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Abundance of antibiotic resistance genes in environmental bacteriophages

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The ecosystem is continuously exposed to a wide variety of antimicrobials through waste effluents, agricultural run-offs and animal-related and anthropogenic activities, which contribute to the spread of antibiotic resistance genes (ARGs). The contamination of ecosystems with ARGs may create increased opportunities for their transfer to naive microbes and eventually lead to entry into the human food chain. Transduction is a significant mechanism of horizontal gene transfer in natural environments, which has traditionally been underestimated as compared to transformation. We explored the presence of ARGs in environmental bacteriophages in order to recognize their contribution in the spread of ARGs in environmental settings. Bacteriophages were isolated against environmental bacterial isolates, purified and bulk cultured. They were characterized, and detection of ARG and *intI* genes including bla_{TEM}, bla_{OXA-2}, *intI1*, *intI2*, *intI3*, *tetA* and *tetW* was carried out by PCR. This study revealed the presence of various genes [*tetA* (12.7 %), *intI1* (10.9 %), *intI2* (10.9 %), *intI3* (9.1 %), *tetW* (9.1 %) and bla_{OXA-2} (3.6 %)] and bla_{TEM} in a significantly higher proportion (30.9 %). bla_{SHV}, bla_{OXA-1}, *tetO*, *tetB*, *tetG*, *tetM* and *tetS* were not detected in any of the phages. Soil phages were the most versatile in terms of ARG carriage. Also, the relative abundance of *tetA* differed significantly vis-à-vis source. The phages from organized farms showed varied ARGs as compared to the unorganized sector, although bla_{TEM} ARG incidences did not differ significantly. The study reflects on the role of phages in dissemination of ARGs in environmental reservoirs, which may provide an early warning system for future clinically relevant resistance mechanisms.

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INTRODUCTION

The ecosystem is continuously exposed to a wide variety of antimicrobials through agricultural run-off, waste water treatment plant effluents and animal-related and anthropogenic activities. Antimicrobials are extensively used in animals such as poultry for treatment and disease control and as growth promoters (Economou & Gousia, 2015). In environmental settings, interactions between environmental and resistant bacteria provide ideal selective and ecological conditions for the emergence of resistant strains, eventually contributing to the spread of antibiotic resistance genes (ARGs) (Murugan *et al.*, 2012; Pruden *et al.*, 2012; Marti *et al.*, 2013; Berglund *et al.*, 2015; Xu *et al.*, 2015). Among different antibiotics, β-lactams are the most commonly and widely used. Within the resistance encoding β-lactamase genes, temoneira (bla_{TEM}) and sulfhydryl variable (bla_{SHV}) types are members of class A extended-spectrum β-lactamases (ESBLs) (Walsh *et al.*, 2007). Apart from these, many other β-lactamase enzymes have been discovered, and their corresponding resistance encoding genes have been reported (Bush & Jacoby, 2010). The OXA-type enzymes, belonging to molecular class D, are another growing family of ESBLs which differ from the TEM and SHV enzymes. They confer resistance to ampicillin and cephalothin and are characterized by high hydrolytic activity against oxacillin and cloxacillin (Bush *et al.*, 1995). The bla_{OXA-1} and bla_{OXA-2} genes are also widespread in enterobacteria (Livermore, 1995). For veterinary, agricultural and human purposes, tetracyclines are another group of antibiotics which are extensively used. Tetracycline compounds are

Two supplementary tables are available with the online Supplementary Material.

excreted after medication via urine and faeces in active form (~70 %) and thus have significant persistence in aquatic environments owing to their high hydrophilicity and low volatility (Daghrir & Drogui, 2013). Integrons are also critical intermediates in the acquisition and expression of resistance genes, and their gene cassettes have played important roles in genome evolution and fluidity within the bacterial kingdom (Boucher *et al.*, 2007).

The contamination of the environment with unabated and extensive use of antibiotics via sewage, water, soil, etc., poses a serious threat to human patients or diseased animals. In this regard, aquatic environments have represented to be potential reservoir for ARGs, as they allow sewage-derived ARGs to persist and spread in the environment (Czekalski *et al.*, 2015). The contamination of such pristine components of the ecosystem with ARGs may create a congenial environment for their transfer to naive microbes that may lead to their entrance into the human food chain.

