Journal of Food Safety and Food Quality 62, Heft 1 (2011), Seiten 1–32

Arch Lebensmittelhyg 62,
6–10 (2011)
DOI 10.2376/0003-925X-62-6


ISSN 0003-925X

Korrespondenzadresse:
annette.johne@bfr.bund.de

[1]Federal Institute for Risk Assessment, Thielallee 88–92, 14195 Berlin, Germany;
[2]Institute of Food Hygiene, Free University Berlin, Germany;
[3]Institute of Food Hygiene, University of Leipzig, Germany;

# Studies on the growth of *Salmonella* in table eggs under different storage temperatures

*Untersuchungen zum Wachstum von Salmonellen in Konsumeiern bei unterschiedlichen Lagerungstemperaturen*

Matthias Greiner[1], Thomas Alter[2], Annette Johne[1], Daniela Schlichting[1], Alexandra Fetsch[1], Juliane Bräunig[1], Annemarie Käsbohrer[1], Sandra Knobloch[3], Peggy Braun[3], Bernd Appel[1]

**Summary**

In Germany salmonellosis is one of the most common foodborne infections in humans. Contaminated table eggs are one of the main sources of the infection. The aim of the study was to obtain experimental data on the impact of temperature conditions on the behaviour of *Salmonella* Enteritidis (SE) in table eggs which may occur under practical conditions. For this purpose, 170 fresh eggs were contaminated by inoculating SE into the albumen of intact eggs and eggs were stored at different temperatures (group A: 5 °C; group B: 25 °C; group C: 25 °C/5 °C in alternating sequences of three-day intervals). Every six days, qualitative and quantitative examinations to detect SE were carried out during the following 30 days. *Salmonella,* which were inoculated into the albumen could be detected over the whole study period, i. e. it was not inactivated; however, only during storage at 5 °C, there was no growth of SE. By qualitative examination, SE could be detected in the egg yolk in all groups within six days after inoculation. By quantitative testing, a considerable bacterial growth was confirmed during storage at 25 °C or changing temperatures (25 °C/5 °C). By alternating storage temperatures, the highest migration rate into the yolk could be detected (64 %), but changing temperatures had no additional effect on the detection or concentration of *Salmonella* in fresh eggs. This study shows that eggs should be chilled immediately after laying; a storage temperature of ≤ 5 °C is recommended.

**Keywords:** storing conditions, albumen, yolk, *Salmonella* Enteritidis

**Zusammenfassung**

Die Salmonellose des Menschen ist eine der häufigsten lebensmittelbedingten Erkrankungen in Deutschland. Hierfür werden u. a. Konsumeier als Infektionsquelle verantwortlich gemacht. Ziel der Studie war die Gewinnung experimenteller Daten zur Frage, welchen Einfluss Temperaturschwankungen auf das Verhalten von *Salmonella* Enteritidis (SE) in Eiern haben, die unter Praxisbedingungen vorkommen können. Hierfür wurden 170 tagfrische Hühnereier durch Inokulation mit SE in das Eiweiß kontaminiert und unter verschiedenen Temperaturbedingungen gelagert (Gruppe A: 5 °C; Gruppe B: 25 °C; Gruppe C: Temperaturwechsel 25 °C/5 °C, jeweils über die Dauer von drei Tagen). Nach jeweils sechs Tagen wurden über einen Zeitraum von 30 Tagen qualitative und quantitative Untersuchungen zum Nachweis des Erregers durchgeführt. Der ins Eiweiß eingebrachte Erreger wurde über den gesamten Versuchszeitraum im Eiweiß nachgewiesen, d. h. er wurde nicht inaktiviert. Eine Vermehrung von SE fand bei Lagerung der Eier bei 5 °C jedoch nicht statt. Bei der qualitativen Untersuchung konnte SE bereits innerhalb von sechs Tagen nach Kontamination bei allen Lagerbedingungen im Eigelb nachgewiesen werden. Bei der quantitativen Untersuchung wurde bei Lagerung bei 25 °C bzw. wechselnden Lagerungstemperaturen (25 °C/5 °C) eine erhebliche Keimvermehrung nachgewiesen. Bei Temperaturschwankungen (Gruppe C) zeigte sich die höchste Migrationsrate in den Dotter (64 %), wobei wechselnde Lagerungstemperaturen keine zusätzliche Wirkung auf den Nachweis oder die Erregerkonzentration bei frischen Eiern hatten. Diese Studie belegt, dass Eier sofort nach dem Legen gekühlt werden sollten; eine Lagertemperatur von ≤ 5 °C wird empfohlen.

