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Distribution of Phenotypic and Genotypic Antibiotic Resistance in *E. coli* Isolates along the Production and Supply Chain of Pork around Hubei Province of China

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RZ designed the project. ZG and CYT collected and processed the samples. SBK, AS, MA and IA performed the experiments and wrote the manuscript.

Key words
Antibiotics, Drug resistance, *E. coli*, Pathogenic isolates, Virulence.

ABSTRACT

Using MacConkey agar, *E. coli* were isolated from 285 samples including 125 tonsil swabs (4-6 weeks old healthy pigs from 5 farms), 80 tissue samples from different slaughter houses (20 each intestine, liver, meat and kidney), and 80 samples from super- and wet-markets (each 20 meat and liver) collected both in summer and winter. Isolates were tested for 15 antibiotics (CRO, AMX, GEN, STR, TET, CHL, CLR, LVX, OFX, GAT, CIP, SXT, AMP, LIN and AZM) according to the disc diffusion method and antibiotic resistant genes {*tet(A)*, *tet(B)*, *tet(C)*, *strA/strB*, *aadA*, *aac(3)IV)*, *aadB*, *sul1*, *sul2* and *sul3*, *blaCMY-2*, *blaTEM* and *blaSHV}* using mPCR. Resistance for LIN was the highest in overall (96.3%, 77/80) isolates as well as from pig farms (100%, 20/20) and different markets (100%, 20/20, 85%, 17/20). Resistance for other antibiotics such as AMX, TET, AMP and SXT was found 82.5% (66/80), 63.7% (51/80), 58.7% (47/80) and 50% (40/80), respectively. The most prevalent ARGs in the isolates recovered from pig farms was bla_{TEM} (100%, 20/20), followed by *blaCMY-2* (80%, 16/20), *tetA* and $tetB$ (60%, 12/20) and $tetC$ (50%, 10/20). *E. coli* became more and more diverse along the PSCP with group B2 being the most prevalent. Besides multiple drug resistance, they share many traits with the human pathogenic isolates based on virulence gene contents that may pose a potential threat to public health.

INTRODUCTION

An increase in the antimicrobial resistance of *E. coli* is a worldwide public health concern which is mainly associated with merciless use of antibiotics (Foley and Lynne, 2008; Geimba *et al*., 2004). Unnecessary and extensive application of antimicrobials in animal feed and veterinary practice have led towards antimicrobial resistance in all bacterial pathogens in general and in *E. coli* in particular (Jiu *et al*., 2016). Multiple drug resistance (MDR) has been developed in these bacterial pathogens including *E. coli* that may be a great challenge to the world after infection. Every year, 25000 deaths in European Uninon and 23000 deaths in US have reported due to MDR bacteria (Jessica *et al*., 2015). This resistance against antimicrobials is due to different factors including transfer of plasmids, transposons and antimicrobial resistant genes. Clinically *E. coli* is divided into three types, commensals, diarrheagenic and extra intestinal pathogenic *E. coli* (ExPEC) (Khan *et al*., 2016). ExPEC are those strains that cause infections outside the intestine such urinary tract infection (UTI), septicemia and meningitis in new born babies (Clermont *et al*., 2011; Johnson *et al*., 2005a, b). There are four phylogenetic groups of *E. coli* (A, B1, B2 and D) (Khan *et al*., 2016). ExPEC mainly belong to group B2 with lesser extent to D while commensals belong to group A and B1. Keeping in view the importance of ExPEC, we carried out this population biological study of *E. coli* to investigate phylogenetic grouping, distribution of virulence genes, antibiotic resistance and antibiotic resistant genes along the PSCP.

MATERIALS AND METHODS

Samples

A total of 285 samples were collected in this study.

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From the pig production system we collected 125 tonsil swabs from 4-6 weeks old clinically healthy pigs from different herds of five intensive pig farms located in Ezhou, Xiantou and Qianjiang of Hubei province (25 tonsil swabs from each farm). Tonsil swab was taken by scraping the tonsil surface thrice with a sterile bamboo tongue-spatula after gently opening the pig mouth with a mouth gag, and immediately put into a sterile plastic tube and transported to laboratory. From the pork supply chain, 160 samples were purchased from different markets located in Yichang and Wuhan of Hubei province, including 80 samples from different slaughter houses (20 each meat, liver, intestine and kidney), 80 samples from different wet and super markets (20 each meat and liver) in both summer and winter. Samples were collected in sterile plastic bags and subjected to laboratory for bacterial isolation.

