https://doi.org/10.22319/rmcp.v12i1.5378

Technical note

Prevalence of the *qnrB*, *qnrA* and *bla*$_{TEM}$ genes in temperate bacteriophages of *Escherichia coli* isolated from wastewater and sewer water from slaughterhouses in the State of Mexico

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Abstract:

Antibiotic resistance genes (ARG) have been described mainly in bacteria, but are known to occur in temperate phages. Prevalence of the *qnrB*, *qnrA* and *bla*$_{TEM}$ genes was identified in *Escherichia coli* strains and temperate phages by lytic cycle induction. From a total of 48 samples collected from drinking water, wastewater and sewer water in slaughterhouses in the State of Mexico, Mexico, 37 contained *E. coli* isolates. Resistance was highest to tetracycline (32/37; 86.4 %), followed by trimethoprim-sulfamethoxazole (19/37; 51.3 %) and ampicillin and nalidixic acid (18/37; 48.6 %). Prevalence of the *bla*$_{TEM}$ gene was 37.8 % in the bacterial isolates and 3.5 % in the phage isolates. The bacterial isolates contained 8.1 % *qnrA* and 29.7 % *qnrB* genes, while the phage isolates contained 2.7 and 24.3 %, respectively. Presence

of ARG in the bacterial isolates was linked to phage DNA, highlighting the significant role it plays in the spread of ARG in the studied slaughterhouses. Understanding the mechanisms of antimicrobial resistance will contribute to developing effective control measures.

Key words: Bacteriophages, *Escherichia coli,* Genes, Resistance.

Received: 13/05/2019

Accepted:31/03/2020

Among the most important medical advances of the last century, antibiotic therapy is a vital resource in the fight against infectious bacterial diseases. However, its widespread use has led to the advent of antibiotic-resistant bacteria[1]. Rapid spread of resistant strains, driven in part by human migration and the increasing industrialization of food and animal production, is a recogized worldwide health problem. One cause of strains developing resistance is the presence of antibiotic resistance genes (ARG), which can be acquired and transferred through mobile genetic elements (MGE) such as bacteriophages[2].

Lysogenic bacteriophages containing ARG have been identified, largely in aquatic environments[2,3]. They have also been reported in fecal samples collected in hospitals from clinically healthy patients, suggesting that these phages may be universally present though undetected in health safety screening programs[4,5]. Various studies of lysogenic bacteriophages have detected ARGs that have claimed the lives of thousands of people and generated millions of dollars in losses worldwide. These studies have also elucidated phages' contribution to the spread of ARGs in the environment[1]. The present study objective was to improve understanding of bacteriophages' role in the spread of ARG by quantifying the prevalence of the bla_{TEM}, *qnrA* and *qnrB* genes in bacterial and phage DNA.

Water samples were collected from municipal slaughterhouses (SLH1, SLH2, SLH3 and SLH4) in the State of Mexico, Mexico, from September to December 2015. Samples were collected based on probabilistic criteria[6], and following official guidelines[7]. A total of 48 samples were collected from the animal processing area of each slaughterhouse: 16 from potable water (PW); 16 from wastewater (WW); and 16 from sewers (SW). Each sample was collected with a swab, which was then rubbed along the edges of the lid surface[7]. Genotypic confirmation of *E. coli* isolation was done using an endpoint PCR to amplify the *uidA* gene (primers listed in Table 1), under established conditions[8]. Antibiotic susceptibility was quantified using fourteen antimicrobial substances in different concentration ranges following the disk diffusion method guidelines of CLSI[9]. Results interpretation was done according to CLSI guidelines[10].

Table 1: Specific primers used in PCR analysis

Primer	Sequence (5'--- 3')	Gene	Reference
UAL1939b	ATGGAATTTCGCCGATTTTGC	*uidA*	Aguilar *et al*. 2015
UAL2105b	ATTGTTTGCCTCCCTGCTGC		
MultiTSO-F_for	CATTTCCGTGTCGCCCTTATTC	*bla*$_{TEM}$	Dallene *et al*. 2010
MULTITSO-T_rev	CGTTCATCCATAGTTGCCTGAC		
QnrAm_F	AGAGGATTTCTCACGCCAGG	*qnrA*	Kraychete *et al*. 2016
QnrAm_R	TGCCAGGCACAGATCTTGAC		
QnrBm_F	GGMATHGAAATTCGCCACTG	*qnrB*	
QnrBm_R	TTTGCYGYYCGCCAGTCGAA		

Phage isolation was done with mitomycin C, and phage lysis verified with the spot-test method and double layer test[11]. Phage DNA was isolated with the phenol-chloroform method[12]. Bacterial DNA removal and the presence of phage DNA were confirmed with an endpoint PCR test in a Multigene^{TM} Mini Personal thermal cycler (Labnet International Inc., Edison, NJ, USA) with *uidA* amplification as a negative control[3].

