# Epidemiological study on prevalence, serovar diversity, multi-drug resistance and CTX-M-type extended-spectrum β-lactamases of *Salmonella spp.* from patients with diarrhea, food of animal origin, and pets in several provinces of China

Running title: CTX-M-type ESBL-producing *Salmonella spp.* in China

Wei Wang,[a] Li Zhao,[b] Yujie Hu,[a] Tania Dottorini,[c] Séamus Fanning,[a,d,e] Jin Xu,[a,#] Fengqin Li[a,#]

NHC Key Laboratory of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, Beijing, China [a]; School of Public Health, Shandong University, Jinan, China [b]; School of Veterinary Medicine and Science, University of Nottingham, LE12 5RD, Sutton Bonington, Nottingham, UK [c]; UCD-Centre for Food Safety, School of Public Health, Physiotherapy and Sports Science, University College Dublin, Belfield, Dublin D04 N2E5, Ireland [d]; Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Stranmillis Road, Belfast BT9 5AG, Northern Ireland [e].

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[#]Corresponding authors:

Professor Jin Xu, NHC Key Laboratory of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, No.7 Panjiayuan Nanli, Chaoyang District, Beijing, China, tel.: (+86) 010 67768526; xujin@cfsa.net.cn.

Professor Fengqin Li, NHC Key Laboratory of Food Safety Risk Assessment, China National Center for Food Safety Risk Assessment, No.7 Panjiayuan Nanli, Chaoyang District, Beijing, China, tel.: (+86) 010 67776356; lifengqin@cfsa.net.cn.

**Abstract:**

A total of 2,283 *Salmonella spp.* isolates were recovered from 18,334 samples including patients with diarrhea, food of animal origin and pets across 5 provinces of China. The highest prevalence of *Salmonella spp.* was detected in chicken meats (39.3%, 486/1,237). Fifteen serogroups and 66 serovars were identified, with Typhimurium and Enteritidis being the most dominant. Most (85.5%, 1,952/2,283) isolates exhibited resistant to ≥ 1 antimicrobial and 56.4% were multi-drug resistant (MDR). A total of 222 isolates harbored extended-spectrum β-lactamases (ESBLs), 200 of which were CTX-M-type that were mostly detected from chicken meat and turtle fecal. Overall, eight $bla_{\text{CTX-M}}$ genes were identified, with $bla_{\text{CTX-M-65}}$, $bla_{\text{CTX-M-123}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-79}}$, and $bla_{\text{CTX-M-130}}$ being the most prevalent. Totally, 166 of the 222 ESBL-producing isolates had amino acid substitutions in GyrA (S83Y, S83F, D87G, D87N, and D87Y) and ParC (and S80I), whilst the PMQR-encoding genes *oqxA/B*, *qepA*, and *qnrB/S* were detected in almost all isolates. Of the fifteen sequence types (STs) identified in the 222 ESBLs, ST17, ST11, ST34, and ST26 ranked among the top 5 in the number of isolates. Our study revealed considerable serovars diversity, high prevalence of co-occurrence of MDR determinants, including CTX-M-type ESBLs, QRDRs mutations and PMQR genes. This is the first report of CTX-M-130 *Salmonella spp.* from patients with diarrhea and QRDRs mutations from turtle fecal samples. Our study emphasizes the importance of actions, both in the health care settings and in the veterinary medicine sector, to control the dissemination of MDR, especially the CTX-M *Salmonella spp.* isolates.

## INTRODUCTION

*Salmonella spp.* infections have been proven to be a major cause of global morbidity and mortality in both humans and animals (1, 2). Worldwide, 93.8 million salmonellosis cases occur annually with 155,000 resulting in death (3). In China, more than 20% of all foodborne illnesses are estimated to be caused by *Salmonella spp.* (4). In 2013, unpublished data from the China CDC surveillance system showed that the carriage rate of human Salmonellosis was 549 per 100,000 people. This is more than 30 times higher than human infections in Europe in 2017 (19.7 per 100,000) and the USA in 2018 (18.3 per 100,000) (5, 6). Moreover, the indiscriminate use of wide-spectrum antibiotics creates an additional threat represented by the appearance and dissemination of multi antibiotic resistance profiles in the pathogen population. Multi drug resistant (MDR) *Salmonella spp.* potentially arising due to selective pressure from sustained antimicrobial exposure, are more likely to be the causative agents of invasive disease (7). Of concern is the increased incidence of infections caused by ESBL-producing organisms, including *Salmonella spp.*, because they are not only resistant to most of the β-lactam antimicrobials but also to other antimicrobial classes, leaving few treatment options with the potential for worse clinical outcomes (8, 9).

During the last decade, the most encountered (particularly in areas of Europe and Asia) ESBLs genes were the CTX-M enzyme family, primarily carried by transferable plasmids and transposons (10). The emergence of CTX-M-type ESBL-producing *Salmonella spp.* has been reported in clinical cases, animals, and food samples worldwide including China (11-13). Worryingly, the CTX-M encoding plasmids and transposons, can also contain resistant genes for other antimicrobials like fluoroquinolones (14). This brings big challenges to clinical treatment as extended spectrum cephalosporins (ESCs) and fluoroquinolones are the drugs of choice for treatment of invasive *Salmonella* infections (15). The mechanism of quinolone resistance has been elucidated as plasmid-mediated quinolone resistance (PMQR) and chromosomal mutations in the quinolone resistance-determining regions (QRDRs) (16, 17). PMQR can be classified into three different resistant mechanisms: AAC(6')-Ib-cr acetylating ciprofloxacin and norfloxacin; Qnr proteins mediating target protection and the OqxAB and QepA mediating drug efflux (17). While, mutations in QRDRs genes encoding DNA gyrase or topoisomerase IV, are also frequently found in

quinolone resistant *Salmonella spp.* isolates (16). The co-occurrence of ESBL (especially the CTX-M encoding plasmids and transposons) and PMQR in *Salmonella spp.* is a cause for concern because plasmids can spread with relative ease between different reservoirs and such spread may be extremely difficult to control.

In this study, we have therefore investigated ESBL-producing *Salmonella spp.* isolates, collected from patients with diarrhea, food of animal origin and pets across 5 provinces of China. We dissected the serovars diversity, high prevalence of antimicrobial resistance, MLST, and co-occurrence of MDR determinants, including CTX-M-type ESBLs and quinolone resistance. Furthermore, we investigated the relatedness of $bla_{\text{CTX-M}}$ genes, amino acid substitutions in QRDRs and determinants of PMQR across serovars and sources and provided evidence of the existence of *Salmonella spp.* harboring $bla_{\text{CTX-M-130}}$ gene and QRDRs mutations in previously undescribed infection reservoirs such as humans, food and pets.

