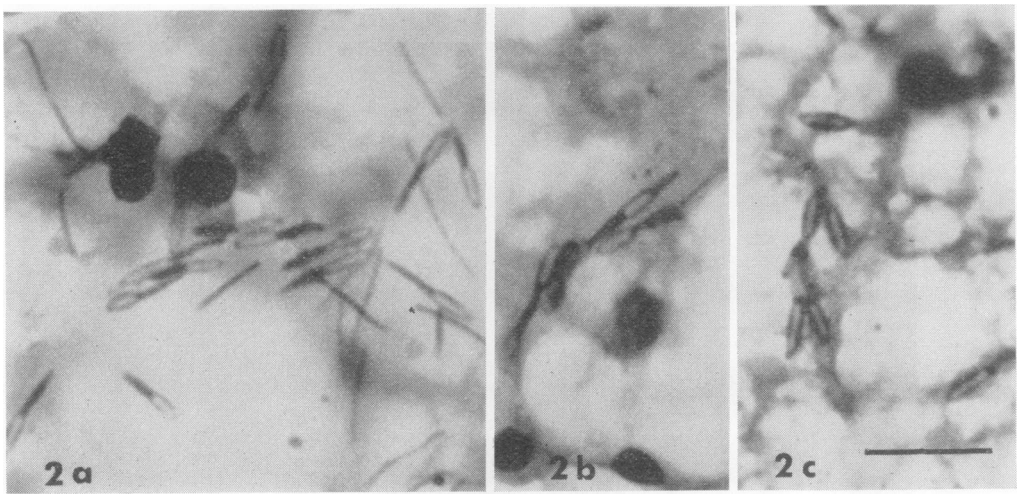

FIG. 2. *Spores of Bacillus piliformis in smears of yolk sac epithelium. (a) Predominantly seen terminal spores attached to thickened heavily stained bacilli. (b) Occasional subterminal spore. (c) Final form of spores. Giemsa stain. Bar = 10 μm.*

amined by aerobic and anaerobic cultural techniques.

Examination of tissue sections prepared from inoculated eggs in which the embryo was dead less than 1 hr revealed scattered foci of *B. piliformis* in the cytoplasm of yolk sac epithelial cells (Fig. 4) and generalized infection of the embryo, including liver, skeletal muscle, kidney, heart, gut, lung, brain, and spinal cord. Though necrosis of the brain and liver was occasionally observed, the presence of *B. piliformis* in the varied embryonic tissues was not generally associated with lesions.

Serial passage was readily attained by inoculating eggs with yolk sac suspensions prepared from eggs in which the embryo died during the previous 18 hr (overnight). The incubation period decreased with passage, and most embryos died between the 3rd and 5th PID by the fourth passage. During the course of 35 serial passages, no change was noted in the morphological or tinctorial properties of *B. piliformis* or in its virulence for embryonated eggs. There was, however, a marked and unpredictable variation in the incubation period from time to time during passage. Numerous titrations were performed on passage material. Though generally falling in the range of $10^{2.0}$ to $10^{3.5}$ ELD_{50} per 0.5 ml of 20% (w/v) yolk sac suspension, extremes of < 10 and $> 10^{4.0}$ were occasionally encountered. An example of a yolk sac suspension titration resulting in high titer is shown in Fig. 5.

The effect of dose and day of embryo death postinoculation upon recoverable infectivity was studied. Groups of 12 eggs, 7-day embryonated, were inoculated with decimal dilutions of a freshly harvested yolk sac suspension pool which titered $5 \times 10^{2.0}$ ELD_{50} per ml and was prepared from eggs in which the embryos were found dead on the 5th PID. Starting on the 4th and extending through the 8th PID, individual yolk sacs from embryos recently dead (within 4 hr) were harvested and stored at −70 C. Each yolk sac was then thawed and titered in 7-day embryonated eggs. As in previous titrations, correlation was noted between dose and survival time of the embryo (e.g., Fig. 5). However, no correlation was noted between the dose or day of embryo death and the quantitative recovery of *B. piliformis*.

To determine whether *B. piliformis* would continue to multiply in eggs after death of the embryo, eggs were subsequently incubated at 37, 30, or 23 C. A large number of 7-day embryonated eggs were inoculated with a 20% suspension prepared from freshly harvested yolk sacs. Embryos found dead on the 4th or 5th PID were randomly grouped and held at these temperatures. Individual yolk sacs from three eggs of each group were harvested daily for 5 days. They were stored at −70 C, then thawed, and titered in 7-day embryonated eggs. No increase in recoverable infectivity was obtained (Table 1).

