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Growth of *Salmonella enteritidis* in Yolk of Shell Eggs from Normal and Seropositive Hens

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ABSTRACT

The growth of *Salmonella enteritidis* inoculated into the yolks of shell eggs from normal and seropositive hens was determined at various temperatures. All eggs were inoculated with approximately 1 colony-forming unit (CFU)/g of yolk. In eggs from normal hens, the organism multiplied with a generation time of 25 min, reaching a density of about 10^8 CFU/g in 12 h at 37°C. A generation time of 3.5 h was observed in eggs incubated at 15.5°C, a temperature frequently used for commercial storage of eggs. Cell density of $>10^7$ CFU/g was reached in 4 d at 15.5°C. No multiplication was observed in eggs incubated at 7°C for 94 d. When inoculated eggs from seropositive birds were incubated at 37°C, the organism multiplied with a generation time of 35 min, reaching a cell density of $>10^6$ CFU/g in 12 h. Raw egg white was detrimental to cells, reducing cell viability 50% in 4 h at 37°C. The limulus amoebocyte lysate test gave a positive reaction with whole liquid egg containing $<10^3$ CFU/g. A protocol is suggested for possible application of this test in epidemiological studies that screen grade A shell eggs for *Salmonella* contamination.

The number of *Salmonella* infections reported annually to the Centers for Disease Control (CDC) has been increasingly gradually and progressively for the past 30 years and is now about 40,000 cases per year. These cases may represent only 1 to 5% of the actual yearly incidence of infection in the United States (*4*). Most strikingly, the incidence of *Salmonella* infection caused by one serotype, *Salmonella enteritidis*, has increased more than sixfold since 1976 in northeastern United States (*13*), with most outbreaks occurring in the summer months (*3*). Recent isolation rates for this serotype have also increased in the mid-Atlantic and south-Atlantic regions, and *S. enteritidis* is now the second most common serotype reported. Epidemiological studies of *Salmonella* outbreaks indicate that grade A whole shell eggs or food that contain such eggs are a major source of *S. enteritidis* human infections in the U.S. and in the United Kingdom (*6*).

The exact mechanism by which grade A table eggs become contaminated, however, is not clear. *S. enteritidis, S. pullorum, S. gallinarum, S. typhimurium,* and *S. heidelberg* have been isolated from the ovaries of naturally infected chickens (*11,12*), and it has been suggested (*13*) that transovarian infection of the developing ovum may have been the mechanism of egg contamination in current outbreaks of foodborne salmonellosis. The infection in laying hens does not seem to cause significant reduction in fertility (*7,10*). Recent experiences of state agencies in the U.S. and in the U.K. (*5*) indicate that a very low frequency of contamination of individual eggs occurs even when eggs are removed from known infected flocks. Undoubtedly, more information is required about the frequency with which infected birds lay infected eggs, the level of microorganisms present in individually infected eggs, the effects of antibody in eggs, and the effect of environmental temperatures on the kinetics of growth of this organism.

This study was undertaken to confirm recent observations (*8,9*) on the growth of *S. enteritidis* in shell eggs from normal birds and to gather additional data on the kinetics of growth in the yolk of shell eggs from seropositive birds. Investigations were also made into the usefulness of the limulus amoebocyte lysate (LAL) assay (*15*) for determining the presence of endotoxin in eggs to facilitate mass screening of retail grade A shell eggs.

MATERIALS AND METHODS

Bacteria and growth conditions

S. enteritidis C398 (81347), isolated from the ovary of a chicken previously inoculated with a human pathogenic isolate by C. Benson (University of Pennsylvania), was provided by CDC. *S. enteritidis* strain 19299-521, used by C. Beard, U.S. Department of Agriculture (USDA), Russell Agricultural Center, Athens, GA, to infect hens was kindly provided by C. Benson. Stock cultures were grown in trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) at 37°C for 18-20 h and maintained at -17°C after addition of glycerol to 40%. Cultures were routinely grown from stock cultures in TSBYE incubated at 37°C for 18-20 h and were transferred three times before use. For inoculation into eggs, the cells were centrifuged (5000 x *g* for 10 min), washed once in sterile saline (0.85% NaCl), and resuspended to an A_{620} of 0.2, or approximately 4×10^8 CFU/ml.

