Detection of *Salmonella* spp. with the BACTEC 9240 Automated Blood Culture System in 2008 - 2014 in Southern Iran (Shiraz): Biogrouping, MIC, and Antimicrobial Susceptibility Profiles of Isolates

Mojtaba Anvarinejad,[1] Gholam Reza Pouladfar,[1] Bahman Pourabbas,[1] Maneli Amin Shahidi,[1] Noroddin Rafaatpour,[1] Mohammad Ali Dehyadegari,[1] Pejman Abbasi,[1] and Jalal Mardaneh[2,*]

[1]Professor Alborzi Clinical Microbiology Research Center, Nemazee Hospital, Shiraz University of Medical Sciences, Shiraz, IR Iran
[2]Department of Microbiology, School of Medicine, Gonabad University of Medical Sciences, Gonabad, IR Iran

[*]*Corresponding author*: Jalal Mardaneh, Department of Microbiology, School of Medicine, Gonabad University of Medical Sciences, Gonabad, IR Iran. Tel/Fax: +98-5157220578, E-mail: Jalalmardaneh@yahoo.com

Abstract

Background: Human salmonellosis continues to be a major international problem, in terms of both morbidity and economic losses. The antibiotic resistance of *Salmonella* is an increasing public health emergency, since infections from resistant bacteria are more difficult and costly to treat.

Objectives: The aims of the present study were to investigate the isolation of *Salmonella* spp. with the BACTEC automated system from blood samples during 2008 - 2014 in southern Iran (Shiraz). Detection of subspecies, biogrouping, and antimicrobial susceptibility testing by the disc diffusion and agar dilution methods were performed.

Patients and Methods: A total of 19 *Salmonella* spp. were consecutively isolated using BACTEC from blood samples of patients between 2008 and 2014 in Shiraz, Iran. The isolates were identified as *Salmonella*, based on biochemical tests embedded in the API-20E system. In order to characterize the biogroups and subspecies, biochemical testing was performed. Susceptibility testing (disc diffusion and agar dilution) and extended-spectrum β-lactamase (ESBL) detection were performed according to the clinical and laboratory standards institute (CLSI) guidelines.

Results: Of the total 19 *Salmonella* spp. isolates recovered by the BACTEC automated system, all belonged to the *Salmonella enterica* subsp. *houtenae*. Five isolates (26.5%) were resistant to azithromycin. Six (31.5%) isolates with the disc diffusion method and five (26.3%) with the agar dilution method displayed resistance to nalidixic acid (minimum inhibitory concentration [MIC] > 32 μg/mL). All nalidixic acid-resistant isolates were also ciprofloxacin-sensitive. All isolates were ESBL-negative. Twenty-one percent of isolates were found to be resistant to chloramphenicol (MIC $\geq$ 32 μg/mL), and 16% were resistant to ampicillin (MIC $\geq$ 32 μg/mL).

Conclusions: The results indicate that multidrug-resistant (MDR) strains of *Salmonella* are increasing in number, and fewer antibiotics may be useful for treating *S. enterica* infections. Routine investigation and reporting of antibiotic MICs in patients presenting with *Salmonella* infections is suggested.

Keywords: Blood, BACTEC Automated System, Antimicrobial Resistance, Minimum Inhibitory Concentration, *Salmonella* spp.

1. Background

Salmonellosis is one of the most common public health problems in many countries, and human salmonellosis continues to be a major international problem, both in terms of morbidity and economic losses (1). *Salmonella* is a Gram-negative, facultatively anaerobic, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. There are two species of *Salmonella*: *S. bongori* and *S. enterica*. The latter species is divided into six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). In California (USA), the prevalence of subspecies II, IIIa, IIIb and IV are reported at 4%, 35%, 33% and 28% respectively

(2). *Salmonella* is spread by humans between countries as a result of foodborne infections, while it is mostly transferred to animals, foods, and the environment via fecal shedding. Faucal or intestinal contagion of carcasses is the main source of human foodborne infections.

