

DISTRIBUTION OF *Salmonella* Enteritidis GENOTYPES AMONG SELECTED BROILER FLOCKS IN BOSNIA AND HERZEGOVINA

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Summary: Cases of human salmonellosis continued decreasing at the EU level in 2012; a total of 92,916 cases were reported by 27 EU Member States. With 91,034 confirmed cases, this represented a 4.7% decrease in comparison to 2011. In the EU, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the serovars most frequently associated with human illness. Human *S. Enteritidis* cases are most commonly connected with the consumption of contaminated eggs and poultry. In 2012, the prevalence of *Salmonella* spp. in broiler flocks was 3.1%. Serovar Enteritidis was isolated in 0.2% of broiler flocks at the EU level. According to the first post-war national monitoring program conducted during 2012 in Bosnia and Herzegovina, the prevalence of *Salmonella* spp. and *S. Enteritidis* in broiler flocks was 10.0% and 8.7%, respectively. To obtain better insight into the epidemiology of the dominant serovar *S. Enteritidis* within the selected broiler flocks, genotyping with pulsed-field gel electrophoresis (PFGE) was performed using *Xba*I with isolates obtained in 2010–2011 from broiler farms located in seven geographical regions with the highest density of them. Due to the apparent similarity of the genotypes found in several broiler flocks, our findings suggest a homogenous population of *S. Enteritidis* circulating among the vast majority of broiler flocks. Secondly, since identical or very similar genotypes were also found in faecal samples from broiler flocks and dust samples from hatcheries, a common source of infection can be indicated.

Key words: *Salmonella* Enteritidis; epidemiology; genotyping; PFGE; poultry; broiler flocks; Bosnia and Herzegovina

Introduction

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) continues to be one of the leading causes of bacterial foodborne diseases throughout the world (1–3). This is mainly attributed to the consumption of contaminated poultry products (4). In addition to health concerns, *Salmonella* spp. affecting animals and humans represents a recognized economic burden due to the chronic effects of

infections. A common reservoir of salmonellas remains in the intestinal tract of predominantly domestic poultry. Animal-to-human transmission occurs when the contaminated poultry and meat products are introduced into the food-production chain. In humans, the symptoms are often mild and self-limiting, although a severe disease with a fatal outcome is possible (5).

The transmission routes of salmonellas are various. Human salmonellosis is mostly associated with the consumption of faecally contaminated foodstuffs, such as eggs and poultry for *S. Enteritidis*, and pork, poultry and beef for the second most common serovar in humans in

the EU, *Salmonella* Typhimurium (3). In children, contact with the infected puppies or turtles (6, 7) and the consumption of raw milk (8) have also been reported to cause salmonellosis. In 2011 and 2012, some unusual sources for human salmonellosis were observed, including contaminated mung bean sprouts in Germany (9), smoked salmon in the Netherlands (10), and watermelons in the UK (11). Such findings indicate the variety of hosts and transmission routes in *Salmonella* epidemiology. Nevertheless, contaminated poultry eggs and meat remain the most common source of human salmonellosis (5, 12).

Knowledge of the dissemination of certain *Salmonella* serovars through the food chain, including primary poultry production, is crucial for the understanding of how food animals and/or food-processing procedures contribute to the contamination of products and subsequent human infections. Traditionally, phenotypic methods such as serotyping and phage typing have been used for the identification of *Salmonella* isolates in outbreak investigations. However, these methods have limited applicability for studies on the transmission of salmonellas because of their poor discriminatory power observed for closely related isolates (13, 14). For the latter, epidemiological studies, based on pulsed-field gel electrophoresis (PFGE), for example, are of great value (15–17). In many laboratories, PFGE has been accepted as the gold standard for the molecular typing of salmonellas during outbreaks of human salmonellosis (18). It was proven useful during the EU multistate outbreak of *Salmonella* Stanley infections in 2011–2012 (19), the *Salmonella* Brandenburg infections in patients hospitalized in a French clinic in 2010–2012 (20), the human *Salmonella* Dublin infection due to contaminated raw milk cheese in France in 2012 (21), and the human *S. Enteritidis* infection caused by contaminated eggs in Slovenia in 2009 (12). According to Uzunović-Kamberović (22) and Hadžiabdić (25), there is an evident lack of information on PFGE typing of *Salmonella* serovars isolated in Bosnia and Herzegovina (BiH). Although a retrospective study on human salmonellosis in the central part of BiH was previously described and provides some data on the prevalence and serovar distribution, their genetic diversity remains unknown (22).

