Colistin Resistance Enterobacteriaceae Isolated from Arthropods in Gifu City, Japan

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Research article

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Abstract

Background

The emergence of antimicrobial-resistant (AMR) bacteria is an important concern for public and livestock health. Arthropods may serve as vectors that disseminate AMR bacteria across different environments. We examined the phenotype and genotype of antimicrobial resistance in *Enterobacteriaceae* collected from arthropods in Gifu city, Japan.

Results

A total of 186 *Enterobacteriaceae* from 94 arthropods were obtained and tested for antimicrobial susceptibility. All isolates were susceptible to the antimicrobial agents tested, except for colistin (39 isolates) and kanamycin (one isolate). The *aph*(3')-Ia gene and amino acid substitutions in the two-component system were responsible for the kanamycin and colistin resistance, respectively.

Conclusion

Overall *Enterobacteriaceae* isolated from the arthropods were susceptible to most of the antimicrobial agents. However, a high prevalence of colistin resistance was observed in the isolates from the arthropods. We suspect that this was a result of the production of antimicrobial peptides by the arthropods rather than selective pressure or exposure to colistin in the environment. Thus, arthropods maybe a potential reservoir of colistin resistant bacteria. These findings could be beneficial to public and livestock health management.

Background

The wide occurrence of antimicrobial-resistant (AMR) bacteria in various fields such as medicine, veterinary, and agriculture is of great concern and necessitates investigation into emerging AMR bacteria in various environments. Arthropods are a group of ubiquitous creatures including *Hexapoda* (insects in a broad sense), *Crustacea* such as pill bugs, *Chelicerata* such as spiders and *Myriapoda* such as centipedes that interact extensively with their environment [1]. They generally harbor complex microbial communities, and both gram-negative (*Enterobacter* spp., *Klebsiella* spp.) and gram-positive bacteria (*Enterococcus* spp., *Staphylococcus* spp.) resistant to antimicrobial agents have been isolated from them [2].

Colistin (CST) is used as a last resort for the treatment of multi-drug-resistant gram-negative bacteria. The initial mode of action for CST is to attach to a negatively charged component of the lipopolysaccharides (LPS) found in gram-negative bacteria cell membranes. It competitively displaces divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids, resulting in the disruption of the outer cell membrane that leads to a leakage of intracellular contents and bacterial cell death [3]. Gram-negative bacteria use different strategies, including genetic mutations in the PhoPQ/PmrAB two-component system and acquisition of the plasmidic mobile colistin resistant gene (*mcr*), to achieve modification of the LPS [4]. The mutations in the PhoPQ/PmrAB two-component system result in the reduction of the binding affinity of CST through the addition of either phosphoethanolamine or 4-amino-4-deoxy-L-arbinose to the lipid A moiety of the LPS which increases the net positive charge, whereas the *mcr* can only lead to addition of phosphoethanolamine to the LPS resulting in an increase in net positive charge but this can confer high CST resistance [5].

Arthropods produce antimicrobial peptides to perform various functions including prevention of bacterial infections and mediating symbiotic relationships between the host and beneficial bacteria [6]. It could therefore be expected that the symbiotic bacteria of arthropods have developed resistance to antimicrobial peptides. Antimicrobial peptides exhibit a similar mode of action to CST against gram-negative bacteria as described above. The difference is that antimicrobial peptides can also translocate into the cytoplasm and interrupt essential intracellular processes resulting in bacteria death [7].

Arthropods may serve as vectors that disseminate AMR bacteria across different environments. For example, houseflies have been associated with the maintenance and dissemination of cephalosporin and colistin resistant *Enterobacteriaceae* [8]. In another study, multi-drug-resistant bacterial species were isolated from cockroaches [9]. *Enterobacteriaceae* are prevalent in the intestinal tract of humans and animals [10]. Members of this family have been opportunistically implicated in blood-stream, intra-abdominal, skin, soft-tissue, and urinary tract infections [11]. We examined the phenotype and genotype of antimicrobial resistance in *Enterobacteriaceae* from arthropods in order to better understand the emergence of AMR bacteria in the environment.

