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Occurrence and antimicrobial resistance of *Salmonella enterica* subsp. *enterica* serovars Typhimurium, Enteritidis, and Typhi isolated from chicken eggs and poultry products

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Abstract: This study was carried out to detect the occurrence and antimicrobial resistance of *Salmonella enterica* subsp. *enterica* serovars Typhimurium, Enteritidis, and Typhi in 252 samples including 100 chicken eggs, 60 processed poultry products (20 nuggets, 20 salami samples, 20 sausages), and 92 poultry giblets (50 livers and 42 gizzards). *Salmonella* spp. and serovars were identified by PCR. The antimicrobial susceptibility testing was performed by disk diffusion method. Forty-seven (31%) of 152 poultry products and 5 (5%) of 100 egg samples were positive for *Salmonella* spp. *Salmonella* spp. was positive in 3.3%, 5%, 18%, and 27% of chicken nugget, sausage, gizzard, and liver samples, respectively. *S.* Typhimurium and *S.* Enteritidis were detected in 21 (8.3%) of samples (11 liver, 4 gizzard, 3 sausage, 3 eggshell) and 2 (0.8%) of samples (2 liver), respectively. All strains isolated from eggs were resistant to erythromycin (100%). Resistance profiles of nalidixic acid (80.7%), tetracycline (76.9%), neomycin (69.2%), cefazolin (36.5%), ampicillin (17.3%), and amoxicillin/clavulanic acid (9.6%) were evident and 86.5% of isolates exhibited multidrug resistance. In conclusion, the contamination rate of *Salmonella* spp. and high antibiotic resistance profiles among the isolates could pose risks for consumers. Effective control programs must be followed in processing and handling.

Key words: *Salmonella*, identification, polymerase chain reaction, antibiotic resistance

1. Introduction

Salmonellosis, being an important public health problem, is one of the most important food-borne diseases worldwide. The primary reservoir of *Salmonella* for humans is the intestinal tract of poultry. Human salmonellosis outbreaks are frequently associated with raw or undercooked poultry, egg, and meat product consumption (1). According to the Centers for Disease Control and Prevention (CDC) 2012 annual report, *Salmonella* was the second most common cause of confirmed outbreaks in the United States (2). It is estimated that annually 93.8 million salmonellosis cases occur in the world and 155,000 of these cases result in death. *Salmonella* comprises more than 2500 known serovars (3). In most developed countries, *S. enterica* serovars Typhimurium and Enteritidis are the most frequently incriminated causal agents of human salmonellosis (4). *S.* Enteritidis was the most common serotype identified among others in 35 countries, followed by Typhi (12 countries) and Typhimurium (8 countries). Serotyping is needed to understand the global epidemiology and surveillance of *Salmonella* as well as the rapid communication of the results (5). In Turkey, the most common serotypes isolated from humans are *S.* Typhimurium and *S.* Enteritidis (6).

Antimicrobial-resistant bacteria are of utmost importance for public health. The use of antimicrobials in human medicine, veterinary medicine, animal husbandry, and agricultural and aquaculture practices plays a key role for the development of resistance (7).

Salmonella spp. isolated from the United States and other countries have revealed an increasing rate of multidrug resistance (8). High prevalence of antimicrobial-resistant bacteria in foods of animal origin creates general concern for the choice of therapeutic agent used for food-borne infections.

This study aimed to determine the prevalence of *Salmonella enterica* serovars Typhimurium, Enteritidis, and Typhi in chicken eggs and poultry products. In addition, the antimicrobial resistance patterns of the isolates were assessed in terms of selecting effective therapeutic agents.

2. Materials and methods

2.1. Sample collection

Totally 200 samples consisting of 100 eggs (50 from markets, 50 from poultry farms), 60 processed poultry products (20 nuggets, 20 sausages, 20 salami samples), and 40 poultry giblets (20 gizzards and 20 livers) were

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examined in the current study. Chicken eggs and poultry samples were collected aseptically in July and August 2014. The samples were transported to the laboratory under aseptic conditions and analyzed immediately within 1–2 h.

2.2. Reference strains

Salmonella Typhimurium ATCC 13311, *Salmonella* Enteritidis ATCC 13076, and *Salmonella* Typhi ATCC 19430 reference strains were used as positive controls for the isolation of *Salmonella*. *Escherichia coli* ATCC 25922 was used for antimicrobial susceptibility testing.

