International Journal of Food Microbiology 129 (2009) 180–186

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Use of pulsed-field gel electrophoresis to characterize the heterogeneity and clonality of *Salmonella* serotype Enteritidis, Typhimurium and Infantis isolates obtained from whole liquid eggs

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ARTICLE INFO

Article history:
Received 16 June 2008
Received in revised form 7 November 2008
Accepted 15 November 2008

Keywords:
Salmonella Enteritidis
Salmonella Typhimurium
Salmonella Infantis
Egg products
PFGE

ABSTRACT

Salmonella is a well-documented pathogen known to occur in a wide range of foods, especially poultry products. The most frequently reported food-sources of human infection are eggs and egg products.
In this study, in order to describe *Salmonella* contamination of egg products, 144 liquid egg samples were collected from 3 different egg-breaking plants during the 3 sampling periods. *Salmonella* detection was performed on raw samples stored at 2 °C for 2 days (*D*+2) and on pasteurised samples stored at 2 °C at *D*+2 and at shelf-life date. *Salmonella* was detected in 130 of the 144 raw egg samples collected and in 11 of the 288 pasteurised egg samples analysed. 740 *Salmonella* isolates were collected and serotyped: 14 serovars were demonstrated. A great diversity, particularly during summer, was noted. The dominant serovars were *S.* Enteritidis, *S.* Typhimurium and *S.* Infantis, mainly found in whole raw egg products.
Typing of 325 isolates of *S.* Enteritidis, 54 isolates of *S.* Typhimurium and 58 isolates of *S.* Infantis was carried out by macrorestriction of the genomic DNA with *Xba*I and *Spe*I enzymes followed by pulsed field gel electrophoresis (PFGE).
The *Salmonella* Enteritidis isolates could be grouped into 3 clusters. Cluster 1 was predominant at all 3 egg-breaking companies during the different sampling periods. This cluster seemed to be adapted to the egg-breaking plants. Cluster 2 was linked to plant 1 and cluster 3 to plant 3.
Two main clusters of *Salmonella* Typhimurium were demonstrated. Cluster A was mainly found at plant 2 during autumn. Plant 3 was contaminated by all the *Salmonella* Typhimurium genotypes but in a more sporadic manner during the three seasons studied. Plant 1 seemed to be less contaminated by *Salmonella* Typhimurium than the others.
Three clusters and 2 genotypes of *Salmonella* Infantis were shown. The main cluster, cluster α, consisted of 75% of the *S.* Infantis isolates and was mainly found during summer at plants 1 and 3. Plant 2 seemed to be less contaminated by *S.* Infantis.
In this study, molecular typing demonstrated that, although certain clusters were common to all three companies, specific clusters, notably of *S.* Enteritidis were present at each plant.

1. Introduction

The number of reported food infection outbreaks involving serotypes of *Salmonella*, specially *S.* Enteritidis and *S.* Typhimurium, has increased steadily over the past three decades (Tauxe, 2002; Wallace et al., 2000). Nevertheless, in France, a recent study on the impact of the implementation of mandatory regulations on *Salmonella* in laying hens has shown that the incidence of salmonellosis decreased by 33% from October 1998 onwards (Poirier et al., 2008).

Salmonella is a well-documented pathogen known to occur in a wide range of foods, especially poultry products. The most frequently reported food-sources of human infection are eggs and egg products (Kimura et al., 2004; Gillespie et al., 2005; Zhang et al., 2006). Most cases of human infection with *S.* Enteritidis result from the consumption of contaminated raw eggs (Mead et al., 1999; Olsen et al., 2000). In 1997, *S.* Enteritidis accounted for 85% of all reported cases of human salmonellosis in Europe, but incidence has declined from this peak (Fisher, 2001; Haeghebaert et al., 2002; Delmas et al., 2006). During this same period, salmonellosis from *S.* Typhimurium remained

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doi:10.1016/j.ijfoodmicro.2008.11.021

relatively stable throughout Europe and the US (Fisher, 2001). In France, in 2003, *S.* Enteritidis and *S.* Typhimurium constituted 39.6% and 30.8% of human salmonella isolates, respectively and *S.* Infantis belonged to the 10 main *Salmonella* serotypes isolated (Weill and Grimont, 2005).

Salmonella isolates are traditionally distinguished by serotyping based on the immuno-chemical structure of the lipopolysaccharide and flagella. This system currently identifies more than 2000 serotypes worldwide (Popoff, 2001). More recently, molecular typing methods have been assessed and adopted for further discrimination of *Salmonella* isolates (for a review of methodologies for typing *Salmonella*, see Winokur, 2003). Pulsed-field gel electrophoresis (PFGE) is currently considered to be the "gold standard" system for the molecular typing of *Salmonella* (Ridley et al., 1998). Use of PFGE with endonuclease *Xba*I has been widely recognized as a sensitive means of fingerprinting *Salmonella* serovars (Lukinmaa et al., 1999; Liebana et al., 2001). Murase et al. (1995) also demonstrated that macrorestriction analysis with two enzymes was a more discriminatory method for subtyping *Salmonella*.

The objective of the present study was to investigate the distribution of *Salmonella* contamination of egg products by analysing the PFGE patterns of *S.* Enteritidis, *S.* Typhimurium and *S.* Infantis found in egg products from three egg-breaking manufacturers during three different seasons.