Materials And Methods

A total of 94 arthropods comprising *Hexapoda* (*n* = 49), *Myriapoda* (*n* = 32), *Chelicerata* (*n* = 10), and *Crustacea* (*n* = 3) were collected at Gifu university and Mount Kinka in Gifu city, Japan between 2016 and 2018 (Table 1). Flying and walking arthropods were caught using net traps and forceps, respectively, then euthanized using carbon dioxide in separate bags [12] and were returned to the laboratory for bacterial isolation from the arthropods' whole body. Bacterial isolation was performed using deoxycholate hydrogen sulfide lactose agar medium and incubated at 37 ºC overnight. API20E (BioMerieux Tokyo, Japan) was used for bacterial identification. The minimum inhibitory concentration was determined using frozen plates (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The following 10 antimicrobial agents were tested: cefotaxime (CTX,

0.5–64 μg/mL), meropenem (MEM, 0.25–32 μg/mL), gentamicin (GEN, 0.5–64 μg/mL), kanamycin (KAN, 1–128 μg/mL), tetracycline (TET, 0.5–64 μg/mL), nalidixic acid (NAL, 1–128 μg/mL), ciprofloxacin (CIP, 0.03–4 μg/mL), colistin (CST, 0.12–16 μg/mL) chloramphenicol (CHL, 1–128 μg/mL), and sulfamethoxazole-trimethoprim (SXT, 2.38/0.12–152/8 μg/mL). Clinical and Laboratory Standards Institute guideline breakpoints [13] were used for all antimicrobials except for CST. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) resistance breakpoints [14] were used for CST.

Table 1
Classification and number of arthropods used in the study

Subphylum	Order	Family	English names	Scientific name	
					Total sample
					No. positive/tested (%positive)
Hexapoda	*Orthoptera*	*Acrididae*	Grasshopper	*Hieroglyphus daganensis*	9/9(100)
		Gryllidae	Cricket	*Meloimorpha japonica*	2/2(100)
		Subtotal			11/11(100)
	Coleoptera	*Lucanidae*	Stag beetle	*Lucanus maculifemeratus*	4/5(80)
		Tenebrionidae	Mealworm	*Tenebrio molitor*	5/7(71.4)
		Tenebrionidae	Darkling beetle	*Allecula melanaria*	0/1(0)
		Scarabaeidae	Dung beetle	*Popillia japonica*	2/2(100)
		Elateridae	Click beetle	*Cryptalaus larvatus*	1/1(100)
		Coccinellidae	Lady beetle	*Harmonia axyridis*	2/2(100)
		Subtotal			14/18(78)
	Hemiptera	*Reduviidae*	Assassin bug	*Agriosphodrus dohrni*	2/5(40)
		Pentatomidae	Stink bug	*Halyomorpha halys*	0/1(0)
		Cicadellidae	Leafhopper	*Orientus ishidae*	0/1(0)
		Subtotal			2/7(29)
	Lepidoptera	*Papilionidae*	Butterfly	*Luehdorfia japonica*	1/1(100)
		Pieridae	White butterfly	*Peris rapae*	0/2(0)
		Saturniidae	Moth	*Antheraea yamamai*	0/2(0)
		Subtotal			1/5(20)
	Dermaptera	*Forficulidae*	Earwig	*Gonolabis marginalis*	5/5(100)
	Diptera	*Tipulidae*	Crane fly	*Tipula aino*	0/1(0)
	Hymenoptera	*Formicidae*	Large ant	*Camponotus japonicus*	0/1(0)
	Mantodea	*Mantidae*	Praying mantis	*Tendora sinensis*	1/1(100)
	Subtotal				34/49(69.4)
Myriapoda	*Polydesmida*	*Eurymerodesmidae*	Millipede	*Eurymerodesmus spp.*	11/11(100)
	Scutigeromorpha	*Scutigeridae*	Centipede	*Thereuonema tuberculata*	13/21(62)
	Subtotal				24/32(75)
Chelicerata	*Araneae*	*Liphistiidae*	Spider	*Heptathela kimurai*	0/10(0)
Crustacea	*Isopod*	*Armadillidiidae*	Pill bugs	*Armadillidium vulgare*	3/3(100)
Total					61/94(64.9)

Whole genome sequencing (WGS) was conducted on one CST-resistant *Enterobacter* spp. and one KAN-resistant *Klebsiella* spp. by Illumina Miseq sequencer (Illumina, California, USA) according to the manufacturer's instructions. Resistance genes and plasmids were investigated using ResFinder [15] and PlasmidFinder [16]. The *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, and *mcr*-5 genes were investigated in all CST-resistant isolates using primers described elsewhere [17–19]. In addition, amino acid substitution in the two-component system genes (*phoP, phoQ, pmrA*, and *pmrB*) associated with CST resistance in *Enterobacter* spp. were investigated using primers (Table 2) based on a sequence of CST-resistant *Enterobacter* spp. using eurofins

genome primer design tool [20]. A comparison of the sequences of the genes; *phoP, phoQ, pmrA*, and *pmrB*, in our CST resistant isolates, CST susceptible isolates, and those of CST susceptible reference sequence *Enterobacter cloacae* ATCC 13047(CP001918) was performed using T-coffee software [21] to identify amino acid substitution.