2.3. Isolation and identification of *Salmonella* spp.

Egg contents and shells were analyzed separately for the isolation of *Salmonella* spp. For this purpose, sterile cotton swabs were used for the eggshell surface. After being wetted with sterile buffered peptone water (BPW; Merck 107228), the swabs were applied to the entire surface of eggs. The swabs were directly inoculated into 10-mL BPW tubes and incubated at 37 °C for 24 h. For *Salmonella* spp. isolation from the egg content, the surfaces of eggs were sterilized by immersion methods with 72% ethyl alcohol (Merck 107017) for 2 min and then air-dried under UV light for 10 min. Egg content was separated from eggshell and albumin and yolk were mixed aseptically (9).

The method proposed by ISO 6579 was used for the isolation and the identification of *Salmonella* spp. from egg, eggshell, and poultry-related samples (10). After the isolation process, five different suspicious *Salmonella* spp. colonies were subcultured on blood agar (Oxoid, CM0271) for confirmatory testing with biochemical methods (indole, methyl red, Voges–Proskauer, citrate, urease, and carbohydrate fermentation tests (TSI)). Finally, isolates detected in the study were stored on Tryptone Soya Broth (Oxoid CM0129) with 5% glycerol at –20 °C in cryovials for PCR verification and antibiotic susceptibility tests.

2.4. PCR assays

Genomic DNA of *Salmonella* spp. isolates were extracted with the Instagene Genomic DNA extraction kit (Bio-Rad, Hercules, CA, USA) as described by the manufacturer. The primers and PCR assay conditions previously described by Aabo et al. and de Freitas et al. were used with minor modifications (11,12). The internal control (240 bp) previously described by Croci et al. was used (13). The *SdfI* gene, *Spy* gene, and *Via*B genes, producing 304-bp, 401-bp, and 738-bp fragments, were used for serotyping Enteritidis, Typhimurium, and Typhi, respectively. The primer sequences of the serotypes of concern are presented in Table 1.

PCR was performed in a reaction mixture with final volume of 50 µL containing 5 µL of template DNA, 5 µL of 10X PCR buffer (Vivantis), 1.5 U of Taq polymerase (Vivantis), 500 µM dNTP mix (Vivantis), 3 mM $MgCl_2$ (Vivantis), and 25 pmol of each primer (Sentromer).

PCR amplification of *Salmonella* spp. was performed with an initial denaturation of 95 °C for 1 min followed by 30 cycles, each consisting of 94 °C for 15 s, 57 °C for 15 s, and 72 °C for 30 s. The final extension cycle was performed at 72 °C for 8 min (Techne TC-512). The multiplex PCR (mPCR) protocols for *S.* Enteritidis, *S.* Typhimurium, and *S.* Typhi consisted of initial denaturation at 95 °C for 2 min and 30 cycles subsequent each consisting 95 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min with a final elongation step of 5 min at 72 °C.

Table 1. *Salmonella* spp., serotypes, the target gene, sequences, and sizes of fragments.

Isolates	Target gene	Target gene GenBank accession number	Primers	Sequence (5' to 3')	Size (bp)	References
Salmonella spp.	Random Fragment	CP011233.1	ST11	AGCCAACCATTGCTAAATTGGCGCA	429	(11)
			ST15	GGTAGAAATTCCCAGCGGGTACTG		
Salmonella Enteritidis	*Sdf*I	CP007323.2	ENT R	TGTGTTTTATCTGATGCAAGAGG	304	(12)
			ENT F	TGAACTACGTTCGTTCTTCTGG		
Salmonella Typhimurium	*Spy*	CP011233.1	TYPH F	TTGTTCACTTTTTACCCCTGAA	401	
			TYPHR	CCCTGACAGCCGTTAGATATT		
Salmonella Typhi	*Via*B	CP012151.1	ViaBF	CACGCACCATCATTTCACCG	738	
			ViaBR	AACAGGCTGTAGCGATTTAGG		
Internal control				GCCTGCAAGTAGCCAACCATTGCTA AATTGGCGCATGCACCAGACTCCCC TTTG	240	(13)

All amplification products were analyzed by agarose gel (1.5%) electrophoresis at 100 V for 45 min (EC250-90, Thermo, USA). The gels were stained with ethidium bromide and visualized under a UV transilluminator (Vilber Lourmat, Marne La Vallee, France).

