# SALMONELLA ENTERITIDIS – PHENOTYPIC AND GENOTYPIC TECHNIQUES

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Today, *Salmonella enterica subspecies enterica serovar Enteritidis (S. Enteritidis)* represents one of the most common serotypes that causes enterocolitis. Since *S. Enteritidis* identification methods are advanced permanently, the following phenotyping methods could be applied for this purpose: biotyping, phagotyping (phage typing – PT), and resistotyping. From methods for genotyping of *S. Enteritidis,* plasmid profile analysis (PP), restriction analysis of the virulence plasmid, ribotyping, pulsed field gel electrophoresis (PFGE), insertion sequences, polymerase chain reaction (PCR), random amplified polymorphic DNA analysis (RAPD) could be applied. On the one hand*, S. Enteritidis* expresses clearly homogenous structure which is reflected by domination of few phagotypes, presence of one plasmid profile in most of strains, merely three clonal lines, as well as a large number of electrophoretic types in a single dendrogram line. Insufficient discrimination of typing systems of *S. Enteritidis* suggests the introduction of new typing methods as well as improvement of the old ones. *Acta Medica Medianae 2009;48(3):31-34.*

***Key words***: *Salmonella Enteritidis*, bacterial typing techniques, identification

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*Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S*. *Enteritidis*) represents one of the most common serotypes that causes enterocolitis (1). Although there is a constant improvement in certain *S*. *Enteritidis* strains identification methods, this serotype, due to its homogeneity, is very difficult for typing, whereas genotyping and phenotyping methods allow typing with certain limitations.

In identification of *S. Enteritidis*, the following phenotyping methods are applied: biotyping, phage typing (PT) and resistotyping. Resistotyping, usually applied for other salmonella serotypes, in case of *S.* Enteritidis, is not informative as the most of strains are susceptible to the majority of antibiotics applied (2). For biotyping of *S. Enteritidis*, 45 biochemical tests are necessary, though they are commonly used for typing of bacteria from *Enterobacteriaceae* genera. However, phagotype (PT) 8 strains, which are most common worldwide (80%), have identical reaction to majority of usual tests, with exception of melibiose-negative reaction, which has usually been associated with phage types PT 13a and 14b (3).

There are several phagotyping methods for identifying of *S. Enteritidis*. Colindale phagotyping method enables classification of this serotype in 27 phagotypes. Still, most of the strains belong to one or two most common phagotypes (4). At the moment, this system requires the collection of 10 phages at least. Until now, two more methods have been developed, which means that there is no international agreement about this issue, and potential confusion about predominant phagotypes in different countries may occur. In order to bring in this technique, it usually takes several months. The advantage of phagotyping is the possibility to type up to 40 strains in 48 hours, while disadvantage is low discriminatory level among strains, as they mostly belong to PT4 and 8 (4). On the other hand, a conversion from one phagotype to another is recorded (5).

Biochemical fingerprinting (Phene Plate System) method, applied for examination of 86 *S. Enteritidis* strains isolated in Germany between 1980 - 1992, identified 23 biochemical phenotypes (BPT). The combination of biochemical fingerprinting and phagotyping methods allowed a classification of 25 phenotypes. Phenotype C2:8 (BPT: PT) was found over an extended period of time, whereas phenotype C4:4 was isolated just between 1988 and 1992 (6). This finding suggests that the combination of certain phenotyping methods could improve their discriminatory power.

Furthermore, the combined application of electrophoresis of multiple locus enzyme method, outer membrane protein analysis, whole cells protein profile (WCPP) analysis, and Fourier Transform Infrared Spectroscopy (FTIR) on *S. Enteritidis* PT 25/17 strains revealed that within one phagotype strains different from basic profile could be found. WCPP and FTIR methods have been confirmed as suitable for additional classification of *S*. Enteritidis (7).

Genotyping methods applied on *S. Enteritidis* are plasmid profile analysis (PP), restriction analysis of the virulence plasmid, ribotyping, pulsed field gel electrophoresis (PFGE), insertion sequences (ISs), polymerase chain reaction (PCR), and random amplified polymorphic DNA analysis (RAPD).

