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SHORT COMMUNICATION

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, H:i 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 – 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 *fli*B-*fli*A and variable region of *flj*B 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; Staten 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 *flj*B gene fragment and intergenic region of the phase I flagellin gene cluster *fli*B-*fli*A fragment were detected according to the procedure recommended by EFSA (EFSA BIOHAZ, 2010). For *Salmonella* Typhimurium two fragments were expected: 1389 bp for *flj*B 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 (*flj*B) 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 antiserum have been used. For one such strain, after third round of phase inversion with addition 0.5 ml of antiserum, 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, 10909". The CT assay for this strain was repeated with the same faulty result. Detailed information about CT assay results are presented in Table I.

Ninety two *S. enterica* subsp. *enterica* 1,4,[5],12:i:- strains, recognized in both (serotyping and microarray)

Table I
Summarized results of CT microarray and multiplex PCR.

Final serovar / antigenic formula identification	No	CT microarray result	Multiplex PCR result
S. enterica 1,4,[5],12:i:-	16	*Salmonella* Typhimurium (10909)	*S.* Typhimurium
S. Typhimurium*	1	*Salmonella* Typhimurium (11933)	
S. Typhmurium ATCC 700720	1	*Salmonella* Typhimurium (10909)	
S. enterica 1,4,[5],12:i:-	92	*Salmonella* 1,4,[5],12:i:- (2717)	Monophasic *S.* Typhimurium
S. enterica 1,4,[5],12:i:- **	1	*Salmonella*, genovar (10397)	
S. Tumodi	1	*Salmonella*, genovar (8232)	*Salmonella* O:4, H:i other than *S.* Typhimurium
S. Gloucester	1	*Salmonella*, genovar (14390)	
S. Tsevie	1	*Salmonella*, genovar (15912)	
S. Farsta	1	*Salmonella* Typhimurium (10909)***	
S. Agama	1	*Salmonella*, genovar (10762)	
S. Lagos	1	*Salmonella*, genovar (9865)	

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

methods as *S. enterica* 1,4,[5],12:i:- in multiplex PCR were recognized as monophasic *Salmonella* Typhimurium, with single 1000 bp product for *fli*B-*fli*A 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* Typhmurium 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 monophasic *Salmonella* from O:4 group, which harbor H:i antigen. It could be done only using molecular biology methods, which allow to identify *Salmonella* serovar, when classical serologic methods fail (Fitzgerald *et al*., 2007; Franklin *et al*., 2011; Junia Jean-Gilles Beaubrun *et al*., 2012; Wattiau *et al*., 2008). Those methods were not developed only for monophasic *Salmonella* Typhimurium identification, but for broad spectrum of *Salmonella* serovars. They are also not routinely used, mostly because of high hardware requirements and high costs for single test. For that reason other techniques, mostly simple PCR-based, were developed, which allow to support conventional serotyping technique, especially in case of difficult strains (monophasic or rough). For example, Herrera-Leon et all developed multiplex PCR for identification of most common phase-I flagellar antigens (Herrera-Leon *et al*., 2004). 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 – *flj*B 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 *flj*B-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 *flj*B gene sequence, which encode second phase of flagellar antigen, what give more information needed for complete strain identification. Together with *fli*B-*fli*A intergenic region typing it gives information about affinity to group of *Salmonella* serovars, which harbor O:4 and H:i antigens. Moreover CT microarray 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.

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

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Literature

Achtman M., J. Wain, F.-X. Weill, S. Nair, Z. Zhou, V. Sangal and S. Brisse. 2012. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathogens* 8: e1002776.

Braun S.D., A. Ziegler, U. Methner, P. Slickers, S. Keiling, S. Monecke and R. Ehricht. 2012. Fast DNA serotyping and antimicrobial resistance gene determination of *Salmonella enterica* with an oligonucleotide microarray-based assay. *PloS One* 7: e46489.

Check-Points B.V. 2014. List of serovars identified by Check&Trace Salmonella.