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were applied by the disk diffusion method standardized by the CLSI (14). Antimicrobial disks (Oxoid) used and their concentrations were as follows: ampicillin (10 µg), tetracycline (30 µg), amoxicillin-clavulanic acid (30 µg), cefazolin (30 µg), erythromycin (15 µg), gentamicin (10 µg), neomycin (10 µg), nalidixic acid (30 µg), enrofloxacin (5 µg), and trimethoprim/sulfamethoxazole (25 µg).

Salmonella isolates obtained and the reference strain (*Escherichia coli* ATCC 25922) were suspended in physiological saline solution to a density approximating a 0.5 McFarland standard. Each of these suspensions were plated on Mueller Hinton Agar (Merck, 105437). The antibiotic disks were placed onto the agar and incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured and were interpreted according to CLSI standards (14).

3. Results

In this study, a total of 72 suspicious colonies were selected and further identified from 252 samples. Fifty-two of the 72 suspicious colonies were found to be positive with phenotypical and biochemical tests and confirmed by PCR. According to these results, 47 (31%) of 152 poultry products and 5 (5%) of 100 egg samples were positive for *Salmonella* spp. (Table 2). *Salmonella* spp. was positive in 3.3%, 5%, 18%, and 27% of chicken nugget, sausage, gizzard, and liver samples, respectively (Table 2). None of the egg contents or salami samples were found to be contaminated with *Salmonella* spp., while 5 (5%) of eggshell samples (4 from farms and 1 from a retail market) were found positive. According to mPCR results, *S.* Typhimurium and *S.* Enteritidis were identified from 21 (11 liver, 4 gizzard, 3 sausage, 3 eggshell) and 2 (2 liver) samples respectively (Table 2; Figure). None of the *Salmonella* isolates were verified as *S.* Typhi.

In the antibiotic susceptibility testing, all of the isolates were found to be resistant to erythromycin (100%). In addition, resistances to nalidixic acid (80.7%), tetracycline (76.9%), neomycin (69.2%), cefazolin (36.5%), ampicillin (17.3%), and amoxicillin/clavulanic acid (9.6%) were evident in our study. The antibacterial susceptibility testing results of *Salmonella* spp. isolates against 10 different antibacterial agents are shown in Table 3. In the present study, resistance to three or more antibiotics was accepted as multidrug resistance. Therefore, 45 (86.5%) isolates were found to exhibit multidrug resistance in this study (Table 4).

Table 2. Distribution of *Salmonella* isolates in the examined samples.

Samples (n)*	No. of samples positive for *Salmonella* spp.		*Salmonella* serovars		
	ISO 6579	PCR	*S.* Typhimurium	*S.* Enteritidis	*S.* Typhi
Total chicken eggs (100)	5 (5%)	5 (5%)	3 (3%)	-	-
Eggshell, obtained from poultry farms (50)	4 (4%)	4 (4%)	3 (3%)	-	-
Eggshell, obtained from retail markets (50)	1 (1%)	1 (1%)	-	-	-
Egg content, obtained from poultry farms (50)	-	-	-	-	-
Egg content, obtained from retail markets (50)	-	-	-	-	-
Processed poultry products (60)	5 (8.3%)	5 (8.3%)	3 (5%)	-	-
Chicken nugget (20)	2 (3.3%)	2 (3.3%)	-	-	-
Chicken sausage (20)	3 (5%)	3 (5%)	3 (5%)	-	-
Chicken salami (20)	-	-	-	-	-
Poultry giblets (92)	42 (46%)	42 (46%)	15 (16.3%)	2 (2.2%)	-
Chicken liver (50)	25 (27%)	25 (27%)	11 (11.9%)	2 (2.2%)	-
Gizzard (42)	17 (18%)	17 (18%)	4 (4.3%)	-	-
Total	52 (21%)	52 (21%)	21 (8.3%)	2 (0.8%)	-

*: Number of samples; -: Not detected.

