**Molecular Methods for Identification of Monophasic Salmonella Typhimurium Strains**

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**Abstract**

Two molecular biology methods were used to differentiate *Salmonella enterica* 1,4,[5],12:i- strains: “Salmonella Check&Trace microarray” (CT) and multiplex PCR (mPCR). For 92 strains in CT result “Salmonella 1,4,[5],12:i-” were obtained. Those strains were confirmed in mPCR as monophasic fljB-lack *Salmonella* Typhimurium. For 17 strains, which in CT assay were recognized as *Salmonella* Typhimurium, the same identification was obtained in mPCR. Reference *Salmonella* strains: Lagos, Agama, Tsevie, Glocester and Tumodi in CT were recognized as *Salmonella* genovar, in mPCR – as *Salmonella* O:4. H1 other than *Salmonella* Typhimurium, the same like *Salmonella* Farsta, recognized incorrectly in CT as *Salmonella* Typhimurium.

**Key words:** microarray, molecular methods, monophasic *Salmonella* Typhimurium, multiplex PCR, serotyping

*Salmonella* is a significant etiologic agent of bacterial intestinal infections in Poland and other European Union countries. According to the data published by NPHI-NIH Department of Epidemiology in the report “Infectious diseases and poisonings in Poland in 2013”, 7577 cases of human *Salmonella* infections were notified in 2013 in our country. Among the most common *Salmonella* serovars, *Salmonella* Enteritidis and *Salmonella* Typhimurium were isolated. Basing on the data collected by European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), similar situation is also observed in other European countries, but as far as the end of the last century *Salmonella* Enteritidis were responsible for about 80% of cases of salmonellosis, whereas since 2000 systematic decrease in the frequency of isolation of serovar Enteritidis is observed. At the same time, especially in recent years, increase of infections caused by *Salmonella* Typhimurium is notified. This is a quite worrysome situation, because in opposition to *Salmonella* Enteritidis strains, which mostly are antimicrobial-sensitive, among *Salmonella* Typhimurium isolates about 60% of strains isolated from people have higher antimicrobial resistance, especially for ampicillin, tetracycline and sulphonamides. Moreover, at the end of the ‘90s in a few European countries and USA outbreaks of food poisoning caused by *Salmonella enterica* subsp. *enterica* 1,4,[5],12:i- were observed. Using methods of molecular biology, this etiologic agent was finally recognized as *Salmonella* Typhimurium. Since the 90’s increasing incidence of monophasic *Salmonella* strains with antigenic formula 1,4,[5],12:i- has been observed. According to the EFSA/ECDC data, *Salmonella* 1,4,[5],12:i- has been the third most common *Salmonella* serovar isolated from human samples in 2011. 90 % of these strains were resistant to ampicillin, streptomycin, tetracycline and sulphonamides in (EFSA, 2012).

An increasing number of *Salmonella* O:4 strains with “i” flagellar antigen, non-agglutinating with any antisera for second-phase of H antigen is also observed in Poland. There is no precise information, of which *Salmonella* serovar this monophasic variant is. It could be monophasic *Salmonella* Typhimurium or one of the other serovars, belonging to the same serogroup, which could have such antigenic formula, i.e. *Salmonella* Lagos, *Salmonella* Agama, *Salmonella* Farsta, *Salmonella* Tsevie, *Salmonella* Glocester or *Salmonella* Tumodi (Grimont and Weill, 2007).

*Salmonella* serovars mentioned above could be recognized in a few ways. The most popular is the conventional serotyping method – determining the presence of somatic and flagellar antigens by slide agglutination

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using specific antisera (Szych and Madajczak, 2010). Then serovar is identified according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Unfortunately, this method has many limitations, connected with antisera quality or atypical forms of Salmonella antigens. Such deviation like monophasic form or roughness could obstruct the determination of antigenic structure and serovar identification. For that reason many alternative – molecular biology based methods have been developed, however not all of them are routinely used. The most precise, and the same most complicated, are microarray-based and sequencing-based methods (Achtman et al., 2012; Braun et al., 2012; Franklin et al., 2011). Some of those methods found application in commercially available tests, like microarray based “Salmonella Check&Trace” (Check-Points BV, Netherlands) or bead-based suspension array as xMAP® Salmonella Serotyping Assay (Luminex, USA) (Fitzgerald et al., 2007; Jean-Gilles Beaubrun et al., 2014; Wattiau et al., 2008). All these methods could be used for differentiation of Salmonella serovars with O:4 and H:i antigens, with undetectable second phase flagellar antigen by traditional methods, but they are a little bit too complicated. For that reason, EFSA in their scientific opinion proposed the simple multiplex-PCR-based method for identification and differentiation of Salmonella Typhimurium and its monophasic variant 4,[5],12:i:-. In this method two genetic markers are used: intergenic region of the phase I flagellin gene cluster fliB-fliA and variable region of fliB gene, which encodes II-phase flagellar antigen (EFSA BIOHAZ, 2010).

