Journal of Infection and Public Health (2016) 9, 499—505

http://www.elsevier.com/locate/jiph

Molecular detection and antimicrobial resistance of *Klebsiella pneumoniae* from house flies (*Musca domestica*) in kitchens, farms, hospitals and slaughterhouses

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Received 22 June 2015; received in revised form 11 November 2015; accepted 11 December 2015

KEYWORDS
Klebsiella pneumoniae;
Musca domestica;
Nosocomial infections;
Vector-borne diseases

Summary Identifying disease vectors and pathogens is one of the key steps in controlling vector-borne diseases. This study investigated the possible role of house flies (*Musca domestica*) as vectors in the transmission of *Klebsiella pneumoniae* in Chaharmahal VA Bakhtiari and Isfahan provinces of Iran. House flies were captured from household kitchens, cattle farms, chicken farms, animal hospitals, human hospitals and slaughterhouses. Isolation of *K. pneumoniae* from external surfaces and guts of the flies was performed using MacConkey agar (MA) and thioglycollate broth (TGB). Identification of the isolates was performed with phenotypic techniques and polymerase chain reaction (PCR). A total of 600 house flies were sampled during the study period from different locations in four different seasons. Overall, 11.3% of

Abbreviations: *K. pneumoniae*, *Klebsiella pneumoniae*; MA, MacConkey agar; TGB, thioglycollate broth; PCR, polymerase chain reaction; *M. domestica*, *Musca domestica*; TSB, tryptic soy broth; CLSI, National Committee for Clinical Laboratory Standards.

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http://dx.doi.org/10.1016/j.jiph.2015.12.012

the captured house flies were positive for *K. pneumoniae*. In Chaharmahal VA Bakhtiari province, the prevalence was 12.7%, while in Isfahan province, 10.0% of the sampled house flies were infected with *K. pneumoniae*. Season-wise, the highest prevalence of infections among the house flies was in summer. The organisms were highly resistant to ampicillin, amoxicillin, cefotaxime and piperacillin. A lowest level of resistance was observed for imipenem/cilastatin. The findings of this study demonstrated that house flies are potential vectors of antibiotic-resistant *K. pneumoniae* in Isfahan and Chaharmahal provinces, Iran. Control efforts for infections caused by this particular bacterium should take *M. domestica* into account.

Introduction

The house fly, *Musca domestica*, is considered to be an important insect pest of both human and domestic animals that disseminate infectious diseases [1,2]. The fly occurs in the same environment with human and animals, and its maggots (larvae) are highly rich in organic matter and have high microbial flora [3,4]. Regular contact of the fly with wastes and animals provide an opportunity to transmit pathogens to both humans and animals [5—7]. Among other pathogens, house flies are known to transmit *Pseudomonas* spp., Enterobacteriaceae, *Staphylococcus aureus*, *Vibrio cholera, Chlamydia trachoma*, *Salmonella* spp. and *Klebsiella* spp. [5,8—15]. These pathogens are carried on the fly's legs and other body parts [16]. There are essentially four different ways in which house flies may transmit infectious microorganisms [1]: (i) on the hairs and surface of its body, (ii) on the glandular hairs on its feet, (iii) by regurgitation of vomitus, and (iv) by passage through the alimentary tract. Therefore, the fly may function as a temporary mechanical vector, or the pathogen concerned may survive for a longer period of time within the fly's body, in many instances with no adverse effect upon the carrier host. This latter possibility provides an opportunity for multiplication of the pathogen.

Klebsiella species are known to be responsible for more than 10% of in-hospital nosocomial infections [9,10,17,18]. Reports indicate that some of these infections, which at times involve antimicrobial-resistant strains, are vectored by insect pests, including house flies and cockroaches [5,7,10,17]. In Iran, such reports on the isolation of antimicrobial-resistant *Klebsiella* species from house flies are lacking. Therefore, the aim of this study was to ascertain the role of house flies in the carriage of antimicrobial-resistant strains of *Klebsiella* species in different locations in Chaharmahal VA Bakhtiari and Isfahan provinces of Iran.

Materials and methods

Study area and sample collection

The present longitudinal study was conducted in Isfahan (32.6333°N, 51.6500° E) and Chaharmahal VA Bakhtiari (32.3256° N, 50.8644° E) provinces located in the central and southwest areas of Iran, respectively (Fig. 1). A total of 600 randomly selected house flies were collected from four house kitchens, four cattle farms, two animal hospitals, four human hospitals, two slaughterhouses and two chicken farms, selected at random in the two provinces. The flies were either captured manually or by sticky trap methods. The fly samples were then transported to the laboratory at the Biotechnology Research Center using separate sterile tubes to prevent cross-contamination between samples. In the laboratory, flies were identified and killed by refrigeration at −20 °C in a cold chamber. They were then placed in 5 ml peptone water and left at room temperature for 5 h before being processed.