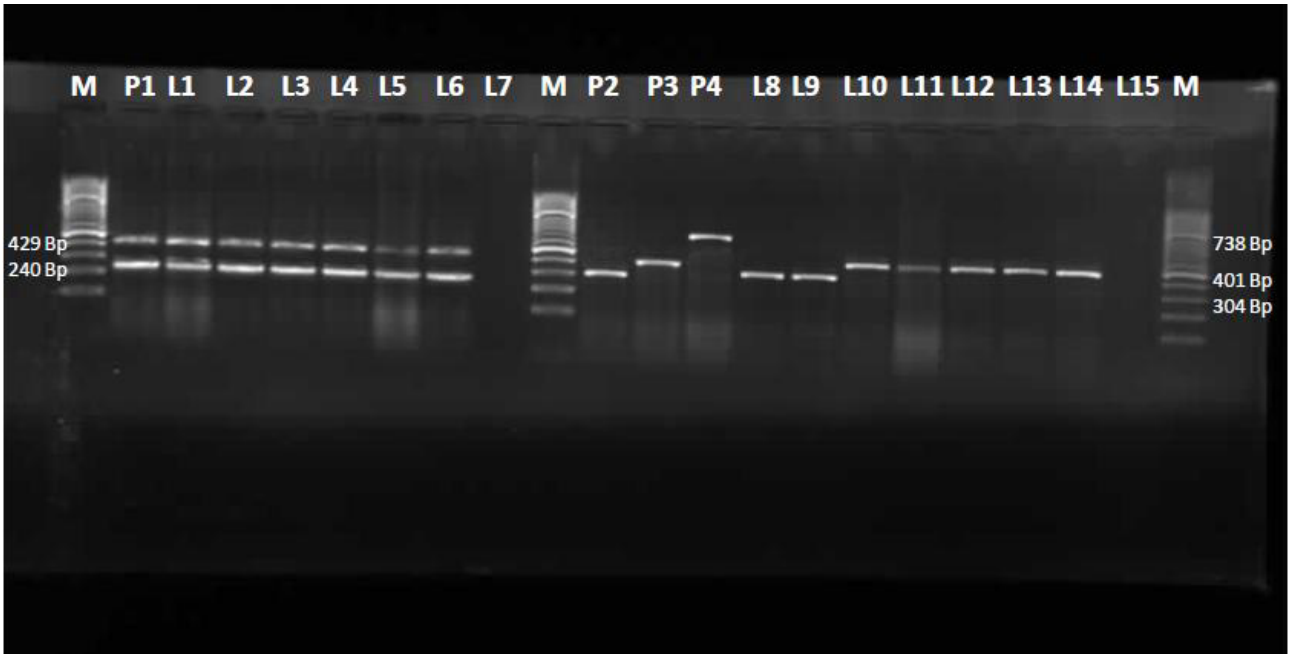

Figure. Identification of *Salmonella* genes from samples by PCR.

Lane M: Molecular weight marker (Gene Ruler 100-bp DNA Ladder Plus, Fermentas); Lane P1: Positive control for *Salmonella* spp. (ATCC 13311, 429 bp); Lane P2: Positive control for *Salmonella* Enteritidis (ATCC 13076, 304 bp); Lane P3: Positive control for *Salmonella* Typhimurium (ATCC 13311, 401 bp); Lane P4: Positive control for *Salmonella* Typhi (ATCC 19430, 738 bp); Lanes 1–6: Some isolates for *Salmonella* spp., 429 bp; Lanes 8 and 9: Some isolates for *Salmonella* Enteritidis, 401 bp; Lanes 10–14: Some isolates for *Salmonella* Typhimurium, 401 bp; Lanes P1–L6: internal control, 240 bp; L7 and L15: Negative control.

Table 3. Antibacterial resistance profiles of *Salmonella* spp. isolated from poultry-related products.

