Virulence Conversion of *Legionella pneumophila* Serogroup 1 by Passage in Guinea Pigs and Embryonated Eggs

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When virulent *Legionella pneumophila* is passaged on supplemented Mueller-Hinton agar, it remains virulent for guinea pigs and embryonated hen eggs for two passages. However, by the fifth passage the cultures become avirulent for guinea pigs. Flagella were not produced by *L. pneumophila* on the first passage on supplemented Mueller-Hinton agar. In contrast, 12 passages on charcoal-yeast extract agar did not result in the reduction of virulence or the loss of flagella of *L. pneumophila*. Growth in supplemented yeast extract broth or on Norit-A-filtered supplemented yeast extract agar also did not result in a reduction of the virulence of *L. pneumophila*. However, *L. pneumophila* did not produce flagella when grown on these two media. Thus, it appears that the production of flagella is not required for the virulence of *L. pneumophila* when administered by the intraperitoneal route of infection. A virulent flagellated form of *L. pneumophila* was recovered by passing an avirulent form six times in guinea pigs. When avirulent *L. pneumophila* was passaged 12 times in embryonated eggs, a nonflagellated form of the bacterium was recovered which had an increased virulence for guinea pigs and embryonated eggs. However, virulent forms were not recovered by passage of avirulent forms on commonly used laboratory media. These results support the suggestion that a suitable host is required for the selection of the virulent form of *L. pneumophila* from avirulent cultures.

Legionella pneumophila was first isolated in 1977 by McDade et al. (9). Since that time several investigators have developed new media in an attempt to improve upon the isolation and growth of this organism (1, 5, 6, 10, 12-15). However, little is known about the effect of these media on the virulence characteristics of *L. pneumophila*. McDade and Shepard were the first to show that within 10 passages on either modified Mueller-Hinton agar (2) or Feeley-Gorman agar (6) *L. pneumophila* lost its virulence for guinea pigs and embryonated eggs (8). However, little has been done to quantitatively evaluate the virulence of *L. pneumophila* after passage on the commonly used laboratory media. Also, little is known about the conditions necessary for recovering virulent strains from avirulent cultures. Recently Wong et al. have reported that a virulent form of *L. pneumophila* can be recovered by passing avirulent cultures in human embryonic lung fibroblast tissue culture (16). In the present study we have shown that virulent strains can be recovered from an avirulent form of *L. pneumophila* serogroup 1 by passage in either guinea pigs or embryonated eggs.

Chandler et al. have recently reported that flagellated *L. pneumophila* can be found in lung tissue sections taken from patients who have

died from legionellosis and have speculated that flagella may be one of the factors which contribute to the rapid spread of infection in the lung (3). We were therefore interested in evaluating the contribution of flagella to the pathogenesis of *L. pneumophila* and in determining whether the recovery of virulent strains from avirulent cultures (hereafter referred to as the avirulent-to-virulent conversion) was associated with production of flagella. Our studies suggest that flagella are not necessary for *L. pneumophila* to establish infection in guinea pigs when the bacteria are administered by the intraperitoneal route.

MATERIALS AND METHODS

Organisms. A virulent strain of *L. pneumophila* serogroup 1 (Philadelphia 2) was obtained from the Centers for Disease Control, Atlanta, Ga. The guinea pig 50% lethal dose (LD₅₀) was 4.1×10^6 colony-forming units. Virulent bacteria were obtained from infected guinea pigs and stored as frozen (-70°C) suspensions.

Animals. Male guinea pigs weighing 250 to 350 g were obtained from local suppliers. Embryonated hen eggs that were 7 to 10 days old were also obtained from local suppliers. Embryonated eggs were maintained in a humidified incubator at 35°C.