The three major mechanisms by which ARGs spread horizontally include conjugation, natural transformation and transduction. Of these, conjugation is the most studied mechanism of horizontal gene transfer (Smillie *et al.*, 2010; Zechner *et al.*, 2012; Arutyunov & Frost, 2013). Transformation of ARGs has been reported on extensively too (Sparling, 1966; Harford & Mergeay, 1973; Prudhomme *et al.*, 2006). Transduction is also a significant mechanism of horizontal gene transfer in naturally occurring environments, but it has been traditionally underestimated. It gained substantial support from studies showing ARGs in abundance in phage particles in nature (Muniesa *et al.*, 2004, 2013; Colomer-Lluch *et al.*, 2011a, b, 2014a, b; Ross & Topp, 2015). A metagenomic analysis of viromes suggests that functional bacterial genes of all types exist in up to 50 to 60 % of bacteriophages and that these particles can serve as reservoirs for genes in diverse environments and act as a mechanism for their transfer among bacteria (Dinsdale *et al.*, 2008). The role of transduction in the transfer of antibiotic resistance among potential pathogens has been demonstrated from *Enterococcus gallinarum* to *Enterococcus faecalis* in *in vitro* experiments (Mazaheri *et al.*, 2011). Other *in vitro* experiments with *Salmonella* spp. transducing bacteriophages demonstrated that ampicillin, chloramphenicol and tetracycline resistance genes could be transduced (Schmieger & Schicklmaier, 1999). Transduction by bacteriophages includes any sort of bacterial DNA, including linear chromosome fragments and mobile elements such as plasmids, transposons, insertion sequences and integrons (Schmieger & Schicklmaier, 1999; Schwarz *et al.*, 2004; Roberts, 2005; Miranda *et al.*, 2013).

The contribution of phages to the spread of ARGs in environmental settings has remained partially explored due to the fact that, although bacteriophage isolation is relatively easier, their characterization involves complicated techniques. In addition to this, further complexity is added on by factors such as high natural abundance of bacteriophages, co-evolution with host, cassette-type constitution of genome and broad host range for a small fraction of phages. Although the role of prophages has been critically cited in literature, the role of generalized transduction, mediated by lytic bacteriophages, has remained only partially explored. In such a case where any gene within a donor can be transferred to a recipient strain has been demonstrated in *Listeria monocytogenes* (Hodgson, 2000), *Salmonella* (Figueroa-Bossi *et al.*, 2001) and *Staphylococcus aureus* isolates (Lindsay & Holden, 2004).

The acquisition of new genes such as ARGs may confer a selective advantage to the bacterial host to increase its ecological fitness and results in clonal expansion providing an ecological success. Thus, understanding the role of bacteriophages in the mobilization of ARGs is critical for environmental health, emergence of resistant strains and development of effective phage therapeutics. Also, it provides an early warning system for future clinically relevant resistance mechanisms. In the present study, we investigated the prevalence of ARGs and *intI* genes in environmental bacteriophage isolates obtained from sewage, soil, water and dung/litter samples and tried to correlate the source with dissemination of these genes.

RESULTS

Isolation and identification of bacteria and bacteriophages

In the present study, diverse bacteria were isolated from samples collected from different environments, viz. soil, sewage, dung/litter, water, etc. (Table 1). Bacteriophages from the same samples were isolated against both Gram-negative and Gram-positive bacteria including *Staphylococcus sciuri*, *Aeromonas hydrophila*, *Escherichia coli*, *Bacillus pumilus*, *Pseudomonas mendocina*, *Paenibacillus* spp., *Sphingobacterium* spp., *Stenotrophomonas* spp., *Acinetobacter baumannii*, *Bacillus firmus*, *Shigella sonnei*, *Bacillus cereus*, *Arthrobacter creatinolyticus*, *Bacillus pichinotyi* and *Caryophanon* spp. Bacteriophages were also enriched from the environmental samples against some bacteria obtained from the National Centre for Veterinary Type Culture Collection (NCVTCC) repository including *Klebsiella pneumoniae*, *Shigella* spp., *Enterobacter* spp., *Serratia marcescens*, *Citrobacter freundii*, *Citrobacter sedlakii*, *Salmonella* Gallinarum and *Escherichia coli*. The bacteriophage isolates obtained were purified and yielded characteristic plaques as described in Table 1. Some phages lead to strong lysis (as indicated by large plaque size) compared to others; plaque size for various phages ranged from 1 to 8 mm diameter in size on nutrient agar (NA). The number of different bacteriophage isolates was observed to be highest against *Bacillus* spp. (19.3 %), followed by *Pseudomonas* spp. (10.5 %), *Salmonella* spp. (10.5 %), *Escherichia coli* (8.8 %) and *Shigella* spp. (8.8 %), although other phages at comparatively lower prevalence rates were also detected (Fig. 1).