Isolation of *Klebsiella* spp. from external surfaces of house flies

Each housefly was transferred to a sterile test tube containing 2 ml of sterile normal saline and shaken thoroughly for 2 min. A fixed volume of these washings were then cultured on to MacConkey agar (MA) (Merck, Germany) plates for primary isolation. The washings were also inoculated into thioglycollate broth (TGB) and both media were incubated overnight at 37 °C. Subcultures were made from TGB onto MacConkey agar plates and incubated overnight at 37 °C. Colonies of *Klebsiella* spp. recovered on the MacConkey agar were identified according to a method described earlier [19]. Briefly, lactose-fermenting colonies were identified

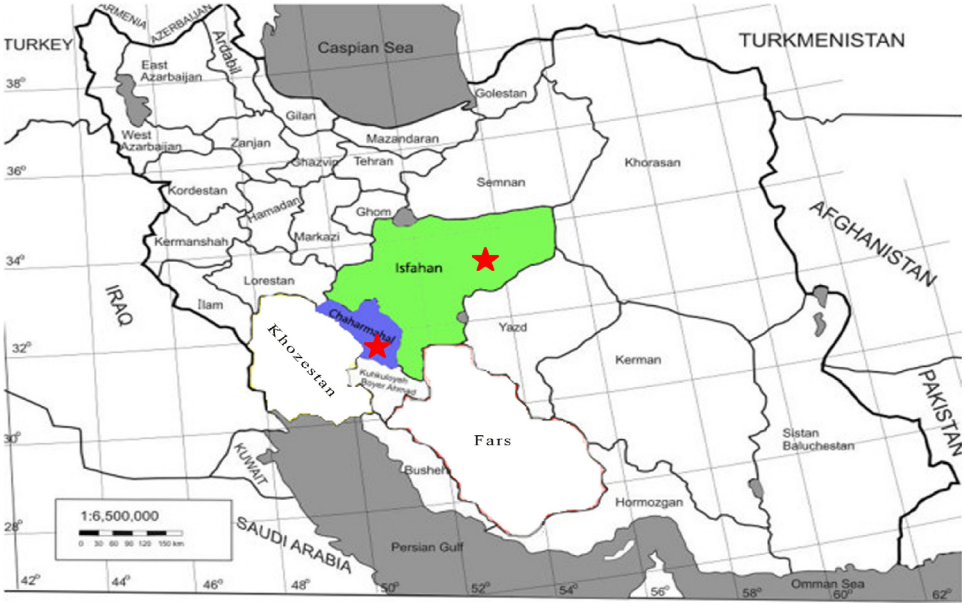

Figure 1 Geographical location of the collection sites Isfahan and Chaharmahal (red stars).

by their macroscopic morphology, Gram reaction and biochemical reactions.

Isolation of *Klebsiella* spp. from the guts of house flies

After external surface washing, the flies were washed in 70% ethyl alcohol for 5 min to decontaminate the external surfaces and dried. The flies were then washed with sterile normal saline to remove traces of alcohol and the gut was dissected out aseptically. The gut was then transferred to a sterile mortar and pestle and emulsified in 20 ml of sterile normal saline. The resulting macerate was processed for bacterial isolation as described above. All isolates were then maintained in Tryptic Soy Broth (TSB, Merck, Germany) for future use.

DNA extraction

Genomic DNA was extracted from all isolates using a CinnaGen DNA extraction kit (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. The extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm according to the method described by Sambrook and Russell [20]. Extracted DNA samples were kept frozen at −20 °C until they were used for molecular analysis using polymerase chain reaction (PCR).

PCR assay

Confirmation of *K. pneumoniae* isolates was performed by PCR using primers Pf: 5-ATT TGA AGA GGT TGC AAA CGA T-3 and Pr1: 5-TTC ACT CTG AAG TTT TCT TGT GTT C-3 for amplification of the 16S rRNA gene, yielding a PCR product of band size 130 bp [21]. *K. pneumoniae* ATCC 700603 was used as a positive control. The amplification reaction was carried out in a total volume of 25 μl, consisting of 1 μM of each of the primers, 2 mM $Mgcl_2$, 200 μM dNTP, 5 μl of 10× PCR buffer, 1 U of Taq DNA polymerase (Fermentas, Germany) and 1 μg of template DNA. Thermal PCR conditions consisted of 5 min of initial denaturation at 95 °C and then 30 cycles of denaturation, each consisting of 1 min at a temperature of 94 °C, 1 min of annealing at 58 °C, 1 min of extension at 72 °C and a final extension was for 5 min at 72 °C. The amplified products were analyzed in 1.5% agarose gel. The electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0) combined in sufficient H_2O and stirred to dissolve). Gels were stained with ethidium bromide. Aliquots of 10 μl of PCR products were applied to the gel. Constant voltage of 80 for 20 min was used for product separation. After electrophoresis, images were obtained in UVItec documentation systems (UK).

