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Early View

Original Research Article

Prevalence, Characterization, and Emergence of Extended-spectrum β-lactamase Producing- and Carbapenem-resistant Gram-negative Bacteria Isolated From Houseflies (*Musca domestica*) in Tunisia

Key words

antibiotic resistance;
ESBL;
integron;
multidrug resistance;
OXA-48 / NDM
carbapenemase
genes

Ghassan Tayh[1]*, Ghaya Jebali[1], Rachid Selmi[1], Randa Jawadi[2], Khaled Kaboudi[2], Monia Daaloul[1], Lilia Messadi[1]*

[1]Univ. Manouba, Institution de la Recherche et de l'Enseignement Supérieur Agricoles, Service de Microbiologie et d'Immunologie, Ecole Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisie, [2]Univ. Manouba, Institution de la Recherche et de l'Enseignement Supérieur Agricoles, Service d'Aviculture et de Pathologie aviaire, Ecole Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisie

***Corresponding author:** lilia_messadi@yahoo.fr

Abstract: Houseflies (*Musca domestica*) live in close contact with humans. They are carriers of human pathogenic bacteria in the digestive tract and on their body. This study aimed to assess the prevalence of antibiotic-resistant Gram-negative bacteria in flies.

Sixty-one isolates were collected from 100 houseflies at three different locations: a laying hen farm, a market, and three houses, comprising 23 *Escherichia coli,* 31 *Klebsiella pneumoniae* and 7 *Pseudomonas aeruginosa*. Antimicrobial sensitivity was determined by the disk diffusion method, and the ESBL-producing isolates were screened by the double-disc synergy test. β-lactamase genes, associated resistance genes, and integrons were studied by PCR.

The ESBL-producing isolates comprised14.8% (9/61) of the isolates, seven *K. pneumoniae* isolates, and two *E. coli* isolates. The highest rate of ESBL-producing strains was observed in houses (7/22; 31.8%), followed by the market (2/43; 4.7%). Multi-drug-resistant bacteria were detected in 19/61 (31.2%) insects. Third-generation cephalosporin-resistant isolates (n= 30) were used to identify the resistance genes. The following resistance genes were identified in the isolates; $bla_{\text{CTX-M-G-1}}$ (76.7%, 23/30), $bla_{\text{SHV-1}}$ (43.3%, 13/30), $bla_{\text{TEM-1}}$ (36.7%, 11/30), bla_{IMP} (16.7%, 5/30), $bla_{\text{OXA-48}}$ (10%, 3/30) and bla_{NDM} (3.3%, 1/30). The quinolone resistance genes *qnrs*, *aac(6')-Ib-cr, qnrB* and *qnrA* were found in 11, 11, 7 and 5 isolates, respectively. Integron 1 (*intI1*) was detected in 15 (50%) isolates, *qacEΔ1+sul1* was identified in ten intI1-positive isolates. Class 2 integron was detected in three isolates.

Houseflies collected from houses and markets may be implicated in the spread of multi-drug resistant bacteria which constitute a considerable threat to human public health. The ESBLs in flies reflect the contamination status of the environment and can be used as indicators of contamination.

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Introduction

The housefly (*Musca domestica*) is common around human and domestic animals and is known as a considerable vector insect that disseminates infections to both humans and animals (1). They often carry and spread pathogenic microbes by their mouth, wings, legs, and body surface, as well as via regurgitation of intestinal contents (2). Regular contact between flies and the

environment and animals provides an opportunity to carry and transport microorganisms to both humans and animals (3). Houseflies are known to transmit several species of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Chlamydia* spp., *Campylobacter* spp., *Pseudomonas* spp., *Vibrio* spp., and *Salmonella* spp. (3-6).

Antimicrobials are important molecules for infections caused by pathogenic bacteria. Fluoroquinolones, third-generation cephalosporins and carbapenems are commonly used to treat gram-negative infections (7). Several reports have demonstrated an increase in resistance to many antimicrobials, and antimicrobial resistance has become a critical public health issue worldwide in recent years (1).

The growth rate of multidrug-resistant (MDR) bacteria as a result of extended-spectrum β-lactamase (ESBL) production in bacteria has become a significant threat to the effectiveness of antibiotics against bacterial infections and increases the limitations of the current antibiotic therapy (8). ESBLs are enzymes produced by several species of bacteria, which can break down penicillin and cephalosporin antibiotic classes, and are encoded by genes often associated with mobile genetic elements that can be transferred to other bacteria (9). Class A β-lactamases are the most common class of β-lactamases and include CTX-M-type, TEM, and SHV. In recent years, CTX-M-type ESBLs have become the most frequently detected genes in gram-negative bacteria worldwide (10,11). The emergence of MDR strains and ESBL-producing bacteria has increased substantially over the last decade and is considered a major concern in human and veterinary medicine (7). Several recent studies have been proved the role of flies as a potential vector of multidrug resistance (MDR) bacteria (1,8,12).

Microbial pathogens in feces can be transmitted to humans or animals through drinking water, food or hands. Flies can be used as an indicator of food contamination, because they can move freely between different habitats, and there is no direct contact between food and feces (13).

Flies can transmit resistant bacteria via regurgitation, translocation from the exoskeleton (e.g. mouth and legs) and defecation. Flies ingest fluids that may be contaminated with bacteria. These bacteria colonize and multiply inside the insect and then transferred by the intestinal rout or regurgitation, which leads to the concept of "transmission by bio-reinforcement". The sharing of their habitats with animals and humans, will support the transmission of antimicrobial resistance (14).

In Tunisia, several studies have reported an increase in antimicrobial resistance in humans, animals and the environment (15-18), while the data on antimicrobial resistance in mechanical vectors are scarce. To our knowledge, there are no reports on the prevalence of MDR bacteria isolated from houseflies in Tunisia. This study aimed to assess the molecular characterization of antibiotic-resistant bacteria isolated from houseflies in Tunisia.