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Inoculation of eggs and enumeration of cells

Fresh grade A shell eggs, purchased from a local Ohio producer, were held at 4°C until used, i.e., within 1 week after laying. Shell eggs from birds, whose sera reacted with Group D *Salmonella* antigen, were kindly provided by C. Beard. All experimental eggs were incubated overnight at the desired temperature to equilibrate the yolk temperature before inoculation with bacteria. Eggs were swabbed with tincture of iodine, and a 1-mm hole was drilled through the shell before inoculation. A 0.1-ml aliquot containing 10^2 cells/ml was aseptically inoculated through the hole, into the yolk, with a Stepper pipette (Vanguard International, Neptune, NJ) equipped with a 23-gauge, 1 in. needle attached to a 1-ml tuberculin syringe. The hole was sealed with DuCo cement and the eggs were returned to the incubator. Five eggs from normal hens and three from seropositive hens were removed from the incubator at intervals and sanitized in 50 ppm household bleach for 5 min; the contents were aseptically placed into tared sterile petri dishes. Yolks were separated from the albumen and reweighed. After vigorous mixing, 0.1-g aliquots of yolk, or appropriate dilutions, were surface-plated onto duplicate xylose lysine desoxycholate (XLD) agar (*14*). If the expected cell count/g was less than 100 colonies, a total of 1 g of yolk was plated onto the surface of ten XLD plates. Colony forming units (CFU) were enumerated after incubation at 37°C for 48 h.

Growth studies in egg white

Egg while (albumen) was aseptically collected from eggs that had been sanitized as described previously. A 200-g test portion of prewarmed egg white, contained in a sterile flask with a stirring bar, was inoculated with *S. enteritidis* to a final concentration of approximately 4 x 10^3 CFU/g. The contents of the flask were mixed by placing the flask on a magnetic stirrer, and the flask was then returned to an incubator at 37°C. Aliquots were removed from the flask after the contents had been equally dispersed by mixing again for 30 s on a magnetic stirrer. The CFU were enumerated by surface spread-plating on XLD agar plates after incubation at 37°C for 48 h.

Limulus amoebocyte lysate test

Limulus amoebocyte lysate (LAL) test kits (E-TOXATE) were purchased from Sigma Chemical Co., St. Louis, MO. All glassware was depyrogenated by heating in air at 150°C for a minimum of 4 h. All test materials and reagents were determined to be endotoxin-free by LAL before the tests were performed. *Salmonella*-contaminated whole liquid egg, inoculated as described above, and uninoculated whole liquid egg were used as test samples for LAL assay using protocols prescribed by the manufacturer.

RESULTS

Growth in shell eggs

The kinetics of growth of *S. enteritidis* introduced into the yolks of shell eggs that were incubated at either 37, 15.5, or 7°C are presented in Fig. 1 and 2. At 37°C, with an initial inoculum of about 1 CFU/g of yolk, the organism multiplied with a generation time of about 25 min and reached a concentration of about 10^8 CFU/g in 12 h (Fig. 1). When similarly infected eggs were incubated at 15.5°C, the organism multiplied with a generation time of 3.5 h, and reached a density of about 10^2 CFU/g in 24 h, and 10^4 CFU/g in 48 h. Cell density of >10^7 CFU/g was reached in 4 d at this temperature (Fig. 2). No multiplication was observed in eggs incubated at 7°C (Fig. 2) up to 94 d, except for two eggs: on day 71, one had between 130 and 160 CFU/g of yolk; on day 85, one of five eggs had a count of 380 CFU/g of yolk.