The aim of our study was to perform a preliminary molecular typing of the selected *S. Enteritidis* isolates by PFGE, isolated in 2010–

2011 from farms located in the major broiler-producing regions in BiH, in order to obtain an overview of their genetic relatedness and diversity, in addition to discovering the possible sources of infection. The obtained data would enable the preparation of the most appropriate *Salmonella* prevention model in broiler flocks.

Materials and methods

S. Enteritidis isolates

In 2010–2011, *S. Enteritidis* strains were isolated from broiler flocks (18 positive samples/ isolates from 1,894 inspected samples) and hatcheries (two positive from 20 inspected) within the scope of a *Salmonella* research program in the primary poultry production in BiH. Broiler flocks and hatcheries originated from the geographical regions (GA 1–7) with the highest density of poultry population (Figure 1); see Table 1 for the distribution of samples, both the inspected and positive, according to their geographical origin. Sampling obtained faecal material from broiler flocks during the last three weeks of breeding ($n = 1,894$) and dust samples from hatcheries ($n = 20$). All the obtained *S. Enteritidis* isolates ($n = 20$) were subjected to PFGE typing.

Isolation and identification

The isolation and identification of *S. Enteritidis* from the faecal material of broilers and the dust samples from hatcheries was performed according to the standardized method (23). Briefly, samples were inoculated at the ratio of 1 : 9 into the non-selective pre-warmed BPW (Buffered Peptone Water; Himedia, India) and enriched for 18 ± 2 h at 37°C . The following day, selective enrichment in MSRV (Modified Semi-solid Rappaport Vassiliadis medium; Himedia, India) was performed and, in the case of swarming, followed by inoculation onto XLD (Xylose-Lysine-Deoxycholate agar; Himedia, India), BG (Brilliant Green agar; Himedia, India) and *Salmonella*-chromogenic agar (Oxoid, UK). *Salmonella*-suspected colonies were further identified by biochemical tests (API Rapid 20E; BioMerieux, France) and serotyped by commercial *Salmonella* antisera. All *S. Enteritidis* isolates were stored at -76°C in a cryo-protective medium for further genotyping by PFGE.