**Schlüsselwörter:** Lagerungsbedingungen, Eier, *Salmonella* Enteritidis

## Introduction

*Salmonella* contamination in table eggs is one of the main sources of *Salmonella* infection in humans, whereof *Salmonella* Enteritidis is the most frequent cause of human salmonellosis cases at EU level. According to EFSA data, the rate of table egg units contaminated with *Salmonella* is estimated at 0.5 % at Community level in 2008 (EFSA 2010).

Contamination of the egg shell surface with *Salmonella* may occur before, during or after laying. *Salmonella* contamination of the egg content can occur by penetrating the shell, or by contaminating the egg before the egg shell is fully formed as a result of an infection of the reproductive system. Several authors reported that there are rarely more than a few hundred *Salmonella* cells detectable in the contents of freshly laid eggs (Humphrey et al., 1991; Gast and Beard, 1992; Gast and Holt, 2000; Chen et al., 2002).

In freshly laid eggs *Salmonella* can be detected in the albumen as well as in the yolk, however, the latter is less frequent (Humphrey et al., 1991). The subsequent increase of these bacteria is affected by length and temperature of storage (Reglich, 1993; Humphrey 1994). Kim et al. (1989) concluded that the storage temperature is the most important factor in determining *Salmonella* growth. One proposed strategy to limit trans-shell migration and internal growth of *Salmonella* in eggs is the refrigeration of eggs. Studies of Miyamoto et al. (1998) showed that the *Salmonella* penetration through the eggshell within 3 h after laying was significantly decreased by cooling the eggs at 4 °C before they were immersed in SE suspension. They deduced that cooling eggs soon after laying seems to depress the penetration of *Salmonella* thereafter. Furthermore, on experimentally inoculated eggs Kim et al. (1989) demonstrated that there was less increase of *Salmonella* in eggs stored at 4 °C than in eggs held at temperatures higher than 4 °C. Wicke (1995) could demonstrate that no penetration of SE (1–10 SE/cm$^2$ egg shell) trough the shell into the albumen occurred during a storage time of 20 days at 10 °C and at 60 % and even 95 % relative humidity. Braun and Fehlhaber (1995) could show the higher the numbers of SE in the albumen the more often the egg yolks are contaminated (i. e. 10 or 230 SE/ml albumen leads to 17 or 54 % positive yolks respectively after four weeks of storage at 20 °C). The risk was relatively low at 7 °C, however the first positive egg yolks were already found after 14 days at 7 °C, at 20 °C and 30 °C the first cells were present in the yolk after 1 or 2 days. Similar results are described by Baker et al. (1990). They observed positive yolks after two days using an initial contamination dose of 50 cells/ml albumen and a temperature of 8 °C.

According to Commission Regulation (EC) No. 1020/2008 amending Annexes II and III to Regulation (EC) No 853/2004, eggs must be stored and transported at a temperature, preferably constant, that is best suited to assure optimal conservation of their hygiene properties, unless the competent national authority imposes national temperature requirements for egg storage facilities and for vehicles transporting eggs between such storage facilities. Storage and transportation at constant temperatures is important to avoid condensation, which would otherwise facilitate the growth of bacteria on the shell and probably ingress into the egg (Commission Regulation EC No 589/2008). In Germany, shell eggs intended for commercial sale must be kept refrigerated at a temperature between 5 °C and 8 °C for transportation and storage from day 18 after laying (BGBl, 2007).

The purpose of this study was to assess the effect of cooling on the hygienic status of table eggs with regard to *Salmonella* Enteritidis (SE) contamination. In this context the question arose whether multiple changes in the storage temperature for fresh table eggs could have an effect on the growth of *Salmonella* Enteritidis in addition to the effect of the (average) temperature.

