MULTI-DRUG RESISTANT GRAM-NEGATIVE ENTERIC BACTERIA ISOLATED FROM FLIES AT CHENGDU AIRPORT, CHINA

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Abstract. We collected flies from Chengdu Shuangliu International Airport to examine for the presence of bacteria and to determine the sensitivity patterns of those bacteria. A total of 1,228 flies were collected from 6 sites around Chengdu Shuangliu International Airport from April to September 2011. The predominant species was *Chrysomya megacephala* (*n*=276, 22.5%). Antimicrobial-resistant gram-negative enteric bacteria (*n*=48) were isolated from flies using MacConkey agar supplemented with cephalothin (20 μg/ml). These were identified as *Escherichia coli* (*n*=37), *Klebsiella pneumoniae* (*n*=6), *Pseudomonas aeruginosa* (*n*=3) and *Aeromonas hydrophila* (*n*=2). All isolated bacteria were tested for resistance to 21 commonly used antimicrobials: amoxicillin (100%), ticarcillin (100%), cephalothin (100%), cefuroxime (100%), ceftazidime 1 (93.8%), piperacillin (93.8%), cefotaxime (89.6%), ticarcillin-clavulanate (81.3%), trimethoprim-sulfamethoxazole (62.5%), ciprofloxacin (54.2%), gentamicin (45.8%), cefepime (39.6%), tobramycin (39.6%), ceftazidime (22.9%), cefoxitin (16.7%), amikacin (16.7%), netilmicin (14.6%), amoxicillin-clavulanate (6.3%) and piperacillin-tazobactam (2.1%). No resistance to meropenem or imipenem was observed. Antibiotic resistance genes among the isolated bacteria were analyzed for by polymerase chain reaction. Thirty of the 48 bacteria with resistance (62.5%) possessed the bla_{TEM} gene.

Keywords: flies, gram-negative enteric bacteria, multi-drug resistance, *Escherichia coli*, *Klebsiella pneumoniae*, China

INTRODUCTION

The synanthropic fly has been regarded as an important vector for human and animal pathogens due to its feeding and breeding behaviors (Graczyk *et al*, 2001). The housefly, *Musca domestica* (Linnaeus, 1758) (Diptera: Muscidae), can disseminate bacteria, protozoa, viruses and helminth eggs (Greenberg, 1968, 1973; Kobayashi *et al*, 1999) which can cause dysentery, infant diarrhea, typhoid, food poisoning, cholera and helminthiasis. Flies may not only act as mechanical vectors, but may also spread antibiotic resistance genes within the microbial community (Fotedar *et al*, 1992; Boulesteix *et al*, 2005). A fly's digestive tract provides a suitable environment for horizontal gene transfers among bacteria leading to spread of resistance and virulence genes

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(Petridis *et al*, 2006; Akhtar *et al*, 2009).

Gram-negative enteric bacteria (GNEB) are common causes of nosocomial infection, infecting nearly every organ and body cavity. Wide spread use of antibiotics to treat GNEB infections has led to the emergence of multi-drug resistant GNEB resulting in great challenges for human health case and the pharmaceutical industry, becoming an important public health problem. Houseflies can transmit a variety of pathogenic GNEB, including *Salmonella* (Greenberg *et al*, 1970; Holt *et al*, 2007), *Escherichia coli* O157:H7 (Ahmad *et al*, 2007), *Yersinia pseudotuberculosis* (Zurek *et al*, 2001), *Shigella* (Levine and Levine, 1991), *Aeromonas hydrophila* (Mcgaughey and Nayduch, 2009). But to our knowledge, there have been no studies of antibiotic resistant GNEB among flies from a non-hospital environment.

In this study, we collects flies from 6 sites around Chengdu Shuangliu International Airport from April to September 2011 to examine for resistance.

MATERIALS AND METHODS

Fly collection and identification

Flies were collected from six sites at Chengdu Shuangliu International Airport, including a restaurant, garbage areas and green areas. The flies were caught with sterilized nets between 10:00 AM to 4:00 PM three times a month from April to September, 2011. Captured flies were taken to the laboratory and identified by species using morphological characteristics after being frozen for 2 hours at -20ºC.