Antibiotics	Diameter of the inhibition zones of *Salmonella* spp. according to the CLSI (mm)*			Antimicrobial resistance profiles of *Salmonella* spp. isolates (n = 30)		
	Susceptible	Intermediate	Resistant	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin	≥17	14–16	≤13	43 (82.6)	-	9 (17.3)
Tetracycline	≥15	12–14	≤11	12 (23.0)	-	40 (76.9)
Amoxycillin/clavulanic acid	≥18	14–17	≤13	41 (78.8)	6(11.5)	5 (9.6)
Cefazolin	≥23	20–22	≤19	11 (21.1)	22 (42.3)	19 (36.5)
Erythromycin	≥23	14–22	≤13	-	-	52 (100)
Gentamicin	≥15	13–14	≤12	36 (69.2)	14 (26.9)	2 (3.8)
Neomycin	≥15	13–14	≤12	5 (9.6)	11 (21.1)	36 (69.2)
Nalidixic acid	≥19	14–18	≤13	9 (17.3)	1 (1.9)	42 (80.7)
Enrofloxacin	≥21	16–20	≤15	14 (26.9)	33 (63.4)	5 (9.6)
Trimethoprim/sulfamethoxazole	≥16	11–15	≤10	48 (92.3)	-	4 (7.7)

*: CLSI, 2014 (14).

Table 4. Multidrug resistance profiles of *Salmonella* spp. isolates.

Number of antibiotics	Antibiotic profiles	Number of isolates
8	AMP, E, ENR, KZ, N, NA, SXT, TE	2
8	AMP, CN, E, ENR, KZ, N, NA, TE	1
7	AMC, AMP, E, KZ, N, NA, TE	1
6	AMP, E, ENR, KZ, N, NA	2
5	AMC, AMP, E, KZ, N	3
5	AMP, E, N, NA, TE	7
5	AMP, E, ENR, N, NA	2
5	E, KZ, N, NA, TE	5
5	E, KZ, NA, SXT, TE	2
5	CN, E, KZ, NA, TE	2
4	AMP, E, NA, TE	2
4	E, KZ, NA, N	1
4	E, N, NA, TE	7
4	E, NA, SXT, TE	1
4	E, KZ, NA, TE	2
3	AMP, E, KZ	2
3	E, NA, TE	3

AMP: Ampicillin; AMC: amoxicillin-clavulanic acid; CN: gentamicin; E: erythromycin; ENR: enrofloxacin; KZ: cefazolin; N: neomycin, NA: nalidixic acid; TE: tetracycline.

4. Discussion

Poultry products and chicken eggs, being among the most popular foods worldwide, are the most incriminated foods for human salmonellosis (15). Determination of the contamination level and the antibiotic resistance of *Salmonella* is of utmost importance to control and to treat *Salmonella* outbreaks.

In our study, 31% (47/152) of poultry products were positive for *Salmonella* spp., including 3.3%, 5%, 18%, and 27% of chicken nugget, sausage, gizzard, and liver samples, respectively. With respect to nugget samples, higher findings were reported by Samaha et al. (16), who isolated *Salmonella* at an incidence rate of 8%, whereas no contamination was reported by Karadal et al. (17). Different contamination rates might be related to different sampling procedures, isolation and identification methods, hygiene conditions in processing plants, and possible contaminations during packing, transport, and storage.

With regard to giblets, our results are in agreement with those of Sodagari et al. (18), who obtained *Salmonella* from 21.6% of poultry livers. However, in earlier studies conducted by Jerngklinchani et al. (19) and Molla and Mesfin (20), 86% of poultry giblets and 34.5% and 42% of livers were found contaminated, respectively, which are higher rates than those of the present study. In contrast, a lower incidence was cited by Abdellah et al. (21), who reported contamination rates of 13.88% and 11.11% for gizzards and livers, respectively. Similarly, Sodagari et al. (18) isolated *Salmonella* spp. from 8.3% of gizzard samples whereas Oral and Türkyılmaz isolated them from 2.4% of liver samples (22). The lower *Salmonella* spp. incidences in processed poultry than giblets found in our study might be due to the heating process.

Salmonella spp. was detected in 5% of eggshells (4% farm and 1% retail market samples) but none of the egg contents in the present study. Similarly, Bayhan Öktem et al. reported *Salmonella* spp. from 6% of chicken eggs (23). However, Çakıroğlu and Gümüşsoy found *Salmonella* spp. in 0.22% of eggshell samples (24), whereas Telo et al. detected them in 1.26% of eggshells but not in the egg content (25). In addition, Humphrey et al. found that 32 egg contents (0.6%) were positive (26). Among the chicken

eggs from poultry farms and marketing channels, Singh et al. reported the prevalence of *Salmonella* to be 3.84% and 5.5%, respectively (9). Eggshell contamination could be attributed to feces, feed, insects, or handling transport or storage materials.

