Turkish Journal of Molecular Biology & Biotechnology (2016) 1: 48-54

ORIGINAL ARTICLE

Turkish JMBB

Molecular typing of clinical *Salmonella* strains by multiplex polymerase chain reaction and determination of antimicrobial resistance patterns in Edirne

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Keywords

Salmonella, serogroup, molecular techniques, susceptibility

ABSTRACT • Background and aims: *Salmonella* is one of the most common pathogens of the gastrointestinal tract for both humans and animals leads to food-borne outbreaks and various infections. Accurate diagnosis and treatment of this important pathogen is only achieved by knowing its serogroup and antibiotic susceptibility. In our study, we aimed to reveal the distribution and antibiotic resistance of Salmonella strains in the community, also examined the effectiveness of multiplex polymerase chain reaction in the identification of Salmonella. **Materials and Methods:** Bacterial identification and antibiotic susceptibility tests of 105 *Salmonella* strains, isolated from the clinical samples (between 2009-2013) in Trakya University Hospital Central Laboratory Microbiology Department, were conducted with VITEK2 (Biomerieux, France) automatized system. Strains were grouped with both slide agglutination by using *Salmonella* polyvalent and group spesific antisera (Plasmatec, UK) and multiplex polimerase chain reaction. Multiplex polimerase chain reaction was performed by using six sets of primers targeting O-antigen sythesising gene regions in A, B, C1, D and E serogroups commonly found in clinical isolates. **Results:** O-grouping results revealed that serogroup D (%68) and C1 (%23) are the most common causes of *Salmonella* originated diarrhea in Edirne, respectively. Multiplex polimerase chain reaction results showed 100% compatibility with serologic grouping. The highest level of resistance found against to ampicillin (%16) among all antibiotics. During four years there is an increasing resistance to cephalosporins, trimetoprim-sulfametoxazol and fluoroquinolones. **Conclusion:** *Salmonella* serogroup D is the most frequent serogroup isolated in Edirne and emerging resistance to several antibiotics might be a serious health problem in the future. According to our study, also, multiplex polimerase chain reaction is a reliable and reproducible method in O-grouping of *Salmonella*.

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* This research project is funded by Trakya University Scientific Research Projects Fund (TUBAP 2012-191).

* Results of this project are partially presented in "Genomes 2014: EMBO Conference on Microbiology after the genomics revolution- Institut Pasteur, France" and "FEMS-6th Congress of European Microbiologists- Maastricht, Netherlands" conferences as posters

INTRODUCTION

Salmonella is one of the most important gastrointestinal tract pathogens for humans and animals leads to food-borne outbreaks, and various infections. Grouping of *Salmonella* according to their O antigens (O-grouping of *Salmonella*) allows both to identify accurately and to obtain epidemiological data. Accurate identification is important to obtain accurate and essential information for future studies on *Salmonella* strains (1,2). Previous studies with several patogens such as *Salmonella, Shigella, Campylobacter, Escherichia coli, Yersinia enterocolitica* give an overview about the distribution of gastroenteritis agents around Edirne (3). However a study spesific for *Salmonella*, which is one of the most common patogen for gastrointestial tract, has not been conducted. This project aimed to reveal the distribution and antibacterial resistance patterns of *Salmonella* strains in the community, also to utilize an accurate, fast and relatively economic molecular identification method in serogrouping. Accurate identification and determination of antibacterial resistance state of *Salmonella* strains will contribute to science by suggesting appropriate diagnostic and treatment methods in *Salmonella* infections.

MATERIALS and METHODS

One hundred and five *Salmonella* strains which were previously isolated and identified from clinical samples, firstly cultured in Brain Heart Infusion Broth for enrichment then inoculated to *Salmonella Shigella* Agar, after the ethics committee approval is obtained for the study (Table 1). After overnight incubation conventional serogrouping was conducted with both *Salmonella* polyvalent and group spesific (*Salmonella* Poly A-I + Vi, *Salmonella* O: 2, O: 4, O: 6,7,8, O: 7,8, O: 8, O: 9, O: 3,10,15, O: 1,3,19) antisera (Plasmatec, UK) using slide agglutination method.

After confirmation of previously identified serogroups DNA extraction procedure was carried out.

Table 1 Distribution of *Salmonella* strains in clinical specimens

Clinical specimen	Number (%)
Stool	89 (%84)
Urine	9 (%9)
Blood	2 (%2)
Abscess	1 (%1)
Other	4 (%4)

Single colony suspended in 50 µl ultra-pure water, boiled in 95°C for 10 minutes, then the suspension centrifugated in 14.000 rpm for 10 minutes and 30 µl supernatant used as DNA template for polymerase chain reaction (PCR) (1).