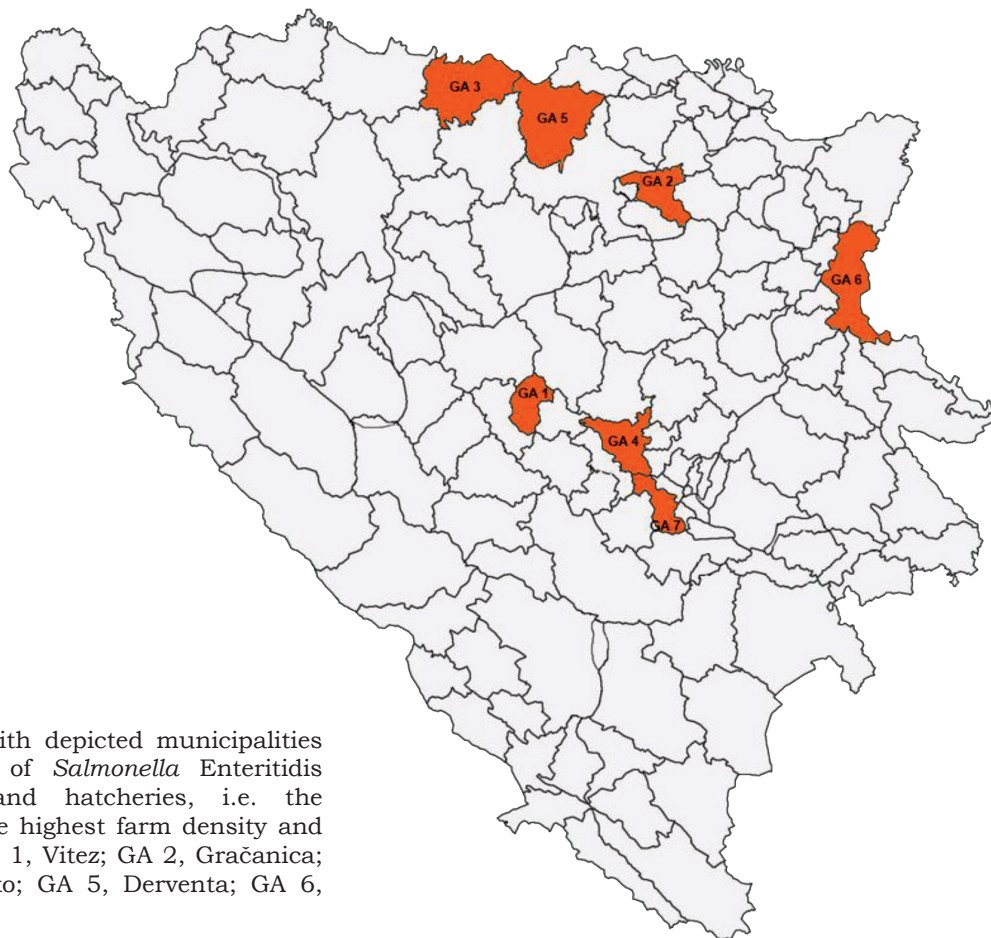


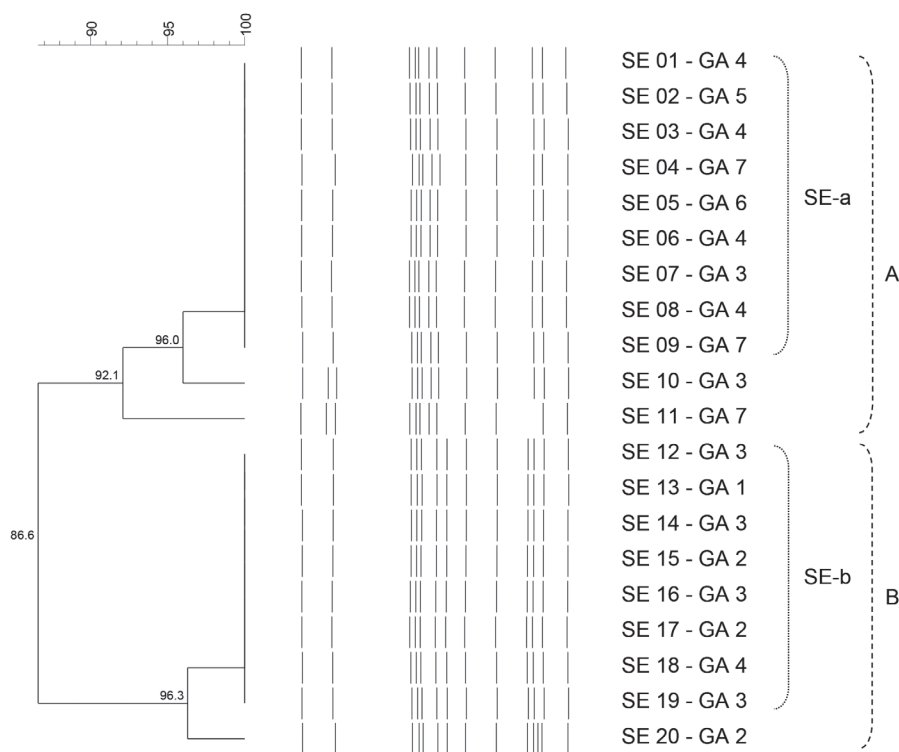
Figure 1: A map of BiH with depicted municipalities showing the distribution of *Salmonella* Enteritidis positive broiler farms and hatcheries, i.e. the geographical areas with the highest farm density and abbreviated as GA 1–7 (GA 1, Vitez; GA 2, Gračanica; GA 3, Srbac; GA 4, Visoko; GA 5, Derventa; GA 6, Zvornik; GA 7, Sarajevo)