Among the methods mentioned above, only plasmid profile analysis of *S*. Enteritidis has not high discriminatory power (8). By examination of *S*. *Enteritidis* strains' plasmid profiles isolated in this area, 12 profiles have been differentiated. However, most of them have had the same plasmid profile with 38MDa plasmid. Furthermore, the difference between the presence of plasmid profiles in epidemical and non-epidemical strains observed for the same time period has not been found (9).

On the other hand, the combination of phagotyping and plasmid profile analysis has been applied with more success. In an assay of 318 strains of *S. Enteritidis*, mostly isolated from poultry in Canada, 12 phage types were identified. However, among these strains, it was possible to differentiate fifteen plasmid profiles (10). From 1989 to 1990, examining plasmid profile of *S. Enteritidis* isolated from Canadian poultry solely, researchers found twelve plasmid profiles against four phage types only (11).

Regarding *S. Enteritidis* PT8, it is considered that the spreading of this predominant clone, with commonly evidenced PP 38 MDa, caused diminishing of discriminatory power for both typing systems. By examination of 203 sporadic and outbreak-related *S. Enteritidis* isolates obtained in Maryland from 1985 to 1990, ten plasmid profiles were identified. PP 55 kba (38 MDa) emerged as a predominant profile; it participated with 86% of whole plasmid profile among isolates of this phage type obtained in the period 1988-1989 (12).

Another study has found that plasmid profile analysis is superior to phage typing method in respect to PT 8 strains as it was possible to describe six different plasmid profiles against only one phage type. On the other hand, by applying two additional phages it was possible to differentiate three strains without 55 kba (38 MDa) plasmid (3). These results confirmed that in some cases plasmid profile analysis and phage typing could be complementary methods. Investigation of 27 *S*. *Enteritidis* phage type's representatives showed that only eleven plasmid profiles could be identified. Two plasmid profiles have been evidenced among fifteen PT4 and PT8 strains, which are the most common phage types in Great Britain. Plasmid profile variations, suitable for outbreak-related researches, have been found at thirteen phage types (13). *S*. Enteritidis PT4 had nine different plasmid profiles (14). Thus, plasmid profile analysis, by these authors, could be considered as an efficient complement to phage typing method.

In some studies, ribotyping has been shown as more discriminatory than plasmid profile analysis and phage typing methods. By investigation of *S*. *Enteritidis* strains isolated in poultry, obtained from two unrelated outbreaks and sporadic isolates of humans, authors have found five different plasmid profiles and four phage types. However, ten ribotypes have been determined. These findings indicate that ribotyping could be applied in differentiation of strains with the same plasmid profile (15).

Ribotyping could be considered as well as a complementary method to the plasmid profile analysis. *S*. *Enteritidis* strains obtained from stool samples of the same two groups of patients at different times in period of four months after infection was analyzed. It was confirmed that sensitivity of ribosomal DNA analysis were enzyme-related. From fourteen various enzymes involved, only *Sma*I and *Sph*I gave different profiles. Differences were determined among groups though all strains of the same group had identical profile. Sixty-six days after the beginning of disease, the *Sph*I profile different from the original isolate obtained from the same patient was observed. Since there was the difference among plasmid profiles related to strains of the mentioned groups (PP 55 4.5 kba and PP 55 4.3 kba) (PP 38 3MDa and PP 38 2.8MDa) it was evidenced that analysis of rRNA gene could be complementary to the analysis of plasmids if they were present (16).