Bacterial isolation and identification

The flies of the same genus and species were batched (20 flies each) and then individually rinsed in 100 ml 0.85% sterile saline solution for 2 minutes. The saline was then diluted 10-fold and plated on MacConkey agar supplemented with cephalothin (Sigma, St Louis, MO) at a concentration of 20 µg/ml and incubated for 24 hours at 37ºC. Two to three colonies were selected from each plate for identification (Donaldson *et al*, 2006). Colonies with typical morphological characteristics were identified by Gram stain and microscopy. The isolates were identified using the automatic ATB Expression system (BioMérieux, Marcy l'Etoile, France) using an ATB ID32 E test strip. Isolates with an identification score >97% were used for antibiotic resistance testing.

Antimicrobial susceptibity testing

The antimicrobial susceptibilities of the isolated GNEB were tested using the ATB G-5 strip susceptibility test for enterobacteria according to the manufacturer's instructions (BioMérieux). We tested susceptibilities to amoxicillin, amoxicillin-clavulanate, piperacillin, piperacillin-tazobactam, ticarcillin, ticarcillin-clavulanate, cephalothin, cefoxitin, cefotaxime, ceftazidime, cefepime, cefuroxime, meropenem, imipenem, ceftazidime 1, trimethoprim-sulfamethoxazole, tobramycin, amikacin, gentamicin, netilmicin and ciprofloxacin.

PCR detection of antimicrobial-resistance genes

All GNEB isolates (n=48) were screened for bla_{TEM}, bla_{SHV} and *ampC* genes. Twenty-four strains with aminoglycoside resistance were screened for *acc(3)-II*, *acc(3)-IV*, *aphA1* and *aphA2* genes. Twenty-six isolates with ciprofloxacin resistance were screened for *qnrB*, *qnrS* and *aac(6')-1b* genes. PCR was performed using the GeneAmp® PCR system 9700 (Applied Biosystems, Carlsbad, CA). The total reaction volume was 15 µl and consisted of 2 X Taq PCR MasterMix (Tiangen Biotech,

China), forward and reverse primers, a DNA template and double-distilled water to the final volume. The PCR cycle conditions were optimized for each primer set. The PCR cycling conditions consisted of initial denaturation at 94ºC for 3 minutes, followed by 30 cycles each of denaturation at 94ºC for 30 seconds, annealing at the temperature optimal for each primer set for 30 seconds (Table 1) and extension at 72ºC for 30 seconds. The amplified PCR product was electrophoresed on 1.0% agarose gel in Tris-acetate-EDTA buffer. D2000 (Tiangen Biotech, China) was used as a molecular weight marker. Positive and negative control bacteria were used for all the PCR assays.

RESULTS

Fly collection

Flies (*n*=1,228) were collected and identified from six sites around Chengdu Shuangliu International Airport. The flies identified were : *Chrysomya megacephala* (*n*=276), *Aldrichina grahami* (*n*=247), *Lucilia sericata* (*n*=211), *Boettcherisca peregrima* (*n*=107), *Muscina stabulans* (*n*=162) and *Bercaea cruenta* (*n*=225) (Table 2).

Bacterial isolation

The GNEB isolated were: *Escherichia coli* (*n*=37, 77%), *Klebsiella pneumoniae* (*n*=6, 13%), *Pseudomonas aeruginosa* (*n*=3, 6%), *Aerommas hydrophila* (*n*=2, 4%) (Table 3). Sixteen of the GNEB isolates (33.3%) were isolated from *C. megacephala.*

Antimicrobial resistance

All isolates (*n*=48) were resistant to 8 or more antibiotics (Table 4). All isolates were resistant to amoxicillin, ticarcillin, cephalothin and cefuroxime. No resistance to meropenem and imipenem was observed. There was variable resistance to ceftazidime 1 (93.8%), piperacillin (93.8%), cefotaxime (89.6%), ticarcillin-clavulanate (81.3%), trimethoprim-sulfamethoxazole (62.5%), ciprofloxacin (54.2%), gentamicin (45.8%), cefepime (39.6%), tobramycin (39.6%), ceftazidime (22.9%), cefoxitin (16.7%), amikacin (16.7%), netilmicin (14.6%), amoxicillin-clavulanate (6.3%) and piperacillin-tazobactam (2.1%).