Statistical analysis

Chi squared ($\chi 2$) tests were performed to determine difference in CST resistance among the group of arthropods. A p-value of < 0.05 was determined to be significant.

Table 2
Primers used to amplify two-component system genes

Gene	Primer name	Sequence (5'-3')
phoP	phoPfoward	CGTACTGGTTGTTGAGGATAACGC
	phoPreverse	TTAGCGTAATTCGAACAGGTAGCC
phoQ	phoQforward	GGGTTCGTTTCTTACTGGCAAC
	phoQreverse	TTAACTATCGTTCAATGTGGGCTG
pmrA	pmrAforward	GTTGAAGACGATCTGTTATTGC
	pmrAreverse	TTTCTGGCTCTCCAGACGGTAG
pmrB	pmrBforward	AGCTGATAAGCGTTATCTGGC
pmrB	pmrBreverse	AGCTGGCGATCAAACCACCTTC

Results And Discussion

Enterobacteriaceae were isolated from 61 of the 94 arthropods: 3 of 3 *Crustacea* samples, 24 of 32 *Myriapoda* samples and 34 of 49 *Hexapoda* samples (Table 1). In total, 186 isolates were obtained: 114 from *Hexapoda*, 64 from *Myriapoda*, and 8 from *Crustacea* (Table 3). The most frequent bacterial species was *Enterobacter* spp. ($n = 61$), followed by *Pantoea* spp. ($n = 26$), *Cedecea* spp. ($n = 18$), *Serratia* spp. ($n = 18$), *Hafnia* spp. ($n = 16$), and *Klebsiella* spp. ($n = 13$). In this study, diverse *Enterobacteriaceae* were isolated from the arthropods. *Enterobacter* spp., the predominant bacteria isolated, have been responsible for intraperitoneal infection in humans [10]. The next most frequently isolated *Enterobacteriaceae* — *Pantoea* spp., *Hafnia* spp., *Serratia* spp., *Cedecea* spp., and *Klebsiella* spp. have been associated with opportunistic infections in humans. *K. pneumoniae* and *K. oxytoca* are known to cause urinary tract infections, pneumonia, and sepsis in patients with compromised immunity [11]. In contrast, some arthropods are known to form symbiotic relationship with these *Enterobacteriaceae* species to enable the breakdown of ingested food such as cellulose for easy digestion and nutrient assimilation [22].

Table 3
Bacterial species isolated from arthropods

Bacteria		Subphylum: Sample No. (Isolate No.)			
Genus	Species	Hexapoda	Myriapoda	Crustacea	Total
Enterobacter	*cloacae*	13(24)	2(4)	1(1)	15(29)
	amnigenus	4(6)	12(20)	0	16(26)
	aerogenes	1(1)	2(2)	0	3(3)
	asburiae	2(3)	0	0	2(3)
	Subtotal	19(34)	16(26)	1(1)	36(61)
Pantoea	*Pantoea* spp.	11(17)	4(5)	2(4)	17(26)
Cedecea	*lapagei*	5(9)	1(2)	1(1)	7(12)
	davisae	3(6)	0	0	3(6)
	Subtotal	8(15)	1(2)	1(1)	10(18)
Serratia	*marcescens*	6(10)	1(2)	0	7(12)
	liquefaciens	0	3(5)	0	3(5)
	fonticola	0	1(1)	0	1(1)
	Subtotal	6(10)	4(8)	0	11(18)
Hafnia	*Alvei*	6(13)	2(3)	0	8(16)
Klebsiella	*pneumoniae*	3(3)	4(5)	0	7(8)
	oxytoca	5(5)	0	0	5(5)
	Subtotal	8(8)	4(5)	0	12(13)
Escherichia	*vulneris*	2(2)	3(3)	0	5(5)
	Coli	0	1(2)	0	1(2)
	hermannii	0	1(1)	0	1(1)
	Subtotal	2(2)	5(6)	0	7(8)
Rahnella	*aquatilis*	3(5)	1(1)	0	4(6)
Citrobacter	*freundii*	2(3)	0	0	2(3)
	youngae	1(1)	2(2)	0	3(3)
	Subtotal	3(4)	2(2)	0	5(6)
Cronobacter	*Cronobacter* spp.	2(5)	0	0	2(5)
Raoutella	*ornitholytica*	0	1(1)	1(1)	2(2)
	planticola	0	1(1)	0	1(1)
	Subtotal	0	2(2)	1(1)	3(3)
Kluyvera	*intermedia*	1(1)	2(2)	0	3(3)
Leclercia	*adecaboxylata*	0	1(1)	1(1)	2(2)
Buttiauxella	*agrestis*	0	1(1)	0	1(1)
Total		34(114)	24(64)	3(8)	61(186)