## RESULTS

***Salmonella spp.* isolates from patients with diarrhea, food of animal origin and pet samples.** A total of 2,283 *Salmonella spp.* isolates were recovered from 18,334 samples (12.5%) **(Table 1)** collected from 5 provinces in China. Of these 2,283 isolates, 1572 (10.8%) of 14,579 were isolated from patients with diarrhea; 660 (19.4%, 660/3,405) were isolated from food of animal origin; and 51 (14.6%, 51/350) were isolated from pet fecal samples. Overall, the prevalence among pets was lower than among food of animal origin ($p < 0.05$), but higher than among patients with diarrhea ($p < 0.05$). However, the prevalence among $\leq 5$ years old patients with diarrhea was significantly higher than among other patients with diarrhea ($p < 0.05$). Finally, the results showed that the prevalence among chicken meat was significantly higher than among the other types of samples ($p < 0.05$).

***Salmonella* serovars and their distribution.** The 2,283 *Salmonella spp.* isolates were serologically divided into 15 serogroups and 66 serovars **(Table S1)**, with serogroup B (n = 1,044, 45.7%) representing the most common serogroup identified, followed by serogroup D1 (n = 684, 30.0%), serogroup C1 (n = 227, 9.9%), serogroup E1 (n = 155, 6.8%), and serogroup C2-C3 (n = 109, 4.8%). These five serogroups comprised of 52 (52/66, 78.8%) serovars and 2219 isolates (2219/2283, 97.2%). The distribution of the serogroups and serovars derived from different sources is shown in **Table S1**. A total of 13 serogroups and 56 serovars were identified from patients with

diarrhea. The distribution of serovars varied among different sources (**Table S1 and Figure 1**).

**Antimicrobial susceptibility testing of 2,283 *Salmonella spp.* isolates.** Out of 2,283 *Salmonella spp.* isolates, 331 (14.5%) were susceptible to all antimicrobial agents tested (pan susceptible), while 1,952 (85.5%) exhibited resistance to at least one compound **(Table 2)**. The top three dominant resistant antimicrobial agents were AMP (64.6%), NAL (62.0%), and TET (58.7%). Notably, three isolates (0.1%, 3/2,283) (two *S.* Enteritidis from ≤ 5 years old patients with diarrhea and one *S.* Indiana from chicken meat) showed resistance to carbapenems (IPM and MEM) **(Table 2 and Table S2)**. Additionally, the isolates cultured from food of animal origin showed significantly higher resistance to NAL (75.9%), CTX (18.3%), and CAZ (14.1%) than those from the other samples ($p < 0.05$). The isolates cultured from pet fecal samples showed significantly higher resistance to TET (80.4%), SAM (62.7%), CHL (60.8%), and SXT (60.8%) than those from the other samples ($p < 0.05$). The isolates cultured from patients with diarrhea showed significantly lower resistance to SAM (26.2%), SXT (23.6%), GEN (16.1%), and CIP (10.0%) than those from the other samples ($p < 0.05$).

Among the 2,283 *Salmonella spp.* isolates, 1,288 (56.4%) were resistant to three or more classes of antimicrobials and these were classified as (MDR) **(Table 2)**. Notably, isolates recovered from pet fecal samples showed highest percentage of resistance to ≥ 3 (80.4%), ≥ 4 (60.8%), ≥ 5 (58.8%), ≥ 6 (33.3%), and ≥ 7 (23.5%) classes of antimicrobials were substantially higher than those from other samples ($p < 0.05$). In total, we identified 159 antimicrobial resistance profiles and 138 MDR profiles. Amongst the MDR profiles, the most common were NAL-AMP-TET (8.0%, n = 183) **(Table S2)**. Besides, the three carbapenem resistant isolates were found to be resistant to all other antimicrobials tested in this study.

The antimicrobial susceptibility phenotypes of isolates among different *Salmonella* serovars are shown in **Table S3**. Most of the isolates found to be resistant to ≥ 1 class of antimicrobials were serovars Indiana (97.9%, 93/95), followed by Enteritidis (97.6%, 656/672), Derby (93.8%, 121/129), Rissen (93.5%, 43/46), Typhimurium (92.0%, 611/664), and Corvallis (91.7%, 22/24). Whilst, MDR profiles were mostly observed in the serovars Indiana (94.7%, 90/95). Serovars with less than 10 isolates were not considered.

**Prevalence of ESBL-producing and *bla*$_{CTX-M}$ positive *Salmonella spp.* isolates.** The prevalence of ESBL-producing *Salmonella spp.* isolates was 9.7% (222/2,283), of these, 102 were isolates collected from patients with diarrhea, 100 from food of animal origin, and 20 from pet fecal samples **(Table 3)**. No ESBL-producing isolates were detected from aquatic product and pigeon fecal samples. The prevalence of ESBL-producing isolates in chicken meat samples (20.0%, 97/486) was lower than in turtle fecal samples (47.6%, 20/42) ($p < 0.05$), but higher than among other samples ($p < 0.05$). Additionally, the ESBL-producing *S.* Indiana isolates were the most detected (96.8%, 92/95). Notably, of these 92 ESBL-producing *S.* Indiana isolates, 90 were recovered from chicken meat samples, 1 from patients with diarrhea and 1 pork samples, respectively.

Of these 222 ESBL-producing isolates, 200 contained the *bla*$_{CTX-M}$ genes (8.8%, 200/2,283) **(Table 3)**. Most of the *bla*$_{CTX-M}$ positive isolates were recovered from chicken meat samples (19.5%, 95/486) significantly higher than among other samples ($p <0.05$). *S.* Indiana isolates were also the serovar harboring most of the *bla*$_{CTX-M}$ genes (95.8%, 91/95), followed by Give (30.8%, 8/26), Saintpaul (16.7%, 3/18), and Thompson (10.0%, 7/70).

**Distribution of *bla*$_{CTX-M}$ genes, gyrA/parC mutations and PMQR among ESBL-producing *Salmonella spp.* isolates.** Overall, 8 *bla*$_{CTX-M}$ alleles (*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-24}$, *bla*$_{CTX-M-27}$, *bla*$_{CTX-M-65}$, *bla*$_{CTX-M-79}$, *bla*$_{CTX-M-90}$, *bla*$_{CTX-M-123}$, and *bla*$_{CTX-M-130}$) were identified among the 222 ESBL-producing *Salmonella spp.* isolates **(Table 4 and Figure 2)**. Approximately 53% (117/222) of ESBL-producing isolates carried one of the *bla*$_{CTX-M}$ genes, while 37% (83/222) were detected co-carrying two of *bla*$_{CTX-M}$ genes **(Figure 2)**. The most prevalent gene was *bla*$_{CTX-M-65}$ with 86 isolates out of the 222 ESBL-producing *Salmonella spp.* isolates harboring *bla*$_{CTX-M-65}$ (38.7%, 86/222), followed by *bla*$_{CTX-M-123}$ (27.9%, 62/222), *bla*$_{CTX-M-14}$ (20.7%, 46/222), *bla*$_{CTX-M-79}$ (19.8%, 44/222), and *bla*$_{CTX-M-130}$ (16.7%, 37/222). The distribution of *bla*$_{CTX-M}$ genes varied across sources and serovars **(Table S4 and Figure 2)**. Notably, the *bla*$_{CTX-M-130}$ gene was mostly detected from patients with diarrhea (25/37) and chicken meat samples (11/37), with Typhimurium, Indiana, and Enteritidis being the dominant serovars. Furthermore, the three carbapenem resistant isolates were also found to be CTX-M-type ESBLs, two of which were *S.* Enteritidis isolates from patients with diarrhea (one carrying the *bla*$_{CTX-M-79}$ and *bla*$_{CTX-M-130}$

genes, and one carrying the *bla*$_{CTX-M-79}$ and *bla*$_{CTX-M-14}$ genes) and one was S. Indiana carrying the *bla*$_{CTX-M-65}$ gene isolate collected from chicken meat.