Media. Bacteria from a frozen spleen suspension were cultured on the following media: charcoal-yeast extract (CYE) agar (5), Mueller-Hinton agar supple-

mented with 0.025% ferric pyrophosphate and 0.025% cysteine (SM-H agar), 1% yeast extract broth supplemented with 0.025% ferric pyrophosphate and 0.025% cysteine (SYE broth) (12), and supplemented yeast extract agar which was prepared by first filtering the SYE broth through Norit-A charcoal before the addition of agar (F-SYE agar).

Cultures were incubated in a humidified incubator at 35°C.

Effect of culture medium on virulence. Bacteria were grown on the various culture media according to the passage schedules listed in Results. After maximum growth was obtained (24 to 72 h), the bacteria were harvested in sterile 0.85% saline. Colony counts were determined by plating serial 10-fold dilutions on CYE agar. Groups of four guinea pigs were injected intraperitoneally with 0.5 ml of appropriate dilutions. Deaths were recorded for 10 days. The guinea pig LD₅₀ was calculated by the method of Reed and Muench (11).

The LD₅₀ for embryonated eggs was determined by injecting 0.1 ml of the appropriate dilutions into the yolk sacs of 7- to 10-day-old embryonated eggs (9).

Avirulent-to-virulent conversion. Bacteria passaged 12 times on SM-H agar were used for the avirulent-to-virulent conversion. For the first guinea pig passage, two guinea pigs were inoculated intraperitoneally with approximately 10¹⁰ colony-forming units of bacteria obtained from the 12th passage on SM-H agar. After 48 h, the spleens were removed and a 10% spleen suspension was made in saline. Each of three plates of CYE agar was swabbed with 0.1 ml of each spleen suspension. When heavy growth was obtained (after 4 to 5 days), the bacteria were removed from the agar with sterile saline and diluted with saline to approximately 10¹⁰ colony-forming units per ml. A 0.5-ml sample of this dilution was injected intraperitoneally into each of two guinea pigs. The bacterial suspension was also checked for flagella by electron microscopy (4). Spleens were removed at 48 h or after signs of infection were noted, i.e., either a fever greater than 40°C or ruffled fur and watery eyes. The spleens were then homogenized and diluted with sterile saline to form a 10% suspension. Samples of each spleen suspension were swabbed onto CYE agar. After confluent growth was obtained, the bacteria were removed with saline from a randomly selected agar plate. A 0.5-ml portion of this suspension, which contained approximately 10¹⁰ colony-forming units per ml, was injected intraperitoneally into each of two guinea pigs. The bacterial suspension was checked for flagella as previously described (4). Passage continued in a like manner with a progressively lower inoculating dosage as the number of virulent bacteria in the culture increased. After six passages in guinea pigs, the LD₅₀ values for guinea pigs and embryonated eggs were calculated.

For the avirulent-to-virulent conversion in embryonated eggs, approximately 10⁹ colony-forming units of bacteria obtained from the 12th passage on SM-H agar were inoculated into the yolk sac of twelve 7- to 10-day-old embryonated hen eggs. The yolk sacs were harvested from those eggs whose embryos died after 3 days and then were plated onto CYE agar. After heavy growth was obtained, the bacteria were removed from the agar surface with saline and diluted to approximately 10⁹ colony-forming units per ml. Twelve 7- to

10-day-old embryonated eggs were injected with 0.1-ml samples of this dilution. The dilution was checked for flagella by electron microscopy. Passage continued in a like manner with a progressively lower inoculating dosage. After 12 passages in embryonated eggs, the LD₅₀ values for guinea pigs and embryonated eggs were calculated.

Estimation of flagella. The approximate percentage of flagellation of *L. pneumophila* was estimated by electron microscopy and scored as negative (-), 1⁺ (<25%), 2⁺ (25 to 50%), 3⁺ (50 to 70%), and 4⁺ (75 to 100%).

RESULTS

Effects of media on maintenance of virulence.