Bacterial DNA extraction was done following a published protocol[13], and phage and bacterial DNA concentration and purity quantified with a spectrometer (Quawell q500). Endpoint PCR was used to detect the *qnrA, qnrB* and *bla*$_{TEM}$ genes, under previously reported conditions[14,15]. Statistical analyses consituted an analysis of variance (ANOVA) and a linear correlation test run using StatCalc ver. 8.2.2 (Copyright © 2016 AcaStat Software). Significance was set at $P<0.05$.

Of the 48 collected samples, 37 (77 %) produced *Escherichia coli* isolates with varying resistance indices (Table 2). Of the 37 isolates, 13 (35.1 %) were from SLH1, while 8 each (21.6 %) were from SLH2, SLH3 and SLH4. The higher ($P<0.05$) number of isolates from SLH1 was probably a function of the larger number of animals pocessed there compared to the other slaughterhouses. A positive correlation (r= 1) was observed between the 19 (51.3 %) isolates from wastewater samples and the 18 (48.6 %) from sewage samples. This is to be expected since both sites exhibit similar conditions which are adequate for bacterial growth.

Table 2: Antibiotic resistance patterns for *E. coli* isolates from wastewater and sewer water collected from municipal slaughterhouses in northern State of Mexico

Antib/Conc (μg)	**SLH1**			**SLH2**			**SLH3**			**SLH4**		
	WW	SW	Total (%)	WW	SW	Total (%)	WW	SW	Total (%)	WW	SW	Total (%)
Ampicillin (10)	4	3	7/18 (38.8)	0	2	2/18 (11.1)	4	1	5/18 (27.7)	2	2	4/18 (22.2)
Amikacin (30)	0	0	0/1 (0)	0	0	0/1 (0)	1	0	1/1 (100)	0	0	0/1 (0)
Carbenicillin (100)	4	3	7/19 (36.8)	2	2	4/19 (21)	3	1	4/19 (21)	2	2	4/19 (21)
Gentamicin (10)	1	1	2/5 (40)	0	0	0/5 (0)	2	1	3/5 (60)	0	0	0/5 (0)
Cefalotin (30)	0	1	1/5 (20)	1	0	1/5 (20)	1	0	1/5 (20)	1	1	2/5 (40)
Cefotaxime (30)	0	0	0/0 (0)	0	0	0/0 (0)	0	0	0/0 (0)	0	0	0/0 (0)
Netilmicin (30)	0	0	0/1 (0)	0	0	0/1 (0)	0	0	0/1 (0)	1	0	1/1 (100)
Cyprofloxacin (5)	0	0	0/3 (0)	1	1	2/3 (66.6)	1	0	1/3 (33.3)	0	0	0/3 (0)
Norfloxacin (10)	0	1	1/3 (33.3)	1	0	1/3 (33.3)	1	0	1/3 (33.3)	0	0	0/3 (0)
Cloramphenicol (30)	7	3	10/23 (43.4)	4	3	7/23 (30.4)	1	1	2/23 (8.6)	2	2	4/23 (17.3)
Trimethoprim-sulfamethoxasole (25)	7	4	11/19 (57.8)	1	3	4/19 (21)	1	0	1/19 (5.2)	2	1	3/19 (15.7)
Nitrofurantoin (300)	0	1	1/6 (16.6)	2	0	2/6 (33.3)	1	0	1/6 (16.6)	2	0	2/6 (33.3)
Nalidixic acid (30)	1	2	3/18 (16.7)	2	3	5/18 (27.7)	2	4	6/18 (33.3)	3	1	4/18 (22.2)
Tetracycline (30)	7	5	12/32 (37.5)	3	4	7/32 (21.8)	3	3	6/32 (18.7)	3	4	7/32 (21.8)

Antib= antibiotic; WW = wastewater; SW = sewer water; Conc = concentration.

The number of isolates with ARG was higher in wastewater than in sewer water ($P<0.05$), and there were no isolates from the potable water samples (Table 3). Most probably due to the wide range of ARG variants and their different resistance mechanisms, the identified bacterial isolates which exhibited intermediate phenotypic resistance and susceptiblility also amplified *bla*$_{TEM}$, *qnrA* and *qnrB.*

Table 3: Phenotype/genotype characterization and relation of bacterial and phage isolates collected from municipal slaughterhouses in northern State of Mexico