Multiplex PCR was performed by using six sets of primers targeting O-antigen sythesising gene regions in A, B, C1, D and E serogroups commonly found in clinical isolates, oriC (P1, P2) *Salmonella* internal control primers were added to each PCR mix (Table 2).

Multiplex PCR was performed in a reaction volume of 25 ml. Each reaction mix contained 1X PCR buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 mM of each primers (F-prt, R-prt, F-rfbJ, R-rfbJ, F-vi, R-vi, F-wzxC1, R-wzxC1, F-tyvD, R-tyvD, F-wzxE, R-wzxE), 0.2 mM of *Salmonella* internal control primers, 2.5 U Taq polymerase and 5 ml of DNA template. Reaction was performed in Thermal Cycler (Bio-Rad Inc.). Agarose gel electrophoresis was conducted in 2% agarose gel using 0.5X TBE buffer and ethidium bromide for staining. Multiplex PCR conditions consisted of primer denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 8 min. *Salmonella paratyphi A, Salmonella typhimurium, Salmonella choleraesuis, Salmonella enteritidis* and *Salmonella newport* representing serogroups A, B, C1, D and E, respectively obtained from the culture collection of Turkish Public Health Association used as positive controls for each PCR.

Table 2 Oligonucleotide primers used for *Salmonella* multiplex PCR

Primers	Target	Sequence (5' to 3')	Amplicon size (bp)
F-rfbJ	B group	CCAGCACCAGTTCCAACTTGATAC	662
R-rfbJ		GGCTTCCGGCTTTATTGGTAAGCA	
F-tyv	D group	GAGGAAGGGAAATGAAGCTTTT	614
R-tyv		TAGCAAACTGTCTCCCACCATAC	
F-prt	A & D group	CTTGCTATGGAAGACATAACGAACC	256
R-prt		CGTCTCCATCAAAAGCTCCATAGA	
F-wzxC1	C1 group	CAGTAGTCCGTAAAATACAGGGTGG	483
R-wzxC1		GGGGCTATAAATACTGTGTTAAATTCC	
F-wzxE1	E group	TAAAGTATATGGTGCTGATTTAACC	345
R-wzxE1		GTTAAAATGACAGATTGAGCAGAG	
P1	oriC	TTATTAGGATCGCGCCAGGC	163
P2		AAAGAATAACCGTTGTTCAC	

Antimicrobial susceptibility tests were conducted with VITEK2 automatized system (Biomeriéux, France) by using gram-negative identification and antibotic susceptibility test cards and the minimal inhibitory concentration (MIC) results analyzed according to The Clinical and Laboratory Standards (CLSI) 2011 criteria.

RESULTS

Salmonella internal control band (163 bp) were shown in all standard strains. Serogroup-specific bands for serogroups A, B, C1, D and E were identified as 256 bp, 662 bp, 483 bp, 615 bp and 345 bp, respectively (Figure 1). All standart strains yielded an internal control *Salmonella*-specific band of approximately 163 bp, Serogroup-specific bands are approximately 256 bp for serogroup A (*S. paratyphi* A), approximately 662 bp for serogroup B (*S. typhimurium*), approximately 483 bp for serogroup C1

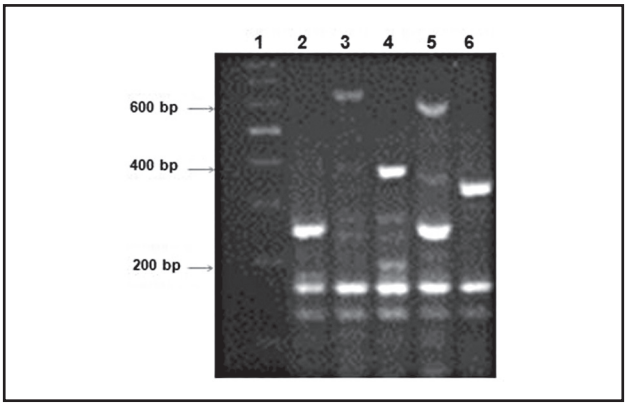

Figure 1 O-grouping of *Salmonella* standart strains by multiplex PCR. All standart strains yielded an internal control *Salmonella*-specific band of approximately 163 bp, serogroup-specific bands are approximately 256 bp for serogroup A (*S. paratyphi* A), approximately 662 bp for serogroup B (*S. typhimurium*), approximately 483 bp for serogroup C1 (*S. cholerasuis*), approximately 615 bp for serogroup D (*S. enteritidis*) and approximately 345 bp for serogroup E (*S. newport*). Lane 1, 100 bp ladder; lane 2 *S. paratyphi* A, lane 3 *S. typhimurium*, lane 4 *S. cholerasuis*, lane 5 *S. enteritidis*, lane 6 *S. newport*.

