Phenotypic and genotypic studies of extended spectrum beta-lactamase (ESBL) resistance among *Salmonella* isolates from poultry sources in Iran

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Abstract:

BACKGROUND: Poultry and poultry products are among the major sources of *Salmonella* infections for humans. Increasing occurrence of antimicrobial resistance among *Salmonella*e has become a serious public health concern. The detection of extended spectrum b-lactamase (ESBL) producers among *Salmonella* spp. has increased in recent years. **OBJECTIVES:** The purpose of this study was to investigate the antibiotic resistance pattern of *Salmonella*, and to understand whether ESBLs were present in *Salmonella* isolated from poultry farms and slaughterhouses from various parts of Iran. **METHODS:** A total of 314 isolates of *Salmonella* spp., 272 of poultry and 42 from human origin, collected during winter 2005-2011 were characterized for antimicrobial resistance and the presence of ESBL genes in this study. Phenotypic Disk diffusion method was performed for detection of antimicrobial susceptibility against 16 antimicrobial agents according to the Clinical and Laboratory Standards Institute's recommendations (CLSI, 2005). To detect the presence of ESBL genes in 30 isolates out of 61 phenotypical resistant isolates, PCR amplification was used by employing specific primers for screening of the CTX-M and CMY groups, respectively. **RESULTS:** The highest resistance to ceforoxime in poultry and cefixime in human isolates was observed, and multidrug resistance (MDR) was seen with a maximum seven antimicrobial agents. The PCR detection of CTX-M and CMY genes in all isolates including five phenotypically ESBL positive isolates was negative. **CONCLUSIONS:** This study revealed the incidence of resistance to cephalosporins and the frequency of MDR among *Salmonella* isolates from poultry farms in Iran. The prevalence of MDR *Salmonella* isolates from poultry are of particular concern as these strains can transmit to humans through the food chain.

Introduction

The infections caused by *Salmonella* are a significant public health problem throughout the world (Su et al., 2005). The importance of poultry and poultry products as sources of *Salmonella* infections in the food chain has attracted extensive research efforts over many years and now the increased occurrence of antimicrobial resistance among both typhoidal and non-typhoidal *Salmonellae* is considered a serious public health concern (Fernandez et al., 2000; Parry and Threlfall, 2008). The use of antimicrobials in animals for therapeutic or prophylactic purposes or as growth promoters, influences the prevalence of resistant bacteria in animal population and increases the risk for transfer of

resistant bacterial strains to human (Viola and DeVincent, 2006; Gyles, 2008). Since the widespread application of beta-lactams against infections caused by members of *Enterobacteriaceae* family in both human and animals, the emergence of resistance to various beta-lactams has been frequently observed in clinical cases worldwide (Gniadkowski, 2001; Dierikx et al., 2010). The extended spectrum beta-lactamases (ESBLs) are typically encoded on large plasmids which can be easily exchanged between strains and species of bacteria (Jacoby and Medeiros, 1991). In a few Iranian studies, the occurrence of resistance to different ESBLs among Gram-negative bacteria recovered from human clinical infections has been investigated but no studies have addressed such occurrence among bacterial isolates in food animals (Mehregan and Rahbar, 2008; Hamidian et al., 2009). The aim of this study was to investigate the presence of ESBL resistance and some relevant genes among *Salmonella* isolates collected from poultry sources in different parts of Iran.

Materials and Methods

Bacterial isolates: In previous surveys on *Salmonella* infections conducted by our laboratory and with the help of Iranian Veterinary Organization (IVO), 314 *Salmonella* isolates were collected from different poultry sources (broiler, breeder, environment, abattoirs) in different geographical areas of Iran and humans from Tehran's hospitals during winter 2005-2011. Samples were isolated from various specimens including fresh feces, cloacal feces, environment, and carcasses. All samples were identified as *Salmonella* according to standard procedures and the respective serogroups and serotypes, where possible, were determined according to the Kauffmann-White scheme (Morshed and Peighambari, 2010; Akbarian et al., 2012). *Salmonella* isolates then were kept at -70°C and liquid nitrogen for further studies.