Table 1. List of samples used for phage isolation

S. no.	Bacteriophage accession no.	Source of sample used for phage isolation	Host bacteria against which phage isolated	Plaque characteristics
1	VTCCBPA3	Animal fair site soil	*Staphylococcus sciuri*	2–3 mm, clear
2	VTCCBPA4	Animal farm soil	*Bacillus*	1–2 mm, clear
3	VTCCBPA5	Village pond	*Aeromonas hydrophila*	2 mm, clear
4	VTCCBPA6	Equine stud farm sewage	*Aeromonas hydrophila*	1–2 mm, turbid
5	VTCCBPA7	Equine stud farm sewage	*Escherichia coli*	1 mm, clear
6	VTCCBPA8	Village pond	*B. pumilus*	1–2 mm, with halo zone of ~6 mm diameter
7	VTCCBPA9	Equine stud farm soil	*Escherichia coli*	1 mm with hazy margins
8	VTCCBPA10	Equine stud farm soil	*Escherichia coli*	2 mm, clear
9	VTCCBPA11	Soil – animal fair site	*Bacillus* spp.	2 mm, with a halo zone of 6 mm diameter
10	VTCCBPA12	Animal fair site soil	*Bacillus* spp.	2–3 mm, clear
11	VTCCBPA13	Animal farm soil	*B. pumilus*	3–4 mm, with halo zone
12	VTCCBPA14	Pond	*Pseudomonas mendocina*	1–2 mm, clear
13	VTCCBPA15	Pond	*Paenibacillus* spp.	5–6 mm with halo zone
14	VTCCBPA16	Animal farm sewage	*Sphingobacterium* spp.	1 mm, clear, circular
15	VTCCBPA17	Animal farm sewage	*Stenotrophomonas* spp.	2 mm clear, with halo zone
16	VTCCBPA18	Animal farm sewage	*Escherichia coli*	Clear, pinpoint
17	VTCCBPA20	Animal farm soil	*Pseudomonas*	8 mm, clear
18	VTCCBPA22	Animal farm sewage	*Pseudomonas*	3 mm, clear
19	VTCCBPA23	Animal farm soil	*Escherichia coli*	1 mm, clear
20	VTCCBPA24	Animal farm sewage	*Acinetobacter baumannii*	1 mm, with halo zone
21	VTCCBPA25	Poultry litter	*Salmonella* Gallinarum	1–2 mm, clear
22	VTCCBPA26	Poultry litter	*Salmonella* Gallinarum	3 mm, clear
23	VTCCBPA27	Poultry litter	*Salmonella* Gallinarum	1–2 mm, clear
24	VTCCBPA28	Poultry litter	*Salmonella* Gallinarum	2 mm, clear
25	VTCCBPA29	Poultry litter	*Salmonella* Gallinarum	2 mm, clear
26	VTCCBPA30	Poultry litter	*Salmonella* Gallinarum	6 mm, clear
27	VTCCBPA32	Equine shed soil	*Bacillus*	4 mm, clear with rough margin
28	VTCCBPA33	Silage pit soil	*B. firmus*	2–3 mm, clear with diffuse margin
29	VTCCBPA34	Silage pit soil	*B. firmus*	2–3 mm, clear with diffuse margin
30	VTCCBPA37	Village sewage	*Shigella sonnei*	1 mm, clear
31	VTCCBPA38	Equine farm soil	*B. cereus* group/*Bacillus thuringiensis*	Clear, pinpoint
32	VTCCBPA39	Equine farm soil	*Arthrobacter creatinolyticus*	Clear, pinpoint
33	VTCCBPA40	Equine farm soil	*Arthrobacter creatinolyticus*	3–4 mm, clear
34	VTCCBPA41	Equine farm soil	*B. pichinotyi*	Clear, pinpoint
35	VTCCBPA42	Equine farm soil	*Caryophanon* sp.	2–3 mm, clear centre with hazy periphery
36	VTCCBPA43	Ganga water/Banaras	*Pseudomonas* spp.	2–3 mm, clear
37	VTCCBPA44	Ganga water/Banaras	*Pseudomonas* spp.	5 mm, clear
38	VTCCBPA46	Ganga mud/Banaras	*Acinetobacter* spp.	1–2 mm, clear
39	VTCCBPA47	Ganga water/Aurihar	*K. pneumoniae*	Pinpoint, clear
40	VTCCBPA48	Ganga water/Aurihar	*K. pneumoniae*	1 mm, clear
41	VTCCBPA49	Ganga water/Aurihar	*K. pneumoniae*	1 mm, clear pinpoint with halo zone
42	VTCCBPA50	Ganga soil/Aurihar	*K. pneumoniae*	1 mm, clear
43	VTCCBPA51	Animal farm soil	*Shigella* spp.	6 mm, clear
44	VTCCBPA52	Animal farm soil	*Enterobacter* spp.	1 mm, clear
45	VTCCBPA53	Animal farm soil	*Enterobacter* spp.	1 mm, clear
46	VTCCBPA54	Animal farm soil	*Enterobacter* spp.	1 mm, clear

Table 1. cont.