In this study, *S.* Typhimurium and *S.* Enteritidis were isolated from 8.3% (3% eggshell, 3% sausage, 11.9% liver, and 4.3% gizzard) and 0.8% (2.2% chicken liver) of samples. In a study conducted by El-Aziz (27), *S.* Typhimurium contamination rates of raw chicken meat, liver, and heart were reported to be 44%, 40%, and 48%, respectively. However, according to Oral and Türkyılmaz, 0.2% of *Salmonella* spp. isolates obtained from liver were serotyped as *S.* Enteritidis (22), whereas Kılınç and Aydın identified *S.* Enteritidis and *S.* Typhimurium from 45.9% and 29.41% of *Salmonella*-positive poultry internal organ samples (28). Abdellah et al. indicated that *S.* Typhimurium was the most frequent serovar (40.35%) among the *Salmonella* serotypes identified from chicken meat and giblet samples (21).

Although low *Salmonella* spp. contamination rates were detected in this study, it is a public health concern to isolate invasive *Salmonella* serotypes such as *S.* Typhimurium. During the poultry production process, internal organs including the liver and gizzard from all chickens are collected into the same pool, which can be a *Salmonella* spp. contamination source for healthy organs. In addition, staff may play a role in cross-contamination with *S.* Typhimurium during slaughtering and evisceration processes. *S.* Typhi is also an indicator of food contamination from an asymptomatic carrier (12).

In studies performed worldwide associated with poultry, remarkable variations are reported in the resistance of *Salmonella* spp. to a wide range of antimicrobial agents. In our study, 86.5% of *Salmonella* isolates exhibited multidrug resistance. Multiresistance profiles of *Salmonella* isolates indicate the need for effective control programs and more prudent use of antibiotics, which is a problem of utmost importance for public health.

In our study, all *Salmonella* isolates (100%) were found to be resistant to erythromycin, followed by nalidixic acid (80.7%), tetracycline (76.9%), neomycin (69.2%), cefazolin (36.5%), ampicillin (17.3%), and amoxicillin/clavulanic acid (9.6%). Cardoso et al. (29) also reported 100% erythromycin-resistant *Salmonella* isolates in their study. High resistance to erythromycin among *Salmonella* isolates was also cited by Yildirim et al. (95%) and Kılınç and Aydın (89.7%), respectively (28,30). With respect to tetracycline resistance, our results are in the same line as those of Yildirim et al. at a value of 67.6% (30). Yildirim et al. also reported neomycin resistance (55.8%) similar to our results (69.2%), whereas a lower resistance rate to neomycin (23%) was reported by Kılınç and Aydın (28,30). In addition, lower resistance rates between 25.4% and 15.4% for nalidixic acid and tetracycline were reported by Iseri and Erol (1).

Oral and Türkyılmaz determined enrofloxacin, oxytetracycline, gentamicin, amoxicillin, neomycin, and trimethoprim/sulfamethoxazole susceptibilities from 97.9%, 93.6%, 76.6%, 72.4%, 38.3%, and 10.6% of isolates, respectively (22). Our results are in line with those of Oral and Türkyılmaz in terms of trimethoprim/sulfamethoxazole.

The high resistance profiles of *Salmonella* isolates against commonly used antibiotics are probably the result of the misuse of these antibiotics in veterinary medicine, which creates problems in the treatment of salmonellosis (30).

In conclusion, contamination rates of *Salmonella* (15%) found in this study suggest that poultry products might be the source of human salmonellosis. Although good manufacturing practices and hazard analysis critical control point concepts have already been introduced to Turkey's poultry industry, mishandling in any step of poultry production might be a cause for the presence of *Salmonella* spp. Each type of internal organ should be separately stored and packed individually for avoiding the cross-contamination risk. Personnel education is also needed. In respect to antimicrobial resistance, the results of this study point to poultry as a potential reservoir of multiresistant *Salmonella* isolates, which are now a serious public health concern. Effort is needed to provide antibiotics to be used following assessment of antibiogram profiles and unlimited access to antimicrobial agents must be avoided to prevent the spread of multiresistant isolates.

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