Since plasmid profile analysis commonly does not allow efficient subtyping of *S. Enteritidis* PT 8, for that purpose, ribotyping was introduced. The investigated strains were obtained from Northeast, Mid-Atlantic and Midwest region of the USA mainly. Most of the strains (15 to 20) examined in that study had identical plasmid profile as well as ribotype which suggested similarity of these strains. By *Acc*I restriction endonuclease, six different profiles were identified, while one of the samples could be subtyped by enzyme *Sma*I. Outbreak-related strains had identical ribotypes. These imply that ribotyping with *Acc*I and *Sma*I provides additional discriminatory power among certain PT 8 strains (17). By restriction analysis of *S. Enteritidis* virulence plasmid obtained from different sources, conservative character of the nucleotide sequences of the virulence plasmid with molecular weight of 38 MDa was confirmed. Therefore, it is concluded that the restriction analysis of this plasmid is not convenient in defining of the outbreak strain (18).

PFGE analysis of *S. Enteritidis* PT 4 strains isolated in England and Wells, in combination with the restriction enzyme *Xba*I, demonstrated nine major profiles with the predominant one (14). Furthermore, with the same enzyme, this technique applied on *S. Enteritidis* PT4 strains, isolated from humans and poultry in Korea, determined ten profiles (19). However, the application of this method on strains with phage types 9a, 13a, 25 isolated in Slovakia, gave only two profiles which did not correspond to determined phage types (20). On the other side, combined application of PFGE patterns with three restriction enzymes (*Xba*I, *Spe*I, and *Not*I), plasmid profile analysis and phage typing to *S. Enteritidis* strains isolated in Taiwan, confirmed the existence of 46 subtypes where discriminatory index was 0.795 (21).

There was an attempt to differentiate *S. Enteritidis* strains by determining IS200 profile which led to the conclusion that *S. Enteritidis* isolates have high level of genome homogeneity. *S*. *Enteritidis* contains both constant and variable insertion regions of the insertion sequence (IS) 200. Examination of *S. Enteritidis* isolated in Switzerland showed that only three clonal lines could be identified by determination of IS200 profile (22). Analysis of rearranged DNA around variable regions and their comparison with phage type have shown that PT 4 human isolates related to an enterocolitis outbreak belong to the same clonal line. Since the application of IS200 has proved three basic clonal lines, a partial differentiation of PT4 and 8 was enabled. PT 4 nearly matched the clonal line (SeCL) I, PT 8 matched SeCL II, and PT NT, 15, and 11 matched SeCl III which could be found rarely after 1983. Taking these findings into account, it was considered that the profile obtained by SE IS200

could be used for investigation of epidemiology of less common serotypes (22). The application of insertion sequences for *S. Enteritidis* strains typing is not always successful. By applying IS200 in combination with ribotyping to fourteen strains of this serotype, the French authors could not carry out typing which was possible by determination of plasmid profile and drug susceptibility assay (23).

Quantitative Polymerase Chain Reaction (qPCR) could also be applied for the analysis of *S. Enteritidis* strains. Part of the plasmid DNA fragment, serotype specific and designated as *PstI/PvuII* located at *S*. *Enteritidis* virulence plasmid was used in development of primers for this reaction (24).

Random amplified polymorphic DNA analysis – RAPD is also implemented in differentiation of *S*. *Enteritidis* isolates. Twenty-nine isolates were subtyped by phage typing, ribotyping, and PFGE on 20 different subtypes. However, RAPD fingerprinting itself has enabled subtyping into fourteen RAPD types. Isolates of *S. Enteritidis* which could not be subtyped in any other way, were differentiated by that method into three subtypes. On the contrary, isolates from the same source were not differentiated by any of subtyping methods (25).

The application of combined methods is not necessarily successful in typing of *S. Enteritidis*. The combination of sero-phage typing, plasmid profile analysis, genome fingerprinting, and ribotyping were used in typing of *S*. *Enteritidis* which caused an outbreak in one region of Spain. Analyzed isolates were phage type A, with plasmid of 38 MDa, and they expressed similar genome fingerprinting and the same ribotype (26).

Four methods were applied in investigation of *S. Enteritidis* strains represented with 33 phage types and one phage susceptible strain which is classified as RDNC (React Did Not Comply). These methods were based on chromosomal typing which allows determining of genome relationships between strains of different phage types. Isolates were observed by IS200, ribotyping, PFGE, and gene probes. Combination of these four genotypic methods resulted in forming of two groups of strains with eight and seven phage types respectively. These groups could be considered as major evolutionary lines of *S. Enteritidis* (27).