As all of the 222 ESBL-producing isolates were resistant to quinolones (149 CIP and 199 NAL resistant isolates, respectively), we further investigated the presence of QRDRs mutations and PMQR genes **(Table 4)**. The sequencing analysis resulted in the identification of seven QRDRs point mutations, five in *gyrA* (S83Y, S83F, D87G, D87N, and D87Y) and one in *parC* (S80I), respectively. The seven QRDR mutations were found in 166 ESBL-producing isolates, including 97 isolates from chicken meats samples, 48 isolates from patients with diarrhea, 18 isolates from turtle fecal samples, and 3 from pork samples. While the *oqxA/B* (n = 218), *qepA* (n = 52), *qnrB* (n = 116), and *qnrS* (n = 222) were found in the 222 ESBL-producing isolates. The *aac(6')-Ib* gene was detected in all 222 isolates, further analysis done by DNA sequencing and BLAST searches confirmed that this gene is the one conferring resistance to aminoglycosides as no known *aac(6')-Ib* variants that could lead to ciprofloxacin resistance were present.

**MLST.** In total, 15 sequence types including ST11, ST13, ST17, ST19, ST26, ST29, ST32, ST34, ST40, ST50, ST155, ST446, ST516, ST588, and ST1544 were identified from 222 ESBL-producing isolates **(Figure 2)**. ST17 (92/222) was the most prevalent sequence type among the ESBL-producing isolates. Notably, comprising all the above and isolates co-carrying two of *bla*$_{CTX-M}$ genes were all identified as serovar Indiana, Enteritidis and Typhimurium. Interestingly, 14 out of 21 *S.* Thompson isolates were found not carrying the *bla*$_{CTX-M}$ genes. Besides, 8 ST516 serovars as Give, 6 ST40 serovars as Derby, 3 ST50 serovars as Saintpaul, 1 ST13 serovar as Agona, 1 ST29 serovar as Stanley, 1 ST446 serovar as Hvittingfoss, and 1 ST588 serovar as Havana were also identified from the ESBL-producing isolates.

## DISCUSSION

Globally, the burden of morbidity, mortality and economic losses from human and animal enteric pathogenic bacteria, including *Salmonella spp.*, is immense, despite the presence of antibiotic drugs (18). Worryingly, the emergence of MDR and ESBLs, especially CTX-M-producing *Salmonella spp.* in humans, animals, pets and foods is increasingly worldwide, including China (1, 2, 4, 5, 8). In this study, we surveyed the prevalence, serovars distribution, MDR profiles and the occurrence of ESBL-producing *Salmonella spp.* in patients with diarrhea from 20 hospitals, food of

animal origin samples from a total of 20 supermarkets, and pet fecal samples from 5 veterinary clinics between 2014, and 2015 from 5 provinces (Beijing, Heilongjiang, Hubei, Jiangxi and Shandong) in China. Furthermore, we investigated the characteristic of CTX-M-type ESBL-producing *Salmonella spp.*, at the genetic level.

Overall, 1,572 *Salmonella spp.* isolates were recovered from 14,579 patients with diarrhea, showing a prevalence of 10.8% among human isolates, which concurs with previous findings obtained in Shanghai (8.2%), but was higher than in the provinces of Beijing (children, 4.3%) and Guangzhou (4.5%) (19-21). Moreover, our results revealed that the prevalence (13.0%) of *Salmonella spp.* in children aged ≤ 5 years old was higher than in adults (8.7%-9.7%) ($p < 0.05$), which is consistent with the literature, showing that children are more susceptible to Salmonellosis (22, 23). Therefore, our findings suggest that the efforts to determine the risk factors causing such high *Salmonella spp.* infections should concentrate upon children ≤ 5 years old in China.

Accordingly, more than 70% of foodborne disease outbreaks in China are attributed to *Salmonella spp.*, and many diseases are linked to the consumption of food of animal origin, especially chicken and pork, being considered as the major reservoirs of *Salmonella spp.* dissemination (24, 25). In our study the prevalence (19.4%) of *Salmonella spp.* in food samples of animal origin concurs with previous findings obtained in China but was higher than in Spain (8.9%) and Poland (5.5%) (26-29). The upper edge of the reported *Salmonella spp.* prevalence range was observed in chicken meats (39.3%), similarly to what was previously found in Henan (38%), but lower than in Shaanxi (54%), and Guangdong (63.6%) (30-32). By contrast, we found a lower prevalence rate (9.0%) of *Salmonella spp.* in pork compared to previous findings in China (26, 30-32). Lower contamination of aquatic products (prevalence rate of 6.4%) was found when compared to previous findings obtained in China, Thailand, and Malaysia, but higher than in Morocco (5, 26, 33-36). Our data shows that food, especially chicken meat is an important reservoir of *Salmonella spp.* contamination and emphasize the importance of monitoring *Salmonella spp.* infections in food-producing animals and the food chain supply.

Recently, more people are into pets and consequently the number of pet shops and pet clinics has increased in China. Notably, pet reptiles and birds have been proven to pose an important zoonotic potential being important reservoirs for

pathogens including *Salmonella spp.*, with patients who are immunocompromised, young children, pregnant women and older adults at the greatest risk for transmission via direct and indirect contact (37, 38). However, pets are generally considered to be of little concern as a source of *Salmonella spp.* to humans (39). Our findings, support the assertion that pets are important reservoirs of infections, specifically we observed an overall prevalence of *Salmonella spp.* in pet fecal samples of 14.6% (pigeon, 15.0%; turtle, 14.5%) lower than in Costa Rica (pigeon, 24.1%) and Korea (turtle, 50%), but higher than that in a previous study in China (pigeon, 4.1%) (40-42). An estimated 11% of all *Salmonella spp.* infections are attributed to animal exposure annually in the USA, with the highest rates of illness and death occurring among children (43). During 1990-2014, a total of 53 live poultry-associated Salmonellosis (LPAS) outbreaks were reported, involving 2,630 illnesses, 387 hospitalizations, and 5 deaths. Since 2007, numerous outbreaks of human *Salmonella spp.* infections linked to contact with animals and their environments have been investigated, including those involving contact with turtles and backyard poultry (44). Taken all together, these findings emphasize the importance in managing and studying animal-associated Salmonellosis outbreaks, as they occur at the intersection of human and animal health.

Our data showed that Typhimurium and Enteritidis were the most common serovars found among patients with diarrhea, which is consistent with the results obtained previously in China and other regions worldwide (3, 5, 19-21). Whilst, Enteritidis and Indiana, Derby and Typhimurium, Thompson and Aberdeen were the most common serovars found in chicken meat, pork and aquatic product, respectively, which was consistent with the literature (8, 31-33). By contrast, previous investigations covering the northern Chinese regions found the serovars Senftenberg, Meleagridis, Hadar, Derby, Corvallis, and Kentuck in other as the most prevalent in chicken meat (26, 30). Such differences may result by variation in temperature, both within and between seasons, local environmental conditions and sampling strategy.