Antibiotic	Isolate source and resistance pattern		Bacterial DNA gene type (count)	Phage DNA gene type (count)
Ampicillin	WW[a]	R= 10	*bla*$_{TEM}$ (7)	*bla*$_{TEM}$ (2)
		I= 1	*bla*$_{TEM}$ (1)	-
		S= 8	*bla*$_{TEM}$ (2)	-
	SW[b]	R= 8	*bla*$_{TEM}$ (3)	*bla*$_{TEM}$ (3)
		I= 2	-	-
		S= 8	*bla*$_{TEM}$ (1)	-
Nalidixic acid	WW[a]	R= 8	*qnrA* (2), *qnrB* (3)	*qnrA* (1), *qnrB* (1)
		I= 7	*qnrA* (1), *qnrB* (3)	*qnrB* (1)
		S= 4	*qnrB* (1)	-
	SW[b]	R= 10	*qnrB* (2)	*qnrB* (2)
		I= 3	*qnrB* (1)	-
		S= 5	*qnrB* (1)	-

WW = wastewater; SW = sewer water; R = resistant; I = intermediate resistance; S = susceptible.
[abc] Different letter superscripts in the same column indicate statistical difference (P<0.05).

A bacteriophage pool was obtained from each bacterial isolate for detection of *bla*$_{TEM}$, *qnrA* and *qnrB*; all the isolates presented temperate phages. The *bla*$_{TEM}$ gene was the most prevalent in all the bacterial and phage isolates (Table 4). These results coincide with a previous study of water samples collected from wastewater treatment plants and slaughterhouses in which *bla*$_{TEM}$ was highly prevalent (80 to 100 %) and had high gene copy densities (± 3.3 $\log_{10}$)[(3)]. The *bla*$_{TEM}$ gene is the most frequently reported worldwide[(16,17)], especially in Gram negative bacteria. This is assumed to be due to its broad dissemination via migratory waterfowl and the large number of β-lactamase enzymes synthesized by bacteria. In the bacterial isolates, prevalence of *qnrA* was 8.1 % and that of *qnrB* was 29.7 %, while in the phage isolates it was 2.7 and 10.8 %, respectively. In the phage isolates these prevalences contrast with previously reported prevalences in wastewater samples[(3,18)]. High *qnrA* prevalences have also been reported in samples obtained from a health center[(4)]. Although the observed prevalence is not high, quinolone resistance has been increasing due to indiscriminate use in veterinary and human medicine[(18)].

Table 4: Distribution of *bla$_{TEM}$*, *qnrA* and *qnrB* in bacterial and phage isolates from samples collected from municipal slaughterhouses in northern State of Mexico

Slaughterhouse/ isolate count	*bla$_{TEM}$*		*qnrA*		*qnrB*	
	Bacterial DNA (%)	Phagic DNA (%)	Bacterial DNA (%)	Phagic DNA (%)	Bacterial DNA (%)	Phagic DNA (%)
SLH1/13	3 (23)	1 (7.6)	1 (7.6)	-	8 (61.5)	2 (15.3)
SLH2/8	4(50)	1 (2.5)	1 (12.5)	1 (12.5)	1 (12.5)	1 (12.5)
SLH3/8	4/(50)	1 (2.5)	-	-	1 (12.5)	1 (12.5)
SLH4/8	3 (37.5)	2 (2.5)	1 (12.5)	-	1 (12.5)	-
Total= 37	14 (37.5)	5 (13.5)	3 (8.1)	1 (2.7)	11 (29.7)	4 (10.8)

The amount of ARG identified in the bacterial DNA and phage DNA was positively correlated (r= 0.99) (Table 4). This correlation coincides with the widely reported presence, in multi-resistant bacteria, of phages capable of disseminating portions of ARG-containing bacterial genome throughout the environment. These phages can transduce ARG to commensal and pathogenic bacteria, providing them new adaptive (short-term) and evolutive (long-term) abilities, and contributing to generation of new resistant pathogenic strains which are potentially fatal.

The highest number of bacterial isolates exhibiting presence of the three evaluated ARG was recorded in SLH1, while in SLH2 all three genes were found in the phage isolates (Table 4). The contamination and unhygienic conditions in the sampled municipal slaughterouses provide many of the factors required for successful viral transduction: temperature, pH, concentration, and bacterial and phage physiological state.

Human lifestyles promote a steady increase in antibiotic resistance among microbes. Consciousness of the consequences of indiscriminate and unnecessary use of antimicrobials is slowly growing but numerous studies still report highly virulent and resistant bacteria in areas of human activity[(1)]. The bacterial and phage isolates analyzed here exhibited the presence of *bla$_{TEM}$*, *qnrA* and *qnrB* in highly variable distributions; at least one of these genes was present in all the sampled slaughterhouses. Phages belonging to the MGE group play a vital role in the transfer of ARG between pathogenic-commensal and pathogenic-pathogenic bacteria[(5)]. Better understanding of the mechanisms of antimicrobial resistance is an important tool in the ongoing development of effective strategies to reduce this phenomenon.

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