S. no.	Bacteriophage accession no.	Source of sample used for phage isolation	Host bacteria against which phage isolated	Plaque characteristics
47	VTCCBPA55	Animal farm soil	*Enterobacter aerogenes*	3 mm, clear with a halo zone
48	VTCCBPA56	Animal farm soil	*Serratia marcescens*	3 mm, clear with a halo zone
49	VTCCBPA57	Animal farm soil	*Citrobacter freundii*	1–2 mm, clear
50	VTCCBPA58	Animal farm soil	*Citrobacter freundii*	1–2 mm, clear
51	VTCCBPA59	Animal farm soil	*Shigella* spp.	3–4 mm, clear with diffuse margins
52	VTCCBPA60	Animal farm soil	*Shigella* spp.	8 mm, clear
53	VTCCBPA61	Animal farm soil	*Citrobacter sedlakii*	1 mm, clear
54	VTCCBPA62	Animal farm soil	*Citrobacter sedlakii*	1–2 mm, clear
55	VTCCBPA63	Animal farm soil	*Shigella* spp.	1–2 mm, clear

Bacteriophage purification, characterization and preservation

Isolated bacteriophages were purified, bulk cultured and preserved in the NCVTCC phage repository. The phage isolates against different bacteria obtained from the same sample were analysed by SDS-PAGE for major protein bands in order to identify them as distinct phages. Members of families *Myoviridae*, *Siphoviridae* and *Podoviridae* were observed when visualized by transmission electron microscopy (Fig. 2). Some phages showed novel characteristics as well (Fig. 2j).

Detection of ARGs and *intI* genes in phage DNA

Of a total of 55 bacteriophages analysed, 23 (41.8 %) showed presence of a single gene, 11 (20 %) showed presence of multiple genes, while 21 (38.2 %) did not show positivity for any of the tested genes. ARG and *intI* genes detected in the phage DNA included bla_{TEM} [17/55 (30.9 %)], *tetA* [7/55 (12.7 %)], *intI1* [6/55 (10.9 %)], *intI2* [6/55 (10.9 %)], *tetW* [5/55 (9.1 %)], *intI3* [5/55 (9.1 %)] and $bla_{\text{OXA-2}}$ [2/55 (3.6 %)] (Fig. 3). The ARGs that were not detected in any of the phage DNA included bla_{SHV}, $bla_{\text{OXA-1}}$, *tetO*, *tetB*, *tetG*, *tetM* and *tetS*.

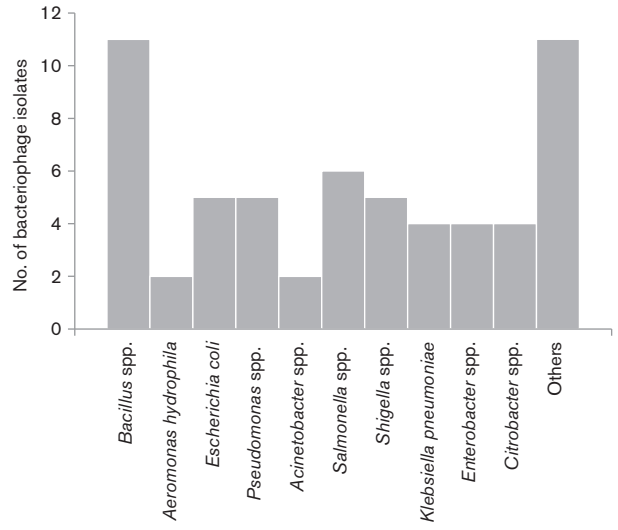

Fig. 1. Number of different bacteriophages versus host bacteria isolated from different sources.

Among detected genes, phage isolates from soil samples were most versatile, as seven different types of genes were found in them (Fig. 4 and Table S1, available in the online Supplementary Material). In soil phages, the occurrence of bla_{TEM} [6/32 (18.75 %)] was the highest, followed by *intI1* [4/32 (12.5 %)], *intI2* [4/32 (12.5 %)], *tetA* [3/32 (9.4 %)], *intI3* [3/32 (9.4 %)], *tetW* [3/32 (9.4 %)] and $bla_{\text{OXA-2}}$ [1/32 (3.1 %)]. The genes including *tetA*, *intI2* and *intI3* were not detected in sewage or dung/litter phage isolates, and *tetW* and $bla_{\text{OXA-2}}$ were additionally not detected in any of the sewage phages. In addition to this, none of the phages isolated from water sources showed the presence of *tetW*, $bla_{\text{OXA-2}}$ and *intI1*.

Altogether, the occurrence of bla_{TEM} differed significantly ($P<0.05$) considering the source (viz. soil, water, sewage and dung/litter) of bacteriophage isolates. The respective abundance was observed to be 7/32 (21.8 %) in soil phages, 1/9 (11.1 %) in water phages, 4/8 (50.0 %) in sewage phages and 5/6 (83.3 %) in dung/litter phages. Also, the abundance of *tetA* differed significantly ($P<0.05$) among four sources of bacteriophage isolates; however, the abundance of *tetW*, $bla_{\text{OXA-2}}$, *intI1*, *intI2* and *intI3* did not differ significantly among four sources of bacteriophage isolates.