In this study two molecular biology methods were used to differentiate Salmonella strains belonging to the O:4 serogroup and sharing the same first flagellar antigen H:i: microarray-based method and recommended by EFSA multiplex PCR.

One hundred and ten Salmonella enterica subsp. enterica strains with O:4 and H:i antigens have been used for this study. Samples were collected in 2007–2012 years in regional Epidemiological Sanitary Stations in Poland and in a private company from the food-quality sector. Moreover, the reference strain Salmonella Typhimurium ATCC 700720 has been used as well as Salmonella Lagos, Salmonella Agama, Salmonella Farsta, Salmonella Tsevie, Salmonella Glocester and Salmonella Tumodi strains obtained from the National Salmonella Centre (Gdańsk, Poland). All strains used for study were reidentified according to the routinely used procedure, based on the determination of biochemical features in classical homemade tube tests and serovar identification by slide agglutination using specific antisera (Biomed Kraków, Poland; Immunolab, Poland; Statens Serum Institute, Denmark) according to the White-Kauffmann-LeMinor scheme (Grimont and Weill, 2007; Szych and Madajczak, 2010). Moreover, the phase inversion was performed to obtain II-phase flagellar antigen in accordance with routine procedure (Szych and Madajczak, 2010).

To perform Microarray-based method, the commercially available test “Salmonella Check&Trace microarray” (CT) (Check-Points BV, Netherlands) was used. Assay was performed according to manufacturer instruction. In multiplex PCR the fliB gene fragment and intergenic region of the phase I flagellin gene cluster fliB-fliA fragment were detected according to the procedure recommended by EFSA (EFSA BIOHAZ, 2010). For Salmonella Typhimurium two fragments were expected: 1389 bp for fliB gene and 1000 bp for intergenic region. For monophasic Salmonella Typhimurium only one product was expected – 1000 bp for intergenic region. Other O:4 strains sharing the “i” antigen have two fragments 1389 bp (fliB) and 250 bp (intergenic region).

One hundred and forty six Salmonella enterica subsp. enterica O:4 strains, that harbor H:i antigen were submitted to the laboratory where this study was performed. After the reidentification process 110 strains were selected for further research. For all strains the second phase of the flagellar antigen was not detected, even in the phase inversion process. The presence of O:1, O:5, O:12 somatic antigens was varied. All other reference Salmonella strains used in the study (serovars: Lagos, Agama, Farsta, Tsevie, Glocester, Tumodi and Typhimurium ATCC700720) were recognized correctly. Seventeen of all monophasic Salmonella strains used in the study were recognized in CT assay as Salmonella Typhimurium. For 16 strains in Salmonella Typhimurium group, “Salmonella Typhimurium (10909)” result was obtained and for one strain (PZH 113/07) “Salmonella Typhimurium (11933)”. Ninety two strains were recognized as “Salmonella 1,4,[5],12:i:-(2717)”. For one strain (PZH 258/12) the result “Salmonella, genovar 10397” was obtained. For the selected strains from the Salmonella Typhimurium group mentioned above, a more precise phase inversion process was performed. Highly concentrated, not-commercially available, H:i antisera have been used. For one such strain, after third round of phase inversion with addition 0.5 ml of antisera, a positive reaction with II-phase antigens H:1,2 was obtained (strain no PZH 113/07). For three more strains phase inversion was unsuccessful. For reference non- Salmonella Typhimurium strains in CT assay “Salmonella, genovar” result was obtained with various code. One of them – Salmonella Farsta was incorrectly recognized as “Salmonella Typhimurium, 10099”. The CT assay for this strain was repeated with the same faulty result. Detailed information about CT assay results are presented in Table 1.

Ninety two S. enterica subsp. enterica 1,4,[5],12:i:- strains, recognized in both (serotyping and microarray)
methods as *S. enterica* 1,4,5,12:i:- in multiplex PCR were recognized as monophasic *Salmonella* Typhimurium, with single 1000 bp product for *flfB-flfA* intergenic region. Strain PZH 258/12 was also recognized as monophasic *Salmonella* Typhimurium. Strain PZH 113/07 was found to be *Salmonella* Typhimurium with two specific multiplex PCR products. The same result was obtained for 16 strains with difference in serotyping and CT microarray and for reference strain *Salmonella* Typhimurium ATCC 700720. For all reference non-*Salmonella* Typhimurium strains, two DNA fragments were detected – 1389 bp and 250 bp, as expected.