Material and methods

Samples collection

One hundred houseflies (*Musca domestica*) were collected between September and December 2019 from three different locations: 34 flies from three laying hen farms (Mornaguia, Tebourba and Sidi Othman) located in a state of Manouba (Northern Tunisia) using sticky traps, 34 flies from a market in El-kadhra cite, located in the Tunis state, using electrocution traps, and 32 flies from three houses in the governorate of Gabes (Southern Tunisia), and Nabeul and Tunis regions (North Tunisia). The collected flies were placed in sterile jars and transported to the Laboratory of Microbiology and Immunology of the Ecole Nationale de Médecine Vétérinaire (ENMV) for further analysis. These samples were used to search for bacterial isolates from the digestive tract of the flies.

Bacterial isolation and identification

Each fly was suspended in alcohol to be disinfected and then washed with distilled water to remove all alcohol. Flies were then transported into a 1.5 ml Eppendorf tube containing Buffered Peptone Water (BPW), crushed using anatomic forceps until a mixture was obtained, and the mixture was incubated overnight at 37°C. A 10 µl loop of broth was plated onto MacConkey agar (Oxoid, Basingstoke, United Kingdom) and MacConkey agar supplemented with cefotaxime (CTX 1 µg/ml) for isolation of non-fastidious gram-negative bacteria. A 10 µl loop of BPW was inoculated onto Xylose Lysine Deoxycholate agar (XLD agar) (Oxoid) and cetrimide agar (Oxoid) for isolation of *Salmonella* spp. and *Pseudomonas aeruginosa*, respectively. The plates were incubated overnight at 37°C. The bacterial isolates were identified by conventional biochemical tests (triple sugar iron agar, urease production, indole production, Simmons citrate agar, motility, and oxidase test) and confirmed by the API 20E System (bioMérieux, France). Bacterial isolates were stored at -20C in brain heart infusion broth supplemented with 20% glycerol.

Antimicrobial sensitivity test

The antimicrobial sensitivity was studied by the disk diffusion method on Mueller-Hinton agar plates according to the guidelines and clinical breakpoints of the Antibiogram Committee of the French Society (CA-SFM)(19) using twenty-one antibiotics discs (Bio-Rad France) comprising (µg/disk) twelve β-lactams and nine non-β-lactam antibiotics: amoxicillin (25), amoxicillin/clavulanic acid (20/10), ticarcillin/clavulanic acid (75/10), cefotaxime (30), ceftazidime (30), cefepime (30), cefoxitin (30), aztreonam (30) ertapenem (10), piperacillin (30), cephalothin (30), cefuroxime (30), chloramphenicol (30), gentamicin (15), nalidixic acid (30), enrofloxacin (5), tetracycline (30), sulfamethoxazole/trimethoprim (1.25/23.75), streptomycin (10) and florfenicol (30). Furthermore, colistin susceptibility was examined using the colispot test (20).

ESBL Phenotype Detection

The double synergy test is the most widely used test for detecting ESBL phenotypes. This was carried out using a disk of cefotaxime, ceftazidime and cefepime around a disk containing clavulanic acid which was placed on Mueller-Hinton agar plates at a distance of 30 mm between the two discs. In this case, confirmation was performed if the zone of inhibition increased by 5 mm with a cefotaxime disk, ceftazidime or cefepime with a clavulanic acid disk(21).

All isolates exhibited resistance to third-generation cephalosporins (cefotaxime and/or ceftazidime) and positive ESBL confirmation disk tests were selected for molecular analyses.

Detection of β-lactamase genes

PCR amplification was performed to detect the presence of the β-lactamase-encoding genes *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{SHV}$ and *bla*$_{CMY}$ (22,23). Carbapenem resistance genes *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ were searched by PCR (24) (Supplementary table 1).

Detection of antimicrobial resistance genes

The presence of genes that confer resistance to tetracycline (*tet(A)* and *tet(B)*), sulfamethoxazole (*sul1*, *sul2*,and *sul3*), aminoglycosides (*aac(3)-I*, *aac(3)-II*, *aac(3)-IV, aadA-1* and a*adA-5* and quinolones (*qnrA*, *qnrB*, *qnrS*, and *aac*(6')-1b) were tested by PCR amplification (25) (Supplementary table 1).

Detection and characterization of integrons

The presence of *intI1* and *intI2* genes was screened in all isolates by PCR. The presence of *qacEΔ1+sul1* genes within the 3'-conserved regions of class 1 integrons was confirmed by PCR. The gene cassettes in the variable region of class 1&2 integrons were screened by PCR and sequencing. The primers used are listed in Supplementary table 1 (25).

Data analysis

Pearson's Chi-square test was used to investigate the significant differences in antimicrobial resistant isolates using SPSS version 20 software (IBM Corporation, Somers, NY). The level of statistical significance was set at $P< 0.05$.

Results

Bacterial species

A total of 61 bacterial strains were obtained from 100 studied flies. The frequency of bacterial isolates in the studied flies was distributed as follows: *Klebsiella pneumoniae* 31 (50.8%) isolates, *Escherichia coli* 23 (37.7%) isolates, and *Pseudomonas aeruginosa* 7 (11.5%) isolates. Most of the isolates 96.7% (59/61) were collected from houses and the market. The frequency of microorganisms according to the sampling place is shown in Table 1.

Antimicrobial sensitivity

According to the susceptible breakpoint, the bacteria were categorized as susceptible (S), intermediate (I), and resistant (R). The non-susceptible isolates were regrouped into resistant and intermediate categories (26).

The highest susceptibility rates against *E. coli* isolates were observed for amoxicillin-clavulanic acid (60.8%), followed by chloramphenicol (56.5%), and tetracycline (43.5 %). The lowest rates were obtained for cefotaxime (8.7%), streptomycin (8.7%), trimethoprim-sulfamethoxazole (8.7%), cefepime (8.6%) and florfenicol (4.3%). All *E. coli* isolates were sensitive to cefoxitin, gentamicin, and colistin (Figure 1).

Figure 1 shows that the highest resistance rates in *K. pneumoniae* were recorded for amoxicillin-clavulanic acid and ticarcillin-clavulanic acid (64.5%), followed by piperacillin (58.1%), cefuroxime (51.6%), ceftazidime (51.6%), cefotaxime (48.4%), cephalothin (48.4%) and aztreonam (42%). The lowest levels of resistance were recorded for chloramphenicol (12.9%) and florfenicol (6.5%). No resistance to gentamicin or colistin was observed.