Antimicrobial resistance in foodborne pathogens such as *Salmonella spp.* is a major concern for public health safety. Still more worrying, is that *Salmonella spp.* isolates conferring resistance to multiple drugs are rapidly increasing globally (8, 28, 34). In Europe, more than half of the *Salmonella spp.* isolates (52.6%) collected from human were found to be susceptible and only 28.6% of the isolates were found to be MDR (45). Conversely, 85.5% of the isolates in our investigation were resistant to at

least one antimicrobial and 56.4% were MDR. A total of 178 isolates related to human infections caused by invasive *Salmonella spp.* collected in five provinces of China between 2007 and 2016 revealed that 53.4% isolates were MDR (46). The high rates of MDR *Salmonella spp.* could pose a significant challenge for the effective treatment of salmonellosis in China. Furthermore, our findings show that *Salmonella spp.* isolates resistant against the conventional first-line antimicrobials (AMP, NAL, CHL, SXT and TET) remains high (23.6%-68.0%) in patients with diarrhea. In comparison, studies performed in patients with diarrhea in the USA, showed lower resistance rates (2.7%-20%) of *Salmonella spp.* isolates to AMP, CHL, and NAL (47). Although isolates showed low resistance rates to some antimicrobials like gentamicin (17.8%) in this study, they should not be used for clinical therapy, as they are not effective both in humans and animals. Overall, these circumstances render China particularly suitable to study the MDR *Salmonella spp.* in the food-chain.

Most of the isolates (> 90%) identified as the serovars Indiana, Enteritidis, Derby, Rissen, Typhimurium, and Corvallis were resistant to at least one antimicrobial which concurs with previous findings obtained in China (20,27,32,33). The most MDR was observed within the above mentioned serovars as well as for the Thompson and London. However, the highest percentage of MDR was observed for the isolates identified as the serovars Kentucky, Typhimurium, and Infantis while the *Salmonella* Enteritidis isolates were more susceptible in humans and animals in the Europe (48). Of note, *Salmonella* Indiana isolates, mainly recovered from chicken meats (93/95), were reported as the second most commonly serovar in China with the highest percentage of MDR (49). More attentions need to be paid to the MDR *Salmonella spp.* isolates especially the *S.* Indiana isolates in China amongst workers in the fields of veterinary medicine, food of animal origin and public health.

In our study, 102 (6.5%) of 1,572 *Salmonella spp.* isolates recovered from patients with diarrhea were identified as ESBL-producing, among which 99 were found to harbor the $bla_{\text{CTX-M}}$ genes. The high presence of $bla_{\text{CTX-M}}$ genes among ESBL-producing isolates was consistent with previous findings obtained from children with diarrhea in China (8). Of interest, most ESBL-producing isolates were found in pet turtle fecal samples (47.6%), but only 3 of these isolates were CTX-M-type. Few, if any, data is currently available about ESBL-producing *Salmonella spp.* isolates in pet turtles. In 2019, thirty-five *Salmonella spp.* isolates

were recovered from 59 pet turtle samples, but none were identified as ESBL-producing (50). Similar to other studies, 20% of the *Salmonella spp.* isolates from chicken meat samples were identified as ESBL-producing, and 97.9% of the ESBL-producing isolates carried the *bla*$_{\text{CTX-M}}$ genes (9, 51, 52). In Asia, the daily animal protein intake increased more than three times between 1960 and 2013 (53). To meet this demand, the scale of broilers farming increased very rapidly. With the high density of birds, the use of antimicrobials as prevention, and treatment during animal growing and husbandry, especially chicken industry, is placing ever greater selection pressure for resistant strains of bacteria to evolve. Widespread misuse and overuse of antimicrobials might have led to the emergency of these MDR and ESBL-producing *Salmonella spp.* strain in food of animal origin.

Our findings highlight the presence of *bla*$_{\text{CTX-M-65}}$, *bla*$_{\text{CTX-M-79}}$, and *bla*$_{\text{CTX-M-130}}$ genes, in addition to the *bla*$_{\text{CTX-M-14}}$ gene, being the most common genes found in patients with diarrhea. Our results suggest that the CTX-M subtypes may have particularly epidemic characteristics in different geographical regions (8, 54-56). In 2019, the *bla*$_{\text{CTX-M-130}}$ gene was firstly found in *Salmonella spp.* isolates recovered from food samples in China (57). However, to the best of our knowledge, this is the first study reporting the detection of *bla*$_{\text{CTX-M-130}}$ gene in *Salmonella spp.* isolates recovered from patients with diarrhea in China. The co-presence of the *bla*$_{\text{CTX-M-65}}$ with *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-24}}$, *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{CTX-M-79}}$, and *bla*$_{\text{CTX-M-90}}$ genes in *Salmonella spp.* isolates from chicken meat obtained in this study has been previously described (58, 59). The *bla*$_{\text{CTX-M-123}}$ gene has been recently detected in *Salmonella spp.* isolates recovered from patients with diarrhea and chicken meat but at lower levels with respect to its ortholog found in 2013 in *Escherichia coli* isolates in China (19, 32, 60). Nevertheless, our findings give evidence for the potential spread of the *bla*$_{\text{CTX-M-123}}$ gene, with high prevalence among chicken meat samples in China. The *bla*$_{\text{CTX-M}}$ genes are known to be carried on transmissible plasmids, facilitating their transmission between different reservoirs such as *Salmonella spp.* and other Enterobacterales (14). This has important implications for understanding of the transmission dynamics and for evaluating control measures targeting the *bla*$_{\text{CTX-M}}$ dissemination between animals and human.

Finally, we also tested for the co-occurrence of quinolone and ESBL resistance traits. Seven QRDRs point mutations, five in GyrA (S83Y, S83F, D87G, D87N, and





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D87Y) and one in ParC (S80I) were found in 166 of the 222 ESBL-producing *Salmonella spp.* isolates from different sources as previously determined (16, 12, 30). Overall, 47.1% of the ESBL-producing isolates recovered from patients with diarrhea, all of which were CTX-M-type, also had QRDRs amino acid substitutions. The prevalence of QRDRs amino acid substitutions among ESBL-producing isolates from patients with diarrhea was consistent with previous reports in Thailand, but in contrast with findings gathered in patients with diarrhea in Senegal featured showing a much lower frequency (14, 55). Notably, all 97 ESBL-producing isolates recovered from chicken meat samples had the QRDRs amino acid substitutions, similarly to the data from Henan province in China (30). To date several investigations have been trying to identify QRDRs mutations in *Salmonella spp.* isolates recovered from turtle, without success (61). To our best knowledge, our results represent the first evidence of QRDRs amino acid substitutions in ESBL-producing *Salmonella spp.* isolates recovered from turtle fecal samples. Furthermore, all 222 ESBL-producing *Salmonella spp.* isolates were found to carry at least three of the PMQR genes including *oqxA/B*, *qepA*, and *qnrB/S*. Of note, it is quite common to have the *oqxA/B* genes on MDR-encoding plasmids, along with other resistance genes such as the ESBL-encoding genes (62). The *qepA* gene was previously detected in *Salmonella spp.* from patients with diarrhea in China, but absent in patients with diarrhea isolates in this study (62). The co-presence of the *qnrB/S* genes, *oqxA/B*, and *qepA* in a single *Salmonella spp.* isolate is seldom reported in the Europe (64). Worryingly, our findings suggest that the incidence of PMQR genes in ESBL-producing *Salmonella spp.* isolates is increasing in China.