The phage isolates from organized farms showed more varied ARG and *intI* genes, as all of these were observed in one or the other phage DNA. However, among phages isolated from unorganized zones, $bla_{\text{OXA-2}}$ was not at all detected (Fig. 5 and Table S1). Bacteriophages from organized farms and unorganized zones showed relatively equal overall incidences of ARG and *intI* gene detection [25/40 (62.5 %) in organized farms vs 9/15 (60 %) in unorganized zones]. bla_{TEM} was detected more in phage isolates from organized farms [15/40 (37.5 %)] as compared to unorganized zone [2/15 (13.3 %)], but the difference was statistically insignificant. Other genes, viz. $bla_{\text{OXA-2}}$, *tetA*, *intI1*, *intI2* and *intI3*, were also detected from phages of both the zones but without any

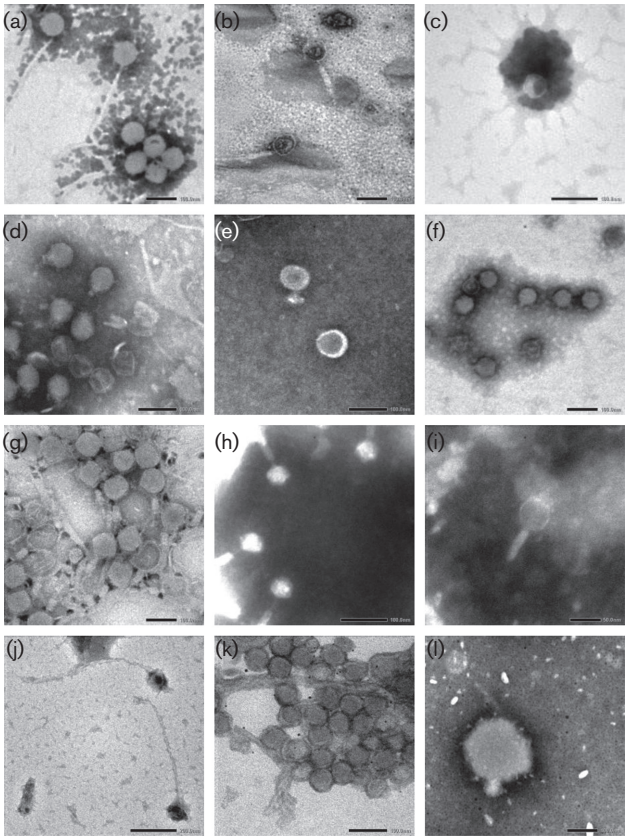

Fig. 2. Transmission electron microscopy pictures of phage isolates. (a) *Siphoviridae* phage against *Staphylococcus sciuri* isolated from soil of animal fair site. (b) *Myoviridae* phage against *B. cereus* isolated from equine stud farm soil. (c) *Myoviridae* phage against *Serratia marcescens* isolated from soil. (d) *Podoviridae* phage against *Shigella* spp. isolated from soil. (e) *Podoviridae* phage against *Escherichia coli* isolated from sewage of equine stud farm showing bla_{TEM}. (f) *Podoviridae* phage against *Aeromonas hydrophila* isolated from pond water showing bla_{TEM}. (g) *Myoviridae* phage against *Escherichia coli* isolated from soil of equine stud farm showing *tetW* and *intI1*. (h) *Myoviridae* phage against *Caryophanon* spp. showing *intI2*. (i) *Myoviridae* phage against *K. pneumoniae* isolated from river water showing bla_{OXA-2}. (j) *Siphoviridae* phage against *Citrobacter sedlakii* isolated from animal dung. (k) *Siphoviridae* phage against *Pseudomonas mendocina* isolated from pond water showing *intI3*. (l) *Podoviridae* phage against *Shigella* spp. isolated from animal dung.

significant difference between the two groups. There was a significant difference between organized and unorganized ($P<0.05$) zones for *tetW* ARG in phage isolates.

DISCUSSION

The indiscriminate use of antibiotics and the emergence of antibiotic-resistant bacteria clearly point towards the spread of ARGs and the involvement of bacteriophages in the environmental settings (Balcazar, 2014). The actual role of bacteriophages in the spread of ARGs has remained

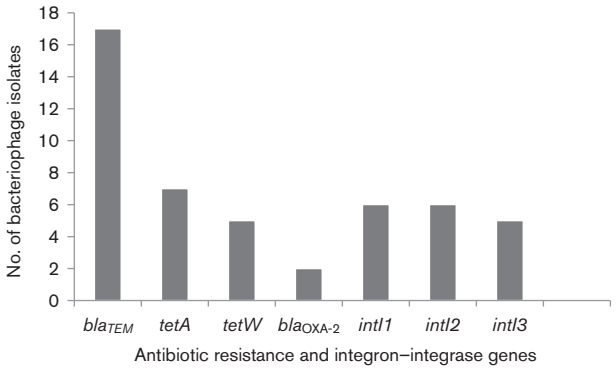

Fig. 3. Comparative abundance of ARG and *intI* genes in phage DNA.

