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RESEARCH ARTICLE

Incidence and Antimicrobial Resistance of *Campylobacter* and *Salmonella* from Houseflies (*Musca Domestica*) in Kitchens, Farms, Hospitals and Slaughter Houses

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Abstract Carriage status of *Campylobacter* and *Salmonella* was investigated in houseflies in Shahrekord and Isfahan provinces of Iran. This was a longitudinal study conducted from June 2013 to May 2014. Flies were collected from household kitchens, animal farms, slaughter houses and hospitals and put in sample bottles filled with peptone water. Bacteria were isolated and DNA was extracted from bacterial isolates using a commercial kit. Confirmation of the organisms was carried out by polymerase chain reaction using primer sets for detection of these pathogens. Out of 600 houseflies 19.5 % (117/600) were positive for *Campylobacter* and 15.8 % (95/600) were positive for *Salmonella* organisms. The recovery frequencies of the two organisms in different locations were similar. Higher proportions of infected flies were obtained during summer whereas low proportions were obtained during winter of all the organisms ($P < 0.05$). The organisms had low to moderate resistance to different antimicrobial agents. It is concluded that houseflies do harbor antimicrobial resistant diarrheagenic pathogens including *Campylobacter* and *Salmonella*, more so during summer. The data support the importance of taking into account the houseflies in future plans aimed at stemming infections caused by these organisms.

Keywords *Salmonella* · *Campylobacter* · Antimicrobial resistance · Houseflies · Polymerase chain reaction · Season · Iran

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Introduction

The two most prevalent pathogens causing food borne gastroenteritis throughout the world are *Campylobacter* spp. and *Salmonella enterica* [1]. Reservoirs for these organisms are animals (domesticated and wild), birds, insects and the environment [2]. Humans are frequently exposed to both of these causing agents when they consume raw or under-cooked food, cross-contaminated food and sometimes through contaminated environment or in contact with infected animals [2, 3]. The public health concern of these organisms is heightened due to existence of antimicrobial resistant strains.

Housefly (*Musca domestica*) is known as carrier of a large number of bacteria and is involved in the transmission of important bacterial agents causing human and animal infections [4, 5]. Its feeding and reproductive habits make it an important mechanical and biological vector for several human and animal pathogens including those causing blindness, nosocomial, enteric and anthropozoonotic infections, as well [5–10]. It also serves as reservoir and disseminator of metazoan parasites of both medical and veterinary significance [7].

The flies, having close association with different forms of excreta and decaying organic matter [11], represent a substantial public health risk whenever they have access to human food. Their body anatomy, secretions they make

and their feeding habits make flies able to pick up and disseminate several pathogens [12, 13]. To that effect, a number of authors have detected bacterial pathogens from houseflies [14–16]. To date, however, the carriage status of such pathogens in housefly population in different parts of Iran is lacking.

Recent studies suggest flies may play an important role in the spread of antimicrobial resistance within the microbial community [5, 17]. Additionally, the development of antibiotic resistance among clinical bacterial isolates and commensal bacteria of people and animals, as well as bacteria in other habitats, raises a concern that flies may be vector competent not only for specific pathogens but also for non-pathogenic bacteria carrying antibiotic resistance genes [18]. Consequently this study was conducted with the aim of determining the presence and antimicrobial resistance pattern of *Campylobacter* and *Salmonella* in the houseflies collected in Shahrekord and Isfahan provinces of Iran.

Material and Methods

Study Area, Design and Sample Collection

This was a longitudinal study conducted from June 2013 to May 2014 (collected weekly) in Isfahan and Shahrekord provinces of central and southwestern Iran respectively. It was conducted on 600 houseflies collected from household kitchens (n = 4), cattle farms (n = 4), chicken farms (n = 2), animal hospitals (n = 2), slaughter houses (n = 2) and human hospitals (n = 4). The flies were collected either by manual capture or by using sticky traps. Following capture the fly samples were transported to the laboratory of Biotechnology Research Center using separate sterile tubes to prevent any contamination due to mixing of the samples. In the laboratory flies were identified and killed by refrigeration in −20 °C cold chamber. They were then placed in 5 ml peptone water and left at room temperature for 5 h before being processed for bacterial isolation.

Isolation of Bacteria from Fly Samples

Flies were examined for the presence of *Campylobacter* and *Salmonella*. For *Salmonella* isolation, swab from peptone water were cultured onto Mac Conkey agar plates and incubated at 37 °C for 24 h. Yellowish non-lactose fermenting colonies were considered as *Salmonella* suspect colonies. These colonies were subcultured on Xylose lysine desoxycholate (XLD) agar selective media. After incubation, typical *Salmonella* colonies with slightly transparent zone of reddish color and a black center were picked up and colonies were considered as those of *Salmonella* and subjected to gram staining and biochemical tests such as Oxidase, Triple Sugar Iron (TSI) agar, Urea broth, Indole, MR-VP, Simon citrate, Motility and Lysine Iron Agar (LIA).