MLST revealed a total of 15 STs identified among the 222 ESBL-producing *Salmonella spp.* isolates. ST17 (92/222) was the most prevalent sequence type, while all the ST17 isolates were serotyped as Indiana, 90 of which were detected in chicken meat isolates from Shandong and Jiangxi province, one from pork and one from a patient with diarrhea. Our data is consistent with previous findings obtained in China, showing that the ST17 *S.* Indiana isolates with the highest percentage of MDR are mainly recovered from chicken, and that chicken is considered as the major reservoir of ST17 *S.* Indiana clone in China (59, 65). Significantly, all isolates co-carrying two of $bla_{\text{CTX-M}}$ genes were serotyped as Indiana, Enteritidis and Typhimurium, while 14 out of 21 ST26 *S.* Thompson isolates were found carrying none of the $bla_{\text{CTX-M}}$ genes.

The MLST results showed that the Indiana, Enteritidis and Typhimurium isolates may pose a serious public health risk.

To the best of our knowledge, we firstly reported the detection of *Salmonella spp.* harboring the $bla_{\text{CTX-M-130}}$ gene from patients with diarrhea and QRDRs mutations from turtle fecal samples. Furthermore, antimicrobial resistance affects the development of the world economy and threats public health. Considering a very high ESBLs prevalence in China, we strongly suggest the government to initiate both clinical or veterinary testing for ESBLs when resistance to first line beta-lactams in *Salmonella spp.* are detected in order to improve monitoring and support effective treatment selection. Based on the concept of One Health, our study emphasizes the importance of a holistic working approach for animal, human, environment and related sectors. Specifically, our results stress the pressing need for investigating antimicrobial usage (AMU) as well as antimicrobial resistance (AMR) across the entire food safety chain, establishment of the national AMU and AMR surveillance network system. The results obtained on AMU and AMR will provide some knowledges for public communications and education. Besides, the rational and prudent use of antimicrobials should be propagandized in the community, health care settings, and animal farms so as to control the dissemination of MDR especially the CTX-M-type ESBLs in *Salmonella spp.* at the national level.

## MATERIALS AND METHODS

**Study setting, sample collection, and bacterial strains.** From January 2014 to December 2015, a *Salmonella spp.* control program has been conducted in China to monitor *Salmonella spp.* infections across different sources and regions. A total of 14,579 fresh fecal samples were collected from patients with acute diarrhea aged from 20 days to 81 years old (5,515 from ≤ 5 years old, 6,654 from 5-59 years old and 2,410 from ≥ 60 years old) at the enteric clinic setting of 20 hospitals in the Chinese provinces of Beijing, Heilongjiang, Hubei, Jiangxi and Shandong. Clinical information of each patient was extracted from the archived medical records. In parallel, 3,405 food samples of animal origin, including 1,237 chicken meat, 1,354 pork and 814 aquatic products were also collected from 20 supermarket outlets, including 10 big departmental stores and 10 local agriculture markets across the afore mentioned five Chinese provinces. Alongside, 350 fresh pet samples, including 290

turtle and 60 pigeon fecal samples were also collected from 5 veterinary clinics across the afore mentioned Chinese provinces.

Both human and pet fecal samples (the weight of each sample was ≥ 1 g) were placed in a sterile tube and then placed in a box maintained at a temperature lower than 4 ℃ and then were immediately transported to the laboratory and subjected to microbiological analysis within 2 h. Fecal samples were cultured by streaking on xylose lysine desoxycholate agar (HopeBio, Qingdao, China) and Chromagar *Salmonella spp.* (CHROMagar Microbiology, Paris, France), followed by incubation at 36 ℃ ± 1 ℃ for 18 h - 24 h. Three suspected *Salmonella spp.* colonies were streaked onto Trypticase soy agar (HopeBio, Qingdao, China) and further incubated at 37 °C for 18 h.

The animal food samples (the weight of each sample was ≥ 250 g) were collected at each sampling site and all were stored inside tightly sealed aseptic bags, surrounded by a biological ice bag, and then placed in a box maintained at a temperature lower than 4 ℃. Samples were also immediately transported to the laboratory and subjected to microbiological analysis within 2 h. All samples were subjected to qualitative analysis for *Salmonella spp.* using an enrichment method described by the National Food Safety Standard of China-Food microbiological examination, *Salmonella spp.* (GB 4789.4-2016).

Finally, confirmation of *Salmonella spp.* isolates recovered from fecal and food of animal origin was done through biochemical and molecular methods. Biochemical characterization was done using API 20E test identification test strips (bioMérieux, Marcy l' Etoile, France), while for the molecular confirmation we performed a PCR assay targeting the *invA* gene (66). For all the confirmed *Salmonella spp.* isolates, serovars were determined by the slide agglutination test, using *Salmonella spp.* antisera (Statens Serum Institute, Denmark) according to the Kauffmann-White scheme. All confirmed *Salmonella spp.* isolates were stored in brain heart infusion broth with 40% [v/v] glycerol (HopeBio, Qingdao, China) at -80 °C. Each sample retained one isolate at last.

**Antimicrobial susceptibility testing.** The antimicrobial susceptibility of all *Salmonella spp.* isolates was determined using the broth dilution method by the Biofosun® Gram-negative panels (Shanghai Biofosun Biotech, China) according to the CLSI guidelines (67). The following antimicrobials were assessed: ampicillin

(AMP, 1–32 mg/L), ampicillin-sulbactam (SAM, 0.25/0.125-32/16 mg/L), ceftazidime (CAZ, 0.25–32 mg/L), cefotaxime (CTX, 0.25–32 mg/L), imipenem (IPM, 0.125–16 mg/L), meropenem (MEM, 0.125–16 mg/L), trimethoprim–sulfamethoxazole (SXT, 0.125/2.38-16/304 mg/L), gentamicin (GEN, 0.25–32 mg/L), tetracycline (TET, 0.25–32 mg/L), ciprofloxacin (CIP, 0.03–64 mg/L), nalidixic acid (NAL, 0.25–128 mg/L), chloramphenicol (CHL, 0.25–32 mg/L). Confirmation of carbapenemase presence was done by agar dilution method expressed as the minimum inhibitory concentration (MIC) values for imipenem and meropenem, followed by the Etest® (bioMérieux, France) test.