Antimicrobial resistance test

Antimicrobial resistance testing of the obtained isolates was performed by the Kirby—Bauer disc

diffusion method on Mueller Hinton agar based on the recommendations of CLSI [22] for aerobic isolates. The following antimicrobial agents were used: ampicillin, amoxicillin, amikacin, cephalexin, ceftazidime, ceftriaxone, ceftizoxime, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, nitrofurantoin, piperacillin, tetracycline and kanamycin (Pattan-Teb, Tehran, Iran).

Statistical analysis

Data generated were subjected to descriptive statistics using Microsoft Excel version 2010 (Microsoft, USA) and expressed in percentages.

Results

Detection of *K. pneumoniae* in captured house flies

The overall prevalence of *K. pneumoniae* in the sampled house fly population was 11.3% ($n = 600$). In Chaharmahal VA Bakhtiari province, 12.7% (38/300) of the sampled house flies harbored *K. pneumoniae*, while in Isfahan province, the prevalence was 10% (30/300). The *K. pneumoniae* recovery proportional frequencies from the 600 flies in different locations were kitchens (6.0, $n = 100$), cattle farms (18.0, $n = 100$), chicken farms (8.0, $n = 100$), slaughterhouses (14.0, $n = 100$), animal hospitals (13, $n = 100$) and human hospitals ($n = 100$). Proportional recovery frequencies of *K. pneumoniae* from 600 houseflies captured in different seasons were spring (10.0, $n = 150$), summer (18.0, $n = 150$), autumn (10.7, $n = 150$) and winter (6.7, $n = 150$).

Antimicrobial resistance profiles of *K. pneumoniae* isolates obtained from house flies

Antimicrobial resistance profiles of *K. pneumoniae* isolates obtained in this study are shown in Table 1. The isolates were found to be resistant to all antibiotics evaluated at different percentages. The organisms displayed high levels of resistance to ampicillin, amoxicillin, cefotaxime and piperacillin. A lowest level of resistance was observed for Imipenem/cilastatin.

Discussion

K. pneumoniae is an important pathogen in both the community and clinical settings, associated with healthcare infections with significant morbidity and mortality [23]. It causes a variety of disease conditions, such as nosocomial pneumonia, septicemia, wound infections and neonatal septicemia [24]. In the present study, the pathogen was recovered from 11.3% of all the house flies collected from different sampling locations ($n = 600$), indicating the ubiquitous nature of the organism and the possible involvement of flies in its epidemiology. This infection rate is low compared to what was reported in Libya for the flies collected in the hospitals, streets and abattoir [25]. The potential of house flies to carry microorganisms has been demonstrated previously [9].

The presence and abundance of *M. domestica*, particularly during the summer, in the locations visited during the current study has been reported by other authors [26,27]. In this work, the highest infection level of the flies with *K. pneumoniae* was detected to occur during the summer. This correlates to the high breeding season of the flies and therefore increases the chances of fly-borne *K. pneumoniae* outbreaks in both human and animal populations [27]. In a study conducted previously [28], the authors found that seasons during which both flies and cases of dysentery are prevalent often coincide. This information is critical, as it may be used in guiding efforts aimed at minimizing *K. pneumoniae* infections attributable to house flies.

The carriage rates of *K. pneumoniae* in this study were found to be 6.0%, 18.0%, 8.0%, 14.0%, 13.0% and 9.0% for house flies collected in kitchens, cattle farms, chicken farms, slaughterhouses, animal hospitals and human hospitals, respectively. The higher proportions of *K. pneumoniae*-positive flies from cattle farms, slaughterhouses and animal hospitals suggest higher contamination levels of these localities. This particular observation could be linked to the presence of organic waste in these areas, which favor the growth and development of both bacterial pathogens and the flies [29]. It has been recently noted that environments rich in decomposing organic matter harbor diverse microbes and serve as suitable substrates for the development of fly populations [30,31].

Nine percent of the house flies captured in the hospital environment were infected with *K. pneumoniae* in this study. This is a significant finding in the public health sense as the contaminated flies can in turn contaminate the patient environment, exposing them to hospital-acquired illnesses [9]. Previous studies isolated similar drug-resistant strains of *Klebsiella* spp. from patients and flies in hospital environments [9,10].