Bacterial isolation

Upon arrival to the laboratory, tonsil swabs were immediately washed with 0.5 ml PBS, and 0.1 ml of them was inoculated on MacConkey agar plate. Out of 200 g meat or tissue samples, 50 g was cut into small pieces and mixed with 450 ml of BHI in a homogenizer (250 Watt, Type MJ-25BM05A, Guangdong Midea Electrical Appliance Co., Ltd., Foshan, China) for 2 min. One ml of homogenized sample was mixed with 9 ml of BHI and incubated at 37°C for 24 h. 0.5 ml of the culture was then inoculated on MacConkey agar plate. The plates were incubated at 37°C for 24 h. Typical lactose fermenting, pink colonies (one colony/sample) were selected for further analysis. *E. coli* was confirmed using standard bacteriological biochemical tests using an API 20E system (bioMerieux, France).

Extraction of DNA

Genomic DNA was extracted from the isolates using E.Z.Nce.A bacterial DNA kit (Omega Bio-Tek, USA).

Antimicrobial susceptibility testing (AST)

The antimicrobial susceptibility of *E. coli* isolates was determined for 15 antimicrobials according to the disc diffusion method using Muller-Hinton agar (MHA, Qingdao hopebio technology Co., China). Interpretation of the results followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Gilani *et al*., 2008). Antimicrobial susceptibility testing was performed for the following antimicrobial agents (antimicrobial abbreviations and breakpoints are shown in parenthesis): Ceftriaxone (CRO, 30 µg), Amoxicillin (AMX, 20 µg), Gentamycin (GEN, 10 µg), Streptomycin (STR, 10 µg), Tetracycline (TET, 30 µg), Chloramphenicol (CHL, 30 µg), Clarithromycin (CLR, 15 µg), Levofloxacin (LVX, 5 µg), Ofloxacin (OFX, 5 µg), Gatifloxacin (GAT, 5 µg), Ciprofloxacin (CIP, 5 µg), Suphamethoxazole+Trimethpram (SXT, 25 µg), Ampicillin (AMP, 10 µg), Lincomycin (LIN, 2 µg) and Azithromycin (AZM, 15 µg) (Table I). Isolates showing resistance to at least three antimicrobial agents belonging to different antimicrobial classes were considered multidrug resistance (MDR) strains.

Table I.- Antibiotics discs, their concentration and size of zone of inhibition.

S. No	Antibiotics	Disc content	Zone of inhibition (millimeter)		
			S	I	R
1.	CRO	30 µg	> 23	20-22	<19
2.	AMX	20 µg	>17	14-16	<13
3.	GEN	10 µg	>15	13-14	<12
4.	STR	10 µg	>15	12-14	<11
5.	TET	30 µg	>15	12-14	<11
6.	CHL	30 µg	>18	13-17	<12
7.	CLR	15 µg	>18	14-17	<13
8.	LVX	5 µg	>31	21-30	<20
9.	OFX	5 µg	>31	21-30	<20
10.	GAT	5µg	>18	15-17	<14
11.	CIP	5 µg	>31	21-30	<20
12.	SXT	25 µg	>16	11-15	<10
13.	AMP	10 µg	>17	14-16	<13
14.	LIN	2 µg	>21	16-20	<15
15.	AZM	15 µg	>18	14-17	<13

CRO, Ceftriaxone; AMX, Amoxicillin; GEN, Gentamycin; STR, Strptomycin; TET, Tetracyclin; CHL, Chloramphenicol; CLR, Clarithromycin; LVX, Levofloxacin; OFX, Ofloxacin; GAT, Gatifolxacin; CIP, Ciprofloxacin; SXT, Sulphamethoxazole+Trimethoprim; AMP, Ampicillin; LIN, Lincomycin; AZM, Azithromycin; S, sensitive; I, intermediate; R, resistance.