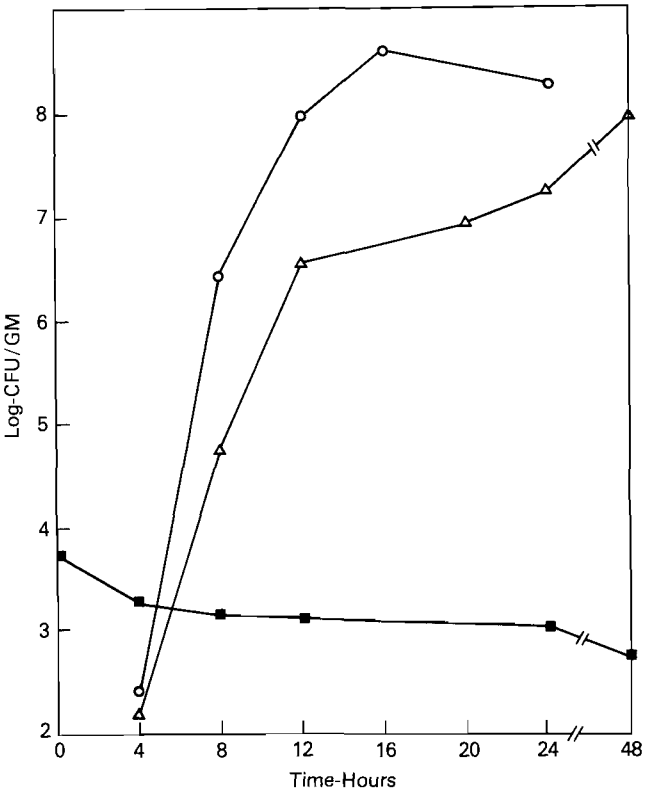

Figure 1. *Kinetics of growth of* Salmonella enteritidis *in the yolk of eggs from normal hens (○), seropositive hens (Δ), and egg white (■) incubated at 37°C.*

If transovarian infection of the developing ovum is the primary mechanism of egg contamination by this serotype, birds laying infected eggs are also likely to be seropositive, and eggs from such birds are expected to contain antibody to *Salmonella enteritidis*. We therefore investigated the outgrowth of low numbers of cells introduced into the yolk of shell eggs from seropositive birds. At 37°C, the organism multiplied with a generation time of 35 min and reached a titer of >6 logs within 12 h (Fig. 1).

Growth in egg white

Egg white did not support growth of the organism at 37°C (Fig. 1). The original inoculum of 4 x 10^3 CFU/g of egg white was reduced 50% in 4 h and 90% in 48 h.

LAL test

Since the frequency and level of contamination by *Salmonella* in eggs from an infected flock is likely to be low, the random sampling of individual eggs for microbiological examination is considered impractical unless eggs can be composited to eliminate large numbers of expected negatives. With the aim of developing such a protocol, we investigated the utility of the LAL assay as a rapid screening test for endotoxin in egg yolk and whole liquid egg.

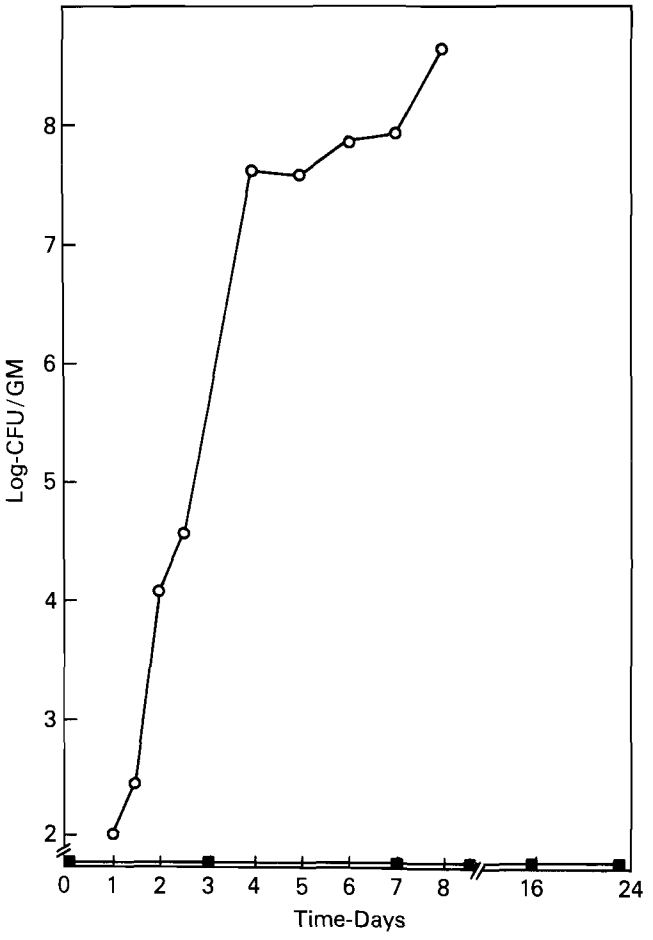

Figure 2. *Effect of temperature on the kinetics of growth of* Salmonella enteritidis *in the yolk of eggs from normal hens. Eggs incubated at 15.5°C (○) and at 7.2°C (■).*

Eggs inoculated with about 1 CFU/g of yolk and incubated at 37°C to allow multiplication of *Salmonella* to two different levels were broken, and serial tenfold dilutions of yolk and homogenized whole liquid egg were tested. The presence of 100-1000 cells/g in either of the test samples gave a positive reaction in LAL assay (Table 1).