For *Campylobacter* isolation swab from peptone water were cultured onto *Campylobacter* Blood Free Selective Agar (Oxoid) plate supplemented with CCDA *Campylobacter* Selective Supplement (Oxoid). All the plates were incubated at 42 °C for 48 h, under microaerophilic condition which was generated by using an anaerobic jar containing a gas generating pack (GasPak EZ Campy, BD). The plates were examined for colonies typical of *Campylobacter* namely, round translucent colonies, raised, convex and glistening, with an entire edge and a tendency to spread along streaking lines. The suspected colonies were then examined for oxidase positive, gram negative, slender, spiral curved rods which also appeared as s-shape and gull-winged shape, with typical corkscrew, twirling and darting movements under hanging drop examination. Two to three colonies were then selected and transferred onto a Columbia Blood Agar (Oxoid) plates with 5 % defribinated horse blood added, incubated at 37 °C for 24 h under aerobic condition. For identification on colonies isolated from the blood agar plates, the authors used standard microbiological and biochemical procedures including gram staining, production of catalase, oxidase, hippurate hydrolysis, urease activity, indoxyl acetate hydrolysis, and susceptibility to cephalotin.

DNA Extraction

Genomic DNA was extracted from all isolates using 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 [19]. Extracted DNA samples were stored frozen at −20 °C until used for molecular analysis using PCR at the Biotechnology Research Center.

PCR Assay

PCR test was performed to confirm the isolated *Salmonella* and *Campylobacter* spp. using methods described previously by Rahn et al. [20] for *Salmonella* and Denis et al. [21] for *Campylobacter*. The primers used for amplification of each of these organisms and their target genes are shown in Table 1. Amplification reactions were carried out in a total volume of 25 µl, consisting of 1 µM of each set of primers, 2 mM $MgCl_2$, 200 µM dNTP, 5 µl of 10X PCR buffer, 1 U of Taq DNA polymerase (Fermentas, Germany) and 1 µg of template DNA. Thermal PCR conditions for *Salmonella* consisted of 35 cycles of 1 min for denaturation at 95 °C, 30 s for annealing at 56 °C, and 30 s

Table 1 Primers used in PCR for detection of *Salmonella* and *Campylobacter* spp. *Salmonella* in housefly samples

Organism (target gene)	Primers sequences	PCR product band size (bp)
Salmonella (invA gene)	F: 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′	284
	R: 5′-TCA TCGCACCGTCAAAGGAACC-3′	
Campylobacter spp. (16S rRNA)	MD16S1: 5′-ATC TAA TGG CTT AAC CAT TAA AC-3′	857
	MD16S2: 5′-GGA CGG TAA CTA GTT TAG TAT T-3′	

for primer extension at 72 °C, followed by a terminal extension at 72 °C for 10 min. The products were then maintained at 4 °C until processed.

Thermal PCR conditions for *Campylobacter* consisted of 10 min of initial denaturation at 95 °C, followed by 35 cycles of denaturation each consisting of 30 s at 95 °C, 30 s of annealing at 59 °C, 1 min of extension at 72 °C and a final extension step of 10 min at 72 °C. The products were then maintained at 4 °C until processed. DNase-free water was used as negative control to confirm the absence of contamination of material and facilities and removal of experimental errors and to prove the exclusion of non-target DNA. The amplified products were analyzed in 1.5 % agarose gel. 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 all components 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 Testing

Antimicrobial resistance test was performed by Kirby-Bauer disc diffusion method on Mueller–Hinton agar (MV1084, HiMedia Laboratories) based on recommendations of CLSI (formerly the National Committee for Clinical Laboratory Standards, NCCLS) [22]. Antimicrobial resistance test for *Campylobacter* was performed on Mueller–Hinton agar supplemented with 5 % lyse sheep blood according to CLSI [22]. The following antibiotics were used in this study: ampicillin, amoxicillin–clavulanic acid, aztreonam, ceftazidime, cefalothin, cefixime, chloramphenicol, ceftriaxone (rocephin), doxycycline, cefotaxime, ciprofloxacin, gentamicin, imipenem/cilastatin, norfloxacin, kanamycin, nalidixic acid, oxytetracycline, streptomycin, and tetracycline and trimethoprim–sulfamethoxazole. *Staphylococcus aureus* and *Escherichia coli* were used as quality control organisms in antimicrobial susceptibility determination. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used for the quality control.