Of the 37 *E.coli* isolates, all were resistant to amoxicillin, piperacillin, ticarcillin, cephalothin, cefotaxime and cefuroxime; all were susceptible to amoxicillin-clavulanate, piperacillin-tazobactam, meropenem and imipenem. One *E. coli* isolate obtained from *L. sericata* in June, 2011 was resistant to 17 antimicrobials: including amoxicillin, piperacillin, ticarcillin, ticarcillin-clavulanate, cephalothin, cefoxitin, cefotaxime, ceftazidime, cefepime, cefuroxime, ceftazidime 1, trimethoprim-sulfamethoxazole, tobramycin, amikacin, gentamicin, netilmicin and ciprofloxacin.

Six *K. pneumoniae* isolates were resistant to amoxicillin, piperacillin, ticarcillin, cephalothin, cefuroxime, ceftazidime and trimathoprim-sulfamethoxazole. No *K. pneumoniae* isolates were resistant to amoxicillin-clavulanate, piperacillin-tazobactam, cefoxitin, ceftazidime, meropenem, imipenem, amikacin or netilmicin.

PCR detection of antimicrobial resistance genes

In the 48 isolates with cephalothin resistance, bla_{TEM} and bla_{SHV} genes were detected in 30 and 9 isolates, respectively, but the *ampC* gene was not detected in any of the isolates (Table 5). Five isolates had both bla_{TEM} and bla_{SHV} genes simultaneously: *E. coli* (1) and *K. pneumoniae* (4). Two *K. pneumoniae* isolates had the bla_{SHV} gene only. bla_{TEM} was the most prevalent β-lactamase gene detected in this study. The bla_{SHV} gene was detected more frequently in *K. pneumoniae* (all isolates).

Table 1
PCR primers and annealing temperatures used in this study.

Target gene(s) or region	Primer name	Sequence	PCR product size (bp)	Annealing temperature (°C)	References
bla_{TEM}	TEM-F	ATG AGT ATT CAA CAT TTC CGT G	861	55	Donaldson *et al*, 2006
	TEM-R	TTA CCA ATG CTT ATT CAG TGA G			
bla_{SHV}	SHV-F	ATG CGT TTA TAT TCG CCT GTG	861	55	Donaldson *et al*, 2006
	SHV-R	TTA GCG TTG CCA GTG CTC GA			
ampC	AMPC-F	ATG ATG AAA AAA TCG TTA TGC	1,143	55	Donaldson *et al*, 2006
	AMPC-R	TTG CAG CTT TTC AAG AAT GCG C			
aac(3)-II	AacC2-F	ACT GTG ATG GGA TAC GCG TC	237	60	Sáenz *et al*, 2004
	AacC2-R	CTC CGT CAG CGT TTC AGC TA			
aac(3)-IV	AacC4-F	CTT CAG GAT GGC AAG TTG GT	286	60	Sáenz *et al*, 2004
	AacC4-R	TCA TCT CGT TCT CCG CTC AT			
aphA1	AphA1-F	ATG GGC TCG CGA TAA TGT C	600	55	Sáenz *et al*, 2004
	AphA1-R	CTC ACC GAG GCA GTT CCA T			
aphA2	AphA2-F	GAA CAA GAT GGA TTG CAC GC	680	55	Sáenz *et al*, 2004
	AphA2-R	GCT CTT CAG CAA TAT CAC GG			
qnrB	qnrB-F	GAT CGT GAA AGC CAG AAA GG	476	55	Kim *et al*, 2009
	qnrB-R	ATG AGC AAC GAT GCC TGG TA			
qnrS	qnrS-F	GCA AGT TCA TTG AAC AGG GT	428	50	Kim *et al*, 2009
	qnrS-R	TCT AAA CCG TCG AGT TCG GCG			
aac(6')-Ib	aacIb-F	TTG CGA TGC TCT ATG AGT GGC TA	482	60	Kim *et al*, 2009
	aacIb-R	CTC GAA TGC CTG GCG TGT TT			

Table 2
Captured flies and number of isolated bacteria.