Drug susceptibility test: The susceptibility of 314 *Salmonella* isolates to a panel of beta-lactam antimicrobial agents was determined by the agar disk diffusion method and the interpretation of results was carried out according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2005). The antimicrobial agents were tested and their concentrations (µg) were: peniciline (10), ampicillin (10), amoxicillin + clavulanic acid (30), piperacillin (100), cephalothin (30), ceftazidime (30), ceftriaxone (30), cefixime (5), cefotaxime (30), cefoxitin (30), cefuroxime (30), ceftizoxim (30), cefepime (30), cefazolin (30), imipenem (10), meropenem (10). All antibacterial disks were provided from Tadbir Fan Azma Co (Tehran, Iran). The ATCC reference strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, ATCC 27853, and *E. coli* ATCC 35218 were used for quality control purposes. In this study, the *Salmonella* isolates with intermediate susceptibility classification were considered not to be resistant to that drug and multi-resistance was defined as resistance to more than one drug.

Detection of ESBL genes by using polymerase chain reaction: The presence of ESBL genes in 30 isolates out of 61 isolates showing resistance patterns to cephalosporins were detected using PCR and specific primers targeted CTX-M and CMY genes family groups. To extract bacterial DNA, one mL of the pure overnight culture of each *Salmonella* isolate was transferred to a clean 1.5 mL microtube containing 100 µL TE buffer, boiled for 10 min, and centrifuged for 10 min at 20000 x g to recover the microorganisms as a pellet. The supernatant was discarded and the pellet was resuspended in 100 µL TE buffer and stored at -20°C for further use.

Primers for CTX-M were (5'-ATGTGCAGYAC-CAGTAARGTKATGGC-3') as forward and (5'-TGGGTRAARTARGTSACCAGAAYSAGCGG-3') as reverse (Hasman et al., 2005). The primers and other materials used in PCR reaction were provided by TAG Copenhagen (Copenhagen, Denmark). Amplification reactions for CTX-M were carried out in a 48 µL reaction volume containing 5 µL 10 x PCR buffer, 0.5 µM (each) dATP, dCTP, dGTP, and dTTP, 0.5 µL of each primer, 1 U (0.1 µL) of super Taq polymerase DNA and 4 µL dH2O. Approximately 2 µL of template DNA was added to the mixture. Positive control included *Escherichia coli* 77-30108-11 Danish strain (serogroup O149). Negative controls (dH2O instead of template DNA) were included in all PCR reaction sets. Amplification was programmed in a thermocycler (Gradient Mastercycler, Eppendorff, Germany) as follows: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72° C for 10 min.

Primers for CMY were (5'-ATGATGAAAAA-ATCGTTATGCTGC-3') as forward and (5'-GCTTT-TCAAGAATGCGCCAGG-3') as reverse (Coudron et al., 2000). The primers and other materials used in PCR reaction were provided by TAG Copenhagen (Copenhagen, Denmark). Amplification reactions for CMY was carried out as described above for CTX-M. Positive controls included a transconjugate from *Klebsiella pneumoniae* for CMY-1 (Korean strain) and *Salmonella* Heidelberg 75-12893-1 Danish strain for CMY-2 gene. Negative controls (dH2O instead of template DNA) were included in all PCR reaction sets. Amplification process was performed as described above for CTX-M.

The amplification products were detected by gel electrophoresis in 1.5% agarose gel at 30 V for 25 min in 1 x TAE buffer.

Results

In drug susceptibility test, 103 (32.8%) out of 314 isolates showed multidrug resistance (MDR). The isolates were resistant to at least one and at most, nine antimicrobial agents (Table 1). Among poultry isolates, after penicillin (100% resistance) the high incidence of resistance to ampicillin, cefuroxim, and meropenem, respectively was observed. High resistance to cefexime was observed among human isolates. No resistance to cefotaxime, ceftizoxime, cefepime, cefixime and imipenem was observed (Table 1). Sixty-one isolates were selected based on the results of the primary phenotypic observations on cephalosporins (resistant isolates) and subjected to confirmatory ESBLs test with a panel of antibiotics (Becton, Dickinson and Company) as follow: cefoxitin, cefepime, ceftazidime, ceftazidime + clavulanic acid, cefotaxime, cefotaxime + clavulanic acid. The results showed that four isolates were resistant to cefoxitin and one isolate was resistant to cefepime.

The PCR detection of CTX-M and CMY genes family groups in all 30 isolates including five phenotypically ESBL positive isolates was negative (Figure 1).

Discussion

The main findings of this study were that: (i) ampicillin resistant *Salmonella* were present in both human and poultry isolates; (ii) resistance to cefuroxime was detected at a high frequency level in poultry isolates; (iii) ESBLs were detected in neither human nor poultry isolates; (iv) MDR type in poultry origin isolates was higher than that of in human isolates.