Statistical Analysis

Statistical analysis was carried out using SPSS statistical software version 17.0 (SPSS Inc. Chicago, IL, USA). Descriptive statistics were computed to determine frequencies of fly samples positive for each of the two bacterial species and frequencies of resistance to different antibimicrobials. Chi square test was used to determine significance of the observed differences in proportions between locations and seasons.

Results and Discussion

Detection of the Organisms Among the Flies

The overall recovery frequencies of *Salmonella* and *Campylobacter* from houseflies were 15.8 % (95/600) and 19.5 % (117/600) respectively. The recovery frequencies of *Salmonella* and *Campylobacter* in the specific provinces were 15.0 % (45/300) and 21.7 % (65/300) respectively in Shahrekord; and 16.7 % (50/300) and 17.3 % (52/300) respectively in Isfahan. The observed differences in these proportions of *Salmonella* were not statistically significant ($P > 0.05$). However, the frequency of *Campylobacter* was significantly different between the two provinces ($P < 0.05$). The sampling location specific recovery frequencies are displayed in Table 2. The recovery frequencies of these organisms were more or less similar in all the sampling locations. Nevertheless, cattle farm, animal hospital and slaughter houses showed significantly higher levels of total recovery frequencies as compared to kitchens, chicken farms and human hospitals ($P < 0.05$). Seasonal recovery frequencies of the organisms are displayed in Table 3. The frequencies were significantly high during summer and low during winter for both the bacteria ($P < 0.05$).

Antimicrobial Resistance

Different proportions of *Salmonella* and *Campylobacter* were found to be resistant to each of the antimicrobials tested (Table 4). Higher frequencies of resistance of

Table 2 Recovery frequencies of *Salmonella* and *Campylobacter* organisms from houseflies captured at different locations in Shahrekord and Isfahan provinces of Iran

Location	Prevalence of enteric bacteria (%)	
	Salmonella	*Campylobacter* spp.
Kitchens (n = 4)	9.0 (9/100)	16.0 (16/100)
Cattle farms (n = 4)	20.0 (20/100)	21.0 (21/100)
Chicken farms (n = 2)	13.0 (13/100)	14.0 (15/100)
Slaughter houses (n = 2)	23.0 (23/100)	27.0 (28/100)
Animal hospitals (n = 2)	20.0 (20/100)	22.0 (23/100)
Human hospitals (n = 4)	10.0 (10/100)	17.0 (17/100)

Table 3 Seasonal recovery frequencies of *Salmonella* and *Campylobacter* organisms from houseflies captured at different locations in Shahrekord and Isfahan provinces of Iran

Season	Prevalence of enteric bacteria (%)	
	Salmonella	*Campylobacter* spp.
Spring	16.7 (25/150)	22.0 (33/150)
Summer	28.7 (43/150)	34.7 (52/150)
Autumn	12.7 (19/150)	14.7 (22/150)
Winter	5.3 (8/150)	6.7 (10/150)

Table 4 Antimicrobial resistance profiles of *Salmonella* spp. and *Campylobacter* spp. isolates against 20 antimicrobial agents

Antimicrobial agent	Proportion of resistant isolates (%)	
	Salmonella (n = 95)	*Campylobacter* (n = 117)
Ampicillin	26.3	24.8
Amoxicillin–clavulanic acid	30.5	19.7
Aztreonam	21.1	17.1
Ceftazidime	35.8	20.5
Cefalothin	23.2	31.6
Cefixime	22.1	21.4
Chloramphenicol	40.0	27.4
Ceftriaxone (Rocephin)	24.2	30.0
Doxycycline	52.6	19.7
Cefotaxime	21.1	24.8
Ciprofloxacin	15.8	33.3
Gentamicin	34.7	21.4
Imipenem/cilastatin	31.6	39.3
Norfloxacin	34.7	30.0
Kanamycin	29.5	24.8
Nalidixic acid	52.6	19.7
Oxytetracycline	52.6	17.1
Streptomycin	52.6	20.5
Tetracycline	52.6	31.6
Trimethoprim–Sulfamethoxazole	30.5	21.4

Salmonella were observed for Doxycycline and of *Campylobacter* for Imipenem/cilastatin.

The role of houseflies as reservoirs of infectious microorganisms has been described by several researchers [11–13, 23]. Because of their habitat preference, mobility, feeding habits, and attraction to residential areas, the flies have a great potential to disseminate bacterial pathogens, including those incriminated to cause human and animal infections [24, 25]. Flying back and forth between different sites [26] the flies transmit the pathogens to surrounding communities both mechanically, via contaminated mouthparts and legs; and biologically, via excretion of ingested microbes either in vomit or feces [27].