Detection of antibiotic resistance genes

A set of multiplex PCRs was used for identifying major resistance genes following the procedure as previously described (Kozak *et al*., 2009). The major genes conferring resistance for tetracycline [*tet(A)*, *tet(B)*, *tet(C)*], streptomycin (*strA/strB*, *aadA* and (*aac(3)IV*), gentamycin (*aac(3)IV*, *aadB*), sulfonamides (*sul1*, *sul2* and *sul3*), and b-lactamases (*blaCMY-2*, *blaTEM*, *blaSHV*) were targeted. Primers and multiplex PCRs conditions used for detection of antibiotic resistance genes are given in the Table II. Multiplex PCR 1 was done using the following thermal cycling conditions: one cycle consisting of 15 min at 95°C, 30 cycles consisting of 1 min at 95°C, 1 min at 66°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C. Multiplex PCR 2 and 3 were done using the following thermal cycling conditions:

Table II.- Primers and conditions used for antibiotic resistance genes.

PCR	Gene	Primer	Sequence	Final conc. of primer (M)	Annealing temp (°C)	Product size (bp)
1	sul1	sul1-F[b]	CGGCGTGGGCTACCTGAACG	0.2	66	433
		sul1-B[b]	GCCGATCGCGTGAAGTTCCG	0.2		
1	Sul2	sulII-L[c]	CGGCATCGTCAACATAACCT	0.3	66	721
		sulII-R[c]	TGTGCGGATGAAGTCAGCTC	0.3		
1	Sul3	sul3-GKa-F[d]	CAACGGAAGTGGGCGTTGTGGA	0.2	66	244
		sul3-GKa-R[d]	GCTGCACCAATTCGCTGAACG	0.2		
2	tet (A)	TetA-L[c]	GGCGGTCTTCTTCATCATGC	0.1	63	502
		TetA-R[c]	CGGCAGGCAGAGCAAGTAGA	0.1		
2	tet (B)	TetBGK-F2[m]	CGCCCAGTGCTGTTGTTGTC	0.2	63	173
		TetBGK-R2[m]	CGCGTTGAGAAGCTGAGGTG	0.2		
2	tet (C)	TetC-L[c]	GCTGTAGGCATAGGCTTGGT	0.5	63	888
		TetC-R[c]	GCCGGAAGCGAGAAGAATCA	0.5		
3	aadA	4F[e]	GTGGATGGCGGCCTGAAGCC	0.1	63	525
		4R[e]	AATGCCCAGTCGGCAGCG	0.1		
3	*strA*/*strB*	strA-F[f]	ATGGTGGACCCTAAAACTCT	0.4	63	893
		strB-R[f]	CGTCTAGGATCGAGACAAAG	0.4		
3	*aac*(3)IV	aac4-L[g]	TGCTGGTCCACAGCTCCTTC	0.2	63	653
		aac4-R[g]	CGGATGCAGGAAGATCAA	0.2		
4	aadB	aadB-L[i]	GAGGAGTTGGACTATGGATT	0.2	55	208
		aadB-R[i]	CTTCATCGGCATAGTAAAAG	0.2		
5	blaTM	GKTEMF[d]	TTAACTGGCGAACTACTTAC	0.2	55	247
		GKTEMR[d]	GTCTATTTCGTTCATCCATA	0.2		
5	blaSHV	SHV-F[j]	AGGATTGACTGCCTTTTTG	0.4	55	393
		SHV-R[j]	ATTTGCTGATTTCGCTCG	0.4		
5	blaCMY-2	CMYF[d]	GACAGCCTCTTTCTCCACA	0.2	55	1000
		CMYR[d]	GGACACGAAGGCTACGTA	0.2		

Table III.- Antibiotic resistance in *E. coli* Isolates along PSCP.