Currently, increasing bacterial resistance to antibiotic agents poses a serious problem throughout the world including Iran. Increased multidrug

Table 1. Antimicrobial resistance pattern of 314 *Salmonella* spp. from poultry (272) and human (42) origin.

Drugs	No (%) of resistant isolates	
	Poultry	Human
Ampicillin	50 (18.3)	1 (2.3)
Penicillin	272 (100)	42 (100)
Amoxicillin	13 (4.7)	0
Amoxicillin clavolanic acid	14 (5.1)	0
Piperacillin	10 (3.6)	1 (2.3)
Cefazolin	16 (5.8)	1 (2.3)
Cefalothin	15 (5.5)	0
Cefuroxime	49 (18.01)	0
Cefotaxime	0	0
Ceftazidime	0	0
Ceftizoxime	0	0
Ceftriaxone	0	0
Cefepime	1 (0.3)	0
Cefoxitin	4 (1.4)	0
Cefixime	0	2 (4.7)
Imipenem	0	0
Meropenem	11 (4.04)	0
Ceftazidime + clavulanic acid	0	0
Cefotaxime + clavulanic acid	0	0

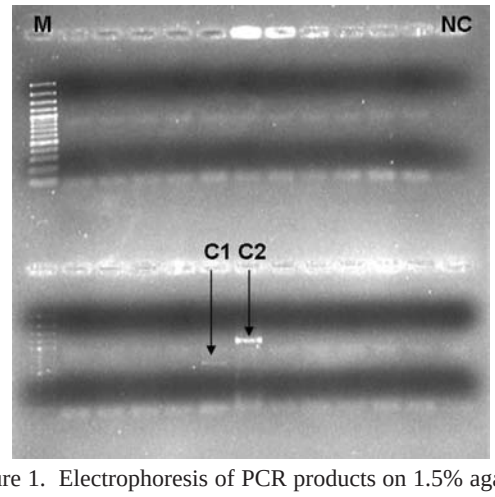

Figure 1. Electrophoresis of PCR products on 1.5% agarose gel. Lanes M, C1, and C2, and NC indicate ladder, positive control (for CTX-M gene), positive control (for CMY gene), and negative control (dH2O instead of template DNA), respectively. Other lanes demonstrate the negative results for tested *Salmonella* isolates.

resistance (MDR) has been reported in *Salmonella* isolates in many countries. In the present study, 32.8% of *Salmonella* isolates were MDR which is in accordance with the previous findings in Iran by Morshed and Peighambari (2010) and Firoozeh et al. (2011) showing 61.2% and 69% of *Salmonella* isolated from human and poultry as being MDR, respectively. In the present study, resistance to some agents such as ampicillin and cefalothin were comparable with previous findings, but frequency of resistance to ESBLs in our study was lower than those of Morshed and Peighambari (2010) and Firoozeh et al., (2011). Morshed and Peighambari (2010) reported 24.1% resistance to cefixime, 17.2% resistance to ampicilin, 6.9% resistance to ceftazidime, 10.3% resistance to cephalothin, and among human isolates, 33.3% resistance to ampicilin and 11.1% resistance to cefalothin. In a recent Iranian study, Firoozeh et al. (2011) found that seven (16.6%) isolates were phenotypically resistant to cefixime, ceftazidime, ceftriaxone and cefotaxime. Use of penicillin, ampicillin, and amoxicillin for the control of clostridia and bacterial enteritis may generate a selective pressure for possession and retention of a beta-lactamase but it is worth noting that cephalosporins have not been approved for use in poultry and poultry production in Iran.