## Materials and methods

### Eggs

A total of 170 eggs from laying hen breeds (Lohmann White LSL) (size L) were collected at the day of lay from one producer in Saxony, who is known to be *Salmonella*-free. Regular testing of producer's premises was carried to verify the *Salmonella*-free status. Eggs were transported to the institute within one hour at room temperature and immediately artificially contaminated with SE.

### *Salmonella* inoculation

The albumen was artificially contaminated (1 ml was injected with a steril syringe through the sterilized eggshell) with 40 cfu (colony forming units) SE phage type 4/6 (RKI-Nr.: 07-00988) per ml which is approximately equal to 1.2x10$^3$ cfu/egg. Twenty non-inoculated (SE-negative) eggs served as negative controls.

### Storage

The eggs were incubated at 5 °C (group A) or 25 °C (group B) or in alternating sequence of three-day intervals at both temperatures (group C, starting at 25 °C). The relative humidity was kept at 40–50 % (experimental setup shown in Table 1).

**TABLE 1:** *Qualitative results of SE detection in egg white and yolk.*

| Group | Day | Temperature | No of temperature changes | N | # positive egg white | # positive yolk |
|---|---|---|---|---|---|---|
| A | 6 | 5 | 0 | 10 | 10 | 4 |
| A | 12 | 5 | 0 | 10 | 10 | 3 |
| A | 18 | 5 | 0 | 10 | 10 | 0 |
| A | 24 | 5 | 0 | 10 | 10 | 4 |
| A | 30 | 5 | 0 | 10 | 10 | 4 |
| B | 6 | 25 | 0 | 10 | 10 | 2 |
| B | 12 | 25 | 0 | 10 | 10 | 0 |
| B | 18 | 25 | 0 | 10 | 10 | 6 |
| B | 24 | 25 | 0 | 10 | 10 | 10 |
| B | 30 | 25 | 0 | 10 | 10 | 9 |
| C | 6 | 25/5 | 1 | 10 | 10 | 4 |
| C | 12 | 25/5 | 3 | 10 | 10 | 3 |
| C | 18 | 25/5 | 5 | 10 | 10 | 6 |
| C | 24 | 25/5 | 7 | 10 | 10 | 9 |
| C | 30 | 25/5 | 9 | 10 | 10 | 10 |

Inoculation into egg white with approximately 1.2 x 10$^3$ cfu/egg. Incubation at 5 °C (group A), or 25 °C (group B) or alternating 25 °C/5 °C (group C).

Journal of Food Safety and Food Quality 62, Heft 1 (2011), Seiten 1–32

### *Salmonella* detection and enumeration

At defined days post contamination (6, 12, 18, 24, 30 days), ten eggs per group were investigated qualitatively and quantitatively for SE. For this purpose, 30 ml of albumen were taken from each egg under aseptic conditions and mixed thoroughly. For quantitative analysis, 5 ml of the mixture was serially diluted and spread on XLD agar (Sifin TN 1196). The plates were incubated for 24 h at 37 °C. For the qualitative analysis, 25 ml egg white was incubated in 225 ml peptone water (Sifin TN 1137 with ferrioxamine Sifin TN 1336) and incubated for 24 h at 37 °C. 100 µl of this suspension was transferred into Rappaport-Vassiliadis enrichment medium (Sifin TN 1227), incubated for 24 h at 42 °C, plated on XLD agar and then incubated again for 24 h at 37 °C before visual inspection (according to ISO 6579). The egg yolk was investigated in the same groups of n=10 eggs per group at each sampling date. After heat-sterilisation of the yolk surface using a gas jet, approximately 8 ml yolk was taken under aseptic conditions and mixed thoroughly. 3 ml of this mixture was used for the quantitative analysis using XLD agar as described above. 5 ml egg yolk was incubated in 45 ml peptone water and then investigated qualitatively as described above.

### Statistical analysis

We conducted logistic regression analysis to investigate the qualitative detection rates as a function of time. Simple multivariable linear regression was used to analyse the quantitative data. The regression models were implemented using SAS (version 9.1).