prejudicially estimated based on metagenomic approaches and the detection of ARGs in phage fractions (i.e. supernatant of samples subjected to centrifugation followed by DNase/RNase treatment to eliminate any non-phage DNA) and not in the purified and cultured bacteriophage isolates (Colomer-Lluch *et al.*, 2011a, b; Marti *et al.*, 2014; Calero-Cáceres & Muniesa, 2016; Subirats *et al.*, 2016). The view is further strengthened from a recent study where the overestimation of ARGs via phages has been reported using bioinformatics tools and correlated experimentally (Enault *et al.*, 2016). The present study was focused on detection of varied ARGs and *intI* genes in purified phage DNA so as to find their actual involvement in the spread of ARGs in the natural environment. The important ARG and *intI* genes studied include bla_{TEM} and bla_{SHV} (which encode for β-lactamase genes that are widespread among Gram-negative pathogens worldwide; Bali *et al.*, 2010; Paterson *et al.*, 2003); bla_{OXA-1} and bla_{OXA-2} (oxacillinases showing hydrolytic activity for isoxazolyl penicillins such as oxacillin, cloxacillin and other penicillins such as methicillin; Bush *et al.*, 1995); *intI1*, *intI2* and *intI3* (genetic platforms which can incorporate ORFs via site-specific recombination and ensure correct expression; Mazel, 2004); and tetracycline resistance genes such as *tetA*, *tetB*, *tetG*, *tetM*, *tetO*, *tetS* and *tetW* (Speer *et al.*, 1992). We carried out a systematic study, where bacteriophages were enriched against naturally occurring culturable bacteria isolated from various environmental samples of soil, sewage, pond/river water and dung/litter, etc. (Table 1). A variety of Gram-negative as well as Gram-positive bacteria were isolated; however, bacteriophages against all of the bacterial isolates of a particular sample were not enriched from the same sample. Bacteriophage isolates obtained included *Bacillus* phages (20 %), *Salmonella* spp. phages (10.9 %), *Pseudomonas* sp. phages (9.1 %), *Escherichia coli* phages (9.1 %), *Shigella* spp. phages (9.1 %) and other phages in comparatively lower abundance (Fig. 1). The results indicated that bacteriophages were abundantly present in the environment and were enriched in the presence of host bacteria.

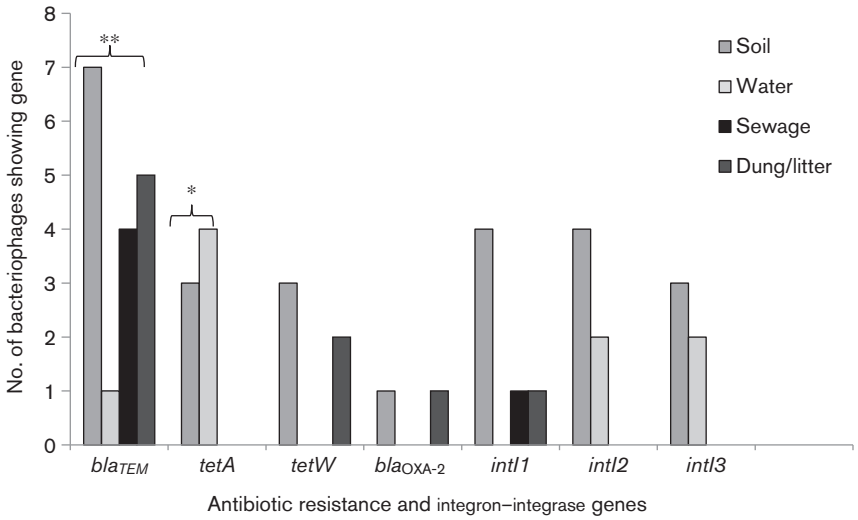

Fig. 4. Source of phage isolation (i.e. soil, water, etc.) versus ARG and *intI* gene abundance/profile.

We observed some of the bacteriophages by TEM for purification and found them to be pure and belonging to different families including *Myoviridae*, *Siphoviridae* and *Podoviridae* (Fig. 2). We analysed the occurrence of ARGs and *intI* genes in phage DNA and found that 61.8 % of all DNA from phages had these genes. Thus, the current study provides a strong indication that ARGs may spread through generalized transducing bacteriophages in the environmental settings. Also, it provides a true quantitative as well as qualitative assessment of phage involvement in ARG dissemination, as the genes have been detected in DNA from purified bacteriophages and not in phage fraction samples (Colomer-Lluch *et al.*, 2011a, b; Ross & Topp, 2015).

bla_{TEM} was observed to be the most abundant ARG overall (Fig. 3) and was comparatively higher in the soil phages.