As shown in Table 1, when spleen suspensions of virulent *L. pneumophila* serogroup 1 were grown on CYE agar, the organisms remained virulent for guinea pigs and embryonated eggs for at least 12 passages (CYE₁₂; the subscript denotes the number of passages on the medium) and remained flagellated. When the spleen suspension was plated on CYE agar (CYE₁) and then was passaged on SM-H agar, the organisms remained virulent for the first and second passages on SM-H agar (CYE₁ → SM-H₁, CYE₁ → SM-H₂; the arrow indicates a transfer from one medium to another). However, after 5 passages on SM-H agar (CYE₁ → SM-H₅), the organisms became avirulent for guinea pigs and remained avirulent for at least 12 passages on SM-H agar. They also lost their flagella on the first passage on SM-H agar (CYE₁ → SM-H₁). Flagella were not produced on any subsequent passages on SM-H agar.

Once the bacteria lost their virulence by passage on SM-H agar, virulence for guinea pigs was not recovered after 17 passages on CYE agar (CYE₁ → SM-H₁₂ → CYE₁₇). Also, after 17 passages on CYE agar (CYE₁ → SM-H₁₂ → CYE₁₇), the bacteria had the same LD₅₀ for embryonated eggs as they had after 19 passages on SM-H agar (CYE₁ → SM-H₁₉). Thus, virulence for guinea pigs and embryonated eggs was not increased by passing avirulent *L. pneumophila* on CYE agar.

Twelve passages in SYE broth (CYE₅ → SYE₁₂) or seven passages on F-SYE agar (CYE₅ → F-SYE₇) did not result in the loss of virulence of *L. pneumophila*. However, passage on both of these media resulted in a loss of flagella. Therefore, it appears that flagella are not required for virulence.

Avirulent-to-virulent conversion. When avirulent *L. pneumophila* from the 12th passage on SM-H agar was passed six times in guinea pigs (GP₆), a form virulent for guinea pigs and embryonated eggs was recovered (Table 2). This fully virulent form was also flagellated (CYE₁ → SM-H₁₂ → GP₆ → CYE₂).

When avirulent *L. pneumophila* from the 12th passage on SM-H agar was passaged in embry-

TABLE 1. Effects of media on the virulence and flagellation of *L. pneumophila* serogroup 1

Cultivation conditions ^a	LD ₅₀ ^b		Flagellation
	Guinea pig	Embryonated egg	
CYE ₁	4.1 × 10 ⁶	<10	4 ⁺
CYE ₁₂	3.8 × 10 ⁶	190	4 ⁺
CYE ₁ → SM-H ₁	7.1 × 10 ⁶	ND ^c	—
CYE ₁ → SM-H ₂	1.1 × 10 ⁷	ND	—
CYE ₁ → SM-H ₅	>2.7 × 10 ⁹	ND	—
CYE ₁ → SM-H ₁₂	>1.3 × 10 ⁹	ND	—
CYE ₁ → SM-H ₁₉	ND	2.5 × 10 ³	—
CYE ₁ → SM-H ₁₂ → CYE ₁₇	>4.5 × 10 ⁹	2.9 × 10 ³	—
CYE ₅ → SYE ₁₂	7.76 × 10 ⁶	ND	—
CYE ₅ → F-SYE ₇	1.07 × 10 ⁶	ND	—

^a The subscripts denote the number of passages on the media. The arrows indicate a transfer from one medium to another.

^b The LD₅₀ is in colony-forming units.

^c ND, Not determined.

onated eggs 12 times (Egg₁₂), a form virulent for guinea pigs and embryonated eggs was also recovered. However, this virulent form recovered from the egg passages was not as virulent for guinea pigs (LD₅₀, 7.9 × 10⁷) as the guinea pig-passaged form (LD₅₀, 5.1 × 10⁶), and it was also nonflagellated (CYE₁ → SM-H₁₂ → Egg₁₂ → CYE₁).