Table 1 Antimicrobial resistance profile of *K. pneumoniae* isolates from houseflies captured in different locations in Iran.

Antimicrobial agent	Disc potency (μg)	Number (%) of isolates resistant ($n = 68$)
Ampicillin	10	55 (80.9%)
Amoxicillin	25	54 (79.4%)
Amikacin	30	16 (23.5%)
Cephalexin	30	31 (45.6%)
Ceftazidime	30	29 (42.6%)
Ceftriaxone	30	32 (47.1%)
Ceftizoxime	30	32 (47.1%)
Cefotaxime	30	54 (79.4%)
Chloramphenicol	30	20 (29.4%)
Ciprofloxacin	5	36 (53.0%)
Gentamicin	10	30 (44.1%)
Imipenem/cilastatin	10	11 (16.2%)
Nitrofurantoin	300	32 (47.1%)
Piperacillin	100	54 (79.4%)
Tetracycline	30	19 (28.0%)
Kanamycin	30	26 (38.2%)

In this study, *K. pneumoniae* was recovered from the gut and external surfaces of the house flies from all sampling sites. This observation is in line with the findings of a previous study whereby a similar bacterium was associated with the gut content and external surface of house flies [32]. Several other authors [33] detected the same pathogen in the guts of field-collected Australian tropical fruit flies.

The presence of this microbe in both the gut and surfaces of flies is of paramount importance in its transmission and possible changes to disease epidemiology. Bacteria in house flies can remain viable for days or weeks in the gut and are expelled in either wounds or food.

Over the years, the epidemiology of invasive pathogens causing a variety of disease conditions, including *K. pneumoniae*, has changed significantly, accompanied by an increase in resistance to many antimicrobial agents, resulting in a reduction in therapeutic options [34—38]. Antimicrobial resistance has become a global clinical and public health concern [39—41] such that some authors consider the surveillance of antimicrobial resistance patterns to be decisive for optimizing treatment and a key for prevention [42]. Overall, in this study, antimicrobial resistance levels among *K. pneumoniae* isolates were higher, remaining above 23.0% for 15/16 of the tested antimicrobials. This is worth noting, as it implies high chances of treatment failures in the future, thereby jeopardizing human and animal health. Several researchers note that inadequate therapy is among the common reasons for antimicrobial resistance [43].

Carbapenemases have been used as last-resort agents against multidrug-resistant Enterobacteriaceae. However, the incidence of carbapenemase-producing Enterobacteriaceae has been increasing significantly, constituting a huge public health threat [44—48]. According to Yigit and others [49] and Bratu and others [45], most of these bacteria, which include *K. pneumoniae*, show resistance to multiple classes of antibiotics, including ampicillin, tetracycline, trimethoprim/sulfamethoxazole, cephalosporins, fluoroquinolones and aminoglycosides. In this study, the prevalence of imipenem resistance among *K. pneumoniae* isolates was high (16.2%). Our result contrasts an observation made in South Africa by Perovic and others [50], who found that *K. pneumoniae* isolates from houseflies were susceptible to carbapenems (95.5%; $n = 2774$). However, the authors found that higher proportions of the isolates were resistant to beta-lactam antibiotics (68.3%), showing resistance to cefotaxime, ceftazidime, and cefepime. They also found higher levels of resistance to ciprofloxacin (46.5%) and piperacillin-tazobactam (33.1%). The high resistance to carbapenem observed in the present study is a cause for concern because these imipenem-resistant isolates are superbugs resistant to all classes of therapeutic agents. The houseflies can potentially transfer the isolates into food for human and animal consumption; the consequence is compromise to antibacterial therapy in colonized or infected individuals.

The findings of this study have shown that antibiotic-resistant *K. pneumoniae* can be vectored by house flies residing in different locations, posing a threat to animal and public health. Control of the housefly population is therefore of great

importance in the reduction and elimination of infections caused by this bacterium. The best approach could be employing the use of larvicides and adulticidals in *M. domestica* breeding sites [51]. Quantitative epidemiological investigations on the importance of fly-borne transmission of *K. pneumoniae* relative to other modes of transmission and studies that evaluate the measures to stem fly-borne transmission should be conducted [28].

Conclusions

The findings of this study have demonstrated that house flies are potential vectors of *K. pneumoniae* in the study area and possibly other parts of Iran. Efforts to control infections caused by this particular bacterium should therefore consider *M. domestica*.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

Not required.

Authors' contributions

All authors contributed equally to this work. All authors read and approved the final manuscript.

Acknowledgment

The authors extend their appreciation to the Baqiyatallah University of Medical Sciences for their important support. The authors would like to acknowledge the valuable contributions of Dr. Erick V.G. Komba and Dr. Eliningaya J. Kweka.

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