Research Note

Influence of a Nonfavorable Environment, Egg White, on Resistance to Heat and Disinfectant, Adhesion, and Virulence of Salmonella Enteritidis

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ABSTRACT

Although liquid egg white may be subjected to limited heat treatment when it is used in the fabrication of various foodstuffs, pathogenic bacteria such as Salmonella Enteritidis could persist in this environment. Liquid egg white is not a favorable medium for Salmonella growth because of its alkaline pH and iron deficiency and the presence of ovotransferrin. Microorganisms adapted to a nonfavorable environment are often more resistant to stresses than are their laboratory-cultured counterparts. The objective of this study was to determine whether Salmonella exposed to an environment mimicking egg white conditions exhibited modified behavior that could have an impact on food safety. A medium resembling egg white (filtrate of egg white with added ovotransferrin) was used as an adaptation treatment to mimic the stress imparted by the egg white environment. There were no changes in resistance to heat and disinfection, in stainless steel adhesion, or in the virulence of Salmonella Enteritidis cultivated in the egg white medium. Egg white conditions do not appear to make Salmonella more virulent or more difficult to inactivate.

Eggs and egg products have been reported as the major sources of confirmed Salmonella-associated food poisoning in France (13). Liquid egg white is used in the fabrication of various foodstuffs such as cakes, meringues, confectionary, sausages, and terrines (24). However, only limited heat treatment of egg white (57°C for 2 to 5 min) is possible because of the thermal fragility of egg white proteins; thus, Salmonella could persist in the treated product.

Liquid egg white is not a suitable medium for bacterial growth. For Salmonella, iron deficiency seems to be the major reason for growth inhibition, which is due to the binding of iron to ovotransferrin (a sequestering protein) (1, 11, 12, 17). Others factors such as alkaline pH (pH 9) (2, 11) and physical contact of ovotransferrin with Salmonella cells are also involved in Salmonella growth inhibition (2, 19, 31).

When bacteria are confronted with a nonfavorable environment, the adapted microorganisms are often more resistant to a range of stresses and more difficult to eradicate than would be predicted from experimental data obtained under laboratory growth conditions (7, 8). Because Salmonella cells are confronted with a nonfavorable environment in egg white, the aim of this study was to assess whether the egg white environment could make Salmonella more pathogenic or more difficult to eradicate from egg products or egg-breaking factories. We evaluated properties important for food safety, including heat resistance, disinfectant resistance, adhesion capacity, and virulence, using an animal model.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The Salmonella strain used was isolated from egg white (Agence Française de Sécurité Sanitaire des Aliments, Paris, France). Cells were conserved at −18°C and propagated at 37°C for 18 to 24 h in tryptic soy broth (TSB; Biokar, Beauvais, France) before use.

Adaptation treatment. Cells were washed three times by centrifugation (6,000 × g for 10 min at 20°C) and then suspended in adaptation media at a density of 10⁶ CFU/ml. Two adaptation media were used, a TSB optimum growth medium (pH 7) as a control and an egg white–resembling medium (EWRM), which is a filtrate of egg white with added ovotransferrin. Filtrate of egg white was obtained from raw liquid egg white using an ultrafiltration unit with a cutoff of 10,000 Da (Amicon, Beverly, Mass.) according to the method of Baron et al. (1). The filtrate is the egg white without protein and has the same pH, glucose, and ionic content (1). Ovotransferrin was added to the filtrate of egg white at 10% of the theoretical concentration found in egg white (1.3 g/liter; Sigma Chemical Co, St. Louis, Mo.). The iron content of egg white has been reported as insufficient to allow ovotransferrin saturation (27). Baron et al. (1) demonstrated that Salmonella