2. Material and methods

2.1. Detection of Salmonella

The *Salmonella* isolates were collected in connection with a project to investigate changes occurring in the microbiological flora of the whole liquid egg during storage. Samples were prepared for this project by 3 egg-breaking plants in north western France during 3 different seasons: winter, summer and autumn. Sixteen samples were collected for each plant and each season giving 144 samples for this experiment. Each raw sample was taken from a container holding 800 kg of mixed liquid egg products coming from the same laying flock and stored at 2 °C. After pasteurisation, another sample was taken from the same container. *Salmonella* detection was performed on the raw products stored at 2 °C, two days after their production (*D* +2) and on the pasteurised products, also stored at 2 °C, at *D*+2 and at shelf-life date (SLD *i.e.* 14 or 49 days depending on the heat treatment applied by the plants) giving a total of 432 samples subjected to *Salmonella* analysis (*i.e.* 144 raw samples at *D*+2, 144 pasteurised samples at *D*+2 and 144 pasteurised samples at SLD).

The *Salmonella* detection procedure used to test the 432 samples was performed by 4 different laboratories according to their own procedures (Protais et al., 2006). At the time of production, each sample of raw or pasteurised product was distributed in 4 batches and sent simultaneously to the 4 laboratories, giving a total of 1728 analysis. These different batches were analysed on the same day of the week. Briefly, 25 g of sample were added to 225 ml of buffered peptone water (AES Laboratory, Combourg, France). After pre-enrichment for 16 to 20 h at 37 °C, two methods of enrichment were used for 18 to 24 h. Each enrichment medium was then seeded on a selective medium. After 18 to 24 h of incubation at 37 °C, suspect colonies were picked out on Kligler-Hajna (AES Laboratory). One to 4 isolates were collected from any whole liquid egg sample positive for *Salmonella* found by any of the 4 laboratories giving a total of 740 isolates. All isolates were serotyped. A selection of *S.* Enteritidis, *S.* Typhimurium and *S.* Infantis isolates were then genotyped by macrorestriction followed by pulsed field electrophoresis.

Table 1
Serovars of *Salmonella* in different samples of egg products

Salmonella serovar	Number of samples contaminated by the different serovars									Number of isolates	Number of typed isolates
	Winter			Summer			Autumn				
	R[1]	*P*[2]	SLD[3]	*R*	*P*	SLD	*R*	*P*	SLD		
Enteritidis	31	4	/	34	/	2	27	/	/	436	325
Typhimurium	9	/	/	4	/	/	12	2	/	81	54
Infantis	5	/	/	15	/	1	2	/	/	82	58
Livingstone	1	/	/	5	/	2	2	/	/	33	/
Virchow	2	/	/	3	/	/	/	/	/	26	/
Hadar	2	/	/	4	/	/	/	/	/	22	/
Braenderup	1	/	/	3	/	/	3	/	/	18	/
Mbandaka	/	/	/	2	/	/	4	/	/	15	/
Tennessee	/	/	/	2	/	/	/	/	/	13	/
Glostrup	1	/	/	/	/	/	/	/	/	3	/
Anatum	/	/	/	1	/	/	/	/	/	3	/
Bredeney	/	/	/	/	/	/	1	/	/	2	/
Concord	/	/	/	1	/	/	/	/	/	1	/
Heidelberg	/	/	/	/	/	/	1	/	/	1	/
Others	/	/	/	2	/	/	/	/	/	4	/
Total	42/48	4/48	0/48	47/48	0/48	5/48	41/48	2/48	0/48	740	437

[1]: sample from whole raw egg.
[2]: sample from pasteurised whole egg.
[3]: sample from pasteurised whole egg stored until shelf-life date (SLD).

2.2. Molecular typing: RFLP/PFGE

2.2.1. DNA extraction

Whole cellular DNA for PFGE was prepared as described previously (Liebana et al., 2001). Briefly, *Salmonella* isolates were grown overnight at 37 °C under gentle agitation in 3 ml of Luria-Bertani broth (Qbiogene, Montreal, Canada; 10 g/L tryptone, 5 g/L of yeast extract, 10 g/L NaCl). one ml of this solution was rinsed twice by centrifugation (8000 *g*, 3 min) with TN (Tris–HCl 10 mM pH 7.6, NaCl 1 M). The bacteria were suspended in TN to an $OD_{600\ nm}$ of 3 and 180 µl of this bacterial suspension was mixed with 180 µl of 1% standard agarose in sterile water kept at 55 °C, then rapidly poured into rectangular Biorad moulds. After removal from moulds, the blocks were placed in 50 ml tubes (Falcon) containing 2 ml of lysis solution (EDTA 0.5 M pH 9, N Lauryol Sarcosine 1%, Proteinase K 1 mg/ml). Lysis was carried out for 24 h minimum under gentle agitation at 50 °C. The lysis solution was then poured away and the blocks washed under gentle agitation at least 6 times with TE (Tris–HCl 10 mM pH 7.6, EDTA 1 mM).

2.2.2. Enzymatic digestion

The enzymatic digestion was performed according to the manufacturer's instructions using either 40 U of *Xba*I, or 40 U of *Spe*I for 6 h at 37 °C.

2.2.3. Electrophoresis

The DNA fragments were separated using a standard 1% agarose gel in TBE 0.5X at 14 °C in a Chef DRIII apparatus (Biorad Laboratories, USA).

The electrophoresis was performed at 6.6 V/cm under an angle of 120° in two steps: (1) initial time of 20 s, final time of 40 s for 12 h, followed by (2) initial time of 7 s, final time of 13 s for 10 h.

2.2.4. Images analysis

Gels were stained with ethidium bromide, visualized on an UV transilluminator and photographs were captured by a digital imaging system GelDoc 1000 (Video gel doc system, BioRad). The obtained patterns were analysed by Bionumerics software (Applied Maths, Sint-Marteen, Belgium). The relation between two given strains was scored using the Dice coefficient of similarity, and strains were grouped by the hierarchical clustering of inter-strain similarities based on the unweighted pair group method with arithmetic averages (UPGMA) (Romesburg, 1984). A variation in band mobility of 1.5% was tolerated. Fragment patterns were interpreted as described by Tenover et al., (1995).