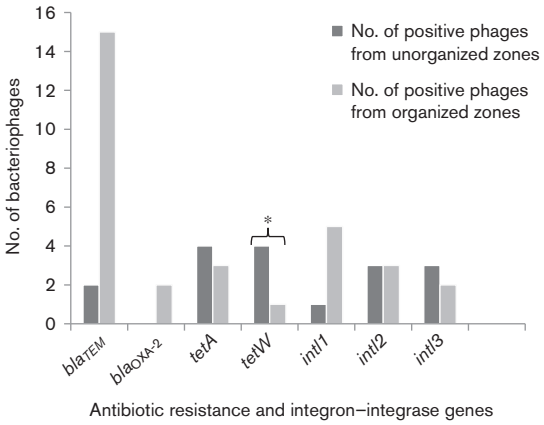

Fig. 5. Relative abundance of ARGs and *intI* genes in phage isolates from organized farms versus unorganized zones.

bla_{TEM} is known to encode for resistance against β-lactam antimicrobial agents. TEM β-lactamases were first found to be encoded by enterobacterial plasmids in 1965, but later on spread to *Pseudomonas aeruginosa* (1969), *Vibrio cholerae* (1973), *Haemophilus* spp. and *Neisseria* spp. (1974) (Livermore, 1995). β-Lactams account for approximately two-thirds, by weight, of all antibiotics administered to humans and include the penicillins, cephalosporins and carbapenems. Widespread and unintended use of β-lactams has lead to the development of various mutant types including ESBLs. In the current study, we observed bla_{TEM} in bacteriophage isolates of *Bacillus* spp., *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas* spp., *Stenotrophomonas* spp., *Salmonella* Gallinarum, *Shigella sonnei* and *Citrobacter sedlakii* (Table S1). Three representative samples showing bla_{TEM} amplicons were sequenced and were found to be 100 % identical to $bla_{TEM\text{-}116}$, which has been previously reported in *Aeromonas hydrophila*, *Aeromonas jandaei* (Balsalobre *et al.*, 2010), *Providencia stuartii* (Lahlaoui *et al.*, 2011), *Escherichia coli* (Vignoli *et al.*, 2005) and some other bacteria and has been associated with ESBL characteristics. However, there are scant reports of bla_{TEM} in bacteriophages. Also, the abundance of bla_{TEM} was highest in the soil phages and least in the phage isolates from water (Fig. 4), which is in concurrence with a previous study, where river water showed a lower copy number of bla_{TEM} genes as compared to urban sewage (Colomer-Lluch *et al.*, 2011a, b). In a recent study, bla_{TEM} was quantified in the soils and has been found to be significantly higher in manured soils post-1940 (Graham *et al.*, 2016). The isolation of ARGs from environmental phages, including soil and water sources, provides an early warning for the future spread of these ARGs to the food chain.

Among tetracycline resistance genes, only *tetA* (efflux pump encoding determinant) and *tetW* (encoding for ribosomal protection protein) genes were detected in the phages.

Tetracycline resistance gene carriage has been previously reported in bacterial communities from lake waters (Yin *et al.*, 2013), river water (Giowanella *et al.*, 2015), animal farms (Kyselková *et al.*, 2015), fish farms (Su *et al.*, 2011) and soil affected by lagoon effluents (Graves *et al.*, 2011). However, it has never been reported in bacteriophages. Among bacteria, *tetA*, *tetB* and *tetC* were found to be more significantly present in *Escherichia coli* isolates from soil (Graves *et al.*, 2011) and *tetW*, *tetQ* and *tetM* in excrements of calves in dairy farm apart from *tetO*, *tetQ* and *tetW* – representing the core tetracycline resistance genes present in all cattle excrements of antibiotic treatment groups (Kyselková *et al.*, 2015). We could observe only *tetA* and *tetW* in phages (Table S1). *tetA* harboured by *Shigella* spp., as reported by Kyselková *et al.* (2015), was also observed in *Shigella* phages in the current study. *tetW* is the most prevalent tetracycline resistance encoding gene in faecal metagenomic DNA and has been reported in hospital effluents as well as animal farms. All these facts point toward its longer persistence in soil and transmission through animals at comparatively higher rate, which may act as genetic reservoirs. Further studies are needed to clarify the hypothesis after following clean management practices and monitoring ARG carriage and persistence in animal farms.

In the current piece of work, we were also able to detect integrons (class I, II and III types) in bacteriophage DNA. Integrons are the genetic platforms that assist evolution of bacteria by allowing gene acquisitions, insertions and recombination (Escudero *et al.*, 2015). *intI1*, *intI2* and *intI3* were abundantly present in soil phage isolates followed by water phage isolates; however, *intI2* and *intI3* were not at all observed in phage isolates from sewage or dung/litter (Fig. 4). Jechalke *et al.* (2015) also observed a substantial increase of *intI1* in soils irrigated with waste water. We observed that apart from *Bacillus* spp. and *Caryophanon* spp. phages, integrons were detected in phages mostly affecting Gram-negative bacteria including *Aeromonas hydrophila*, *Escherichia coli*, *Stenotrophomonas* spp., *Acinetobacter baumannii*, *Salmonella* Gallinarum, *Shigella* spp., *Pseudomonas* spp., *K. pneumoniae*, *Enterobacter* spp. and *Citrobacter sedlakii*. Thus, the current research work emphasizes the strong involvement of Gram-negative bacteria and corresponding bacteriophages in dissemination of integrons and suggests that the phages altogether act as reservoirs and facilitators for ARG dissemination in the environment.