Eggs with varying aged embryos (5 through 11 day) were inoculated with 20% yolk sac suspension which had been frozen and thawed once. All appeared equally susceptible to infection as measured by day of death postinoculation and demonstration of *B. piliformis* in yolk sac smear preparations.

The effect of freeing *B. piliformis* from the host

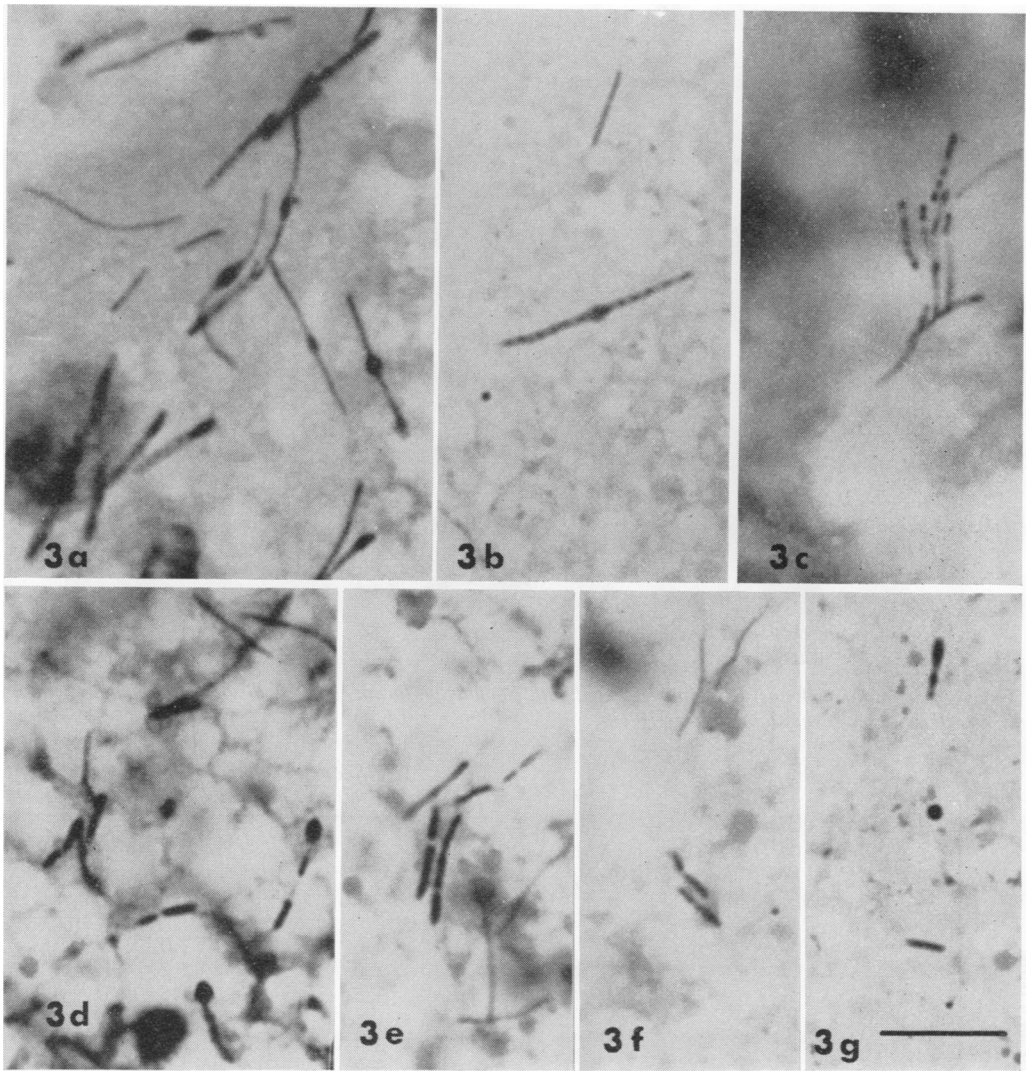

FIG. 3. *Forms of Bacillus piliformis seen less frequently but in same yolk sac smear as those shown in Figs. 1 and 2. (a) Terminal and subterminal monilial swellings in bacilli which vary greatly in length. (b) Subterminal monilial swelling in elongated bacillus showing numerous nucleoids. (c) Beaded bacilli. (d and e) Banding of bacilli. (f and g) Club-shaped and short form of bacillus. Giemsa stain. Bar = 10 μm.*

cell upon titration results was studied. Microscopic examination of stained smears of freshly harvested yolk sac suspension revealed that a large number of yolk sac epithelial cells, filled with *B. piliformis*, were still intact. Such a suspension was subjected to constant shaking with glass beads. A comparison of rough titrations (100-fold dilution) in 7-day embryonated eggs after 5 and 60 min of shaking at 23 C revealed little difference in the quantitative recovery of *B. piliformis* (Table 2).