The present study demonstrates occurrence at different proportions of *Campylobacter* (19.5 %) and *Salmonella* (15.8 %) in houseflies captured in Isfahan and Shahrekord provinces of central and southwestern Iran. At varying frequencies, flies collected from all the locations were positive for these potential enteric pathogens. It is likely that the flies picked up the organisms from the contaminated surrounding environments [11]. The possible sources of the organisms in the farm, animal hospital and slaughter house environments could be feces of infected animals whereas in household kitchens and human hospitals the flies could have picked up the organisms from animals' feces and garbage bins. Some earlier workers have reported carriage of *Campylobacter* in flies found in and around chicken farms [12, 28, 29]. However, a study in Malaysia [30] was not able to recover *Campylobacter* from houseflies in an animal ward and a cafeteria but was able to isolate *Salmonella* from flies captured in these locations. This observation could be associated with differences in levels of contamination at different locations with the organisms in question which implies variations in sources of exposure to the organisms among the flies.

It was noted in the present study that houseflies derived from all the sampling locations were unequally infected with *Salmonella*. This observation is suggestive of unequal levels of contamination in the different locations. This is as the result of higher level of contamination with the organisms in farms, animal hospital wards and slaughter houses originating from the animal feaces, as opposed to hospital wards and household kitchens where hygiene is always observed. The current observation needs to be validated by employing a longer study duration and involvement of a large number of locations to increase precision.

The present study investigated and revealed carriage of bacterial pathogens on both, the external body parts and in the gut of houseflies. These findings indicate that the flies may act as mechanical and biological vectors of bacterial pathogens [31]. Having this microbe found in both the gut

and surface of flies is of paramount importance in its transmission and possibly change of disease epidemiology. Bacteria in houseflies can remain viable for days or weeks in the gut and expelled either in wound or food.

Healthcare-associated infections are among the challenges that the medical professionals face. Their frequencies remain unacceptably high and are associated with excess morbidity, mortality and increased healthcare costs [32]. The flies are likely to disseminate the bacteria they carry to patients and/or workers through contaminating food and/or water.

Houseflies are of common occurrence in livestock farms with varying numbers depending on season. Their contamination with potential human and animal pathogens has been reported earlier [30]. In the current study the flies collected from cattle farms were found infected with both the two bacterial species searched for i.e. *Campylobacter* and *Salmonella*.

The present results reveal exposure of the flies, in farm environments, to these and possibly other microorganisms. A study by Shane et al. [33] found that houseflies confined in a Horsfall isolator containing chickens were positive for and excreting *Campylobacter*. The authors also revealed that, in turn, contaminated flies were able to transmit the organisms to *Campylobacter*-free chickens.

Many infectious diseases in temperate countries display seasonality, exhibiting patterns associated with weather conditions [34–37]. Such diseases include bacterial infections caused by *Campylobacter* and *Salmonella* spp., which display seasonal peaks in summer, alternating with low background levels of infection [38–44]. Understanding these seasonal trends in infectious diseases is important for improving disease surveillance.

Detection of *Campylobacter* and *Salmonella* in houseflies inhabiting the household kitchens is of great concern. This is because the two bacterial species, which are among the leading diarrheagenic bacteria worldwide, are transmitted through food. According to Dawkins et al. [45] once *Campylobacter* is introduced in the kitchen, the organism contaminates surrounding work areas. Since *Campylobacter* infections are known to require a low infectious dose [31] it is suggested that their transmission by flies may be the most important source of infection in the kitchen and in commercial food establishments [15]. This could also hold true for infections caused by *Salmonella* species.

Both *Campylobacter* [46–48] and *Salmonella* [49–51] have been reported to display resistance to different antimicrobial agents. This has included those antimicrobial agents known to be of choice for treatment of infections attributed to the two organisms, which include fluoroquinolones and macrolides for *Campylobacter* [48, 52] and fluoroquinolones and third-generation cephalosporins for *Salmonella* [49, 50, 53]. Isolates of *Campylobacter* and *Salmonella* derived from houseflies in the present study were also resistant, at different frequencies, to a number of commonly used antimicrobials in the veterinary and human medicine fields. The resistance levels were low to moderate unlike in other studies where up to 100 % resistance was found for some antimicrobials [51]. Observations that these organisms are resistant to different antimicrobials, including those which were effective against them, are indicative of the effects of their indiscriminate use [51]. Several studies suggested that some strains of *Salmonella* and *Campylobacter* showed resistance to imipenem [54–57].

Conclusion

The authors conclude that housefly may be considered as important vector of antimicrobial resistant bacterial species causing gastrointestinal diseases including *Campylobacter* and *Salmonella*. The carried organisms are resistant to a number of antimicrobials at different levels. Thus, future plans aimed at stemming infections caused by these zoonotic organisms should take flies into account.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest with respect to the research, authorship and/or publication of this article.

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