Table 1: Distribution of samples according to the selected geographical regions

Geographical region	Broiler flocks (faecal samples)		Hatcheries (dust samples)	
	<i>Number of samples</i>			
	All	Positive	All	Positive
GA 1	100	1 (SE 13)	-	-
GA 2	280	3 (SE 15, SE 17, SE 20)	-	-
GA 3	574	6 (SE 07, SE 10, SE 12, SE 14, SE 16, SE 19)	-	-
GA 4	494	4 (SE 03, SE 06, SE 08, SE 18)	10	1 (SE 01)
GA 5	88	1 (SE 02)	-	-
GA 6	86	1 (SE 05)	-	-
GA 7	272	2 (SE 09, SE 11)	10	1 (SE 04)
<i>Total</i>	<i>1,894</i>	18	<i>20</i>	2

Note: For details on geographical regions, see Figure 1. The names of the positive samples (*S. Enteritidis* isolates) are given in parentheses and were assigned according to the results of genotyping (see Figure 2).

Figure 2: Dendrogram of 20 *Salmonella* Enteritidis pulsotypes showing the genetic relatedness of isolates (SE 01–20) obtained in 2010–2011 from broiler farms ($n = 18$) and hatcheries ($n = 2$; isolates SE 01 and SE 04) located in seven geographical areas of BiH (GA 1–7) with the highest farm density. A and B denote two clusters obtained according to the arbitrary 90% cut-off value for grouping by genotype similarity; Cluster A is subdivided into the SE-a group and two less similar isolates and cluster B into SE-b and one isolate



PFGE typing

Prior to PFGE, *S. Enteritidis* isolates were recovered on blood agar medium and subjected to PFGE typing according to the PulseNet standardized one-day protocol, using the restriction endonuclease *Xba*I (24). The obtained fragments were electrophoretically separated under the following conditions: 20 h at 6 V/cm and 14°C, with pulse times from 2 s to 64 s employing the CHEF-DR II system (BioRad, USA). PFGE profiles were subjected to computer-assisted analysis with BioNumerics software (version 6.6; Applied Maths, Belgium). For normalization, a molecular-sized standard *Salmonella* Braenderup strain H9812 (ATCC BAA-664) was used. For the construction of a similarity matrix, the band-based Dice coefficient with optimization and band-matching tolerance set to 1% was employed. A dendrogram was created using the UPGMA algorithm to enable the estimation of epidemiological relatedness among *S. Enteritidis* isolates.

Results

The prevalence of *Salmonella* spp. in broiler flocks during 2010–2011 was 2.9%, with a 1.0% prevalence of *S. Enteritidis*. Respecting

the arbitrary 90% cut-off value for clustering by genotype similarity, a PFGE dendrogram of 20 selected *S. Enteritidis* isolates revealed two major clusters: A and B (Figure 2).

Cluster A (isolates SE 01–11) could be subdivided into SE-a group (isolates SE 01–09) with identical genotypes and two other isolates (SE 10 and SE 11; SE 10 was more similar to SE-a than to SE 11) differing in one or two bands from SE-a. Similarly, cluster B (isolates SE 12–20) could be subdivided into an SE-b group (isolates SE 12–19) with identical genotypes and isolate SE 20 differing in one band from SE-b. Regarding the origin of isolates, *S. Enteritidis* from GA 3 ($n = 6$) and GA 4 ($n = 5$) were found in both clusters A and B, but isolates from GA 1 ($n = 1$) or GA 2 ($n = 3$) in B, and isolates from GA 5 ($n = 1$), GA 6 ($n = 1$) or GA 7 ($n = 3$) in A.

Interestingly, an *S. Enteritidis* isolate from one of the hatchery dust samples (SE 04) and a faecal isolate from broiler flock (SE 09), both from GA 7, showed identical genotypes (the third isolate, SE 11, from GA 7 showed a two-band difference to SE 04 and SE 09). An identical banding pattern was also observed for the second dust isolate (SE 01) when compared to faecal isolates from broiler flocks (SE 03, SE 06 and SE 08) from GA 4 in cluster A, but one isolate from GA 4 (SE 18) clustered in B. In general, no marked dissimilarities could be observed among all the 20 *S. Enteritidis* isolates.