Repeated rapid freezing (−70 C) and thawing did not destroy the infectivity nor decrease the titer of pooled yolk sac suspension which had been frozen once. Only spores and intensely stained short rod forms were seen in Giemsa-stained smears of yolk sac suspension after three cycles of freezing and thawing.

The effect of increased protein or carbohydrate in the diluent upon recovery of *B. piliformis* was examined. Samples of a fresh, rapidly prepared yolk sac suspension in PBS were titered in 7-day embryonated eggs using three diluents: 10% unheated horse serum in PBS, 5% sucrose in PBS,

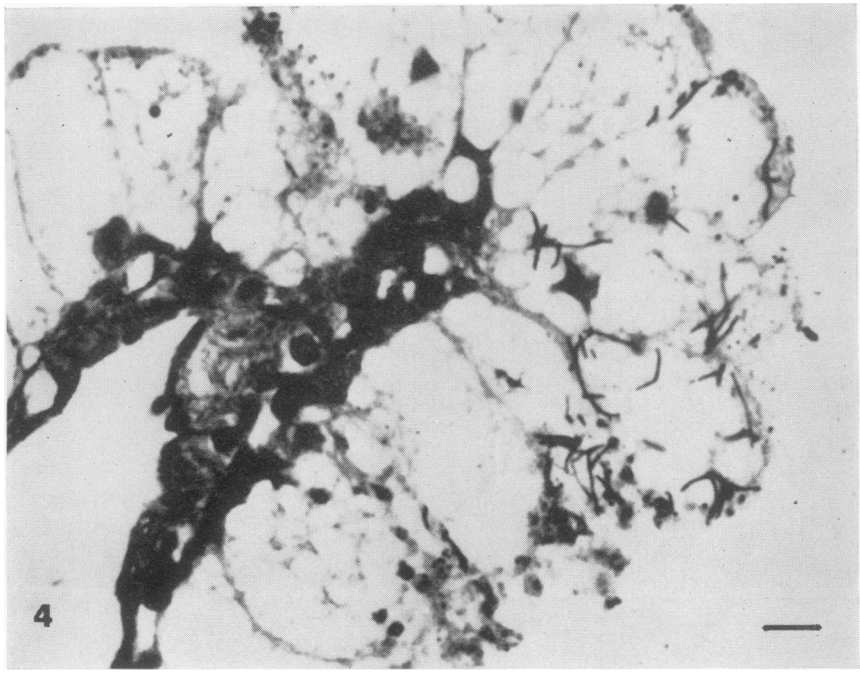

FIG. 4. *Section of yolk sac epithelium infected with Bacillus piliformis. The bacilli appear randomly within the cell. Warthin-Starry silver stain. Bar = 10 μm.*

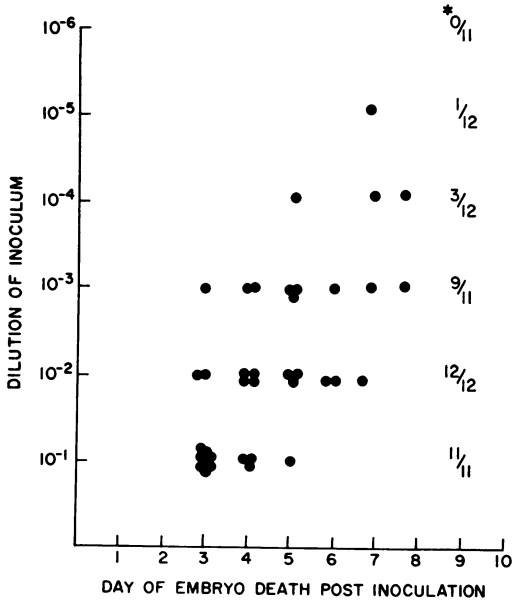

FIG. 5. *Titration of 10th passage Bacillus piliformis in 7-day embryonated eggs. Titer equals 3.6 log ELD_{50} per 0.5 ml of 20% (w/v) yolk sac suspension. Each dot represents an embryo death.* Number of embryo deaths per number of eggs inoculated.*