Echeita M.A., S. Herrera, J. Garaizar and M.A. Usera. 2002. Multiplex PCR-based detection and identification of the most common Salmonella second-phase flagellar antigens. *Research in Microbiology* 153: 107–113.

Echeita M.A., S. Herrera and M.A. Usera. 2001. Atypical, *fljB*-negative *Salmonella enterica* subsp. *enterica* strain of serovar 4, 5, 12: i:- appears to be a monophasic variant of serovar Typhimurium. *Journal of Clinical Microbiology* 39: 2981–2983.

Echeita M.A and M.A. Usera. 1998. Rapid identification of *Salmonella* spp. phase 2 antigens of the H1 antigenic complex using. *Research in Microbiology* 149: 757–761.

European Food Safety Authority (EFSA). 2012. Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010. *EFSA Journal* 10: 1–442.

European Food Safety Authority Panel on Biological Hazards (EFSA BIOHAZ). 2010. Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. *EFSA Journal* 8: 1–48.

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDPC). 2014. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA Journal* 12: 3547–3859.

Fitzgerald C., M. Collins, S. van Duyne, M. Mikoleit, T. Brown, and P. Fields. 2007. Multiplex, bead-based suspension array for molecular determination of common *Salmonella* serogroups. *Journal of Clinical Microbiology* 45: 3323–3334.

Franklin K., E.J. Lingohr, C. Yoshida, M. Anjum, L. Bodrossy, C.G. Clark, A.M. Kropinski and M.A. Karmali. 2011. Rapid genoserotyping tool for classification of *Salmonella* serovars. *Journal of Clinical Microbiology* 49: 2954–2965.

Grimont P.A.D and F.X. Weill. 2007. Antigenic formulae of the Salmonella serovars. WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France (9th ed.). Paris: WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur.

Herrera-Leon S., J.R. McQuiston, M.A. Usera, P.I. Fields, J. Garaizar and M.A. Echeita. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. *Journal of Clinical Microbiology* 42: 2581–2586.

Jean-Gilles Beaubrun J., C.-M. Cheng, K.-S. Chen, L. Ewing, H. Wang, M.C. Agpaoa and D.E. Hanes. 2012. The evaluation of a PCR-based method for identification of *Salmonella enterica* serovars from environmental samples and various food matrices. *Food Microbiology* 31: 199–209.

Jean-Gilles Beaubrun J., L. Ewing, K. Jarvis, K. Dudley, C. Grim, G. Gopinath and D.E. Hanes. 2014. Comparison of a PCR serotyping assay, Check&Trace assay for Salmonella, and Luminex Salmonella serotyping assay for the characterization of *Salmonella enterica* identified from fresh and naturally contaminated cilantro. *Food Microbiology* 42: 181–187.

Madajczak G. and J. Szych. 2010. Evaluation of Premi® Test Salmonella kit for identification of not-typabe by conventional methods Salmonella. *Medycyna Doświadczalna i Mikrobiologia* 62: 29–36.

Szych J. and G. Madajczak. 2010. Infections and poisonings caused by aerobic bacteria (in Polish) In: M. Jagielski (ed.), *Ethiology, clinical picturture and diagnostics of alimentary canal acute infections* and food poisonings (in Polish) (I., pp. 17–92). Warsaw: Fundacja Pro Pharmacia Futura.

Wattiau P., M. Van Hessche, C. Schlicker, H. Vander Veken and H. Imberechts. 2008. Comparison of classical serotyping and PremiTest assay for routine identification of common *Salmonella enterica* serovars. *Journal of Clinical Microbiology*, 46: 4037–4040.

Wattiau P., T. Weijers, P. Andreoli, C. Schliker, H. Vander Veken, H.M.E. Maas and P. Vos. 2008. Evaluation of the Premi Test Salmonella, a commercial low-density DNA microarray system intended for routine identification and typing of Salmonella enterica. *International Journal of Food Microbiology*, 123: 293–298.

Zakład Epidemiologii NIZP-PZH. 2013. Contagious infections and poisonings in Poland.