Discussion

The occurrence of numerous human salmonellosis cases indicates the need for related epidemiological studies and effective control measures, which should not be diminished by the reports on human campylobacteriosis as the most common zoonosis in 2012 with 214,268 confirmed cases in the EU (3, 9–12). An effective control program for *Salmonella* spp. in the primary production very often seems to be the only possibility for a reduction of the bacterial load in food animals. In Bosnia and Herzegovina, the prevalence of *Salmonella* spp. in broiler flocks was 2.9% in 2010–2011, determined in the present preliminary study dated with sampling to a two-year period prior to 2012 when the first official sampling in BiH was initiated. At the EU level, the prevalence was 3.1% in 2012 and 3.2% in 2011, which showed a decrease in comparison to the 4.1% prevalence in 2010 (1, 3).

The two most common serovars found in broiler and layer flocks are *S. Enteritidis* and *S. Typhimurium* (2, 3). The reported average prevalence of the two target serovars in broiler flocks at the EU level continued to decline from 0.7% in 2009 and 0.4% in 2010 to 0.3% in 2011 (1, 5). At the EU level, the prevalence of *S. Enteritidis* in broiler flocks was 0.2% in 2011 and 2012 (1, 3). In 2010–2011, the prevalence of *S. Enteritidis* in broiler flocks in BiH was 1.0% (*this study*). In 2012, the first official monitoring program for salmonellas in BiH was launched, yielding insight into the prevalence of *Salmonella* spp. (10.0%) and *S. Enteritidis* (8.7%) in broiler flocks (25). Such an evident disproportion in the prevalence data (2.9% vs. 10.0% of *Salmonella* spp. and 1.0% vs. 8.7% of *S. Enteritidis* obtained in the preliminary study in 2010–2011 vs. official monitoring in 2012, respectively) could have several reasons. Specifically, a different sampling strategy (sampling performed by the farmers themselves in 2010–2011 vs. expert sampling in 2012) and a different sampling method employed (sampling of fresh faeces into collection containers in 2010–2011 vs. sampling by boot socks according to the adopted sampling legislation).

In BiH, the prescribed method for the detection of *Salmonella* spp. in faecal material is the horizontal standardized microbiological method (23). This method, followed by serotyping, enables

only limited options for a wider epidemiological research on the circulation of selected serovars. Therefore, the source-infection relations and the possibilities for intervention could only be discovered by the use of molecular typing methods, e.g. MLST, MLVA and PFGE that have been the most promoted in recent years (18). A standardized PFGE protocol has been routinely used in several laboratories (12, 26, 27) as promoted by EFSA and ECDC at the EU level. The initiative for protocol standardization and routine use of PFGE in epidemiological studies will lead toward the construction of a large database of pulsotypes, enabling prompt reactivity during outbreaks and adding markedly to the ensuring of public health (28).

Knowing the importance of *S. Enteritidis* for the poultry industry and public health, with 44.4% of all the reported serovars in human-confirmed cases (1, 3), the research on genetic diversity among circulating *S. Enteritidis* isolates in selected broiler flocks of BiH was initiated. It was shown by using PFGE that their genetic diversity was limited (86.6% similarity between the two discovered genetic clusters A and B); thus, they are closely related. According to the criteria by Tenover (29), an isolate is considered to be closely related to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with a single genetic event, i.e. point mutation or insertion/deletion. Such changes typically result in two or three band differences. Clustering of the pulsotypes could, therefore, indicate a dominant genotype circulating within geographical regions of BiH and a common source of infection. Since the vast majority of broiler farms are located in the regions that were selected for our study, intervention by taking the proper biosecurity measures could reduce *Salmonella* prevalence in these flocks and possibly lower the number of human salmonellosis cases. In several other studies, a continuous circulation of certain *Salmonella* serovars within poultry flocks has also been observed (26, 30). Taking into account the identical genotypes of *S. Enteritidis* isolates obtained in GA 4 and GA 7 from the hatchery (dust samples; SE 01 and SE 04, respectively) and broiler flocks (faecal samples; SE 03/06/08 and SE 09, respectively), vertical transmission of *S. Enteritidis* seems to be a reasonable explanation, consistent with other studies (26, 31, 32). Since the sampled parent flocks were negative for *S. Enteritidis* in

2010–2011, a more detailed sampling strategy would better explain, i.e. confirm or exclude, the vertical transmission of *S. Enteritidis* from parent flocks to progeny. This is strongly supported by the fact that the corresponding hatcheries are delivering one-day-old chickens to the majority of *S. Enteritidis*-positive broiler farms.