TABLE 1. *Effect of additional incubation after embryo death upon recovery of Bacillus piliformis*

Temp (C)	Days of incubation				
	1	2	3	4	5
23	4.0[a]	2.0	2.0	1.9	2.2
	3.0	3.1	2.2	1.6	2.7
	3.5	1.5	3.5	1.3	2.2
30	3.4	<1.0	3.3	1.2	2.2
	3.0	2.6	2.3	1.2	1.0
	<1.0	2.6	2.2	2.0	
37	1.8	1.8	2.2	2.5	<1.0
	<1.0	2.9	2.6	2.2	2.7
	2.0	2.5	2.9	1.7	

[a] Titers of individual yolk sacs expressed as log 10 ELD_{50}/0.5 ml of 20% yolk sac suspension (w/v).

and PBS only. Numerous filamentous forms of *B. piliformis* were present, both intra-and extracellular, in a Giemsa-stained smear of this yolk sac suspension. Neither the addition of horse serum nor sucrose affected the titer.

Motility of *B. piliformis* was observed with phase microscopy. The yolk sac of a recently

dead embryo was harvested and gently washed in PBS warmed to 37 C. Small bits of yolk sac epithelium were excised and pressed between a cover slip and slide. The edges were sealed with a mixture of equal parts of melted paraffin and vaseline, and the slide was examined immediately by phase microscopy (1,250×). Slow but distinct forward and backward movement of extracellular filamentous rods was observed. In the absence of obstruction, the bacilli quickly traversed the field of vision.

Studies to determine the stability of the vegetative phase of *B. piliformis* were hampered by the ever present resistant stage, the spore. Yolk sac suspension remained infective for embryonated eggs for 4½ yr when stored at −70 C; when allowed to dry, it was infective for 2 yr at 23 C, the longest time tested. Yolk sac suspension, repeatedly subjected to freeze and thaw and containing only spores as evidenced by microscopic examination of stained smears, was infective for embryonated eggs after heating to 56 C for 1 hr but not after 80 C for ½ hr.

None of the antibacterial agents tested (Table 3) was completely inhibitory. Streptomycin, erythromycin, penicillin, and chlortetracycline were active. *B. piliformis* was resistant to chloramphenicol and sulfamethazine. Passage of *B. piliformis* in the presence of either of these two agents was successful with no detectable effect on the organism.

Tyzzer's disease was readily induced in weanling rabbits stressed with cortisone and given yolk-sac-passaged *B. piliformis* by mouth. Each of four rabbits which received *B. piliformis* died or was killed while moribund by the 10th day. Typical lesions of Tyzzer's disease were seen, and *B. piliformis* was recovered from the liver of each rabbit in embryonated eggs. None of the control littermates died or showed evidence of Tyzzer's disease prior to their being killed on the 10th day. Lesions of Tyzzer's disease were not present in

TABLE 2. *Effect of increased time of shaking with glass beads upon recovery of Bacillus piliformis from yolk sac suspension*

Time of shaking (min)	Dilution of inoculum	Embryo mortality		Mean survival time (days)
		No.	Per cent	
5	Undiluted	10/10[a]	100	4.0
	10^{-2}	4/10	40	6.5
	10^{-4}	2/7	29	10.0
60	Undiluted	9/9	100	4.6
	10^{-2}	4/10	40	9.5
	10^{-4}	0/9	0	

[a] Number of dead embryos per number of eggs inoculated.

TABLE 3. *Effect of antibacterial agents on Bacillus piliformis infection in 7-day embryonated eggs*

Antibacterial		Embryo mortality[a]			
Agent	Dose per egg (mg)	1st Trial	2nd Trial	Total	Per cent
Erythromycin	0.25	3/13	2/10	5/23	22
	0.025	7/12	5/8	12/20	60
Penicillin-G	5,000[b]	2/9	2/9	4/18	22
	500[b]	7/13	5/10	12/23	52
Streptomycin	5.0	1/15	1/9	2/24	8
	0.5	7/11	5/8	12/19	63
Chlortetracycline	1.0	5/11	3/8	8/19	42
	0.1	6/15	5/10	11/25	44
Chloramphenicol	0.25	10/10	6/6	16/16	100
	0.025	10/11	7/7	17/18	94
Sulfamethazine	2.0	10/10	9/9	19/19	100
	0.2	10/10	8/8	18/18	100
Control		14/14	9/9	23/23	100

[a] Number of dead embryos per number of eggs inoculated.
[b] Measured in units.

these animals, and attempted isolation of *B. piliformis* from the liver of these rabbits was unsuccessful.