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growth after 1 day at 30°C in EWRM was comparable to the growth observed in egg white. EWRM contains the major inhibition factors of egg white for *Salmonella*, i.e., alkaline pH (pH 9), presence of ovotransferrin, and iron deficiency, and therefore mimics egg white conditions. EWRM was used as adaptation medium instead of egg white because many experiments involved cells recovered by centrifugation, which was not possible in egg white. Comparison between *Salmonella* growth at 30°C in TSB, in egg white, and in EWRM were performed to find the optimal incubation conditions that allowed cells to adapt. Samples were removed every hour, serial decimal dilutions in peptone water were prepared, and 1 ml of each dilution was plated onto tryptic soy agar (TSA; Biokar) plates. After incubation at 37°C for 24 h, the number of CFU was determined. After adaptation treatments, cells were subjected to different tests to determine whether treatment in EWRM caused adaptation or other modifications.

**Resistance to heat and disinfectants.** After adaptation treatments, cells were washed (6,000 × g, 10 min, 20°C) in 0.1 M potassium phosphate (pH 7) and then suspended in this buffer at a density of 10⁸ CFU/ml. For heat challenge, 1-ml samples of the two cultures were transferred into tubes prewarmed in a 57°C water bath. Some samples were removed every minute and diluted in 9 ml of peptone water. After decimal dilutions, bacteria were counted on TSA inoculated by the pour plate technique.

For disinfectant challenge, 1-ml samples of the two cultures were transferred into tubes containing different disinfectants (Table 1) provided by four egg-breaking factories and diluted following the manufacturers’ recommendations. The test was run at 30°C, and 1-ml samples were removed every 5 min and diluted in 9 ml of neutralizing solution as recommended by the manufacturers. Cells counts were performed as for heat resistance.

For both resistance challenges, experiments were replicated using three independently grown cultures for each adaptation treatment. After enumeration of bacteria, the data were analyzed by linear regression. The decimal reduction time (D-value), defined as the time required to kill 90% of the initial population, was calculated as the negative reciprocal of the slope of the survivor curve. The correlation coefficient (r²) for all trials was 0.95 ± 0.03, indicating that the survivor plots (log number versus time) were linear and D-values could be calculated from the slopes of the line and then compared using Student’s t test.

**Adhesion of Salmonella cells to stainless steel.** Prior to the adhesion experiments, stainless steel slides (AISI 304, Goodfellow, Cambridge Science Park, UK) were cleaned according to the method of Bouttier et al. (6). To mimic the conditions existing in an egg-breaking factory, the sterile slides were then immersed in egg white for 24 h at 30°C. The conditioned slides were rinsed twice in a filtrate of egg white (to remove egg white protein) and placed into petri dishes.

After adaptation treatments, cells were washed three times (7,000 × g, 15 min, 12°C) in 1.5 mM NaCl. The cell pellets were diluted to obtain a bacterial suspension of 10⁷ CFU/ml, and 30 ml of each suspension was transferred onto petri dishes containing the conditioned slides. After 4 h of contact at 30°C, the slides were rinsed six times by immersion in 9 ml of tryptone salt to remove the nonadherent bacteria. The slides were then transferred to 9 ml of tryptone salt and sonicated for 10 min to resuspend the adherent cells. Preliminary experiments showed that this treatment did not affect cell viability. The bacteria were counted on TSA inoculated with the pour plate technique. Experiments were replicated using three independently grown cultures for each medium, and the results were compared using Student’s t test.