Arthropod-derived *Enterobacteriaceae* isolated in this study were susceptible to all antimicrobial agents tested except CST and KAN. CST resistance was observed in 39 (29%) of the 134 isolates, excluding *Cedecea* spp., *Hafnia* spp. and *Serratia* spp. which have intrinsic resistance. The 39 isolates resistant to CST were found in *Enterobacter* spp. (25/61, 41.0%), *Pantoea* spp. (6/26, 23.1%), *Klebsiella* spp. (7/13, 53.8%) and *Escherichia* spp. (1/8, 12.5%). In *Enterobacter* spp., CST resistance was found in *Hexapoda*— grasshopper (7), cricket (2), mealworm (1), dung beetle (1), butterfly (1) and *Myriapoda*— millipede (1), centipede (1). In *Pantoea* spp., CST resistance was found in *Hexapoda*— grasshopper (2), earwig (2). In *Klebsiella* spp., CST resistance was found in *Hexapoda*— cricket (1), mealworm (1), assassin bug (1), earwig (1) and *Myriapoda*— millipede (2), centipede (1). In *Escherichia*

spp., CST resistance was only found in *Hexapoda*– earwig (1), but no CST resistance in *Crustacea* (Table 4). The percentage of CST resistance was significantly high in *Enterobacter* isolates from *Hexapoda* (19/34, 55.9%) than *Myriapoda* (6/26, 23.1%: $P < 0.05$). Hexapods are obligate hosts of bacterial species, more so than any other group of arthropods [23], and as such, these bacterial species have developed resistance to survive the defense mechanism produced by the host [24].

Table 4
Colistin resistance observed in Enterobacteriaceae isolated from arthropods

Subphylum	English name	*Enterobacteriaceae* species: No. of Positive Samples (Isolate No.)											
		Enterobacter spp.			*Pantoea* spp.			*Klebsiella* spp.			*Escherichia* spp.		
		No. tested	CST resistant	%	No. tested	CST resistant	%	No. tested	CST resistant	%	No. tested	CST resistant	%
Hexapoda	Grasshopper	9(19)	7(11)	57.9	5(9)	2(3)	33.3	0	0	0	1(1)	0	0
	Cricket	2(3)	2(2)	66.7	1(1)	0	0	1(1)	1(1)	100	0	0	0
	Mealworm	1(1)	1(1)	100	0	0	0	1(1)	1(1)	100	0	0	0
	Dung beetle	2(2)	1(1)	50	0	0	0	0	0	0	0	0	0
	Click beetle	1(2)	1(1)	50	0	0	0	1(1)	0	0	0	0	0
	Stag beetle	2(2)	0	0	0	0	0	0	0	0	0	0	0
	Lady beetle	0	0	0	1(1)	0	0	0	0	0	0	0	0
	Butterfly	1(4)	1(3)	75	1(1)	0	0	0	0	0	0	0	0
	Assassin bug	1(1)	0	0	0	0	0	3(3)	1(1)	33.3	0	0	0
	Earwig	0	0	0	3(5)	2(3)	60	2(2)	1(1)	50	1(1)	1(1)	100
	Subtotal	19(34)	13(19)	55.9	11(17)	4(6)	35.3	8(8)	4(4)	50	2(2)	1(1)	50
Myriapoda	Millipede	12(18)	1(3)	16.7	2(3)	0	0	3(4)	2(2)	50	1(2)	0	0
	Centipede	4(8)	1(3)	37.5	2(2)	0	0	1(1)	1(1)	100	4(4)	0	0
	Subtotal	16(26)	2(6)	23.1	4(5)	0	0	4(5)	3(3)	60	5(6)	0	0
Crustacea	Pill bugs	1(1)	0	0	2(4)	0	0	0	0	0	0	0	0
Total		36(61)	15(25)	41	17(26)	4(6)	23.1	12(13)	7(7)	53.8	7(8)	1(1)	12.5
CST- colistin													