*Salmonella spp.* isolates expressing resistance to cephalosporins (CAZ or CTX) were further screened to detect their ESBL productivities, which was done by a combination disc diffusion test by cefotaxime and ceftazidime discs, with and without clavulanic acid (HopeBio, Qingdao, China) according to CLSI guidelines (67). *Escherichia coli* ATCC™25922 and *Klebsiella pneumoniae* ATCC™ 700603 were applied as reference strain in antimicrobial susceptibility tests (AST). All identified isolates were preserved in Brain Heart Infusion Broth (HopeBio, Qingdao, China) containing 40% [v/v] glycerol in -80 ℃ for subsequent study.

**DNA purification.** The identified ESBL-producing *Salmonella spp.* isolates were incubated for 18 h - 24h at 37 ℃ in Luria-Bertani broth (HopeBio, Qingdao, China). A commercial bacterial DNA extraction kit (Bacterial DNA Kit D3350, Guangzhou, China) was used to extract pure genomic DNA from the bacterial culture. A Qubit® 3.0 fluorometer (Thermo Fisher Scientific, NH, USA) was used to detect the quality of DNA. DNA samples were diluted into a concentration of 50 mg/L with sterile deionized water for subsequent PCR assay.

**PCR and DNA sequencing.** Genomic DNA extracted from the ESBL-producing *Salmonella spp.* isolates was further screened for the $bla_{\text{CTX-M}}$ gene cluster by PCR (68). In addition, all ESBL-producing *Salmonella spp.* isolates were screened via PCR amplification for the presence of QRDRs (*gyrA*, *gyrB*, *parC*, and *parE*) and PMQR determinants [*qepA*, *aac(6')-Ib*, *oqxA*/*B*, and *qnrA*/*B*/*C*/*D*/*S*] *Salmonella spp.* (69-75). All PCR products were commercially sequenced (Thermo Fisher Scientific China, Shanghai, China) and subsequently analyzed by DNAstar (DNAstar Inc., Madison, WI, USA) and then, the resulted DNA sequences were blasted with reference sequences from NCBI.

**MLST.** MLST of all ESBL-producing *Salmonella spp.* isolates was performed following the protocols described at the MLST website (http://mlst.ucc.ie/mlst/dbs/Senterica/documents/primersEnterica_html). Seven conserved housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) of *Salmonella enterica* were amplified and sequenced at Thermo Fisher Scientific (China) Co. Ltd (Shanghai, China). Sequences were submitted to the *Salmonella* MLST database website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) to assign the sequence types (STs).

**Statistical analysis.** Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago USA) software. Differences between proportions were tested using chi-square test. A $p$ value < 0.05 was considered to be statistically significant.

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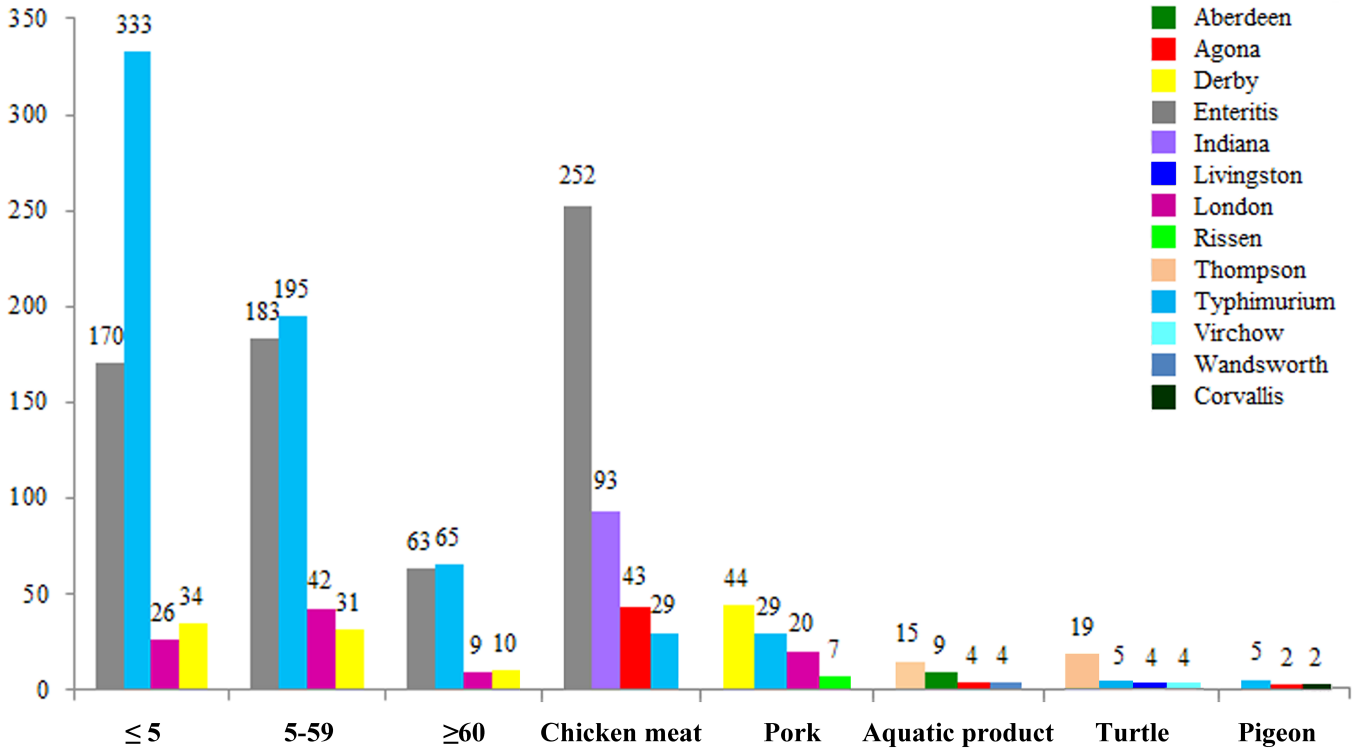
**Figure legends-**

Figure 1 Prevalence of top 4 *Salmonella* serovars among the total isolates recovered from patients with diarrhea, food of animal origin and pet samples in China. On the Y-axis the number of isolates is indicated. Colors indicate different serovars. Each of the histograms represents the number of isolates for each serovar. On the X-axis the sample classes (patients with diarrhea, food of animal origin and pets) are shown. Patient age distribution is indicated (≤ 5, 5-59, and ≥ 60 years old).

Figure 2 Dendrogram of the whole ESBL-producing *Salmonella spp.* cohort. Phylogenetic tree (minimum spanning tree) based on seven loci of 222 ESBL-producing *Salmonella spp.* isolates recovered from patients with diarrhea, animal of food origin and pet samples. The phylogenetic tree was developed by the MEGA5 (www.megasoftware.net) and visualized by Evolview (www.evolgenius.info/evolview/). Sequence types (STs) are indicated by means of colors marked in the branches and backgrounds of isolates names. The $bla_{\text{CTX-M}}$ genes (CTX-M genes), serovars and sample types (sources) are texted or color-coded in the following rings.