## Results and discussion

### Qualitative detection of SE in egg yolk

The qualitative results (presence/absence) of SE in the three experimental groups incubated at different temperatures are shown in Table 1. It was possible to detect SE in egg yolk already six days after inoculation in some eggs in all experimental groups (Fig. 1). We investigated the empirical detection rate ("P", which is the proportion of SE positive eggs out of batch sizes of ten) in relation to days post inoculation ("day") during incubation at constant 25 °C. The resulting logistic regression model was

$$\text{logit}(P) = -4.07 + 0.243 * \text{day}$$

The p-values for the coefficients were <0.001. Setting P=0.25 and solving the regression formula for "day" resulted in 12.2 days, which can be interpreted as the yolk membrane time (YMT), i. e. the time at which 25 % of egg yolks are contaminated, for a temperature of 25 °C. This interpretation follows the definition given in the *Salmonella* Risk Assessment Report of the USDA/FSIS report (2005). The USDA/FSIS model for YMT, based on experimental data by Humphrey and numerically provided by Whiting et al. (2000), is

$$\text{log10time[days]} = 2.0872 - 0.0426 * \text{Temp [°C]} + \text{error}$$

according to the USDA/FSIS report. Using this model, we would expect an YMT (25 °C) = 10.5. Thus, the USDA/ FSIS model predicts that after 10.5 days we would expect growth (qualitative detection of SE) in more than 25 % of the eggs. Our experiment was not designed to challenge the YMT model and our results are not deviating markedly from the predictions by the YMT model.

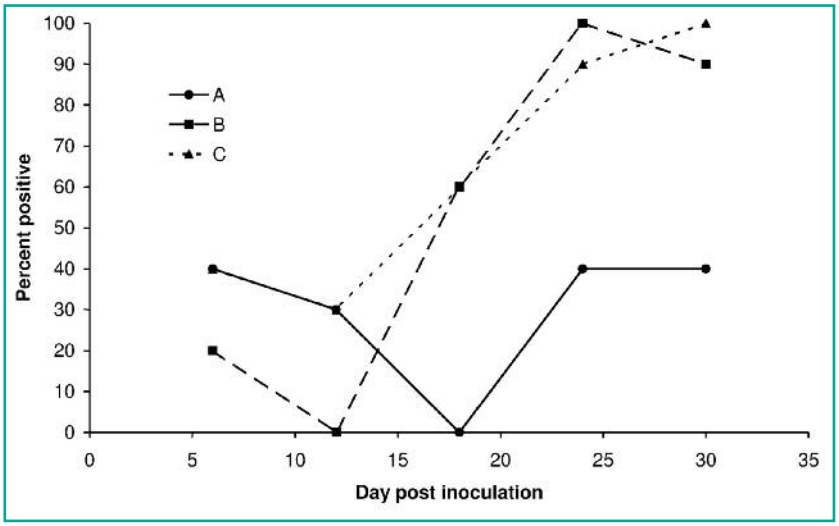


**FIGURE 1:** *Detection rate (P) of SE in the egg yolk in three experimental groups (A, B, C, see Table 1 for detailed results).*

