Recovery in embryonated chicken eggs of viable but non-culturable *Salmonella*

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Abstract

*Salmonella paratyphi B* and *Salmonella typhimurium* were starved in seawater microcosms respectively for 12 and 6 years. After starvation periods, major part of water was evaporated in each microcosm and salt crystals appeared. No culturable cells were obtained in selective and non-selective media. Salty suspension of each microcosm was inoculated in the yolk sacs of embryonated chicken eggs. Culturable cells were detected in a large proportion of eggs.

**Key words:** Resuscitation, seawater, VBNC, resistance.

Introduction

*Salmonella* is an enteropathogenic and food-borne agent, which causes diarrhea and enteritis in humans. *Salmonella enterica* serovar Enteritidis is the cause of a worldwide increase in human salmonellosis associated with the consumption of contaminated eggs. Research indicates that serovar Enteritidis is organ invasive and efficiently contaminates eggs produces. The ability to enter a viable but nonculturable (VBNC) state has been described for several enteric pathogens. The VBNC hypothesis has been the subject of much interest and debate, especially since it has formed the basis of questions about the potential threat of bacteria, which cannot be detected by standard microbiological testing methods of public health. Many studies showed that VBNC bacteria are capable of retaining virulence. A variety of types of resuscitation methods were examined including nutrient addition, with rich or dilute media; temperature shifts and temperature shifts plus nutrient addition. These resuscitation techniques are performed on a wide range of numbers of non-culturable cells, both in the presence and in the absence of culturable cells. While recovery of pathogens from environmental sources is important in determining the etiology of an outbreak, of greater concern from a public health perspective is in vivo recovery and retention of pathogenicity by non-culturable cells. The recovery of non-culturable cells when introduced into an in vivo system and maintenance of pathogenicity of non-culturable cells in an in vivo system have been reported. In this paper, we describe experiments, which elucidate the conditions required for the resuscitation in the yolk sacs of embryonated eggs of VBNC forms of *Salmonella*. The experiments were carried out with *Salmonella typhimurium* LT2 and *Salmonella paratyphi B* strains isolated from Tunisian healthy careers, given by Pasteur institute of Tunisia. The two strains were kept in nutrient agar tube at -20°C. The culture was incubated at 37°C in nutrient broth during 18 hours with shaking (150 rpm). Cells were then collected by centrifuging for 6 min at 8,000 rpm, washed three times in sterile seawater (7,000× g for 12 min) and immediately suspended in 1-litre bottles containing 500 ml of filter-sterilized seawater. The two strains, *Salmonella paratyphi B* and *Salmonella typhimurium* were starved in seawater microcosms.

Material and Methods

**Cultural media used:** Nutrient broth (NB) was used for general bacterial culture. 8 g of dehydrated medium (5 g of peptone of gelatin, 3 g of animal chair extract) was suspended in one litre of double distilled water, distributed in 100 ml Pyrex flasks, autoclaved at 120°C for 15 minutes and the pH was adjusted until 6.9.

Nutrient agar (NA) was implicated for general bacteria culture. 23 g of dehydrated medium (5 g of gelatin peptone, 3 g of animal chair extract, 15 g of agar) was suspended in one litre of double distilled sterilized water, mixed and boiled in Marie bath for one to two minutes. The medium was autoclaved for 15 minutes at 120°C and distributed in sterile Pyrex plates.

**Selective medium for Salmonella-Shigella (SS) was implicated for Salmonella culture. 60 g of dehydrated medium (5 g of animal chair extract, 5 g of mixture of peptone, 10 g of lactose, 8.5 g of mixture of salt bile, 8.5 g of sodium thiosulfate, 8.5 g of ferric citrate, 8.5 g of sodium citrate, 13.5 g of agar, 0.025 g of neutral red, 0.33 g of green brilliant) was suspended in one litre of double distilled water and homogenate was then boiled for two minutes. The solution was adjusted until pH 7 and distributed in Pyrex plate.**

A system for enterobacteria identification Api 20 E galleries contained 20 microtubes of dehydrated substrates. The microtubes were inoculated with bacterial suspension. The reactions produced during the incubation period were detected by spontaneous color reaction. Analytic Catalogue did the reading of reactions.

**Bacterial strains and microcosms preparation:** The experiments were carried out with *Salmonella typhimurium* LT2 and *Salmonella paratyphi B* strains isolated from Tunisian healthy careers, given by Pasteur institute of Tunisia. The two strains were kept in nutrient agar tube at -20°C. The culture was incubated at 37°C in nutrient broth during 18 hours with shaking (150 rpm). Cells were then collected by centrifuging for 6 min at 8,000 rpm, washed three times in sterile seawater (7,000× g for 12 min) and immediately suspended in 1-litre bottles containing 500 ml of filter-sterilized seawater. The two strains, *Salmonella paratyphi B* and *Salmonella typhimurium* were starved in seawater microcosms.
respectively for 12 and 6 years, 15 microcosms were used for each strain and 30 microcosms containing sterilized seawater were used as control microcosms. The different microcosms were closed by sterilized cotton, protected by sterilized cellulose paper, which permit a progressive evaporation of water. All microcosms were put at obscured experimental room, belonging to Bacteriology Department (University of Pharmacy of Monastir, Tunisia). The holding temperature in the experimental room is 20±2°C during wintertime and 25±2°C during summer period.