Four clinical types of *Salmonella* infections may be distinguished: (1) gastroenteritis, (2) bacteremia, (3) enteric fever, and (4) the carrier state in persons with previous infections. The antibiotic resistance of *Salmonella* is an increasing public health emergency, as infections from resistant bacteria are more difficult and costly to treat (2, 3). The resistant bacteria in animals can transfer to humans via three pathways: consumption of contaminated meat or other food, direct contact with animals, or through the

environment (4). Resistance is caused by the use of antibiotics in humans and in animal husbandry (5). There has been no evidence of resistance of *Salmonella* to fluoroquinolones, but with the consumption of antimicrobials from animal feed, resistance against this class of antibiotics has been observed, and the rate of resistance in animals, foods, and humans is quickly rising in several countries (2, 3). Consumption of antibiotics by animals can lead to the transmission of resistant bacteria between animals, which then spreads to humans through food (6). The high incidence of treatment failure and severity of infection can cause extended disease duration and increased rates of infection, hospitalization, and death (2, 3).

Antimicrobial resistance in bacteria such as *Salmonella* is either genetically inherent or the result of the bacterium being exposed to antibiotics (7, 8). Most of the antibiotic resistance has emerged as a result of mutations or through the transfer of genetic material between bacteria (9). A broad variety of biochemical and physiological mechanisms are responsible for the development of resistance (5, 7). During the last decade, some treatments have become ineffective, and this may lead to the spread of certain infections in the future. Drug resistance is created by the incorrect use of antimicrobials, and develops when a bacterium has mutated; the inappropriate use of antibiotics in humans and in veterinary medicine leads to higher rate of resistance (10). Third-generation cephalosporins are commonly used for the treatment of *Salmonella* spp. invasive infections or severe diarrhea because of their bactericidal properties and a low prevalence of resistance to them (11). However, increasing resistance to cephalosporins has been reported worldwide for *Salmonella* spp.

β-lactamases are distributed among a wide range of bacteria, with clinical significance over a wide geographic area (12). The emergence of extended-spectrum β-lactamases (ESBLs) in *Salmonella* is important, as this will limit the treatment options and could result in an isolate with a remarkable selective advantage. Understanding the antimicrobial susceptibility pattern of *Salmonella* is crucial, both from the clinical treatment perspective and the public health outlook. In recent decades, Salmonellosis has risen considerably, both in incidence and severity. Attempts to prevent and control this bacterial infection are critical because of the high number of reported human cases and thousands of deaths every year worldwide. Increased antimicrobial resistance has made empiric antimicrobial therapy for such potentially fatal infections quite limited. Current studies recommend either third-generation cephalosporins or fluoroquinolones as the therapeutic choices; however, rising evidence of the emergence of resistance to these antimicrobials is a serious concern (13).

2. Objectives

The aims of the present study were: 1) to investigate the isolation of *Salmonella* spp. with the BACTEC 9240 automated system from blood samples during 2008 - 2014 in Shiraz, southern Iran, 2) to detect the subspecies, 3) to perform biogrouping, 4) to characterize the minimum inhibitory concentration (MIC) by the agar dilution method, and 5) to characterize the antimicrobial susceptibility pattern of isolates by the disc diffusion method.

3. Patients and Methods

3.1. Patients

The inclusion criteria for all patients with a clinical suspicion of bacterial infection were two or more of the following clinical signs of infection: fever (> 38°C) or temperature instability, respiratory distress, apnea, irritability, lethargy, poor peripheral circulation, tachycardia, hypotension, poor feeding, abdominal distention, and diarrhea.

3.2. Isolation and Confirmation of Salmonella spp. Isolates

The required blood specimens were collected aseptically before the commencement of antibiotic treatment. BACTEC fluorescent series 9240 instruments (Becton Dickinson, USA) were used for the rapid detection of bacteria from the blood samples. The specimens were collected in BACTEC standard culture vials for aerobic and facultative anaerobic bacteria. The bottles were loaded into the BACTEC machine within 30 minutes of sample collection. Whenever the machine gave an alert signal, the specific bottle was removed and Gram staining and subcultures were done on microbiological media, including blood agar, chocolate agar, and MacConkey's agar. The isolates were identified as *Salmonella* based on Gram staining, the oxidase test, the catalase test, motility, triple-sugar iron (TSI) fermentation, and colony morphology. For the final confirmation, biochemical tests embedded in the API-20E biochemical kit system (bioMerieux, France) were used. A total of 19 *Salmonella* spp. isolated consecutively from blood samples of patients with the BACTEC 9240 system between 2008 and 2014 in Shiraz, Iran, were tested.