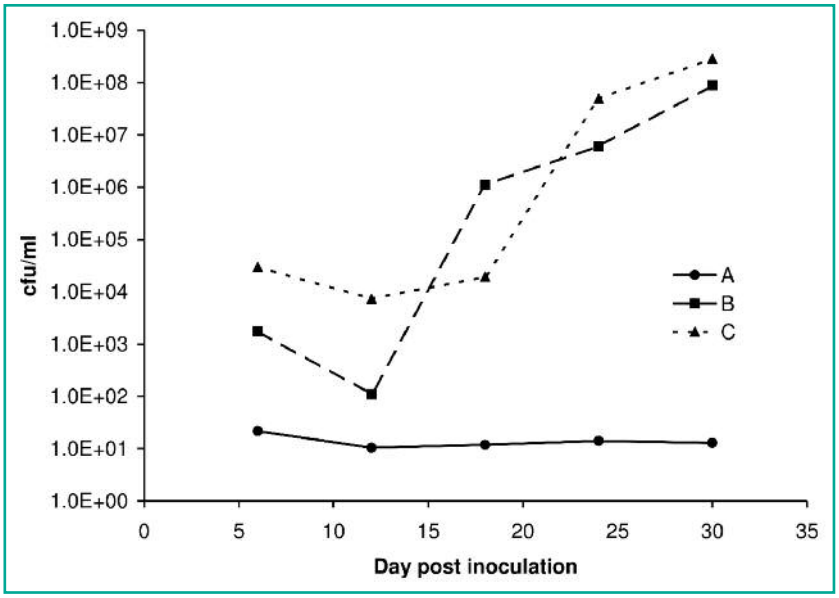


**FIGURE 2:** *Mean number of SE in the egg white in three experimental groups (A, B, C).*

### Quantitative detection of SE in the egg white and in the yolk

In groups B and C considerable growths occurred in the egg white (Fig. 2). The growth in egg yolk is shown as individual counts (Fig. 3). It can be seen that the steep increase in growth rate in the albumen occurred after about twelve days post inoculation. These findings are consistent with the interpretation of the YMT as described above.

In the yolk, quantitative detectable growth of SE was found in groups B and C at day 24 and day 30 (Fig. 3).

The results clearly show that growth occurs at 25 °C (group B and group C) whereas effectively no growth occurs at 5 °C (group A).

### Effect of temperature changes

Visual inspection of the growth curves of group B and C (Fig. 2) suggests that multiple changes of the environmental temperature (e. g. 25 °C/5 °C as in group C) might have a positive effect on the growth in addition to the pure temperature effect. The time under growth temperature conditions is less for group C compared to group B. Therefore, it was expected that growth observed in group C would lag behind the growth observed in group B. Surprisingly, this effect was not seen in our data. The effect of temperature changes on the qualitative detection was investigated using the model

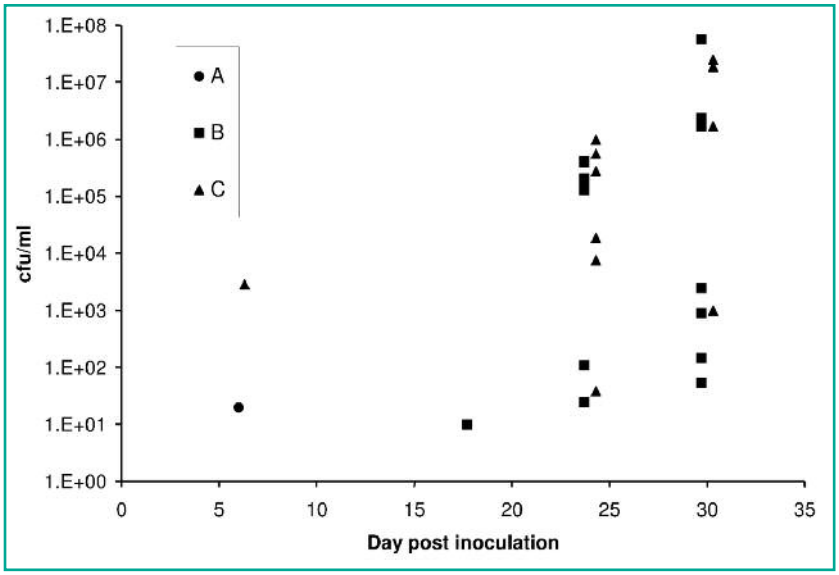


**FIGURE 3:** *Quantitative findings of SE in the egg yolk in three experimental groups (A, B, C). Note that the symbols for group B and C for 24 and 30 days post inoculation are plotted slightly below and above their x-values to allow visual differentiation.*