In *P. aeruginosa* isolates, total resistance was detected for cefoxitin, amoxicillin-clavulanic acid, and cephalothin. It should be noted that there is a high resistance to cefuroxime and chloramphenicol (85.7%), cefotaxime, ticarcillin-clavulanic acid, aztreonam, nalidixic acid, florfenicol, tetracycline, and trimethoprim-sulfamethoxazole (71.4%). All isolates were sensitive to piperacillin, cefepime, gentamicin, streptomycin, colistin, and enrofloxacin (Figure 1).

Table 2 shows that the antimicrobial resistance profile of bacterial isolates was related to sampling locations**.** The difference of antimicrobial resistance in the isolates between the three sites didn't reach statistical significance except for amoxicillin, enrofloxacin, tetracycline and trimethoprim-sulfamethoxazole. The resistance rate of these antibiotics was high in the house and the differences was statistically significant.

The ESBL-positive isolates among the 61 studied isolates were 9 (14.8%) strains, composed of seven *K. pneumoniae* isolates and two *E. coli* isolates. The highest rate of ESBL strains was observed in houses (7/22; 31.8%), followed by the market (2/43; 4.7%). The ESBL-producing strains were absent on laying hen farms. This difference in ESBL rates considered statistically significant (0.031). Among the 61 strains tested, 19 (31.2%) were multi-drug resistant (MDR). These 19 multi-resistant strains are composed of 9 strains of *K. pneumoniae* with a rate of 29% (9/31), 5 strains of *E. coli* (5/25; 21.7%), and 5 strains of *P. aeruginosa* (5/7; 71.4%). Noteworthy, all ESBL strains were MDR.

Table 3 shows that ESBL-producing strains are more resistant to most antibiotics than non-ESBL strains. These differences are statistically significant except for amoxicillin, cefoxitin, ertapenem, chloramphenicol, and florfenicol.

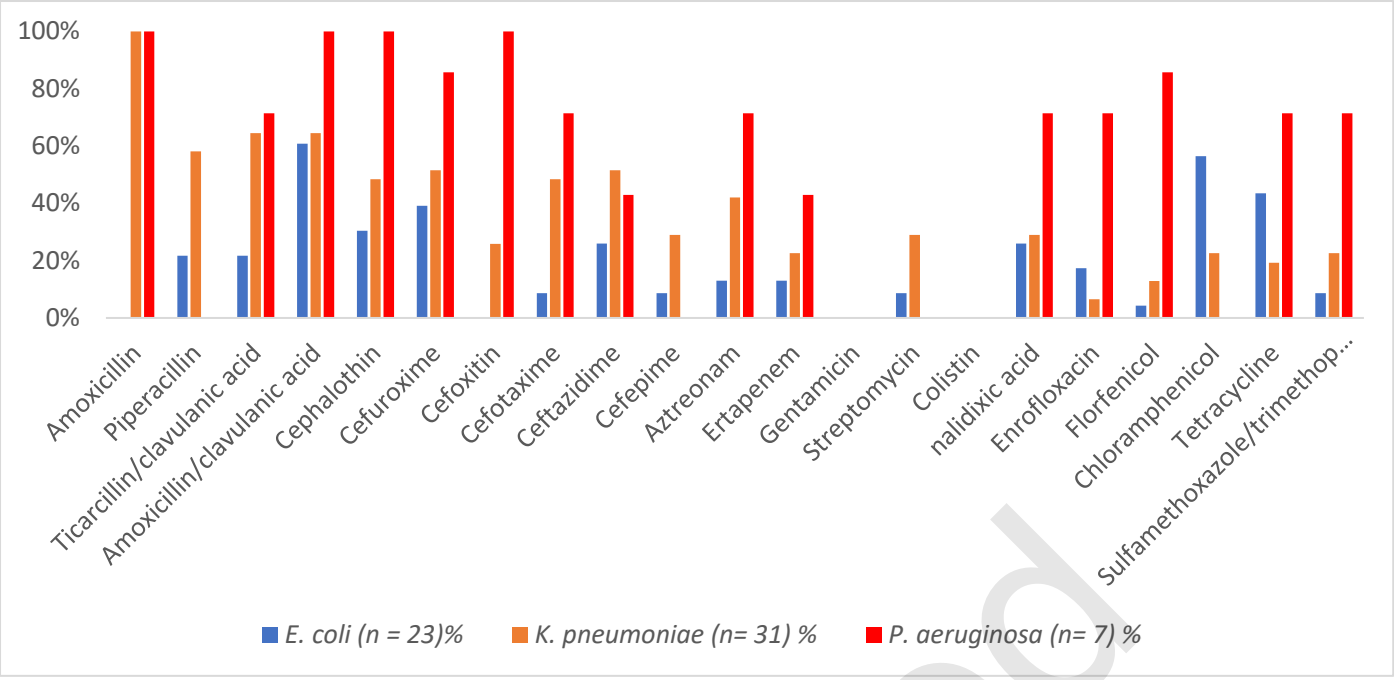

Figure 1: Antibiotic resistance rate in the bacterial strains

Table 1: Distribution of bacterial species according to collection sites

Bacterial species	Number of bacterial strains			Total
	Houses	Market	Farm	
E. coli	11	10	2	23
K. pneumoniae	7	24	0	31
P. aeruginosa	0	7	0	7
Total	18 (29.5%)	41 (67.2%)	2 (3.3%)	61 (100%)

Table 2: Frequency of not susceptible strains according to collection locations

Antibiotics	Not susceptible (I+R) %			*P*-value
	Collection places			
	Houses (n=22)	The market (n=43)	Frams (n=2)	
Amoxicillin	18 (81,8)	34 (79,1)	0	0,012
Piperacillin	10 (45,5)	13 (30,2)	0	0,503
Ticarcillin/clavulanic acid	10 (45,5)	20 (46,5)	0	0,554
Amoxicillin/clavulanic acid	12 (54,5)	29 (67,4)	1 (50)	0,549
Cephalothin	10 (45,5)	19 (44,2)	1 (50)	0,234
Cefuroxime	10 (45,5)	20 (46,5)	1 (50)	0,982
Cefoxitin	0	15 (34,9)	0	0,095
Cefotaxime	7 (31,8)	12 (27,9)	0	0,899
Ceftazidime	8 (36,4)	16 (37,2)	0	0,433
Cefepime	7 (31,8)	4 (9,3)	0	0,088
Aztreonam	8 (36,4)	13 (30,2)	0	0,234
Ertapenem	0	10 (23,3)	0	0,087
Gentamycin	0	0	0	a
Streptomycin	7 (31,8)	4 (9,3)	0	0,083
Colistin	0	0	0	a
Nalidixic acid	10 (45,5)	10 (23,3)	1 (50)	0,401
Enrofloxacin	8 (36,4)	3 (6,9)	0	0,018
Florfenicol	9 (40,9)	13 (30,2)	1 (50)	0,243
Chloramphenicol	1 (4,5)	7 (16,3)	0	0,440
Tetracycline	12 (54,5)	8 (18,6)	1 (50)	0,011
Sulfamethoxazole/trimethoprim	7 (31,8)	7 (16,3)	0	0,048