DISCUSSION

Several media have been developed in an attempt to improve the isolation and growth of *L. pneumophila* for the purpose of investigating the metabolism and the pathogenic mechanisms of this organism (1, 5, 6, 10, 12–15). The possibility that these media may alter the characteristics of *L. pneumophila* was first suggested by McDade and Shepard (8). They found that the virulence of *L. pneumophila* for guinea pigs and embryonated eggs was lost by passage on either modified Mueller-Hinton agar or Feeley-Gorman agar. However, Wong et al. recently reported that virulence for guinea pigs can be maintained for 12 passages on CYE agar (16). Our study confirms the results of Wong et al. that virulence for guinea pigs, as well as embryonated hen eggs, can be maintained when the bacteria are passaged on CYE agar. In addition, we have shown that virulence for guinea pigs is maintained after passage either on F-SYE agar or in SYE broth. However, *L. pneumophila* lost both its flagella and its virulence for guinea pigs

after five passages on SM-H agar. Of the media tested, only CYE agar supported the growth of virulent, flagellated *L. pneumophila*.

From these results, it is apparent that CYE agar is, at present, the best medium for maintaining both the virulence and flagellation of *L. pneumophila*. Since *L. pneumophila* will remain viable and virulent after storage on CYE agar for several months at either room temperature (7) or 4°C (unpublished observation), CYE agar may be used to maintain bacteria without the loss of virulence after extended passage on CYE medium.

Although the SYE broth and F-SYE agar do not support the production of flagella by *L. pneumophila*, the bacteria do remain virulent when cultured in these media. The SYE broth therefore provides a medium which can be used to investigate the metabolism and possible toxin production of virulent *L. pneumophila*. Since the F-SYE agar is a clear medium and contains no charcoal, it could be used to screen for any *L. pneumophila* bacteriophages which are not specific for flagella and could possibly be used for antibiotic sensitivity testing of virulent strains.

The loss of virulence by *L. pneumophila* after passage on SM-H agar is not permanent. Recently Wong et al. have reported the recovery of virulence by passage in human embryonic lung fibroblast tissue culture (16). Our studies show that virulence can also be recovered by passage in guinea pigs and embryonated hen eggs. Our

TABLE 2. Avirulent-to-virulent conversion of *L. pneumophila* serogroup 1^a

Cultivation conditions	LD ₅₀		Flagellation
	Guinea pig	Embryonated egg	
CYE ₁ → SM-H ₁₂ → GP ₆ → CYE ₂	5.1 × 10 ⁶	9	4 ⁺
CYE ₁ → SM-H ₁₂ → Egg ₁₂ → CYE ₁	7.9 × 10 ⁷	28	—

^a See Table 1 for an explanation of symbols.

results also confirm the observation by Wong et al. that virulence is not recovered by passage on CYE medium. The requirement for passage in guinea pigs, embryonated eggs, or tissue culture cells for the recovery of virulence supports the view that the host may be selecting for a virulent form of *L. pneumophila* (16).

It has been suggested by Chandler et al. (3) that flagella may contribute to the rapid spread of infection by *L. pneumophila* in the lungs and that, therefore, flagella may contribute to the pathogenesis of *L. pneumophila*. Our results indicate that flagella are not required for the full expression of virulence by *L. pneumophila* when administered by the intraperitoneal route. When the virulent, flagellated *L. pneumophila* cells were cultured on F-SYE agar, they lost their flagella with no reduction in their virulence for guinea pigs. Bacteria which were recovered from guinea pigs injected with F-SYE agar-grown *L. pneumophila* remained nonflagellated when grown on CYE medium. Also, when avirulent bacteria were transferred from SM-H₁₂ to CYE agar and incubated at 25°C, they were approximately 25% flagellated, but there was no increase in their virulence (unpublished observations). However, these studies were performed with the intraperitoneal route of infection rather than with infection by the upper respiratory tract, which is proposed to be the natural route of infection caused by *L. pneumophila*. Studies are continuing to evaluate the contribution of flagella in establishing lung infections caused by *L. pneumophila*.

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