**Virulence studies: mouse model.** After the adaptation treatments, cells were washed three times (8,000 × g, 5 min, 20°C) in phosphate-buffered saline (PBS). The bacterial pellets were diluted to obtain seven different inoculum doses: 1 to 10⁶ cells per mouse. Mice (C57BL/6, Centre d’Elevage R, Janvier, Le Genest Saint Isle, France) were infected intraperitoneally with a 0.1-ml dose, five mice per dose per adaptation treatment. Mice were kept in individual cages in two rooms corresponding to two adaptation treatments to prevent cross-infection, and two mice per room were inoculated with sterile PBS (controls). Each day, the dead mice were removed and preserved at −20°C, and after 4 weeks, all surviving mice were euthanized. The spleen and liver of each mouse were aseptically removed and homogenized in a mortar with 2 ml of PBS. Serial dilutions of each homogenate were made in tryptone salt and plated on TSA and Hektoen agar (bioMérieux, Marcy l’Etoile, France). Colonies were confirmed as *Salmonella Enteritidis* using slide agglutination. The absence of *Salmonella* in control mice was checked by enrichment procedures following the European standard V-08-053. LD₅₀ titers (the dose that kills 50% of the animals) were calculated according to the method of Reed and Muench (23) from results obtained from five mice per inoculum dose.

**Virulence studies: chicken model.** After the adaptation treatments, cells were washed three times by centrifugation (8,000 × g, 5 min, 20°C) in PBS. The bacterial pellets were diluted to obtain an inoculum dose of 10⁶ cells per ml. Newly hatched layer chickens (ISA, Quintin, France) were randomized in three isolated chambers (20 chickens per chamber) corresponding to two adaptation treatments and one control (no inoculation) to prevent cross-infection. Chickens were inoculated orally at 1 day of age (to limit the role played by barrier flora) with a 0.5-ml inoculum dose. A large number of cells were inoculated because the oral route is less sensitive than the intraperitoneal route (18). Twelve days post-infection, all chickens were euthanized, and the cecum, liver, and spleen were collected aseptically to enumerate *Salmonella* cells. Serial dilutions of each homogenate were made in tryptone salt and plated on Rambach agar (bioMérieux). The typical colonies were confirmed by slide agglutination testing. To confirm that control chickens had no *Salmonella* cells, we utilized enrichment culture as for control mice. The number of *Salmonella* cells recovered from the organs of chickens in the two adaptation treatment groups were compared using Student’s t test.

**RESULTS AND DISCUSSION**

The behavior of *Salmonella* cells was comparable in EWRM and egg white (Fig. 1). Therefore, EWRM appears to be a valid model medium to mimic the unfavourable conditions of egg white for *Salmonella*. An incubation period of 10 h was chosen for adaptation treatments. This period was considered appropriate for studying the consequences of EWRM exposure because it mimics real-life

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<th>Disinfectant</th>
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<tr>
<td>I</td>
<td>Lauryl propylene diamine</td>
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<tr>
<td>II</td>
<td>Polyhexamethylene biguanide</td>
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<tr>
<td>III</td>
<td>Sodium hydroxide</td>
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<td>IV</td>
<td>Glutaraldehyde</td>
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conditions (e.g., egg products may be left 12 h between breaking and heat treatment).

**Resistance to heat and disinfectants.** The D-values of cells cultivated in TSB and those cultivated in EWRM were not significantly different for the heat and disinfectants tested (Table 2). Prokaryotic cells respond to sublethal environmental or chemical stresses by inducing specific sets of proteins that confer protection against subsequent exposure to the stress. Proteins associated with one stress also can be induced during other stresses, and a cross-protection effect has been demonstrated (8, 15, 28). However, *Salmonella* cells cultured in medium without iron (10, 30) and during heat shock (29) did not produce the same proteins. Our results indicate that egg white conditions (iron deficiency, ovotransferrin presence, and alkaline pH) did not modify the behavior of *Salmonella* cells subjected to a 57°C heat shock (temperature of egg white heat treatment) or of *Salmonella* cells subjected to the action of the primary disinfectants used in egg-breaking factories.