Anti-biotics	No. of *E. coli* isolates resistant				
	Total (%)	PF (%)	SH (%)	WM (%)	SM (%)
	n=80	n=20			
LIN	77(96.25)	20(100)	20(100)	20(100)	17(85)
AMX	66(82.5)	19(95)	19(95)	16(80)	12(60)
TET	51(63.75)	13(65)	18(90)	11(55)	9(45)
AMP	47(58.75)	13(65)	14(70)	9(45)	11(55)
SXT	40(50)	8(40)	16(80)	8(40)	8(40)
CHL	39(48.75)	12(60)	13(65)	8(40)	6(30)
CLR	29(36.25)	11(55)	4(20)	10(50)	4(20)
STR	20(25)	5(25)	6(30)	6(30)	3(15)
GEN	14(17.5)	2(10)	6(30)	2(10)	4(20)
OFX	12(15)	1(5)	7(35)	3(15)	1(5)
CIP	12(15)	1(5)	6(30)	3(15)	2(10)
LFX	10(12.5)	1(5)	6(30)	2(10)	1(5)
AZM	7(8.75)	3(15)	1(5)	0(0)	3(15)
CRO	5(6.25)	0(0)	4(20)	0(0)	1(5)
GAT	3(3.75)	0(0)	0(0)	2(10)	1(5)

PF, pig farms; SH, slaughter house; WM, wet market; SM, super market. For abbreviations of antibiotics, see Table I.

one cycle consisting of 15 min at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C. Multiplex PCR 4 and 5 were done using the following thermal cycling conditions: one cycle consisting of 15 min at 94°C, 30 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and one cycle consisting of 10 min at 72°C.

RESULTS

Antibiotic resistance

Antimicrobial resistance patterns were different according to origin of *E. coli* isolates. Resistance for LIN was the highest in overall (96.3%, 77/80) isolates as well as from pig farms (100%, 20/20) and different markets (100%, 20/20, 85%, 17/20) (Table III). Resistance for other antibiotics such as AMX, TET, AMP and SXT was found 82.5% (66/80), 63.7% (51/80), 58.7% (47/80) and 50% (40/80), respectively. Resistance for GAT and CRO was found the lowest in all the isolates. Resistance for other antibiotics was found different in the isolates obtained from the pig farms and different markets. Multiple

antibiotic resistance was found (85%, 68/80) in over all isolates, being higher in the isolates from pig farms and slaughter houses (95%, 19/20) followed by super (85%, 17/20) and wet (75%, 15/20) as shown in Figure 1.

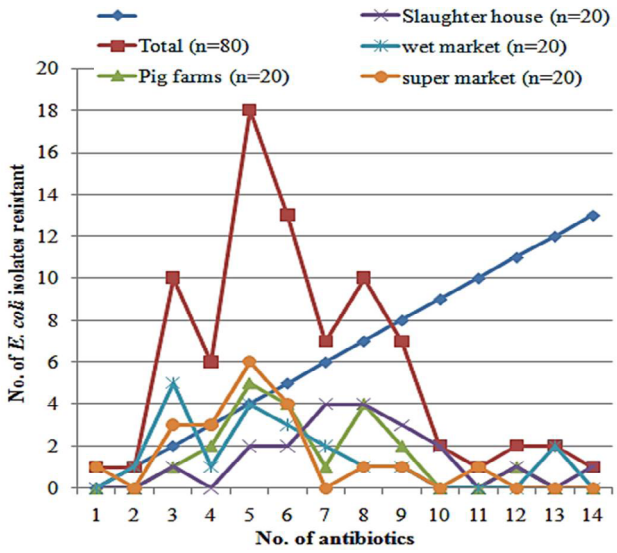

Fig. 1. Multiple drug resistance in *E. coli* isolates along the PSCP.

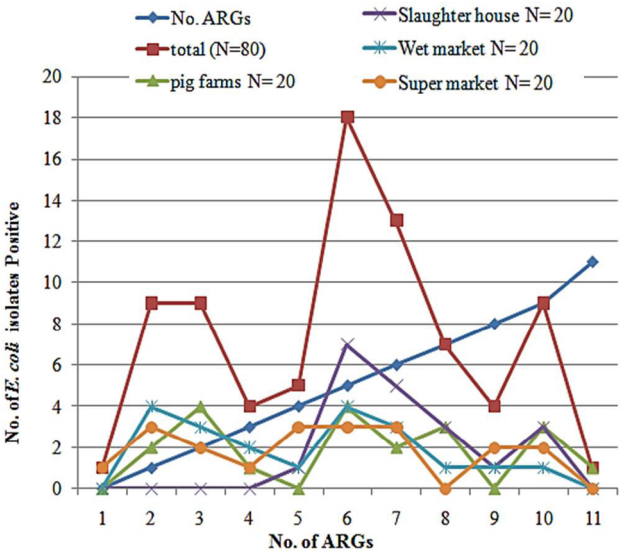

Fig. 2. Number of isolates having ARGs along the PSCP.