Reviviscence essay of starved Salmonella in nutrient broth: After starvation of Salmonella paratyphi B for 12 years and that of Salmonella typhimurium for 6 years, the major part of water was evaporated in each microcosm and salt crystals appeared. A volume of 0.2 ml of salty water was spread on nutrient agar (NA) plate and selective medium for Salmonella-Shigella (SS) and incubated at 37°C for 48 hours. 0.2 ml of infected suspension was also added to 100 ml of nutrient broth, incubated for 10 days at 37°C and inoculation in NA and SS media was realized every day. The biochemical activities were followed by inoculation in Api 20 E galleries.

Reviviscence essay of starved Salmonella in the yolk sac of embryonated eggs: An essay of resuscitation was realized in non-embryonated eggs. 40 eggs from specific-pathogen-free chickens, living without male strain, were used. One millilitre of each Salmonella suspension microcosms and also of control microcosms was injected with a 1-ml syringe (needle dimensions, 0.9 mm and 40 mm). 20 negative-control eggs were inoculated with sterilized distilled water. The eggs were then incubated at 37°C. After incubation for 12, 48, 96 h, one week and 10 days, the eggshells were broken. The vitellus fluid was harvested with a syringe, and 0.2 ml was spread on nutrient agar and SS medium. The plates were incubated for 48 h at 37°C. A second essay of resuscitation was realized in embryonated eggs; seven-day-old embryonated eggs from specific-pathogen-free chickens strain were used. One millilitre of each Salmonella suspension microcosms and also of control microcosms was injected into a yolk sac as described previously. Negative-control eggs were inoculated with sterilized distilled water.

Results

Change of culturability in seawater microcosms: Fig. 1 shows changes in culturable counts of S. typhimurium and S. paratyphi B strains exposed to sterile seawater. After 12 years of starvation of Salmonella paratyphi B and 6 years of Salmonella typhimurium, no colonies were observed in selective and non-selective media. After resuscitation in nutrient broth for 48 hours to 10 days the starved strains developed rough colonies on nutrient agar medium (Figs. 2 and 3).

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Recovery of VBNC Salmonella in embryonated eggs: Recovery was not obtained in non-embryonated eggs. Culturable Salmonella organisms growing in NA and SS were successfully recovered in the embryonated eggs inoculated with 6-years-starved Salmonella typhimurium and 12-years-starved Salmonella paratyphi B cells. Fig. 4 shows the percentage of eggs presenting Salmonella recovery according to the incubation period at 37°C. Smooth colonies formed by S. typhimurium and S. paratyphi B after resuscitation in the yolk sac of embryonated eggs are shown in Fig. 5.

Discussion
In this study, recovery of VBNC cells was not obtained in non-embryonated eggs but it was successfully observed in embryonated eggs. Eggs contain an adequate amount of nutrients to support the growth of Salmonella. Although antimicrobial agents such as conalbumen and lysozyme are present in the albumen, both are neutralized when the yolk and white are homogenized, thus allowing microbial growth. Gast and Holt 14 also suggested that the ability of Salmonella to grow rapidly in liquid whole eggs is a characteristic of various strains. This could explain why Salmonella is able to rapidly multiply in homogenized eggs despite the inhibitory effects. The embryonated-egg model has been successfully used to recover VBNC Legionella pneumophila cells.15 This agree with the experimental results of Cappelier et al. 16, in which passage in embryonated eggs is the preferable model for recovery of the VBNC stage of Campylobacter. The embryonated-egg model can be considered an animal model in which the animals have reduced defences. Recovery of VBNC C. jejuni cells after intestinal passage in mice 17, 18 or in 1-day-old chicks 19 has already been described. In contrast, Medema et al. 20 and Van de Giessen et al. 21 were unable to recover VBNC cells in animal models. Oliver and Bockian 22 showed that injections of VBNC Vibrio vulnificus cells into mice killed the animals and concluded that VBNC V. vulnificus cells remain virulent, at least for some time after entry into the VBNC state, and are capable of causing fatal infection after recovery in vivo. Other authors have shown that VBNC E. coli cells retain pathogenicity, with cells being able to produce enterotoxin 11, 22, 23 and maintain virulence plasmids. 24, 25 By analyzing spore counts in the feces of mice administered spore suspensions, it has been also shown that it is possible that spores of Bacillus subtilis could germinate in the gastrointestinal tract 17. Our findings indicate that VBNC forms of Salmonella resist in salty water during many years. Moreover, the VBNC stage can be resuscitated after passage through embryonated eggs. During our bibliography study, we did not find any research concerning the resistance of VBNC pathogenic bacteria for many years in environmental microcosms. VBNC cells of Salmonella in the present study could be compared to the spores.

References