*a No statistics are computed because values are constant

Table 3: Comparison of antibiotic resistance rates among ESBL (n= 9) and non-ESBL *E. coli* (n=23) and *K. pneumoniae* (n=31)

	ESBL (n = 9)		Non-ESBL (n =45)		*P*-value
Antibiotics	S (%)	I+R (%)	S (%)	I+R (%)	
Amoxicillin	0 (0%)	9 (100%)	15 (33,3%)	30 (66,7%)	0,084
Piperacillin	0 (0%)	9 (100%)	31 (68,9%)	14 (31,1%)	0,000
Ticarcillin/clavulanic acid	0 (0%)	9 (100%)	29 (64,4%)	16 (35,6%)	0,000
Amoxicillin/clavulanic acid	0 (0%)	9 (100%)	20 (44,4%)	25 (55,6%)	0,030
Cephalothin	0 (0%)	9 (100%)	32 (71,1%)	13 (28,9%)	0,000
Cefuroxime	0 (0%)	9 (100%)	29 (64,4%)	16 (35,6%)	0,000
Cefoxitin	9 (100%)	0 (0%)	37 (82,2%)	8 (17,8%)	0,171
Cefotaxime	0 (0%)	9 (100%)	40 (88,9%)	5 (11,1%)	0,000
Ceftazidime	0 (0%)	9 (100%)	32 (71,1%)	13 (28,9%)	0,000
Cefepime	0 (0%)	9 (100%)	43 (95,6%)	2 (4,4%)	0,000
Aztreonam	0 (0%)	9 (100%)	38 (84,4%)	7 (15,6%)	0,000
Ertapenem	9 (100%)	0 (0%)	38 (84,4%)	7 (15,6%)	0,205
Gentamycin	9 (100%)	0 (0%)	45 (100%)	0 (0%)	a
Streptomycin	2 (22,2%)	7 (77,8%)	41 (91,1%)	4 (8,9%)	0,000
Colistin	9 (100%)	0 (0%)	45 (100%)	0 (0%)	a
Nalidixic acid	2 (22,2%)	7 (77,8%)	37 (82,2%)	8 (17,8%)	0,000
Enrofloxacin	3 (33,3%)	6 (66,7%)	40 (88,9%)	5 (11,1%)	0,000
Florfenicol	6 (66,7%)	3 (33,3%)	31 (68,9%)	14 (31,1%)	0,988
Chloramphenicol	9 (100%)	0 (0%)	42 (93,3%)	3 (6,7%)	0,425
Tetracycline	2 (22,2%)	7 (77,8%)	36 (80%)	9 (20%)	0,001
Sulfamethoxazole/trimethoprim	2 (22,2%)	7 (77,8%)	43 (95,6%)	2 (4,4%)	0,000

a No statistics are computed because values are constant

Molecular assay

Thirty isolates; 9 *E. coli*, 15 *K. pneumoniae* isolates, and 6 *P. aeruginosa* were third-generation broad-spectrum cephalosporin-resistant (ceftazidime and/or cefotaxime) and were introduced for further molecular characterization.

Characterization of β-lactamase genes

The prevalence of β-lactamase genes identified among these isolates were the following (Table 4): $bla_{\text{CTX-M-G1}}$ (76.7%, 23/30), $bla_{\text{SHV-1}}$ (43.3%, 13/30), $bla_{\text{TEM-1}}$ (36.7%, 11/30), bla_{IMP} (16.7%, 5/30), $bla_{\text{OXA-48}}$ (10%, 3/30), and bla_{NDM} (3.3%, 1/30).

Identification of antimicrobial agent-coding genes

A variety of antibiotic resistance genes were detected among the broad-spectrum cephalosporin-resistant isolates: the Plasmid-Mediated Quinolone Resistance Determinants *qnrs, qnrB* and *qnrA* were found in 13, 9 and 6 isolates, respectively. The *aac(6')-Ib-cr*, *aadA-1*, *aac(3)-II* and *aadA-5* genes were found in 19, 16, 12, and 4, respectively of aminoglycoside-resistant isolates. Twenty of the 30 isolates harbored *sul* genes (*sul1*: (n = 20) and *sul1*+*sul2*: (n = 5)). Tetracycline genes were detected in twelve isolates: *tetA* in 7 isolates and *tetB* in five isolates.

Integrons and arrangement of genes cassettes

The *intI1* and *intI2* genes were searched by PCR to detect the class 1 & 2 integrons among the isolates**.** The class 1 integron has been detected in fifteen (50%) isolates, and *qacEΔ1+sul1* genes were identified in ten *intI1*-positive isolates. The class 2 integron was detected in three isolates. Gene cassette arrangement in integron-1: *dfrA17 + aadA5* was present among three *E. coli* isolates, and *dfrA12 + aadA2* was detected in three *K. pneumoniae*. Moreover, *aadA1* was present in the class 2 integron among two *E. coli* isolates (Table 4).