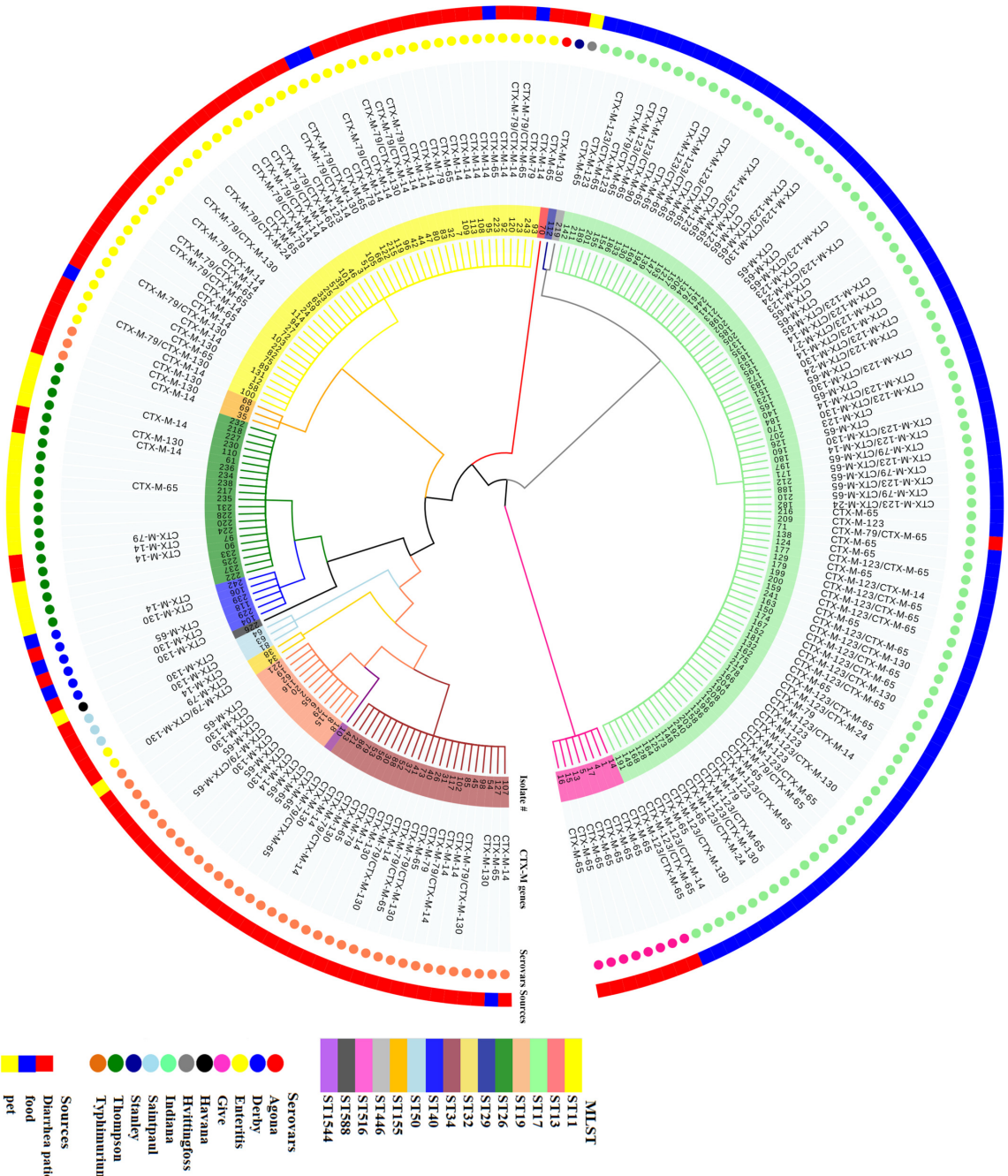


**Table 1 Prevalence of *Salmonella spp.* isolates recovered from patients with diarrhea, food of animal origin, and pets in China.**

| Sources | | No. of samples tested | No. of positive samples | % Prevalence |
|---|---|---|---|---|
| Patients with diarrhea | ≤5 | 5,515 | 717 | 13.0 |
| | 5-59 | 6,654 | 646 | 9.7 |
| | ≥60 | 2,410 | 209 | 8.7 |
| | Total | 14,579 | 1,572 | 10.8 |
| Food of animal origin | Chicken meat | 1,237 | 486 | 39.3 |
| | Pork | 1,354 | 122 | 9.0 |
| | Aquatic product | | | |
| | Freshwater fish | 349 | 22 | 6.3 |
| | Saltwater fish | 309 | 17 | 5.5 |
| | Shrimp | 156 | 13 | 8.3 |
| | Total | 814 | 52 | 6.4 |
| | Total | 3,405 | 660 | 19.4 |
| Pets | Turtle | 290 | 42 | 14.5 |
| | pigeon | 60 | 9 | 15.0 |
| | Total | 350 | 51 | 14.6 |
| Overall | | 18,334 | 2,283 | 12.5 |

Patient age distribution is indicated (⩽5, 5-59, and ⩾60 years old).







Antimicrobial Agents and Chemotherapy

**Table 2 Antimicrobial resistance of *Salmonella spp.* isolates recovered from patients with diarrhea, food of animal origin, and pets sin China**