Regarding the considerable number of isolates and a limited value of PFGE in the epidemiological analysis of *S. Enteritidis*, due to high homogeneity among strains, this preliminary research is a significant innovation in the molecular typing of *S. Enteritidis* by PFGE in BiH. Therefore, it is a valuable contribution to future research on the epidemiology of salmonellas. This is strongly supported by our recent research in which an 85.0% similarity was observed among *S. Enteritidis* isolates obtained from broiler and laying-hen flocks (25), confirming the high homogeneity among *S. Enteritidis* isolates. It is also clear that a multiple approach that involves strict biosecurity measures, the education of poultry producers, consistent hygiene measures in the food-processing plants, and coordinated research by veterinary and public health experts is the only model for the prevention of *Salmonella* spp. contamination. In this regard, more attention should be given to establishing a national genotype database, enabling prompt reactions to food-related outbreaks, since such collaboration at the national level has already yielded promising scientifically verified results in Slovenia (12). The presented preliminary study calls for a wider coordinated research on *Salmonella* spp. epidemiology in BiH, indicating the emerging need for inclusion of molecular typing protocols in microbiological laboratories for the prevalent food-born pathogens, with the main goal of minimizing the incidence of human infections.

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RAZŠIRJENOST GENOTIPOV *Salmonelle* enteritidis V IZBRANIH REJAH PITOJNIH PIŠČANCEV V BOSNI IN HERCEGOVINI

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Povzetek: Leta 2012, ko je 27 držav članic prijavilo skupaj 92.916 primerov salmoneloz, se je v EU nadaljevalo upadanje števila primerov salmoneloz pri ljudeh. V primerjavi z letom 2011 je bil v letu 2012, ko je bilo 91.034 primerov tudi potrjenih, opazen 4.7% upad. *Salmonella* enteritidis in *Salmonella* typhimurium sta serovara, ki sta v EU najpogostejše povezana z obolenji pri ljudeh. Primeri *S. enteritidis* pri ljudeh so najpogostejše posledica uživanja okuženih jajc in perutninskega mesa. V rejah pitovnih piščancev je bila leta 2012 prevalenca *Salmonella* spp. 3.1 %. Serovar enteritidis je bil v EU izoliran pri 0.2 % rej pitovnih piščancev. Glede na rezultate prvega povojnega programa monitoringa, ki se je izvajal leta 2012, sta bili v Bosni in Hercegovini prevalenci *Salmonella* spp. in *S. enteritidis* v rejah pitovnih piščancev 10.0 % in 8.7 %. Namen našega dela je bil pridobiti boljši vpogled v epidemiologijo dominantnega serovara *S. enteritidis* v izbranih rejah pitovnih piščancev. Izolate, ki so bili pridobljeni v letih 2010–2011 na farmah pitovnih piščancev iz sedmih geografskih območij z največjo gostoto farm, smo tipizirali z metodo pulzne gelske elektroforeze (PFGE) ob uporabi encima *Xba*I. Zaradi očitne podobnosti genotipov, ki smo jih našli v različnih rejah pitovnih piščancev, rezultati kažejo na homogeno populacijo *S. enteritidis*, ki kroži med večino rej. Ker smo enake ali zelo podobne genotipe našli tudi v vzorcih fecesa iz rej pitovnih piščancev in v vzorcih prahu iz valilnic, lahko sklepamo tudi o skupnem viru okužbe.

Ključne besede: *Salmonella* enteritidis; epidemiologija; genotipizacija; PFGE; perutnina; reje pitovnih piščancev; Bosna in Hercegovina