According to the data published by EFSA/ECDC monophasic *Salmonella* Typhimurium is one of the most frequent serovars isolated from human, animal food and feed samples in the last years (EFSA and ECDPC, 2014). Unfortunately it is impossible to distinguish it with conventional methods from other and ECDPC, 2014). Unfortunately it is impossible to distinguish it with conventional methods from other

<table>
<thead>
<tr>
<th>Final serovar / antigenic formula identification</th>
<th>No</th>
<th>CT microarray result</th>
<th>Multiplex PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em> 1,4,5,12:i:-</td>
<td>16</td>
<td><em>Salmonella</em> Typhimurium (10909)</td>
<td>S. Typhimurium</td>
</tr>
<tr>
<td><em>S. Typhimurium</em></td>
<td>1</td>
<td><em>Salmonella</em> Typhimurium (11933)</td>
<td></td>
</tr>
<tr>
<td><em>S. Typhimurium</em> ATCC 700720</td>
<td>1</td>
<td><em>Salmonella</em> Typhimurium (10909)</td>
<td></td>
</tr>
<tr>
<td><em>S. enterica</em> 1,4,5,12:i:-</td>
<td>92</td>
<td><em>Salmonella</em> 1,4,5,12:i:- (2717)</td>
<td>Monophasic <em>S. Typhimurium</em></td>
</tr>
<tr>
<td><em>S. enterica</em> 1,4,5,12:i:- **</td>
<td>1</td>
<td><em>Salmonella</em>, genovar (10397)</td>
<td></td>
</tr>
<tr>
<td>S. Tumodi</td>
<td>1</td>
<td><em>Salmonella</em>, genovar (8232)</td>
<td><em>Salmonella</em> O:4, H:i other than <em>S. Typhimurium</em></td>
</tr>
<tr>
<td>S. Gloucester</td>
<td>1</td>
<td><em>Salmonella</em>, genovar (14390)</td>
<td></td>
</tr>
<tr>
<td>S. Tisie</td>
<td>1</td>
<td><em>Salmonella</em>, genovar (15912)</td>
<td></td>
</tr>
<tr>
<td>S. Farsta</td>
<td>1</td>
<td><em>Salmonella</em> Typhimurium (10909)**</td>
<td></td>
</tr>
<tr>
<td>S. Agama</td>
<td>1</td>
<td><em>Salmonella</em>, genovar (10762)</td>
<td></td>
</tr>
<tr>
<td>S. Lagos</td>
<td>1</td>
<td><em>Salmonella</em>, genovar (9865)</td>
<td></td>
</tr>
</tbody>
</table>

* Strain PZH 113/07 initially identified as *S. enterica* 1,4,5,12:i:-; ** Strain PZH 258/12; *** Incorrect identification

Moreover, Echeita *et al.* developed a PCR procedure for H1 antigenic complex identification and for other antigens (Echeita *et al.*, 2002; Echeita and Usera, 1998). Both techniques could be used also for monophasic *Salmonella* Typhimurium identification, but under condition – *flfB* gene presence. Present studies, similar to the results of Echeita *et al.* showed that epidemic *S. enterica* subsp. *enterica* 1,4,5,12:i:- strains are *flfB*-negative variants of *Salmonella* Typhimurium (Echeita *et al.*, 2001). For that reason other techniques must be used for such strains identification, for example EFSA-recommended two-genes multiplex PCR (EFSA BIOHAZ, 2010). Check&Trace microarray mentioned previously is also useful for monophasic *Salmonella* strains serotyping, including *S. enterica* subsp. *enterica* 1,4,5,12:i:- serovar (Check-Points BV, 2014). Validation of this test was the subject of a few studies, also for diagnostically difficult *Salmonella* strains (Jean-Gilles Beaubrun *et al.*, 2014; Madajczak and Szych, 2010; Wattiau *et al.*, 2008). In previous studies usefulness of CT assay was confirmed, also for monophasic strains, but not for monophasic *Salmonella* Typhimurium (Madajczak and Szych, 2010). Present study, confirmed similar capability of both tests to differentiate *Salmonella* Typhimurium from non-*Salmonella* Typhimurium strains, however multiplex PCR could also confirm the presence of *flfB* gene sequence, which encode second phase of flagellar antigen, what give more information needed for complete strain identification. Together with *flfB-flfA* intergenic region typing it gives information about affinity to group of *Salmonella* serovars, which harbor O:4 and H:i antigens. Moreover CT microaray gave faulty identification for *Salmonella* Farsta, correctly recognized in multiplex PCR. Unfortunately it was impossible to validate both tests for identification of monophasic *Salmonella* strains O:4 and H:i. Such
strains were not isolated during the 6 years of our study, 
but according to principle of operation of both tests, it 
is probable, that they could be correctly recognized in 
multiplex PCR and unrecognized in CT assay.

Comparing these two methods, another important 
factor should be taken into consideration, that is the 
costs of equipment needed to perform both tests. CT 
assay needs dedicated only for this technique (Tube 
Array) reader. Multiplex PCR could be performed with 
standard thermocycler available in many laboratories. 

Multiplex PCR seems to be a better method then 
Check&Trace microarray for differentiation of mono-
phasic Salmonella Typhimurium and other Salmonella 
which harbor O:4 and H:i antigens.

Acknowledgments

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