| Antimicrobials | No. of isolates resistant to the tested antimicrobial agents (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Patients with diarrhea | | | | Food of animal origin | | | | Pets (n=51) | Overall (n=2283) |
| | ≤5 (n=717) | 5-59 (n=646) | ≥60 (n=209) | Total (n=1572) | Aquatic product (n=52) | Chicken meat (n=486) | Pork (n=122) | Total (n=660) | | |
| Ampicillin | 509(71.0) | 425(65.8) | 135(64.6) | 1069(68.0) | 22(42.3) | 261(53.7) | 83(68.0) | 366(55.5) | 40(78.4) | 1475(64.6) |
| Cefotaxime | 84(11.7) | 63(9.8) | 29(13.9) | 176(11.2) | 2(3.8) | 114(23.5) | 5(4.1) | 121(18.3) | 3(5.9) | 300(13.1) |
| Ceftazidime | 51(7.1) | 27(4.2) | 15(7.2) | 93(5.9) | 1(1.9) | 90(18.5) | 2(1.6) | 93(14.1) | 2(3.9) | 188(8.2) |
| Ciprofloxacin | 83(11.6) | 57(8.8) | 17(8.1) | 157(10.0) | 8(15.4) | 136(28.0) | 38(31.1) | 182(27.6) | 14(27.5) | 353(15.5) |
| Nalidixic Acid | 387(54.0) | 374(57.9) | 126(60.3) | 887(56.4) | 19(36.5) | 399(82.1) | 83(68.0) | 501(75.9) | 28(54.9) | 1416(62.0) |
| Ampicillin-Sulbactam | 212(29.6) | 144(22.3) | 56(26.8) | 412(26.2) | 15(28.8) | 236(48.6) | 46(37.7) | 297(45.0) | 32(62.7) | 741(32.5) |
| Gentamicin | 139(19.4) | 89(13.8) | 25(12.0) | 253(16.1) | 1(1.9) | 108(22.2) | 31(25.4) | 140(21.2) | 13(25.5) | 406(17.8) |
| Chloramphenicol | 281(39.2) | 170(26.3) | 54(25.8) | 505(32.1) | 9(17.3) | 163(33.5) | 59(48.4) | 231(35.0) | 31(60.8) | 767(33.6) |
| Trimethoprim-Sulfamethoxazole | 204(28.5) | 127(19.7) | 40(19.1) | 371(23.6) | 25(48.1) | 182(37.4) | 64(52.5) | 271(41.1) | 31(60.8) | 673(29.5) |
| Tetracycline | 473(66.0) | 410(63.5) | 134(64.1) | 1017(64.7) | 29(55.8) | 148(30.5) | 104(85.2) | 281(42.6) | 41(80.4) | 1339(58.7) |
| Imipenem | 2(0.3) | 0(0.0) | 0(0.0) | 2(0.1) | 0(0.0) | 1(0.2) | 0(0.0) | 1(0.2) | 0(0.0) | 3(0.1) |
| Meropenem | 2(0.3) | 0(0.0) | 0(0.0) | 2(0.1) | 0(0.0) | 1(0.2) | 0(0.0) | 1(0.2) | 0(0.0) | 3(0.1) |
| Pan susceptible | 100(13.9) | 121(18.7) | 34(16.3) | 255(16.2) | 18(34.6) | 43(8.8) | 8(6.6) | 69(10.5) | 7(13.7) | 331(14.5) |
| ≥ 1 class of antimicrobials | 617(86.1) | 525(81.3) | 175(83.7) | 1317(83.8) | 34(65.4) | 443(91.2) | 114(93.4) | 591(89.5) | 44(86.3) | 1952(85.5) |
| ≥ 3 classes of antimicrobials | 407(56.8) | 357(55.3) | 118(56.5) | 882(56.1) | 24(46.2) | 252(51.9) | 89(73.0) | 365(55.3) | 41(80.4) | 1288(56.4) |
| ≥ 4 classes of antimicrobials | 295(41.1) | 198(30.7) | 73(34.9) | 566(36.0) | 18(34.6) | 197(40.5) | 66(54.1) | 281(42.6) | 31(60.8) | 878(38.5) |
| ≥ 5 classes of antimicrobials | 220(30.7) | 135(20.9) | 50(23.9) | 405(25.8) | 13(25.0) | 178(36.6) | 49(40.2) | 240(36.4) | 30(58.8) | 675(29.6) |
| ≥ 6 classes of antimicrobials | 141(19.7) | 90(13.9) | 32(15.3) | 263(16.7) | 6(11.5) | 146(30) | 36(29.5) | 188(28.5) | 17(33.3) | 468(20.5) |
| ≥ 7 classes of antimicrobials | 65(9.1) | 43(6.7) | 10(4.8) | 118(7.5) | 0(0.0) | 110(22.6) | 27(22.1) | 137(20.8) | 12(23.5) | 267(11.7) |
| ≥ 8 classes of antimicrobials | 20(2.8) | 13(2.0) | 3(1.4) | 36(2.3) | 0(0.0) | 29(6.0) | 3(2.5) | 32(4.8) | 1(2.0) | 69(3.0) |
| ≥ 9 classes of antimicrobials | 2(0.3) | 0(0.0) | 0(0.0) | 2(0.1) | 0(0.0) | 1(0.2) | 0(0.0) | 1(0.2) | 0(0.0) | 3(0.1) |

n means total number of isolates tested for susceptibility in different samples. Patient age distribution is indicated (≤5, 5-59, and ≥60 years old).

**Table 3 Prevalence of ESBLs-producing and *bla*$_{CTX-M}$ positive isolates in *Salmonella spp.* isolates recovered from patients with diarrhea, food of animal origin, and pets in China (n=2,283).**

| Sources | | Number of isolates | Number of ESBLs-producing isolates (%) | Number of *bla*$_{CTX-M}$ positive isolates (%) |
|---|---|---|---|---|
| Patients with diarrhea | ≤5 | 717 | 46(6.4) | 46(6.4) |
| | 5-59 | 646 | 38(5.9) | 36(5.6) |
| | ≥60 | 209 | 18(8.6) | 17(8.1) |
| | Total | 1,572 | 102(6.5) | 99(6.3) |
| Food of animal origin | Aquatic product | 52 | - | - |
| | Chicken meat | 486 | 97(20.0) | 95(19.5) |
| | Pork | 122 | 3(2.5) | 3(2.5) |
| | Total | 660 | 100(15.2) | 98(14.8) |
| Pet | turtle | 42 | 20(47.6) | 3(7.1) |
| | pigeon | 9 | - | - |
| | Total | 51 | 20(39.2) | 3(5.9) |
| Serovars | Agona | 75 | 1(1.3) | 1(1.3) |
| | Derby | 129 | 6(4.7) | 5(3.9) |
| | Enteritis | 672 | 49(7.3) | 47(7.0) |
| | Give | 26 | 8(30.8) | 8(30.8) |
| | Havana | 1 | 1/1 | - |
| | Hvittingfoss | 1 | 1/1 | - |
| | Indiana | 95 | 92(96.8) | 91(95.8) |
| | Saintpaul | 18 | 3(16.7) | 3(16.7) |
| | Stanley | 36 | 1(2.8) | 1(2.8) |
| | Thompson | 70 | 21(30.0) | 7(10.0) |
| | Typhimurium | 664 | 39(5.9) | 37(5.6) |
| Overall | | 2,283 | 222(9.7) | 200(8.8) |

- means no detection Patient age distribution is indicated (≤5, 5-59, and ≥60 years old).

**Table 4 Prevalence of *bla*$_{CTX-M}$ genes, *gyr*A/*par*C mutations and PMQR genes in ESBLs-producing *Salmonella spp.* isolates recovered from patients with diarrhea, food of animal origin, and pets in China (n = 222).**

| Genes | | Number of isolates | Percentage of isolates |
|---|---|---|---|
| *bla*$_{CTX-M}$ | *bla*$_{CTX-M-14}$ | 46 | 20.7 |
| | *bla*$_{CTX-M-24}$ | 6 | 2.7 |
| | *bla*$_{CTX-M-27}$ | 1 | 0.5 |
| | *bla*$_{CTX-M-65}$ | 86 | 38.7 |
| | *bla*$_{CTX-M-79}$ | 44 | 19.8 |
| | *bla*$_{CTX-M-90}$ | 1 | 0.5 |
| | *bla*$_{CTX-M-123}$ | 62 | 27.9 |
| | *bla*$_{CTX-M-130}$ | 37 | 16.7 |
| *gyr*A mutations | S83Y | 23 | 10.4 |
| | S83F | 95 | 42.8 |
| | D87G | 9 | 4.1 |
| | D87N | 94 | 42.3 |
| | D87Y | 8 | 3.6 |
| *par*C mutations | S80R | 90 | 40.5 |
| PMQR genes | *qnrB* | 116 | 52.3 |
| | *qnrS* | 222 | 100.0 |
| | *qepA* | 52 | 23.4 |
| | *aac(6')-Ib* | 222 | 100.0 |
| | *oqxA* | 218 | 98.2 |
| | *oqxB* | 218 | 98.2 |