Antibiotic resistance genes (ARGs)

The prevalence of ARGs in *E. coli* isolates varied by origin of isolates and was higher in isolates from Slaughter houses (56.6% isolates) and pig farms (35.8% of isolates), which is consistent with the phenotypic data (Table III). The PCR results of ARGs are shown in Figures 1 and 2. Overall, the most common ARG was *blaTEM* (98.7%, 79/80), followed by *tetA*(58.7%, 47/80), *tetB*(55%, 44/80) and *blaCMY-2* (52.5%, 42/80) (Table IV). The most prevalent ARGs in the isolates recovered from pig farms was *blaTEM* (100%, 20/20), followed by *blaCMY-2* (80%, 16/20), *tetA* and *tetB* (60%, 12/20) and *tetC* (50%, 10/20). The isolates recovered from slaughter houses were also found different in ARGs as they possess higher prevalence of *blaTEM* (100%, 20/20), *blaSHV* (90%, 18/20), *sulI* (80%, 16/20), *tet(A)*(75%,15/20) and *tet(B)* (70%, 14/20). Likewise, the isolates from wet markets were higher in *blaTEM* (100%) followed by (55%, 11/20), *tetA* (50%, 10/20) and *blaSHV* and *sulI* (35%, 7/20). While the isolates from super market were higher in *blaTEM* (95%, 19/20) followed by *tetA* and *tetC* (50%, 10/20), *and blaCMY-2and sulI* (40%, 8/20). Interestingly all the isolates recovered from super market were found positive for *blaTEM* (100%).

Table IV.- Antibiotic resistant genes (ARGs) in *E. coli* isolates along PSCP.

ARGs	Total (%)	PF (%)	SH (%)	WM (%)	SM (%)
	n=80	n=20			
tetA	47 (58.75)	12(60)	15(75)	10 (50)	10(50)
tetB	44 (55)	12 (60)	14(70)	11(55)	7 (35)
tetC	30 (37.5)	10 (50)	6 (30)	4 (20)	10(50)
aadA	21 (26.25)	5 (25)	6 (30)	4 (20)	6 (30)
strA/strB	17 (21.25)	5 (25)	4 (20)	4(20)	4 (20)
aac(3)IV	6 (7.5)	1 (5)	1 (5)	3 (15)	1 (5)
blaTEM	79 (98.7)	20 (100)	20 (100)	20 (100)	19 (95)
blaSHV	18 (22.5)	3(15)	3 (15)	7 (35)	5 (25)
blaCMY-2	42 (52.5)	16(80)	18 (90)	0 (0)	8(40)
Sul1	39 (48.75)	8 (40)	16 (80)	7 (35)	8 (40)
Sul2	18 (22.5)	3 (15)	7 (35)	3(15)	5(25)
Sul3	37 (46.25)	8 (40)	16 (80)	8 (40)	5(25)
aaddB	2 (2.5)	0(0)	0(0)	1 (5)	1 (5)

PF, pig farms; SH, slaughter house; WM, wet market; SM, super market.