In Iran, patients referred to hospitals with *Salmonella* infections are usually treated with ciprofloxacin, co-amoxiclav (amoxicillin + clavulanic acid) or cephalosporins (Tajbakhsh et al., 2012). Several Iranian studies have identified ESBLs in various members of Enterobacteriacea such as *Klebsiella*, *Escherichia coli*, and *Salmonella* spp. (Feizabadi et al., 2006; Hamidian et al., 2009; Ghafurian et al., 2011; Moghaddam et al., 2011) but there is no published data focused on presence of ESBLs in avian bacterial pathogens. In 2010, Ranjbar et al. reported the first CTX-M ESBL-producing S. Enteritidis and *S. Infantis* isolates in Iran. Hamidian et al. (2009) found both *bla*CTX-M-15 and *bla*TEM in two isolates and only *bla*TEM in one isolate from 129 *Salmonella* spp. recovered from patient with diarrhea in hospitals of Tehran. It was the first report of *bla*CTX-M-15 in Iran. Increased detection of ESBLs producing *Salmonella* isolated from poultry has been reported in other countries including Brazil, France, Italy, Japan, and the Netherlands (Weill et al., 2004; Hasman et al., 2005; Chiaretto et al., 2008; Fernandes et al., 2009; Dierikx et al., 2010; Shahada et al., 2010). ESBLs are rare in S. enterica strains compared to other *Enterobacteriaceae* such as *E. coli* and *Klebsiella pneumoniae* (Morris et al., 2006). However, there have been an increasing number of reports on ESBLs containing *Salmonella* strains throughout the world (Morris et al., 2006; Lee et al., 2009). ESBLs are detected extensively in bacterial population isolated from human patients in different medical centers but are not frequently reported in the bacterial population circulating in animals. This finding could be indicative of less frequency of these enzymes in animals than in humans but this notion has not been broadly investigated (Carattoli, 2008). Therefore, it is important to screen the occurrence of resistance among bacteria from animals and foods, as these bacteria (or their mobile elements carrying resistance genes) can spread through food products to humans. Unfortunately, there is no surveillance program on the administration of antimicrobial drugs in Iranian poultry industry. Improved regulatory criteria will help the rational administration of antimicrobial drugs in infection control programs which, consequently, will prevent spread of antimicrobial resistance in Iran.

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مطالعه فنوتیپی و ژنوتیپی مقاومت بتالاکتامازهای وسیع الطیف در جدایه‌های سالمونلای بدست امده از منابع طیور در ایران

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چکیده

زمینـه مـطالعه: طیور و محصولات طیور یکی از مهمترین منابع عفونت سالمونلا برای انسان میباشند. افزایش وقوع مقاومت‌های آنتی بیوتیکی در بین جدایه‌های سالمونلا به عنوان یک خطر جدی برای بهداشت عمومی مطرح شده است. جستجوی تولید کننده‌های بتالاکتامازهای مقاوم در بین گونه‌های سالمونلا در سال‌های اخیر افزایش یافته است. **هدف:** هدف از این مطالعه، جستجوی الگوهای مقاومت جدایه‌های سالمونلا و ردیابی حضور ژن‌های مقاوم به بتالاکتامازهای وسیع الطیف (ESBLs) در فارم ها و کشتارگاه های طیور در نقاط مختلف ایران بود. **روش کار:** تعداد ۳۱۴ جدایه سالمونلا شامل ۲۷۲ جدایه طیوری و ۴۲ جدایه انسانی در طی سال های ۲۰۰۵-۲۰۱۱ جمع آوری و برای تعیین الگوهای مقاومت آنتی بیوتیکی و حضور ژن‌های بتالاکتامازهای وسیع الطیف مورد مطالعه قرار گرفتند. تعیین حساسیت ضد میکروبی جدایه‌ها بر اساس روش استاندارد دیسک دیفوژیون نسبت به ۱۶ عامل ضد میکروبی انجام پذیرفت. برای ردیابی ژن‌های ESBLs روش PCR با استفاده از پرایمرهای ویژه جستجوکننده گروه CTX-M و CMY برای ۳۰ جدایه از ۶۱ جدایه مقاوم در آزمایش‌های فنوتیپی به کار گرفته شد. **نتایج:** بر اساس نتایج حاصله، بیشترین مقاومت آنتی بیوتیکی در بین جدایه های طیوری به سفوروکسیم و در بین جدایه‌های انسانی به سفیکسیم مشاهده گردید. همچنین مقاومت چندگانه به آنتی بیوتیکها حداکثر به ۷ دارو بود. آزمایش PCR انجام شده برای تمامی جدایه‌ها از لحاظ حضور ژن CTX-M و CMY منفی بود. **نتیجه گیری نهایی:** نتایج حاصله از این مطالعه نشان داد پدیده مقاومت چندگانه دارویی در بین جدایه‌های سالمونلای طیور به علت احتمال انتقال از طریق زنجیره غذایی از اهمیت ویژه‌ای برای سلامت عمومی برخوردار می‌باشند.

واژه‌های کلیدی: سالمونلا، مقاومت آنتی بیوتیکی، ESBS، ایران.

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