3.3. Biogrouping of Salmonella spp.

In order to characterize the biogroups and subspecies, the following biochemical tests were used: ortho-Nitrophenyl-β-galactoside (2 hours), and acid production from lactose, arabinose, xylose, sorbitol, mannose, dulcitol, salicin, inositol, raffinose, adonitol, glycerol, and malonate. For long-term storage, the purified isolates were saved in tryptic soy broth (TSB) with 20% glycerol (Merck Co., Germany) at -20°C.

3.4. Antibiotic Susceptibility

Susceptibility testing to 24 antimicrobial agents (MAST Co., UK) was determined with diffusion methods according to the CLSI recommendations (16). The agents were: imipenem (IMP, 10 μg), meropenem (MEM, 10 μg), piperacillin-tazobactam (PTZ, 100/10 μg), ciprofloxacin (CIP, 5 μg), co-trimoxazole (SXT, 1.25/23.75 μg), amikacin (AK, 30 μg), gentamicin (GM, 10 μg), tobramycin (TB, 10 μg), ceftriaxone (CRO, 30 μg), cefixime (CFM, 5 μg), cefotaxime (CTX, 30 μg), cefepime (CPM, 30 μg), ceftazidime (CAZ, 30 μg), aztreonam (ATM, 30 μg), ticarcillin (TC, 75 μg), augmentin (AUG, 30 μg) , piperacillin (PRL, 100 μg), nalidixic acid (NA, 30 μg), tetracycline (T, 30 μg), azithromycin (ATH, 15 μg), cephalexin (CFX, 30 μg), ampicillin (AP, 10 μg), cefuroxime (CXM, 30 μg), and chloramphenicol (C, 30 μg).

3.5. Phenotypic Detection of ESBL in Salmonella spp.

Combination disc diffusion method: All *Salmonella* spp. isolates were screened for ESBL production according to the CLSI recommendations by using the confirmatory disk diffusion methods (14). Ceftazidime (30 μg) and ceftazidime + clavulanic acid (30 μg + 10 μg) discs, and cefotaxime (30 μg) and cefotaxime + clavulanic acid (30 μg + 10 μg) discs (Mast, UK) were placed at a distance of 25 mm on a mueller-hinton agar (MHA) plate, then incubated with a bacterial suspension of 0.5 McFarland turbidity standards. The plates were then incubated overnight at 37°C. A $\geq$ 5 mm increase in the diameter of the inhibition zone for the combination disc versus the ceftazidime disc confirmed ESBL production. The ESBL-producing strain *K. pneumoneae* ATCC 700603 and the non-ESBL-producing strain *E. coli* ATCC 25922 were used as positive and negative controls, respectively.

3.6. MIC Determination

3.6.1. Agar Dilution Method

The MICs of nalidixic acid, ampicillin, chloramphenicol, ciprofloxacin, co-trimoxazole, and ceftriaxone were determined by the standard agar dilution method according to CLSI guidelines. All *Salmonella* spp. isolates were processed for reduced susceptibility to the abovementioned antimicrobials by agar incorporation. First, 10 μL of a 0.5 McFarland bacterial suspension (final concentration = 106 CFU/mL) was spotted on the MHA containing each respective antibiotic. These were allowed to air-dry for approximately 5 minutes, and were then incubated at 35°C. The plates were examined at 24 hours and 48 hours for any growth. The lowest concentration of each antibiotic that inhibited the bacterial growth was considered the MIC. The breakpoint concentrations for the tested antimicrobials were chosen according to the CLSI guidelines. *E. coli* ATCC 25922 was used as a control strain.

4. Results

Of the total of 19 *Salmonella* spp. isolates recovered from blood samples by the BACTEC automated system, all belonged to the *Salmonella enterica* subsp. *houtenae*. All 19 of the tested isolates were sensitive to gentamicin, chloramphenicol, meropenem, imipenem, tobramycin, amikacin, piperacillin, ceftriaxone, aztreonam, cefepime, ceftazidime, cefixime, cefuroxime, cephalexin, and cefotaxime. Respectively, 31.5% and 26.5% of the isolates displayed resistance to nalidixic acid and azithromycin. The resistance profiles of the strains are shown in Table 1. When the MIC results were interpreted, all of the tested isolates were sensitive to ciprofloxacin and ceftriaxone. Table 2 depicts the MICs of the various antimicrobials. All isolates were ESBL-negative. Seven isolates showed resistance to two or more antimicrobial agents, and three of these were resistant to four or more antibiotics (Table 3).