Analysis of *S. Enteritidis* multiple-locus enzymes has evidenced fourteen electrophoretic types (ET). These types were unequally distributed in salmonella dendogram. Ten of fourteen electrophoretic types (1, 5, 6, 9, 10, 12–15, 17) were in line A, and the other electrophoretic types were in three additional lines. ET 7 belongs to line C, ET 3 and 4 forming line H, and ET 2 belongs to line L; each of them has distant relationship with line A. This analysis revealed that *S. Enteritidis* strains are genotypic heterogeneous and could be presented with many highly divergent phylogenetic lines (28).

Electrophoresis of multiple-locus enzymes defined *S. Enteritidis* as poly-phylogenetic serotype closely connected with salmonellas which contain flagellar *g* antigen: *S. Gallinarum* and *S. Pullorum*. It is indicated that *S. Enteritidis* takes the oldest and leading position among these salmonella serotypes (29). Also, similarity between genomes of *S. Enteritidis* and *S.* is determined and it is considered that these two serotypes have been created recently from mutual origin (30). Evolution of *S. Dublin* included modification of phase one of flagellar antigen, loosing of epitope *m* and obtaining of epitope *p*, the changes in plasmid virulence structure and relationship with cattle infections (30). On the other side, *S*. *Enteritidis* genom is similar to genom of some *S*. Typhimurium strains which is confirmed by chromosomal mapping of these two bacteria (31).

On the one hand, *S. Enteritidis* demonstrated highly homogeneous structure due to domination of few phage types, presence of one plasmid profile among the majority of strains, existence of three clonal lines only, as well as numerous electrophoretic types in a single dendrogram line. Additionally, *S. Enteritidis* exhibit genetic similarity with salmonellas which contain similar flagellar antigens, while some electrophoretic types are far away from line A. On the one hand, we have clearly genetic homogeneity of the majority of strains, and on the other distinct genetic heterogeneity among the rest of strains; hence, it could be the reason for difficulties in subtyping of *S. Enteritidis* with known pheno- and genotyping methods. Facing with insufficient discrimination ability of systems for typing of *S. Enteritidis* requires the introduction of new ones and further improvement of existing strain typing methods.

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# SALMONELLA ENTERITIDIS - TEHNIKE FENOTIPIZACIJE I GENOTIPIZACIJE

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Danas je Salmonella enterica subspecies enterica serovar Enteritidis (S. Enteritidis) jedan od najzastupljenijih serotipova uzročnika enterokolitisa. S obzirom da se metode identifikacije S. Enteritidis stalno usavršavaju, od fenotipskih metoda mogu se primeniti biotipizacija, fagotipizacija (Phage typing - PT) i rezistotipizacija. Od genotipskih metoda tipizacije na S. Enteritidis se primenjuju analiza plazmidskog profila (PP), restrikciona analiza plazmida virulencije, ribotipizacija, elektroforeza u pulsirajućem polju jednosmerne struje (Pulsed Field Gel Electrophoresis - PFGE), insercione sekvence, lančana reakcija polimeraze (Polymerase Chain Reaction – PCR), analiza strukture slučajno odabrane sekvence DNK (Random Amplified Polymorfic DNA Analysis - RAPD). S. Enteritidis sa jedne strane ispoljava izrazito homogenu strukturu koja se manifestuje dominacijom nekoliko fagotipova, ispoljavanjem jednog plazmidskog profila u većini sojeva, postojanjem samo tri klonalne linije, kao i većim brojem elektroforetskih tipova u samo jednoj liniji dendrograma. Suočavanje sa nedovoljnom diskriminacijom tipizirajućih sistema kod S. Enteritidis upućuje nas na uvođenje novih metoda i poboljšavanje već postojećih za tipizaciju sojeva. *Medica Medianae 2009; 48(3): 31-34.*

***Ključne reči**: Salmonella Enteritidis, tehnike tipizacije bakterija, identifikacija*