TABLE 1. *Reactivity of* Salmonella*-contaminated egg yolk and liquid whole egg in LAL test.*

Test samples	*Salmonella* cells/g	Highest dilution positive in LAL
Egg yolk	6.4×10^7	10^{-5}
	3×10^4	10^{-2}
Whole liquid egg	9.3×10^8	10^{-7}
	1.4×10^3	10^{-1}

DISCUSSION

There are several ways in which shell eggs can become contaminated, externally or internally. Epidemiological investigations of recent outbreaks of foodborne illness caused by egg-associated *S. enteritidis* suggest that external contamination of eggs by chicken feces which contain this organism is an unlikely source. Most commercially produced eggs are collected in rollaway systems and eggs belts that minimize contamination, and these foodborne disease outbreaks have been associated with sanitized, grade A shell eggs which met federal and state standards for shell quality. Furthermore, gut colonization of chicken with salmonellae is usually associated with contaminated animal feeds. However, no *S. enteritidis* was isolated from animal feeds in recent surveys (*13*). Even if salmonellae were to be deposited in the pores of shells, they are walled-off from the highly nutritious yolk by shell membrane and albumen; any migrants from this source are usually unable to proliferate in the albumen (*1*). Our results clearly show that albumen in vitro not only suppressed growth but was detrimental to the cells. The multiplication of *S. enteritidis* inoculated into the albumen of shell eggs was reported (*9*); however, the growth, which occurred after prolonged incubation, was probably due to the union of the yolk and shell membrane, allowing the bacteria to utilize yolk nutrients.

The alternative possibility of transovarian transmission resulting in egg contamination is strongly suggested by isolation of this organism from breeder-multiplier flocks. Like *S. pullorum* and *S. gallinarum, S. enteritidis* can colonize the ovary and the peritoneum of naturally infected chickens (*11*), and the infection of laying birds does not always cause significant reduction in fertility (*7,10*). Thus, infection in either the peritoneum or the ovary may allow contamination of the egg yolk before shell formation, thereby facilitating vertical transmission of the disease.

It is common industry practice to store eggs at 13-16°C. This recommendation is primarily for eggs in market channels where lower temperatures may produce moisture condensation on the shell when cold eggs are brought to room temperature. It is thought that moisture on the shell may facilitate penetration by any pathogen on the shell into the internal contents of the egg.

Our data on the kinetics of growth of *S. enteritidis* in the yolk of shell eggs from normal birds show that any temperature abuse or storage of eggs at 16°C will allow rapid amplification of even low numbers of cells to levels high enough to cause human infection. Similarly, growth was not inhibited in shell eggs obtained from seropositive birds with antibody titers of 1:40 to 1:80 against *S. pullorum* antigen. Although we did not measure antibody titers in the yolk of these eggs, previous studies (*2*) with *S. pullorum* and *S. gallinarum* have shown the presence of agglutinating and nonagglutinating antibodies in the yolks of orally infected birds. However, neither cellular nor humoral mechanisms have been demonstrated that will inactivate salmonellae in the yolks of unfertilized eggs. In view of the common industry practice to operate egg collection belts after a 12-h light cycle, contaminated eggs may be exposed to ambient temperatures for many hours. The high incidence of *S. enteritidis* outbreaks during summer months may, in part, result from such a temperature abuse, which allows rapid multiplication of these organisms to levels high enough to cause human infection. *Salmonella* at a level of 10^8 CFU/g of yolk in artificially contaminated eggs have been shown to survive any conventional cooking methods (*8*).

At present, the random microbiological analysis of

retail eggs to determine contamination, even for epidemiological investigations, is considered impractical because of the expected very low frequency level of contamination and the inability to trace contaminated eggs to an individual producer. This inference is based on the finding that <0.5% of eggs from an infected flock may contain the organism. Our result on the use of LAL, which gave a positive test with whole liquid egg containing <1000 cells/g, may facilitate mass screening of grade A table eggs. For example, sanitized eggs can be broken out aseptically and 1.0 ml test portions from individual yolks can be refrigerated for subsequent analysis. A composite of 20-50 eggs, after overnight incubation at 37°C, can be tested by LAL assay to eliminate most of the expected negatives. Refrigerated portions of yolks from LAL-positive composites can be used to enumerate cells. Such analysis will provide more detailed information on the frequency and the level of contamination.

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