Flies	No. (%) of captured flies	No. (%) of isolated bacteria
Chrysomya megacephala	276 (22.5)	16 (33.3)
Aldrichina grahami	247 (20.1)	4 (8.3)
Lucilia sericata	211 (17.2)	10 (20.8)
Boettcherisca peregrima	107 (8.7)	3 (6.3)
Muscina stabulans	162 (13.2)	7 (14.6)
Bercaea cruenta	225 (18.3)	8 (16.7)
Total	1,228 (100)	48 (100)

Table 3
Distribution of antibiotic resistant bacteria isolated from flies.

Bacteria	No. (%) of isolated bacteria
Escherichia coli	37 (77)
Klebsiella pneumoniae	6 (13)
Pseudomonas aeruginosa	3 (6)
Aeromonas hydrophila	2 (4)
Total	48 (100)

Twenty-four isolates were resistant to aminoglycosides: tobramycin (*n*=19), amikacin (*n*=8), gentamicin (*n*=22) and netilmicin (*n*=7). The *acc(3)-II* gene was detected in 16 isolates (66.7%), but the *acc(3)-IV* gene was not detected in any of the isolates. The *aphA1* and *aphA2* genes were detected in 3 and 4 isolates, respectively (Table 5).

In this study, the *qnrB*, *qnrS* and *aac(6')-Ib* genes were detected in 26 isolates possessing ciprofloxacin resistance. Two isolates had the *qnrB* gene, 6 isolates had the *qnrS* gene and 5 isolates had the *aac(6')-Ib* gene. Five isolates with the *aac(6')-Ib* gene were further analyzed by digestion with BstF5I (New England Biolabs, Ipswich, MA) to identify the *aac(6')-Ib-cr* gene (Park *et al*, 2006). None of the 5 isolates could be digested with BstF5I; therefore, they all possessed the *aac(6')-Ib* gene. One *E.coli* isolate had both the *qnrS* and *aac(6')-Ib* genes simultaneously. Two *K. pneumoniae* isolates had both the *qnrB* and *aac(6')-Ib* genes simultaneously (Table 5).

DISCUSSION

Flies come in contact with animal excreta, food, water and humans. They are suspected reservoirs and vectors for human and animal pathogens. Moreover, recent studies suggest flies may play an important role in the spread of antimicrobial resistance genes within the microbial community (Fotedar *et al*, 1992; Boulesteix *et al*, 2005). Macovei and Zurek (2006) detected antibiotic-resistant and potentially virulent enterococci in houseflies from food settings. Flies have been found to carry multi-drug resistant bacteria in hospital environments and may play a role in transmission of human pathogens within hospitals (Boulesteix *et al*, 2005). In our study, multidrug-resistant *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. hydrophila* were isolated from 6 species of flies in a non-hospital environment. The species of

Table 4
Resistance to antimicrobials among isolated bacteria.

Antimicrobial	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. hydrophila*	Total no. (%)
Amoxicillin	37	6	3	2	48 (100)
Amoxicillin-clavulanate	0	0	3	0	3 (6.3)
Piperacillin	37	6	1	1	45 (93.8)
Piperacillin-tazobactam	0	0	1	0	1 (2.1)
Ticarcillin	37	6	3	2	48 (100)
Ticarcillin-clavulanate	30	5	3	1	39 (81.3)
Cephalothin	37	6	3	2	48 (100)
Cefoxitin	4	0	3	1	8 (16.7)
Cefotaxime	37	4	0	2	43 (89.6)
Ceftazidime	11	0	0	0	11 (22.9)
Cefepime	18	1	0	0	19 (39.6)
Cefuroxime	37	6	3	2	48 (100)
Meropenem	0	0	0	0	0 (0)
Imipenem	0	0	0	0	0 (0)
Ceftazidime 1	35	6	3	1	45 (93.8)
Trimethoprim-sulfamethoxazole	20	6	3	1	30 (62.5)
Tobramycin	17	1	0	1	19 (39.6)
Amikacin	8	0	0	0	8 (16.7)
Gentamicin	20	2	0	0	22 (45.8)
Netilmicin	7	0	0	0	7 (14.6)
Ciprofloxacin	22	2	1	1	26 (54.2)

multi-drug resistant bacteria isolated and types of antibiotic resistance genes seen in this study were similar to reports from the hospital setting (Ding *et al*, 2009).