Table 4: Characteristics of the 30 beta-lactamases-producing isolates recovered from houseflies

Bacterial strains	Codes	Place	ESBL	β-lactamase genes	Other resistance genes detected	Class 1 integron			Class 2 integron	
						Int1	*qacEΔ1 + sul1*	*Gene cassette in VR**	*Int2*	*Gene cassette in VR**
K. pneumoniae	G1	Houses Gabes	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*qnrB, aac(6')-Ib-cr, aac(3)-II, sul1, sul2, tetA*	+				
E. coli	G24	Houses Gabes	-	$bla_{CTX-M-G1}$	*qnrA, aac(3)-II, aadA-1, sul1*					
K. pneumoniae	G2	Houses Gabes	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*aac(6')-Ib-cr, aac(3)-II, aadA-1, sul1, sul2, tetA*	+				
E. coli	G25	Houses Gabes	-	$bla_{CTX-M-G1}$, bla_{TEM-1}, bla_{OXA-48}	*qnrA, aadA-1, sul1, tetA*				+	*aadA1*
K. pneumoniae	G3	Houses Gabes	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*qnrB, qnrS,aac(6')-Ib-cr, aac(3)-II, aadA-1, sul1, sul2, tetA*	+	+		+	
K. pneumoniae	G4	Houses Gabes	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*qnrB, qnrS,aac(6')-Ib-cr, aadA-1, sul1, sul2, tetA*	+				
E. coli	G5	Houses Tunis	+	$bla_{CTX-M-G1}$	*qnrA, qnrS,aac(6')-Ib-cr, sul1, tetB*					
E. coli	G6	Houses Tunis	+	$bla_{CTX-M-G1}$	*qnrA, qnrS,aac(6')-Ib-cr, sul1, tetB*					
E. coli	G26	Houses Tunis	-	$bla_{CTX-M-G1}$, bla_{TEM-1}, bla_{IMP}, bla_{OXA-48}	*qnrA, qnrS,aadA-5, tetB*	+	+			
K. pneumoniae	G7	Houses Tunis	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*qnrB, qnrS,aac(6')-Ib-cr, aac(3)-II, aadA-1,*	+	+	*aadA2 + dfrA12*		
E. coli	G27	Houses Tunis	-	$bla_{CTX-M-G1}$, bla_{TEM-1}, bla_{IMP}	*qnrS,aadA-5, sul1, tetB*	+	+	*dfrA17 + aadA5*		
K. pneumoniae	G8	Market	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*aac(3)-II, aadA-1, sul1, sul2, tetA*	+	+	*dfrA12 + aadA2*		
K. pneumoniae	G9	Market	+	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{TEM-1}	*qnrA, qnrS,aac(6')-Ib-cr, aac(3)-II, aadA-1*	+	+	*dfrA12 + aadA2*		
K. pneumoniae	G10	Market	-	bla_{TEM-1}						
E. coli	G28	Market	-	$bla_{CTX-M-G1}$, bla_{IMP}	*qnrS,aac(3)-II, aadA-5, tetA, tetB*	+	+	*dfrA17 + aadA5*	+	*aadA1*
K. pneumoniae	G11	Market	-		*aac(6')-Ib-cr, aadA-1, sul1,*					
K. pneumoniae	G12	Market	-	bla_{SHV-1}, bla_{IMP}	*qnrB, aac(6')-Ib-cr,*					
E. coli	G29	Market	-	$bla_{CTX-M-G1}$	*qnrB, qnrS,*	+				
E. coli	G30	Market	-	$bla_{CTX-M-G1}$, bla_{OXA-48}	*aadA-5, sul1*	+				
K. pneumoniae	G13	Market	-	$bla_{CTX-M-G1}$	*qnrB, aac(6')-Ib-cr, aadA-1, sul1,*	+	+			
K. pneumoniae	G14	Market	-	$bla_{CTX-M-G1}$	*aac(6')-Ib-cr, aac(3)-II, aadA-1, sul1,*					
K. pneumoniae	G15	Market	-	$bla_{CTX-M-G1}$	*qnrB, aac(6')-Ib-cr*					
K. pneumoniae	G16	Market	-	$bla_{CTX-M-G1}$	*aac(6')-Ib-cr, aac(3)-II, aadA-1, sul1,*	+	+			
K. pneumoniae	G17	Market	-	$bla_{CTX-M-G1}$, bla_{SHV-1}, bla_{IMP}	*qnrB,aac(3)-II, aadA-1, aadA-5*					
P. aeruginosa	G18	Market	-	$bla_{CTX-M-G1}$	*qnrS,sul1, aadA-1,*					
P. aeruginosa	G19	Market	-	$bla_{CTX-M-G1}$, bla_{NDM}	*qnrB, aac(6')-Ib-cr, aac(3)-II, aadA-1,*					
P. aeruginosa	G20	Market	-	bla_{SHV-1}	*qnrS,aac(6')-Ib-cr, sul1*	+				
P. aeruginosa	G21	Market	-	bla_{SHV-1}	*aac(6')-Ib-cr, sul1*	+	+			
P. aeruginosa	G22	Market	-	bla_{SHV-1}	*qnrS,aac(6')-Ib-cr, sul1*	+	+			
P. aeruginosa	G23	Market	-	bla_{SHV-1}	*aac(6')-Ib-cr, sul1, aadA-1,*	+	+			

* VR, Variable region

Discussion

Houseflies are in close association with humans, and they can be involved in major public health problems (14). They can hold bacterial pathogens on their external body surfaces, and they can pick up the bacteria by the mouth, legs, wings, and other body parts through the feeding process, thus being able to transfer pathogens to people (27). Several studies have described the role of flies in the transmission of antimicrobial-resistant bacteria, suggesting that flies may be useful as representatives for the one-health indicator in the spread of antibiotic resistance (28,29).

House flies move and spread in all areas involving farms and civilian areas. In the present study, we investigated the antimicrobial resistance in *E. coli*, *K. pneumoniae* and *P. aeruginosa* in flies. This study focused on the genotypical and phenotypical characteristics of the broad-spectrum cephalosporin-resistant *K. pneumoniae*, *E. coli*, and *P. aeruginosa* isolates in houseflies collected from laying hen farms, a market, and houses in Tunisia.

The objective of the study is to provide data on antimicrobial resistance in different locations (farms and urban areas) and evaluate the possible dissemination of antimicrobial resistance between animals and humans by houseflies. To the best of our knowledge, this is the first study to report the antimicrobial resistance among gram-negative species in houseflies in Tunisia.