DISCUSSION

The failure to obtain growth in a variety of cell-free media from known virulent egg-passaged material emphasizes the requirement of living cells for the propagation of *B. piliformis*. This finding reinforces the histologic evidence seen in tissue sections from diseased rabbits and inoculated embryonated eggs, wherein *B. piliformis* is seen only in apparently viable cells. Though it is remotely possible that *B. piliformis* is incidental to the morbid process, the induction of typical Tyzzer's disease of rabbits by oral administration of high egg-passaged *B.piliformis* and the subsequent reisolation of the organism from the diseased rabbits indicates a primary causative role of *B. piliformis*.

The initial isolation of *B. piliformis* from diseased rabbit liver suspension in embryonated eggs was dependent upon preventing the entry of other enteric bacteria into the portal circulation. A combination of timed exposure to infection and the administration of a nonabsorbable antibacterial agent, sulfaquinoxaline, was used successfully. Under these circumstances, *B. piliformis* was isolated from the liver of a rabbit within 72 hr of exposure to contaminated bedding and prior to the development of liver lesions. The use of chloramphenicol seemed beneficial in the subsequent reisolation of *B. piliformis* from the liver of experimentally induced cases of Tyzzer's disease.

Considerable evidence suggests that our results were mainly dependent upon the stable phase or spore. According to Smadel and Jackson (16), concentrations of the order of 10^6 rickettsia per g of yolk sac tissue are required in order to recognize with assurance the organisms in stained smears. Though we experienced no difficulty in demonstrating *B. piliformis* in yolk sac smears throughout the course of these studies, the recoverable infectivity consistently fell below 10^3 ELD_{50} per g of yolk sac tissue. The unstable nature of the filamentous vegetative phase, an apparent characteristic of *B. piliformis* (4, 6) is probably reflected in the unpredictable and generally low titers experienced during this study. All attempts to increase the recoverable infectivity of *B. piliformis* from embryonated eggs failed. Similarly, Craigie (4) found titrations impracticable and restored to the mean survival time of embryos as a quantitative procedure. This approach was useful only when working with a nonsporing mutant strain, however. Here again, the vegetative form rapidly lost its infectivity in vitro, and no method was found to halt this loss in the fluid state. Moreover, freeze and thaw of this strain resulted in a 99% or more loss in residual infectivity.

Differences noted in antibacterial sensitivity of *B. piliformis* in different laboratories may indicate strain differences or may reflect differences in technique. For example, Craigie (4) concluded that penicillin, the only antibiotic tested, was very effective in that 1,000 units, given within 8 hr of infection, consistently prevented the infection in embryonated eggs whether using sporing or nonsporing strains of *B. piliformis*. He also noted (5) that penicillin, administered orally to mice injected with sporing *B. piliformis* by the intraperitoneal route, was effective in markedly altering the course of the disease. Takagaki and associates (18), however, tested penicillin, colimycin, erythromycin, chloramphenicol, streptomycin, tetracycline, and a variety of compounds containing sulfa. They concluded that only tetracycline showed a protective and therapeutic effect upon mice experimentally infected with *B. piliformis*. We found a graded effect of streptomycin, erythromycin, penicillin, and chlortetracycline and no effect of chloramphenicol or sulfamethazine in embryonated eggs. It is possible that other factors should be considered such as *B. piliformis* challenge dose, antibiotic concentration, the presence or absence of spores in the challenge dose, and the ability of the antibiotic to reach adequate intracellular levels.

Evidence for the motility of *B. piliformis* was first provided by Craigie (4) who studied mouse isolates in yolk sac epithelial cells by phase microscopy. Peritrichous flagella of *B. piliformis* were subsequently demonstrated in liver tissue of diseased mice (7) and rats (10) by electron microscopy. Our rabbit isolates appear motile by phase microscopy and, in this respect, are similar to the mouse isolates.

The taxonomic position of *B. piliformis* remains unknown. Further characterization is necessary before classification can be attempted. In addition, the relationship of the mouse, gerbil, and rabbit isloates needs to be determined. However, there is general agreement that *B. piliformis* is associated with a naturally occurring, often fatal, enterohepatitis of mice, rats, gerbils, rabbits, and primates; it is an obligate intracellular, gram-negative, motile bacterium which forms spores and is sensitive to certain antibiotics.

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