(*S. cholerasuis*), approximately 615 bp for serogroup D (*S. enteritidis*) and approximately 345 bp for serogroup E (*S. newport*). Lane 1, 100 bp ladder; lane 2 *S. paratyphi* A, lane 3 *S. typhimurium*, lane 4 *S. cholerasuis*, lane 5 *S. enteritidis*, lane 6 *S. newport*.

Multiplex PCR results showed 100% compatibility with serologic grouping (4). After confirmation of the success of multiplex PCR over conventional methods, the method is utilized for all clinical strains (Figure 2). Lane 1 representing DNA ladder, lanes 2, 7, 8 group C1, lanes 3, 4, 5, 6 group D, lane 9 negative control, lane 10 positive control.

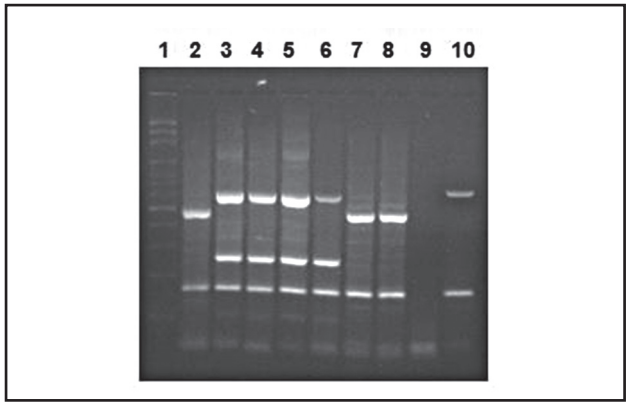

Figure 2 O-grouping of clinical *Salmonella* strains by multiplex PCR. Lane 1 representing DNA ladder, lanes 2, 7, 8 group C1, lanes 3, 4, 5, 6 group D, lane 9 negative control, lane 10 positive control.

O-grouping results revealed that serogroup D (%68) and C1 (%23) are the most common causes of *Salmonella* originated diarrhea in Edirne, respectively. In addition, 8 serogroup B (8%) and 1 serogroup E (1%) were found in our study, however there were not encountered any strain belonging serogroup A.

According to the VITEK2 antibiotic susceptibility test results an increase has been noticed in resistance to the certain antibiotics between 2009-2013. The highest level of resistance found against to ampicillin (%16) among all antibiotics. Moreover, ampicillin resistant four strains showed resistance (two intermediate) to amoxicillin–clavulanate combination. Also, two strains found resistant, four strains found intermediate resistant to piperacillin tazobactam combination. While in 2009 all *Salmonella* strains were susceptible to cephalosporins, in 2012 two strains showed resistance to third generation cephalosporins and in 2013 two strains showed resistance to both third and fourth generation cephalosporins. In addition, among four years

Table 3 Rates of resistance to various antibiotics of *Salmonella* strains

	Years				
Antibiotics	**2009**	**2010**	**2011**	**2012**	**2013**
Ampicillin	11% R	4% R	16% R	24% R	27% R
Amoxicillin/Clavulanate	6% I	-	5% I	3% I 7% R	-
Piperacillin/Tazobactam	-	4% R	5% I 5% R	10% I	-
Ceftazidime	-	-	-	7% R	18% I
Ceftriaxone	-	-	-	7% R	18% R
Cefepime	-	-	-	-	18% R
Fluoroquinolones	11% I	-	5% R	7% I 3% R	18% R
Trimethoprim/Sulfamethoxazole	11% R	-	5% R	3% R	18% R

I: Intermediate resistant. R: Resistant. -: There was not encountered any resistant strain. *: The data include the first 6 months of the year.

six strains found resistant to trimetoprim – sulfametoxazol and nine strains found resistant (six intermediate) to fluoroquinolones (Table 3).