Table 1. Susceptibility Pattern of *Salmonella enterica* Subsp. *houtenae* Isolated From Blood Specimens

Antibiotic	Total Resistant, No. (%)[a]
NA	6 (31.58)
PRL	4 (21.05)
ATH	5 (26.32)
AP	3 (15.79)
AUG	3 (15.79)
TC	2 (10.53)
T	2 (10.53)
TS	3 (15.79)
C	4 (21.05)

[a] Total number of isolates: 19.

5. Discussion

Salmonellosis is a major public health problem in Iran. Isolation of *Salmonella* spp. from different specimens occurs throughout the year. The distribution of *S. enterica* subsp. *houtenae* (IV) infections in this study is in agreement with reports from other countries, such as the United States, showing that subspecies *houtenae* (IV) is a more common subspecies isolated from human blood specimens (15). Our report and those of others (2) clearly show that *Salmonella* subsp. *houtenae* (IV) is capable of causing serious infections, including bacteremia. Unfortunately, despite recommendations to public health agencies regarding the potential risk for acquisition of *Salmonella* infections from exotic sources (e.g. foods, animals), the number of infections in Iran caused by subspecies associated

Table 2. Minimum Inhibitory Concentrations of *S. enterica* Subsp. *houtenae*[a]

MIC, μg/mL	CIP	CRO	NA	TS	AP	C
$\leq$ **0.03**	5	6	-	3	-	-
0.06	3	13	-	6	-	-
0.125	4	-	7	7	-	-
0.25	7	-	1	-	1	-
0.5	-	-	2	-	1	-
1	-	-	-	-	8	-
2	-	-	-	-	6	15
4	-	-	-	-	-	-
8	-	-	4	-	-	-
16	-	-	-	-	-	-
$\geq$ **32 ($\geq$ 4.76 for co-trimoxazole)[b]**	-	-	5	3[b]	3	4
Total, No.	19	19	19	19	19	19

[a] The resistance pattern of the antimicrobial agents is based on the breakpoints of the CLSI ([14]).
[b] The co-trimoxazole MIC of three isolates was $\geq$ 4.76.

Table 3. Antibiotic-Resistance Patterns of *S. enterica* Subsp. *houtenae* Isolates

Antibiotic-Resistance Patterns	No.[a]
Patterns With Disc Diffusion Method	
PRL, AP, AUG, TC, T, TS, C	2
PRL, AP, AUG, C	3
NA, TS	1
NA, ATH	3
Patterns With MIC Method	
AP, TS, C	2
AP, C	1
NA, TS	1

[a] Total number of isolates was 19.

with these sources does not appear to be abating. Abbott et al. ([2]), at the US center for disease control and prevention (CDC), reported a high frequency of extraintestinal infections for the Arizona group between 1967 and 1976.

In the last decade, there have been some reports of nalidixic acid, ampicillin, and co-trimoxazole resistance in *Salmonella*. In our study, six (31.5%) isolates displayed resistance to nalidixic acid with the disc diffusion method, and five (26.3%) with the MIC method (MIC > 32 μg/mL). However, as many as all nalidixic acid-resistant isolates were ciprofloxacin-sensitive on disc diffusion and MIC testing. Resistance to nalidixic acid is a surrogate marker for ciprofloxacin resistance, as therapeutic failures have been documented in clinical cases where ciprofloxacin has been used for treatment of infections caused by nalidixic acid-resistant strains. Our data showed that 16% of isolates are resistant to co-trimoxazole. Seven isolates of *S. enterica* in our study showed resistance to two or more antimicrobial agents and three of these were resistant to four or more antibiotics.