DISCUSSION

Because of intensive pig farming and vast supply chain in China, special attention needs to be given to pathogenic bacteria including *E. coli* (Cai *et al*., 2005; Normile, 2005). The ExPEC often belong to phylogenetic groups B2 and D (Clermont *et al*., 2000). These groups include potent human ExPEC isolates causing UTI, bacteremia and meningitis. UTI is one of the most common bacterial infections among women. It is primarily caused by ExPEC from the patient's own fecal flora (Tan *et al*., 2011). The external sources of the ExPEC in humans are unknown. Pigs and pork

meat may serve as a potential source. An increase in the antimicrobial resistance in bacterial pathogens including *E. coli* is a worldwide public health concern which is mainly associated with merciless use of antibiotics in production animals, and their subsequent transmission to human through animal food and food products (Foley and Lynne, 2008; Geimba *et al*., 2004). There are a variety of mechanisms through which these pathogens get resistance and among them ARGs is the most important. As for as antibiotic resistance is concerned, our study revealed that *E. coli* isolates along the PSCP are highly resistant for LIN (96.3%, 77/80) followed by AMX (82.5%, 66/80), TET (63.7%, 51/80), AMP (58.7% , 47/80) and SXT (50%, 40/80). Resistance for GAT (3.75%, 3/80) and CRO (6.25%, 5/80) was found the lowest in all the isolates. The isolates also revealed high MDR (85%, 68/80) along the PSCP. The prevalence of ARGs in *E. coli* isolates varied by origin of isolates and was higher in isolates from slaughter houses (56.6% isolates) and pig farms (35.8% of isolates), which is consistent with the phenotypic data. Overall, the most common resistance genes were *blaTEM* (98.7%, 79/80), followed by *tetA* (58.7%, 47/80), *tetB* (55%, 44/80) and *blaCMY-2* (52.5%, 42/80). Different studies have described different antibiotic resistance in *E. coli* isolates. In one of the Canadian study, *E. coli* isolates from pork have been found high resistance for TET (31.5%) followed Sulfisoxazole (24.4%), AMP and STR (12.2%) and Kanamycin (9.8%) while among the ARGs, the most prevalent was *tet (A)* (24.4%) followed by *aadA* (22%), *blaTEM* (14.6%), *tetB* (12.2%), *sul1*, *sul3* and *strA/B* (9.8%). *E. coli* isolates from diseased pigs from Guandong province have been found to have high multiple drug resistance (89%), and the highest resistance was found for sulphamethoxazole (95%), followed by tetracyclin (94%), chloramphenicol (89%) and streptomycin (84%) (Wang *et al*., 2010). Another study conducted in Shangdong province of China describing the spread of extended spectrum beta-lactamase (ESBL) resistance producing *E. coli* isolates from pigs to environment. These ESBL were found resistant to multiple drugs besides carrying both CTX-M and TEM resistant genes. These isolates from both pig farms and environment were found to carry the same CTM-X genes which is a clear indication of their transmission from farms to environment (Gao *et al*., 2015). Similarly, *E. coli* isolates from chicken in Western China have revealed high resistance trend for nalidix acid and ciprofloxacin, and decreasing resistance trend for gentamycin. About 49.8% isolates were resistant to more than eight antibiotics. Among the ARGs, *tetA*, *tetB* and *blaTEM* were constantly over 89.9% while *aac3-II* was 28.6% (Wang, 2013). A recent study on *E. coli* isolates from apparently healthy pigs in Japan have revealed high resistance for oxytetrayclin (62.4%) followed by dihydrosterptomycin (44.8%), trimethoprim (28.8%), Ampicilin (24.8%) and Chloramphenicol (20.8%). Our findings regarding antibiotic resistance and distribution of ARGS in *E. coli* isolates along the PSCP are not in consistent with the previous studies as mentioned earlier which may be due to many reasons including use of different antibiotics, different source of isolation and different location. However, there is an increasing and alarming trend in antibiotic resistance all over the world in the general and in China in particular which needs further surveillance for the control of this alarming situation.

CONCLUSION

Different phenotypic and genotypic antibiotic resistance prevail in *E. coli* isolates along the PSCP. Resistance for LIN was the highest in overall (96.3%, 77/80) isolates followed by AMX, AMP. The most prevalent ARGs in the isolates recovered from pig farms was *blaTEM* (100%, 20/20), followed by *blaCMY-2* (80%, 16/20), *tetA* and *tetB* (60%, 12/20) and *tetC* (50%, 10/20). These isolates possess multiple drug resistance which is a matter of great concern for public health.

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Statement of conflict of interest

All authors declare no conflict of interest.

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