DISCUSSION

Salmonella strains that cause foodborne and waterborne infections are important human and animal pathogens and some strains are capable to cause sistemic infections such as typhoid and paratyphoid fever in healthy human. Moreover, strains that normally cause gastroenteritis in healthy human can lead to systemic infections and death, in patients with underlying disease and immunosuppressive drug users. *Salmonella* strains are transmitted through fecal-oral route therefore *Salmonella* remains a serious health problem in developing countries which has infrastructure problems. Contamined food delivery which are the main source for infection and the developing antibiotic resistance creates problem in the prevention of *Salmonella* infection in developed countries as well. The frequency and distribution of *Salmonella* species vary according to geographical regions of Turkey. According to the study conducted in 2002 in Edirne by Tugrul et al., *Salmonella* (%5) was found as the the most common pathogen in gastrointestinal tract and most common serotypes noted as *S. enteritidis* and *S. typhimurium*, respectively (3). Yazıcı et al. detected the frequency of *Salmonella* as %2.5 in 200 patients who are admitted to Adnan Menderes University Hospital, Aydin, and pre-diagnosed with gastroenteritis between 2007-2008 (5). In another study conducted by Oguzturk et al. in Cumhuriyet University Medicine Faculty Emergency Department, Sivas, between May-November 2005, 150 patients with gastroenteritis were investigated in order to determine the agents and they found *Salmonella* (%6.7) as the most common bacterial cause of gastroenteritis (6).

In many countries surveillance system has been established to prevent the conversion of a possible outbreak to an epidemy. Most of these survelliance projects which are trying to predict possible outbreaks are based on conventional serotyping or phage typing.

Outbreaks are usually clustered around a few serotypes therefore making further typing becomes more important. However, due to limited laboratory facilities in some regions of our country, typing of *Salmonella* strains usually remains insufficient. However, accurate typing; helps to establish a relationship between infection with bacterial serotype, provides information about the sources of contamination, allows accurate evaluation of the outbreaks, contributes to epidemiological studies and also allows the discovery of new serotypes (7).

In our study, the multiplex PCR technique was used for this purpose in addition to conventional serotyping. Cross-reactions with other enteric bacterias were observed with conventional serogrouping, whereas such problem has not been faced with multiplex PCR thanks to internal control band and group specific primers which demonstrates the method's reliability and reproducibility.

Lim and Thong, accomplished O-grouping of 67 *Salmonella* strains with multilplex polymerase chain reaction (mPCR) and divided clinically important strains into 5 groups (A, B, C1, D and E). Also they found %100 compatibility with conventional serogrouping results and evaluated the mPCR as useful for epidemiological studies (1).

In the study carried out in Colombia by Munoz et al, 18 reference *Salmonella enterica* strains identificated with multiplex PCR, sensitivity and specificity was found 95.5% and 100% respectively. Thus, the technique evaluated as fast, sensitive, reliable (2).

Salmonella infections usually remain self limited and antimicrobial therapy is not needed. However in severe invasive infections antibiotic usage is a must and emerging resistance to several antibiotics is a serious concern of public health (8). In our study, an increase has been noticed in resistance to

the certain antibiotics between 2009-2013 and the highest level of resistance found against to ampicillin. Morever, between 2011-2013 an increasing resistance observed against to both fluoroquinolones and trimethoprim/sulfamethoxazole combination. The relatively high rate of resistance in 2009 may be due to an outbreak with a resistant strain. Another striking finding is, until 2012 all *Salmonella* strains were susceptible to cephalosporins, however in 2012 two strains showed resistance to third generation cephalosporins and in 2013 two strains showed resistance to both third and fourth generation cephalosporins. As fluoroquinolones and third generation cephalosporins are the first choice antibiotics in the treatment of severe *Salmonella* infections, increasing resistance may be a major health problem in the future.

Ozdemir and Acar have found at least one antibiotic resistance in 30% of 42 clinical *Salmonella* isolates obtained from seven different regions of Turkey. Maximum resistance rates were observed against to ampicillin and nalidixic acid. (9)

Ballal et al. studied on antibiotic susceptibility of enteric patogens isolated from clinical specimens between years 2005-2013 in India. 54 non-typhoid *Salmonella* detected and *S. typhimurium* was found as the most prevalent serotype. Although the antibiotic susceptibility results showed lower rate of resistance development compared to other enteric bacteria, an increase has been noticed in resistance against to ampicillin, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole (10).

According to the study carried out by Abdullahi et al in Nigeria, 43 (29 *S. enteritidis*, 14 *S. typhimurium*) out of 108 clinical *Salmonella* isolates were found as non-typhoidal strains. Ampicillin and chloramphenicol resistance rates were found as 82.8% and 41.4% for *S. enteritidis* and 92.9% and 42.9% for *S. typhimurium*, respectively (11).

According to our study, multiplex PCR evaluated as a reliable and reproducible method in O-grouping of *Salmonella* strains. *Salmonella* serogroup D is the most frequent serogroup found in Edirne. Emerging resistance to several antibiotics (especially to fluoroquinolones and third generation cephalosporins) might be a serious health problem in the future. Determination of the profile of *Salmonella* strains and antimicrobial resistance in Edirne and its region may help to future studies.

*The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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