$$\text{logit}(P) = -4.07 + 0.24*\text{day} + 1.94*C - 0.08*\text{day.C}$$

where "P" and "day" are defined as before, "C" is an indicator of the group membership, C = 1 for an observation from group C and C = 0 else and "day.C" is an interaction term. The model was fitted on all data except group A. The p-values for the intercept and coefficient for "day" were <0.001 and the p-values for the coefficients for "C" and "day.C" were 0.17 and 0.33, respectively, and thus non-significant. After inclusion of a squared term "day^2", the p-values for "C" and "day.C" remained non-significant. These results do not support the interpretation that temperature changes enhance the detection rate beyond the detection rate observed under favourable temperature. A similar analysis was conducted using the quantitative SE data for egg white (Fig. 2). Let "logW" denote the natural logarithm of the cfu's counted in egg white. We investigated the linear model

$$\text{logW} = 2.15 + 0.36 * \text{day} - 0.02*C + 0.08*\text{day.C} + \text{error}$$

which gave p-values for the coefficients "C" and "day.C" of 0.993 and 0.432, respectively. Again, after inclusion of a squared term "day^2", the p-values for "C" and "day.C" remained non-significant. In correspondence with qualitative analysis, these results do not support the hypothesis that the temperature change had any effect on the growth.

## Conclusions

Our data support previous empirical information on a positive effect of temperature on the growth of SE in fresh eggs. No growth was detected at storage condition of 5 °C although SE was qualitatively detectable at this temperature level throughout the whole 30-day observation period.

SE (contamination dose of $1.2x10^3$ cfu/egg) showed a long persistence in the albumen (at least 30 days). A migration of SE from the albumen into the egg yolk was already detectable after six days at all storage conditions. Even at 5 °C (group A) a migration of SE was relatively frequently noted (30 %); SE was detectable in albumen, ranging from <10 cfu/ml to $5x10^1$ cfu/ml, however, no growth of SE could be observed in the egg yolk.

At 25 °C, 54 % of the egg yolks were SE positive (group B). Whereas in the albumen a rapid growth of SE at day 18 with counts up to $1.0x10^7$ cfu/ml rising further to $7.3x10^8$ cfu/ml at day 30 could be detected, the growth of SE in the egg yolk increased later at day 24 (up $1.3x10^5$ cfu/ml) and up to $5.8x10^7$ cfu/ml at the end of the storage.

Varying storage temperatures (25 °C/5 °C/25 °C/5 °C/25 °C; group C) caused the highest migration rate (64 %). A massive growth of SE in the albumen and egg yolk was detected at day 24 (up to $1.8x10^8$ cfu/ ml albumen; up to $1.0x10^6$ cfu/ml yolk).

Our data do not contradict the yolk membrane time (YMT) model used by the USDA/FSIS to define a lag time during which growth of SE is unlikely to occur after inoculation of fresh eggs. The data of our study suggest that a change of temperature (between 25 °C and 5 °C) has no effect on detectability and growth rate of SE in fresh eggs. When changing storage temperatures, the highest migration rate from SE into the nutrition-rich yolk was shown. It can be concluded that cooling of eggs will no longer have an inhibiting effect on SE if these eggs were prior stored at room temperature for several days.

Our results confirm earlier studies (Braun and Fehlhaber, 1995; Braun et al. 1999, Braun et al. 2005) and considering data of Wicke (1995) or Kim at al. (1989), a consequent and constant chilling of eggs after laying with an optimal storage temperature of ≤ 5 °C should be introduced. The growth of SE and the process of trans-shell migration can be inhibited even in the case of a possible temperature variation during storage with subsequent water condensation on the shell. Storing eggs at low temperatures may improve the safety of eggs and minimize the consumer's risk of a SE infection.

Journal of Food Safety and Food Quality 62, Heft 1 (2011), Seiten 1–32

## References


**Baker R. C. (1990):** Survival of *Salmonella* enteritidis on and in shelled eggs, liquid eggs, and cooked egg products. Dairy, Food and Environm Sanitation 10: 273–275.

**Braun P, Fehlhaber K (1995):** Migration of *Salmonella* enteritidis from albumen into egg yolk. Int J Food Microbiol 25: 95–99.

**Braun P, Fehlhaber K, Wicke A (1999):** *Salmonella* enteritidis invades the egg through the shell. World Poultry Special 11: 23–24.