The isolation of bacteria from fly bodies was performed by crushing the flies and to avoid contamination with bacteria from the fly body surface, the disinfection of fly bodies with alcohol was carried out. Bacterial strains of *E. coli*, *K. pneumoniae* and *P. aeruginosa* were isolated in this study and have importance in veterinary and human medicine. Furthermore, multidrug resistance isolates, particularly extended-spectrum beta-lactamase-producing strains, are a major public health concern.

In the present study, *K. pneumoniae*, *E. coli*, and *P. aeruginosa* were detected in 50.8%, 37.7%, and 11.5% of the flies. Our results were compatible with other studies reporting that *K. pneumoniae* and *E. coli* have the highest isolation rates (28-30).

Antimicrobial resistance is one of the most serious public health threats of our time worldwide. Microorganisms protect themselves against antimicrobial drugs by developing spontaneous mutations or by acquiring mobile resistance genes, making the antimicrobial ineffective against pathogens and infections harder to treat, leading to increased illness and death associated with infectious diseases in animals and humans (31). The antimicrobial resistance problem currently causes currently about 700,000 deaths every year, and the estimated number could grow to 10 million by 2050 (32). Recently, studies have identified antibiotic resistance in the housefly and proved that it plays an important role in disseminating antibiotic-resistant bacteria (1).

In this study, the isolated bacteria were resistant to various antibiotics, with most of the isolates being resistant to amoxicillin, amoxicillin/clavulanic acid, cephalothin, cefuroxime, ceftazidime, nalidixic acid, florfenicol, and tetracycline. Our findings demonstrate a high resistance to antimicrobial agents that have been applied in the treatment of humans and animals. From the three studied areas, antibiotic-resistant isolates were more present in the houses than in other areas, probably because houses are over-stuffed with healthy and sick people who carry diverse bacteria, particularly antibiotic-resistant bacteria.

ESBL enzymes make commonly used antibiotics ineffective. ESBL-producing *Enterobacterales* are a global worry in healthcare settings and the community. They can expand fast and cause or complicate infections in healthy humans (9). In the present study, the-ESBL producers rate was 14.8% (n = 9), and similar results were reported in Germany (12.9%, n = 163) and in the Netherlands (15.0%, n = 73) (33,34). This difference of ESBL rates in sampling places is statistically significant, and the highest rate of ESBL strains was observed in houses which encourages the transmission of ESBL-producing isolates in the community. The high rates of these strains in houses might alarm to cleanliness status of the houses. The house flies habitually feed on feces and food. During this feeding process, pathogens adhere to their mouthparts, wings, legs, and other body surfaces; subsequently, the flies transport these pathogens back to humans. Then, it is very important to respect biosecurity rules, especially for the management of household waste, food conservation, etc.

In the present study, the highest overall rate of Enterobacteriaceae strains producing ESBLs was observed in homes (31.8%). On the other hand, Wetzker and al. (2019) in Germany showed that there was no significant variation in the rate of ESBL-positive strains according to the sites studied in an urban area (hospitals, zoo, and residential areas).

The finding of 31.2% of multidrug-resistant isolates reflects the increase in multi-drug resistance in comparison with those of Poudel, et al. (35), Carramaschi, et al. (36) who reported a prevalence of MDR among houseflies of 9% and 5.0%, respectively. The isolated *Pseudomonas* spp. were more frequently multiple-resistant (71.4%) than *K. pneumoniae* and *E. coli.* These results were similar to those of Rahuma, et al. (37)in Libya and following a study in Iran that reported highly resistant *P. aeruginosa* isolates obtained from houseflies to all 15 antibiotics tested, with a resistance rate ranging from 45.3 % to 100.0 % (38).

The comparison of resistance rates in ESBL and non-ESBL isolates was shown to be higher among ESBL isolates to most antimicrobial agents than to non-ESBL isolates with statistical significance. In our study, there are limited treatment options available for ESBL pathogens except for cefoxitin, ertapenem, gentamicin, colistin, and chloramphenicol, which are currently recommended as empiric therapy for infections caused by these bacteria.

These findings were correlated with those of Gutiérrez-Gutiérrez and Rodríguez-Baño (39) who found carbapenems, β-lactam-β-lactam inhibitor combinations, cephamycins, temocillin, gentamycin, colistin, and chloramphenicol the most effective treatments for bacterial infections. As a result, the monitoring of ESBL-producing isolates is considered essential in combination with control measures.

In the present study, we found that *bla*$_{\text{CTX-M-1}}$ is the most prevalent ESBL gene identified among the isolates. This result is in agreement with the findings of German and Spanish studies (7,33), which demonstrated that *bla*$_{\text{CTX-M-1}}$ is the most predominant ESBL gene detected in *Enterobacteriaceae* from houseflies.

Several studies have indicated that flies play a significant role in the spread of antibiotic-resistance genes to other bacteria (7,40). In our study, the presence of ESBL-producing bacteria isolated from houseflies that exhibited resistance to most antimicrobials confirms the ability of the houseflies to disseminate resistance genes carried via mobile genetic elements. Furthermore, two-thirds of the isolates harbored plasmid-mediated quinolone resistance determinants, *qnrs*, *qnrB* and *qnrA*. Qnr genes were carried on the same plasmids that harbored ESBL genes (41). The dissemination of plasmids harboring ESBL and qnr genes in the bacterial community will increase and complicate the antimicrobial resistance issue. Moreover, the presence of ESBL-producing pathogens in houseflies suggests that humans and their inhabitant environments are colonized by these bacteria and the flies are carriers and transmitters of resistant microorganisms in different environments.

There are three main mechanisms of carbapenem resistance in gram-negative bacteria: enzyme production, the efflux pump system, and reduced expression of porins. The plasmid or integron-mediated carbapenemases (ß-lactam-hydrolyzing enzymes) are the main resistance mechanism (42). The presence of *bla*$_{\text{IMP}}$ and *bla*$_{\text{OXA-48}}$ was identified among *E. coli* and *K. pneumoniae* in association with *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{TEM-1}}$. These results are in agreement with recent studies that demonstrated that carbapenemase is associated with cefotaximase *bla*$_{\text{CTXM-1}}$ and *bla*$_{\text{TEM-1}}$ from flies (36,43). In Tunisia *bla*$_{\text{OXA-48}}$ demonstrated as the most significant carbapenemase genes in hospital settings in *Enterobacteriaceae* and are often associated with the ESBL genes *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{TEM-1}}$ (44-46). *Bla*$_{\text{IMP}}$ carbapenemase was detected with *bla*$_{\text{CTX-M-1}}$ among *Enterobacteriaceae* isolated from wild boar (*Sus scrofa*) in Tunisia (47). The *P. aeruginosa* strain G19 was *bla*$_{\text{NDM-1}}$ positive. Published studies from Tunisia demonstrated that gram-negative bacteria express the *bla*$_{\text{NDM-1}}$ gene in carbapenem-resistant bacteria (48,49).