The *mcr*-1-5 genes were not detected in any of the CST-resistant isolates. The absence of *mcr*-1-5 gene in the CST-resistant isolates observed in this study prompted us to investigate the PhoPQ/PmrAB two-component system. We examined amino acid substitution in the PhoPQ/PmrAB two-component system in CST-resistant *Enterobacter* isolates and compared them to those of CST-susceptible *Enterobacter* strains. Our investigation showed various amino acid substitutions: three amino acid substitution in phoP (L129I, F141L, H207Q), five in phoQ (V102I, L133I, M298L, S448A, G464S), four in pmrA (A19G, S21A, N89T, L146Q) and four in pmrB (H132S, A172T, Q271V, R276Q). The amino acid substitutions observed in phoP, phoQ, and pmrA in this study corresponded with those reported by Uechi *et al.* [25]. On the other hand, the amino acid substitutions observed in this study were different from those reported in another study by Nawfal Dagher *et al.* [26]. We could not clarify the specific amino acid substitution responsible for the CST resistance observed. Arthropods rarely have direct contact with CST because of restrictions on CST usage for humans and pigs in Japan. Hence, the observed CST resistance was unexpected and as such it is not plausible to attribute this CST resistance to selective pressure or exposure to CST in the environment. Most arthropods have antimicrobial peptides in their hemolymph that serve as a defense mechanism and plays an important role in fostering symbiotic relationships with beneficial bacteria [6]. It therefore stands to reason that symbiotic bacteria may have developed resistance to antimicrobial peptides. The initial mode of action of antimicrobial peptides and CST against gram-negative bacteria involves binding to the LPS [6], this may have led to the development of cross-resistance to CST [27].

One isolate of *K. oxytoca* from a butterfly in this study showed resistance to KAN. WGS analysis revealed that the resistance gene *aph*(3')-Ia was located on the chromosome of *K. oxytoca*. The *aph*(3')-Ia gene was first discovered on transposon Tn*903* in *Escherichia coli* [28] and has subsequently been found on plasmids and chromosomes of clinical and veterinary *Enterobacteriaceae* isolates [29–30]. The presence of *aph*(3')-Ia in arthropods may suggest the possibility that arthropods received this bacterium from human and/or domestic animals or vice versa.

In previous studies, *Enterobacteriaceae* isolated from cockroaches showed resistance to more than two antimicrobial agents [9, 31]. In Japan, *Enterobacter* spp. isolated from companion animals showed resistance to CTX (33.3%), GM (23.3%), TC (40%), CPFX (43.3%), and CP (46.7%) and were

reported to be extended spectrum beta-lactamase (ESBL) producers [32]. In addition, *Klebsiella* spp. isolated from companion animals were resistant to aminoglycosides and quinolones and were reported to be extended beta-lactamase producers [33]. However, susceptibility to antimicrobial agents were high, as expected in this study. The high susceptibility observed indicate an absence or low prevalence of AMR bacteria and minimal antimicrobial agents' pollution in the immediate environment of the arthropods investigated.

The present study did not compare the isolation rate of bacteria on the external surface and alimentary tract of the arthropods. However, there was no significant difference between the isolation rate of bacteria on the external surface and the alimentary tract of cockroaches in a previous report [9].

Conclusion

In conclusion, the *Enterobacteriaceae* isolated from the arthropods were susceptible to most of the antimicrobial agents. However, high prevalence of CST resistance was observed in the isolates from the arthropods. We suspect that this was a result of the production of antimicrobial peptides by the arthropods rather than selective pressure or exposure to CST in the environment. These findings could be beneficial to public and livestock health management as well as present novel approaches to exploring bacterial adaptations to its environment and the impact this has on the larger ecosystem.

Abbreviations

AMR- Antimicrobial-resistant

CHL- Chloramphenicol

CIP- Ciprofloxacin

CST- Colistin

CTX- Cefotaxime

EUCAST- The European Committee on Antimicrobial Susceptibility Testing

GEN- Gentamicin

KAN- Kanamycin

LPS- Lipopolysaccharides

MEM- Meropenem

NAL- Nalidixic acid

SXT- Sulfamethoxazole-trimethoprim

TET- Tetracycline

WGS- Whole genome sequencing

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data

The sequence datasets generated and analyzed during the current study have been deposited into DDBJ/EMBL/GenBank databases (https://www.ncbi.nlm.nih.gov/nuccore/)under the accession number: MN944620 to MN944643 and MN944719 (*pmrB* gene), MN944644 to MN944668 (*pmrA* gene), MN944669 to MN944693 (*phoP* gene), and MN944694 to MN944718 (*phoQ* gene).

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

MY and MS performed sample collection and laboratory work. JOO processed and analyzed sequence data. JOO and TA wrote the manuscript. All authors read and approved the manuscript.

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