**Adhesion capacity.** Adhesion to stainless steel slides conditioned with egg white was similar for cells cultivated in TSB or in EWRM, about $10^3$ adherent CFU/cm² (initial suspension of $10^7$ CFU/ml). Bouttier et al. (6) obtained similar adhesion results for another strain of *Salmonella* using similar experimental conditions but with slides conditioned with water (our slides were conditioned with egg white to mimic the stainless steel surfaces in egg-breaking factories). Bacterial adhesion to inert equipment surfaces could promote bacterial contamination of food products during processing (9). The adhesion capacity is dependent on environmental factors (pH, temperature, and medium), bacteria (surface properties, flagella, concentration, physiological state, metabolism, and nutritional stress), and physicochemical characteristics of both the microbial cells and the inert surfaces (4, 16). However, our results indicate that the ability of *Salmonella* Enteritidis to adhere to stainless steel surfaces of egg-breaking factories was not modified by conditions encountered during growth in EWRM.

**Virulence studies.** The virulence genes of many pathogenic bacteria are induced by environmental stress, especially nutritional stress. The ability of bacteria to survive these conditions contributes to their virulence (22).

The cells cultivated in TSB or EWRM prior to mouse inoculation were equally virulent in this study (Table 3). The difference between the LD$_{50}$ of cells grown in TSB and that of those grown in EWRM was not significant because the difference is affected by the inaccuracy of the dose inoculated into each mouse (confidence limits were calculated according to the method of Reed and Muench (25)). The spleens of dead mice contained a large number of *Salmonella* cells: $10^6$ cells per g of spleen. Our model was reproducible with little variability among mice challenged with the same inoculum dose. The LD$_{50}$ values were comparable to those obtained by others using the same model (18, 20, 26). Bhatnagar et al. (5) demonstrated an increase in the pathogenicity of Francisella tularensis when cells were cultured under iron-deficient conditions. McDermid et al. (21) observed a decrease in virulence in mice for *Salmonella* Enteritidis cells cultivated in medium with an alkaline pH. Under our experimental conditions, culture in EWRM did not provoke changes in the virulence of *Salmonella* Enteritidis cells in challenged mice.

The mouse model was used because the pathogenesis of the disease in mice infected with *Salmonella* Typhimurium or *Salmonella* Enteritidis closely resembles that of the disease in humans (23). Because the intraperitoneal challenge affords the best reproducibility and the best control of inoculum dose, only the terminal phase of infection was tested. Different steps such as survival at gastric pH, the penetration of gastrointestinal mucosa, and the invasion of the disease in mice infected with *Salmonella* Typhimurium or *Salmonella* Enteritidis are induced by environmental stress, especially nutritional stress. The ability of bacteria to survive these conditions contributes to their virulence (22).

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Peyer’s patches were not encountered by cells in the intraperitoneal challenge. To complete our experiments, we studied a chicken model with oral inoculation. This type of model has not often been used (3, 14) but could give an indication of the capacity for survival of Salmonella cells at gastric pH and the ability of these cells to penetrate intestinal epithelial cells.

There were no significant differences between the two adaptation treatment groups in the numbers of Salmonella cells found in cecum, liver, and spleen after 12 days of infection (Fig. 2). The high number found in the cecum (10^7 cells per g) corresponds to the first step of infection. After oral contamination, the Salmonella cells penetrate and invade the epithelial cells. The contamination of organs such as the liver (10^1.8 cells per g) and spleen (10^2 cells per g) corresponds to the second step of infection. Incubation in EWRM did not increase the ability of Salmonella cells to colonize the digestive tract (first step) and internal organs (second step) of chickens.

Results obtained with these two animal models indicate that the virulence of Salmonella Enteritidis was not affected by prior incubation in EWRM. Changes provoked by egg white conditions probably were not sufficient or durable enough to provide an advantage or a disadvantage to cells during infection.

The adaptation of Salmonella Enteritidis to an unfavorable environment that mimicked egg white conditions did not result in any modification of these cells in their resistance to heat or disinfectants, adhesion to stainless steel, or virulence. The model medium used for these experiments contained the major egg white inhibition factors for Salmonella. These results are important for the safety of processed egg products because the stresses imparted by egg white do not appear to make Salmonella cells more virulent or harder to control.

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REFERENCES