Integrons are responsible for carrying antibiotic resistance genes. They are transferred by mobile genetic elements, such as transposons and plasmids (50). The present study found that 50% of the isolates were positive for class I integrons. In the Czech Republic study, they detected class I integrons in 18 (12%) out of 147 *E. coli* isolates collected from 240 flies on a dairy farm (51). YOSBOONRUANG, et al. (50) isolated 70 *E. coli* from 60 houseflies collected from the hospital, among which 21 (36.8 %) were positive for a class 1 integron gene. Integrons in the isolates carried by them are critical genetic elements that can be disseminated to other bacteria in the environment and humans. The significance of integron is ability to express and transfer antimicrobial resistance gene cassettes. Bacteria may become MDR by receiving these genes (52).

Among the integron-positive isolates, three different gene cassettes were found including a gene encoding resistance to trimethoprim (*dfrA12*, *drfA17*), and aminoglycosides (*aadA2*, *aadA5*). The occurrence of *dfrA* in associated with *aadA* in *Enterobacteriaceae* has been reported in Tunisia from food and human sources (22,53). These findings indicate that houseflies and food represent a reservoir of this type of integron-positive isolates that could potentially be transferred to humans through the flies.

Conclusion

This is the first report regarding the antimicrobial resistance and the prevalence rates of MDR bacteria isolated from houseflies in Tunisia. This study considers significant data on bacteria carried by houseflies and provides important data on their role in the spread of antimicrobial resistance. The flies studied carried bacteria of clinical relevance essentially MDR *Enterobacteriales* and *P. aeruginosa*. We demonstrated the presence of ESBL-producing *E. coli* and *K. pneumoniae* in 14.8% of the collected strains from houseflies caught in Tunisia. We confirmed dominant plasmid-mediated ESBL-type *bla*$_{\text{CTX-M-1}}$. The presence of plasmid-encoding ESBLs with plasmid-mediated quinolone genes associated with integrons in MDR strains may highlight the possible risk of dissemination of multi-resistant bacteria through houseflies (*Musca domestica*). The presence of a high number of ESBL-producing pathogens in houseflies explains that they could easily colonize and be disseminated into different environments and food through these insects and consequently could colonize humans, and animals. To better understand the medical importance of antimicrobial resistance in houseflies, further studies should cover strains and data from environmental, animal and human samples.

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Data availability. The datasets generated during and analyzed during the current study are available in this manuscript.

Ethics approval statement. Not applicable.

Authors' Contributions: Ghassan TAYH designed the study, performed the experimental work (the microbiological and

molecular tests), collected the data, analyzed and interpreted the data and drafted the manuscript. Ghaya JEBALI collected samples and helped in performing the experimental part (microbiological tests) of the manuscript. Rachid SELMI, Randa JAWADI and Khaled KABOUDI collected samples. Monia DAALOUL participated in the project design. Lilia Messadi designed and supervised the study, and contributed to the final writing and editing the manuscript. All authors read and approved the final version of the manuscript.

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Razširjenost, karakterizacija in pojavnost gramnegativnih bakterij z razširjenim spektrom, ki proizvajajo β-laktamaze in so odporne proti karbapenemom, izoliranih iz hišnih muh (*Musca domestica*) v Tuniziji

G. Tayh, G. Jebali, R. Selmi, R. Jawadi, K. Kaboudi, M. Daaloul, L. Messadi

Izvleček: Hišne muhe (*Musca domestica*) živijo v tesnem stiku z ljudmi. V prebavnem traktu in na telesu so prenašalke človeških patogenih bakterij. Namen te študije je bil oceniti razširjenost proti antibiotikom odpornih gramnegativnih bakterij pri muhah.
Od 100 hišnih muh na treh različnih lokacijah: na farmi kokoši nesnic, na tržnici in v treh hišah, je bilo zbranih 61 izolatov, od tega 23 *Escherichia coli*, 31 *Klebsiella pneumoniae* in 7 *Pseudomonas aeruginosa*. Antimikrobna občutljivost je bila določena z disk difuzijsko metodo, izolati, ki proizvajajo ESBL, pa so bili pregledani s sinergijskim testom z dvema diskoma. Geni za β-laktamaze, z njimi povezani geni za odpornost in integroni so bili raziskani s PCR.
Izolati, ki proizvajajo ESBL, so predstavljali 14,8 % (9/61) izolatov, sedem izolatov *K. pneumoniae* in dva izolata *E. coli*. Največ sevov, ki proizvajajo ESBL, je bilo opaženih v hišah (7/22; 31,8 %), sledila je tržnica (2/43; 4,7 %). Bakterije, odporne proti več zdravilom, so bile odkrite pri 19/61 (31,2 %) žuželk. Za določitev genov odpornosti so bili uporabljeni izolati, odporni proti cefalosporinom tretje generacije (n = 30). Pri izolatih so bili ugotovljeni naslednji geni odpornosti: $bla_{\text{CTX-M-G-1}}$ (76,7 %, 23/30), $bla_{\text{SHV-1}}$ (43,3 %, 13/30), $bla_{\text{TEM-1}}$ (36,7 %, 11/30), bla_{IMP} (16,7 %, 5/30), $bla_{\text{OXA-48}}$ (10 %, 3/30) in bla_{NDM} (3,3 %, 1/30). Gene za odpornost proti kinolonom *qnrs*, *aac(6')-Ib-cr*, *qnrB* in *qnrA* so našli pri 11, 7 in 5 izolatih. Integron 1 (*intI1*) so odkrili pri 15 (50 %) izolatih, *qacEΔ1+sul1* pa pri desetih intI1-pozitivnih izolatih. Integron razreda 2 so odkrili pri treh izolatih.
Hišne muhe, zbrane v hišah in na tržnicah, so lahko vpletene v širjenje bakterij, odpornih proti več zdravilom, ki predstavljajo veliko grožnjo javnemu zdravju ljudi. ESBL pri muhah odražajo stanje onesnaženosti okolja in se lahko uporabljajo kot kazalniki onesnaženosti.