**Braun P, Meyer K, Reglich K, Wicke A, Fehlhaber K (2005):** Investigations for risk assessment on the behaviour of *Salmonella* enteritidis in hen's eggs. In: Smulders FJ, Collins JD (Eds.), Risk management strategies: monitoring and surveillance. Wageningen Academic Publishers 2005, 274.

**Bundesgesetzblatt (BGBL 2007):** Verordnung zur Durchführung von Vorschriften des gemeinschaftlichen Lebensmittelhygienerechts. Bundesgesetzblatt Jahrgang 2007 Teil I Nr. 39: 1832.

**Chen HQ, Anantheswaran RC, Knabel SJ (2002):** Effect of rapid cooling on the growth and penetration of *Salmonella* enteritidis into egg contents. J Food Safety 22 (4): 255–271.

**COMMISSION REGULATION (EC) No 589/2008** of 23 June 2008 laying down detailed rules for implementing Council Regulation (EC) No 1234/2007 as regards marketing standards for eggs. Official Journal of the European Union 24.06.2008, L 163/6-L 163/21.

**COMMISSION REGULATION (EC) No 1020/2008** of 17 October 2008 amending Annexes II and III to Regulation (EC) No 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin and Regulation (EC) No 2076/2005 as regards identification marking, raw milk and dairy products, eggs and egg products and certain fishery products of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Union 18.10.2008, L 277/14.

**EFSA (2010):** Trends and sources of zoonoses and zoonotic agents and food-borne outbreaks in the European Union in 2008. EFSA Journal 8 (1) 1496: 45.

**Gast RK, Beard CW (1992):** Detection and enumeration of *Salmonella* enteritidis in fresh and stored eggs laid by experimentally infected hens. J Food Protect 55 (3): 152–156.

**Gast RK, Holt PS (2000):** Deposition of phage type 4 and 13a *Salmonella* enteritidis strains in the yolk and albumen of eggs laid by experimentally infected hens. Avian Dis 44 (3): 706–710.

**Humphrey TJ, Whitehead A, Gawler AH, Henley A, Rowe B (1991):** Numbers of *Salmonella* enteritidis in the contents of naturally contaminated hens' eggs. Epidemiol Infect 106 (3): 489–496.

**Humphrey TJ (1994):** Contamination of egg shell and contents with *Salmonella* enteritidis: a review. Int J Food Microbiol 21 (1–2): 31–40.

**ISO 6579 (2002+Amd 1:2007):** Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.

**Kim CJ, Emery DA, Rinke H, Nagaraja KV, Halvorson DA (1989):** Effect of time and temperature on growth of *Salmonella* enteritidis in experimentally inoculated eggs. Avian Dis 33 (4): 735–742.

**Miyamoto T, Horie T, Baba E, Sasai K, Fukata T, Arakawa A (1998):** *Salmonella* penetration through eggshell associated with freshness of laid eggs and refrigeration. J Food Protect 61 (3): 350-353.

**Reglich K. (1993):** Experimentelle Untersuchungen zum Verhalten von *Salmonella* enteritidis im Eiklar. Leipzig, Germany, Univ., Veterinärmed. Fak. Diss.

**U.S. Department of Agriculture (USDA), Food Safety Inspection Service (FSIS) (2005):** Risk assessments of *Salmonella* Enteritidis in shell eggs and *Salmonella* spp. in egg products. USDA/FSIS report 2005.

**Whiting RC, Hogue A, Schlosser WD, Ebel ED, Morales RA, Baker A, McDowell RM (2000):** A quantitative process model for *Salmonella* Enteritidis in shell eggs. J Food Science 65 (5): 864–869.

**Wicke, A. (1995):** Experimentelle Untersuchungen zum Einfluß exogener Faktoren auf das Penetrationsverhalten von *Salmonella* enteritidis durch die Schale von Hühnereiern. Leipzig, Germany, Univ., Veterinärmed. Fak. Diss.



**Address of corresponding author:**
Dr. Annette Johne
Federal Institute for Risk Assessment
Diedersdorfer Weg 1
12277 Berlin
Germany
annette.johne@bfr.bund.de