These results indicate that multidrug-resistant (MDR) strains of *Salmonella* are rising, and fewer antibiotics may be useful for treating *S. enterica* infections. The disc diffusion method using the current CLSI-recommended breakpoints to test antimicrobials is a reliable assay, and the results conform with the agar dilution method. Routine investigations and reporting of co-trimoxazole, nalidixic acid, ciprofloxacin, ceftriaxone, chloramphenicol, and ampicillin MICs in patients presenting with *Salmonella* infections are suggested. The observations of the present study imply that second- and third-generation cephalosporins, fluoroquinolones, and aminoglycosides represent a reserve of antimicrobials that have therapeutic potential for the treatment of *Salmonella* that are resistant to the current choice of drugs (i.e. ampicillin, nalidixic acid, co-trimoxazole, azithromycin, and chloramphenicol) in the future. Clinical efficacy trials are warranted in order to reach a conclusion in this regard.

In this study, five isolates were resistant to azithromycin. The emergence of resistance to azithromycin may occur before physicians begin using this antibiotic. Recommendations for azithromycin testing against *Salmonella* would facilitate the ability of

clinical laboratories to issue reports about this antibiotic with confidence, and would allow a more accurate susceptibility profile to emerge. A number of studies have observed a rise in the MIC of azithromycin [16, 17]. Therefore, it would be useful to ensure the uniformity of methods employed for testing. By using disc diffusion and agar dilution for susceptibility testing, as well as materials and methods readily available in a general clinical microbiology laboratory, we observed a close correlation between MICs and zone size. *S. enterica* infections have exhibited a gradual decline in susceptibility to traditional antimicrobials, a trend that is concerning in light of this pathogen's broad host range (animals and humans) and its potential to spread antibiotic-resistance determinants to other pathogenic bacteria.

It is imperative to effectively monitor the transmission of *Salmonella* through the food chain, in order to implement effective control measures [18]. In the present study, 21% of isolates were found to be resistant to chloramphenicol (MIC $\geq$ 32). Resistance to chloramphenicol in most European countries is less than 10%, with the exception of Greece, where 40% of *Salmonella* spp. isolates were resistant to this antibiotic in 2007 [19]. In our study, 16% of isolates were resistant to ampicillin (MIC $\geq$ 32). Researchers in Croatia reported that 4.5% of *Salmonella* spp. isolates were resistant to ampicillin, while 14% isolates were resistant to it in Austria and Greece, and 45% were resistant in Estonia [19]. Antibiotic utilization by humans, and the release of antibiotics into the environment, can promote antibiotic resistance in any location [5]. Antibiotic resistance occurs via different mechanisms, such as antibiotic usage in medical and veterinary medicine (e.g., aquacultures, pets, pest control in agriculture, growth support for animals, biocides in toiletries) that lead to distribution of resistance genes through other pathogenic and non-pathogenic bacteria [20].

Salmonella is spread by the trade of live animals, infected animal feed products, and non-heat-treated animal products within and between different countries. It is also spread by humans between countries throughout the world as a result of foodborne infections. Fluoroquinolones are generally regarded as first-line therapy for salmonellosis in adults. These groups of antibiotics are inexpensive and have good oral absorption, are well-tolerated, and are effective in the majority of *S. enterica* strains. Third-generation cephalosporins are used for children with severe *Salmonella* infections. Chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole are infrequently used as alternatives, but result in greater side effects. The MDR strains of *Salmonella* with resistance to cephalosporins and quinolones can be treated with other antibiotics, but those are usually more expensive and more

toxic [21]. Exposure of normal microflora to antimicrobials may increase the number of resistance factors, which can transfer resistance to pathogenic bacteria [20]. There is a direct relationship between antimicrobial use and development of resistance in *Salmonella*.

Horizontal gene transfer (HGT) plays a main role in the progress and diffusion of resistance to β-lactam antibiotics among enteric bacteria, in both community- and hospital-level infections [5]. There was no evidence of *Salmonella* resistance in humans with consumption of fluoroquinolones in our study, but resistance with consumption of antibiotics in animal feed has been observed, and the rate of resistance in animals, food, and humans has quickly increased in several countries [21]. Unfortunately, there is not enough information regarding the incidence rates of bacterial foodborne illnesses in developing countries (especially emerging infectious diseases caused by *Enterobacteriaceae*) [22-24]. We hope that this report will stimulate further epidemiologic studies and investigations into the antimicrobial susceptibility of *Salmonella* infections, and that this information can be used to generate more effective strategies to be implemented by public health agencies, the veterinary industry, and the food industry in order to reduce the extent of disease caused by this organism.

Acknowledgments

Footnotes

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