Recent research indicates horizontal gene transfer occurs commonly in the housefly digestive tract (Petridis *et al*, 2006; Akhtar *et al*, 2009). Petridis *et al* (2006) reported horizontal transfer of antibiotic resistance and virulence genes among *E. coli* isolates can occur in house fly guts. Plasmid-mediated horizontal transfer of resistance among *Enterococcus faecalis* isolates in the housefly alimentary canal was reported by Akhtar *et al* (2009). These results suggest flies may not only act as mechanical vectors but may also provide a suitable environment and selective pressure for the origin bacterial strains, resulting in new properties, including acquired virulence and antibiotic resistance in fly digestive tracts (Akhtar *et al*, 2009). Some GNEB are important reservoirs of antibiotic resistance genes in *E. coli* and *K. pneumoniae* (Donaldson *et al*, 2006). In our study, multi-drug resistant *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. hydrophila* were isolated from flies, which could act as donors of resistant genes to other pathogenic bacteria sharing the same environment such as *Salmonella*. It is possible flies contribute to antibiotic resistance gene spread due to their ecological characteristics. In some countries, antibiotics used in humans are forbidden to be used in animals. Flies may easily comes

Table 5
Prevalence of antibiotic resistance genes in isolated bacteria.

Species	No. of isolates	Number of isolates with resistance genes detected by PCR									
		bla_{TEM}	bla_{SHV}	*ampC*	*aac(3)-II*	*aac(3)-IV*	*aphA1*	*aphA2*	*qnrB*	*qnrS*	*aac(6')-Ib*
E.coli	37	25	1	0	14	0	3	4	0	5	3
K.pneumoniae	6	4	6	0	2	0	0	0	2	0	2
P. aeruginosa	3	1	2	0	0	0	0	0	0	1	0
A. hydrophlila	2	0	0	0	0	0	0	0	0	0	0
Total	48	30	9	0	16	0	3	4	2	6	5

PCR, polymerase chain reaction.

into contact with humans and animals, especially on livestock farms. If horizontal resistance gene transfer occurs in the fly's gut, it would be more dangerous to humans than livestock. Antibiotic resistance can be shared within the bacterial community by gene transfer; antibiotic resistance needs to be viewed as an ecological problem (Ebrahim, 2010).

In this study, all GNEB isolates were resistant to between 8 and 16 commonly used antibiotics, such as amoxicillin, gentamicin and ciprofloxacin. The high level of multi-drug resistant GNEB in flies found in our study has not been reported in previous studies. Antibiotic misuse by patients without a prescription is commonplace in China. It may assumed high levels of multi-drug resistant bacteria are present in the environment. When commonly used medicines lose their effectiveness in treating infections, patients must accept longer treatment periods and higher costs. This will increase health-care costs and the financial burden to families and society.

This study attempted to determine the mechanisms of multi-drug resistance. All isolates (n=48) were screened for the presence of bla_{TEM}, bla_{SHV}, *ampC* resistance genes. Twenty-four isolates with aminoglycoside resistance were screened for the presence of *aac (3)-II*, *aac (3)-IV*, *aphA1* and *aphA2* genes. Twenty-six isolates with ciprofloxacin resistance were screened for *qnrB*, *qnrS* and *aac (6')-Ib* genes. The bla_{TEM} gene (62.5%) was common (62.5%) in bacteria resistant to beta-lactam antibiotics, consistent with a previous reports from hospital setting (Ding *et al*, 2009). Some isolates did not carry resistance genes. Further research is necessary to determine the mechanisms of multi-drug resistance in these other isolates.

In conclusion, our study found flies collected from non-hospital environments carried multi-drug resistant GNEB, which are opportunistic pathogens to humans. This drug resistance may be transfered other pathogens. Flies are not only mechanical vectors for pathogens but may also contribute to pathogen evolution. Effective control measures for flies are necessary.

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