Ključne besede: odpornost proti antibiotikom; ESBL; integron; odpornost proti več zdravilom; karbapenemazni geni OXA-48 / NDM

Supplementary table 1: Primers used in this study for the detection antimicrobial resistance genes of gram-negative isolated from housefiles
AT: Annealing temperature

Assay	Target gene	Primers	Sequences (5'-3')	AT (°C)	Amplicon size (bp)	Reference
ESBL	*bla*$_{CTX-M-grp\ 1}$	Bla CTX-M 1 F	ATGGTTAAAAAATCACTGCG	49	876	(54)
		Bla CTX-M 1 R	TTACAAACCGTCGGTGAC			
	bla$_{SHV}$	Bla SHV F	CACTCAAGGATGTATTGTG	54	885	(22)
		Bla SHV R	TTAGCGTTGCCAGTGCTCG			
	ba$_{TEM}$	Bla TEM F	ATTCTTGAAGACGAAAGGGC	50	1150	(22)
		Bla TEM R	ACGCTCAGTGGAACGAAAAC			
Cephalosporinase	*bla*$_{CMY}$	Bla CMY F	ATGATGAAAAAATCGATATG	55	1146	(23)
		Bla CMY R	TTATTGCAGTTTTTCAAGAATG			
Carbapenemase	*bla*$_{OXA-48}$	OXA-48-F	GCGTGGTTAAGGATGAACAC	56	438	(55)
		OXA-48-R	CATCAAGTTCAACCCAACCG			
	bla$_{NDM-1}$	NDM-1 F	GGTTTGGCGATCTGGTTTTC	56	621	(55)
		NDM 1 R	CGGAATGGCTCATCACGATC	56		
	Bla$_{KPC}$	KPC F	CGTCTAGTTCTGCTGTCTTG		798	(24)
		KPC R	CTTGTCATCCTTGTTAGGCG			
	bla$_{IMP}$	IMP-F	GGAATAGAGTGGCTTAAYTCTC	56	203	(24)
		IMP-R	GGTTTAAYAAAACAACCACC			
	bla$_{VIM}$	VIM-F	GATGGTGTTTGGTCGCATA	52	390	(24)
		VIM-R	CGAATGCGCAGCACCAG			
Colistin	*mcr-1*	CLR F	CGGTCAGTCCGTTTGTTC	54	309	(56)
		CLR R	CTTGGTCGGTCTGTAGGG			
Tetracycline	*TetA*	tetA F	GTAATTCTGAGCACTGTCGC	62	937	(25)
		teA R	CTGCCTGGACAACATTGCTT			
	TetB	tetB F	CTCAGTATTCCAAGCCTTTG	53	416	(25)
		tetB R	CTAAGCACTTGTCTCCTGTT	57		
	TetC	tetC F	TCTAACAATGCGCTCATCGT	56	570	(25)
		tetC R	GGTTGAAGGCTCTCAAGGGC			
Quinolones	*aac(6)'-Ib-cr*	aac(6)'-Ib-cr F	TTGCGATGCTCTATGAGTGGCTA	53	482	(57)
		aac(6)'-Ib-cr R	CTCGAATGCCTGGCGTGTTT			
	qnrA	qnrA F	AGAGGATTTCTCACGCCAGG	57	580	(58)
		qnrA R	TGCCAGGCACAGATCTTGAC			
	qnrB	qnrB F	G**C**MATHGAAATTCGCCACTG	57	264	(58)
		qnrB R	TTTGCYGYYCGCCAGTCGAA			
	qnrS	qnrS F	GCAAGTTCATTGAACAGHGGT	57	428	(58)
		qnrS R	TCTAAACCGTCGAGTTCGGCGCG			
Sulfamide	*Sul1*	Sul1 F	TGGTGACGGTGTTCGGCATTC	62°C	789	(25)
		Sul1 R	GCGAGGGTTTCCGAGAAGGTG			
	Sul2	Sul2 F	CGGCATCGTCAACATAACC	50° C	722	(25)
		Sul2 R	GTGTGCGGATGAAGTGAG			
Aminoglycoside	*aac(3)-II*	aac(3)-II F	ACTGTGATGGGATACGCGTC	57°C	300	(59)
		aac(3)-II R	CTCCGTCAGCGTTTCAGCTA			
	aadA(1-2)	aadA(1ou2) F	GCAGCGCAATGACATTCTTG	54	300	(25)
		aadA (1ou2) R	ATCCTTCGGCGCGATTTTG			
	aadA(5)	aadA(5) F	CTTCAGTTCGGTGAGTGGC	53	500	(25)
		aadA(5) R	CAATCGTTGCTTTGGCATAT			
Integrons	*IntI1*	Int1 F	GGGTCAAGGATCTGGATTTCG	62	483	(25)
		Int1 R	ACATGCGTGTAAATCATCGTCG			
	IntI2	IntI2 F	CACGGATATGCGACAAAAAGGT	62	788	(25)
		IntI2 R	GTAGCAAACGAGTGACGAAATG			
	qacEΔ1	qacEΔ1 F	GGCTGGCTTTTTCTTGTTATCG	62	287	(25)
		qacEΔ1 R	TGAGCCCCATACCTACAAAGC			
	qacEΔ1 + sul1	qacEΔ1 + sul1 F	GGCTGGCTTTTTCTTGTTATCG	63	1125	(25)
		qacEΔ1 + sul1 R	GCGAGGGTTTCCGAGAAGGTG			
	Variable region of the integron class 1	RV(IntI1) F	GGCATCCAAGCAGCAAG	55	Variable	(25)
		RV(IntI1) R	AAGCAGACTTGACCTG A			
	Variable region of the integron class 2	RV(IntI2) F	GATGCCATCGCAAGTACGAG	60	Variable	(25)
		RV(IntI1) R	CGGGATCCCGGACGGCATGCACGA			