The antibiotic misuse in farms, anthropogenic activities, effluent discharge, pesticide usage in farms and industrial activities are all concerned with discharges either to land soils or to river water. As such, emerging antimicrobial resistance is a global concern; the surveillance for the possible expansion of this phenomenon in the environment may help to provide a suitable explanation. The current report is the first to detect ARG and *intI* genes in pure bacteriophage isolates against environmental bacteria and clearly points toward the persistence of ARGs in soil, sewage, water and dung/litter, thus providing a proof of prevalence of ARGs in phages and incriminating them for a strong role in dissemination of ARGs.

METHODS

Sample collection, bacterial isolation and identification. Samples of sewage, soil and dung/litter were collected randomly from different habitats like animal farms, animal fair sites and areas of animal intervention from Hisar located in the state of Haryana, India (29.1492° N, 75.7217° E). Water samples from village ponds from above location and Ganga River, Banaras, UP, India (25.3176° N, 82.9739° E), as well as sediment soil from river bank were also collected. All samples (Table 1) were collected in sterilized plastic bags/bottles during October 2013 to November 2015, brought to the ICAR-National Research Centre on Equines, NCVTCC, and stored in a refrigerator at 4 °C until further processing.

Samples were diluted in PBS; bacteria were isolated using the spread plate method and purified further on NA. The bacterial genomic DNA was extracted from broth cultures using ZR fungal/bacterial DNA miniprep kit (Zymo Research) as per the manufacturer's protocol and used for PCR amplification of the *16S rDNA* gene (Ladrón *et al.*, 2003); amplicons of *16S rDNA* were cloned into pGEM-T Easy vector, sequenced commercially and analysed for homology by using BLAST software (Altschul *et al.*, 1990).

Bacteriophage enrichment, isolation and purification. Bacterial isolates obtained from various samples were characterized, grown individually overnight in nutrient broth (NB) at 37 °C and used for the enrichment of lytic bacteriophages from the respective samples. For the water and sewage samples, 40 ml of suspension was taken, and for soil and dung/litter samples, 1 g was added to 40 ml PBS. The suspension obtained was mixed with 5× NB and an exponentially growing bacterial culture and incubated under shaking conditions at 37 °C overnight. This was followed by centrifugation at 9400 ***g*** and filtration through a 0.22 µm PVDF membrane filter. The bacteriophage activity was detected using spot assay on NA seeded with the host bacterium. The filtrate qualifying spot tests were serially diluted and plated upon enrichment host by the double-agar overlay technique. A clearly separated plaque was excised and purified using SM buffer [5.8 g l^{-1} NaCl, 2.0 g l^{-1} $MgSO_4$, 50 mM Tris/HCl (pH 7.5) and 5 ml l^{-1} presterilized 2 % gelatin] and replating. Plaque characteristics were recorded, and phage titre was determined.

Bacteriophage bulk culture and preservation. The concentrated stocks of phage isolates were prepared as indicated previously (Anand *et al.*, 2016). Briefly, 2 ml of exponentially growing host bacteria was pelleted and suspended in SM buffer. Phage suspension (10^9 p.f.u.) was added to it, and co-incubation followed for 20 min with subsequent addition of NB and incubation for 8 to 12 h at 37 °C. Afterwards, chloroform was added, and centrifugation was carried out at 6470 ***g*** for 10 min. Pancreatic DNase I and RNase A (1 µg ml^{-1}) were added to the supernatant and incubated for 30 min at room temperature followed by addition of NaCl and PEG 8000 (1 M and 10 % w/v), respectively. The bacteriophage pellet was dissolved in SM buffer, and repurification was done by extraction with chloroform (1 : 1 v/v). The isolated bacteriophage preparation was reposited in the NCVTCC, and accession numbers were obtained as indicated in Table 1.

Electron microscopy. Electron microscopy was performed by placing phage solution on carbon-coated copper grids for 5 min followed by negative staining with 1 % phosphotungstic acid (pH 7 for 10 s). Electron micrographs were taken on a JEM-1400 (JEOL) transmission electron microscope operating at 80 kV.

Phage DNA isolation and PCR for ARGs. To rule out the possibility of non-phage DNA contamination, an aliquot of purified bacteriophage was evaluated using conventional PCR of the eubacterial *16S rDNA* gene (Ladrón *et al.*, 2003). Phage DNA was extracted using QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol. Conventional PCRs for ARGs and *intI* genes were performed as described previously and indicated in Table S2.

Statistical analysis. The data were analysed using GraphPad Prism 7 statistical software and the chi-square test. Differences were considered significant at $P<0.05$.

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