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Short communication

Identification of antimicrobial resistance genes in intestinal content from Coyote (*Canis latrans*)

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Abstract

Antibiotic resistance has become a global public health concern in the last few years. Given the widespread rate of recurrence, increasing attention is being turned toward environmental pathways that potentially contribute to antibiotic resistance genes (ARGs) dissemination outside the clinical realm. In this study, a metagenome analysis of intestinal virus-like particle fraction (VLPs) from a wild coyote (*Canis latrans*) revealed for the first time, multiple ARGs, such as B-lactamases and multidrug efflux pumps. Description of ARGs presence in natural environments is critical to understand the emergence of resistant strains.

Key words: antimicrobial resistance, metagenome, Canis latrans

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Table 1. Antibiotic resistance genes identified in virus like particles (VLPs)*

Gene	Identity (%)	Function
*mdt*ABC	81.4	The MdtABC tripartite complex confers resistance against novobiocin and deoxycholate.
*pmr*F	79.66	Required for Lipid A modification to resist the antimicrobial activity of antibiotics such as polymyxin
*mex*K	81.3	MexK system effluxed tetracycline and erythromycin together with outer membrane protein channel OprM.
*amp*H	81.35	Class D β-lactamases *E. coli*.
$bla_{CMY-104}$	85.78	Serine beta-lactamase with a substrate specificity for cephalosporins
bla_{CMY-59}	85.02	AmpC type beta-lactamase
$bla_{CMY-157}$	86.47	Class C beta-lactamase CMY-157
*acr*AB	86.41	AcrAB-TolC is Major multidrug efflux pump in *E. coli*.
*acr*D	81.05	Aminoglycoside efflux pump component.
*mdf*A	76.66	MdfA is a multidrug/proton antiporter with a remarkably broad substrate specificity profile
*acr*EF	77.89	Tripartite efflux system (AcrEF-TolC) involved in the efflux of indole and organic solvents
*mex*EF- *opr*N	89.08	MexEF is the multidrug inner membrane transporter of the MexEF-OprN multidrug efflux complex.
*msb*A	83.13	MsbA is a multidrug resistance transporter that belongs to a superfamily of transporters.
*mdt*K	78.08	Multidrug efflux pump, that confers resistance to norfloxacin, ciprofloxacin, doxorubicin, trimethoprim, chloramphenicol and fosfomycin.
*kpn*EF	76.34	Mutation in KpnEF resulted in increased susceptibility to cefepime ceftriaxon colistin erythromycin rifampin tetracycline and streptomycin.
*mdt*GH	79.16	Multidrug resistance system that confers resistance to norfloxacin and enoxacin.
*cat*B4	100	Chloramphenicol acetyltransferase

* ARGs identification was completed used the most well-known ARGs databases (Papp et al. 2022)

Introduction

Metagenomic analyses has been used to estimate the incidence of ARGs in different antibiotic-contaminated and natural ecosystems (Li et al. 2020). Metagenomic analysis of hospital wastewater revealed ARGs in bacteriophages, showing the significant role of this agents as reservoirs and dissemination vehicles (Subirats et al. 2016). ARGs has been also reported inside of prophages, inside of clinical E. coli strains genomes (Schroeder et al. 2002). These data suggests that lateral transfer of ARGs by phage-mediated transduction could be an important contributing factor in the global spread of antibiotic resistance. The contribution of bacteriophages to the maintenance and spread of ARGs has been broad described in antibiotic contaminated ecosystems, however, in other environments this analysis has not been realized. In this pilot study, metagenomic analyses of VLPs recuperated from intestinal content from coyote (*Canis latrans*) were performed, showing the presence of diverse ARGs.

Materials and Methods

Samples

Three samples of intestinal content from recently deceased coyote, found in Janos Biosphere Reserve, Chihuahua, Mexico, during routine inspection. The samples were collected directly from intestine, placed in sterile plastic containers, and frozen until processing.

Metagenome analysis of Virus-Like Particles (VLPs)

DNA from VLPs was isolated using a protocol described by Summer (2009). The DNA samples were sequenced on a high-throughput DNA sequencer system (Illumina HiSeq™ 2000, San Diego, CA). Low-quality reads were filtered, and contiguous sequences were assembled using the online software metaSPAdes (Nurk et al. 2017). ARGs identification was performed with the Abricate software version 1.0.1., using the databases: AMRFinderPlus, CARD, Resfinder, ARG-ANNOT, and Plasmid-Finder (Papp et al. 2022). ViromeQC was used to quantify non-viral contamination (Zolfo et al. 2019).

Supplementary table 1. Antibiotic resistance genes identified in virus like particles (VLPs)*

	SEQUENCE	GENE	IDENTITY (%)	ACCESSION	PRODUCT
1	NODE_02	*mdt*A	77.18	U00096:2154015-2155263	MdtA is the membrane fusion protein of the multidrug efflux complex mdtABC.
2	NODE_02	*mdt*B	81.4	U00096:2155262-2158385	MdtB is a transporter that forms a heteromultimer complex with MdtC to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex.
3	NODE_02	*mdt*C	81.08	U00096:2158385-2161463	MdtC is a transporter that forms a heteromultimer complex with MdtB to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex. In the absence of MdtB MdtC can form a homomultimer complex that results in a functioning efflux complex with a narrower drug specificity. mdtC corresponds to 3 loci in *Pseudomonas aeruginosa* PAO1 (gene name: muxC/muxB) and 3 loci in *P. aeruginosa* LESB58.
4	NODE_02	*bae*S	80.57	AP009048:2165012-2166416	BaeS is a sensor kinase in the BaeSR regulatory system. While it phosphorylates BaeR to increase its activity BaeS is not necessary for overexpressed BaeR to confer resistance.
5	NODE_02	*bae*R	82.7	AP009048.1:2166412-2167135	BaeR is a response regulator that promotes the expression of MdtABC and AcrD efflux complexes.
6	NODE_02	*yoj*L	80.26	U00096.3:2308615-2306971	YojL mediates resistance to the peptide antibiotic microcin J25 when it is expressed from a multicopy vector. YojL can pump out microcin molecules. The outer membrane protein TolC in addition to YojI is required for export of microcin J25 out of the cell. Microcin J25 is thus the first known substrate for YojI.
7	NODE_02	*pmr*F	79.66	U00096:2367070-2368039	PmrF is required for the synthesis and transfer of 4-amino-4-deoxy-L-arabinose (Ara4N) to Lipid A which allows gram-negative bacteria to resist the antimicrobial activity of cationic antimicrobial peptides and antibiotics such as polymyxin. *pmr*F corresponds to 1 locus in Pseudomonas aeruginosa PAO1 and 1 locus in *Pseudomonas aeruginosa* LESB58.
8	NODE_04	*mdt*M	79.1	U00096.3:4568519-4567286	Multidrug resistance protein MdtM
9	NODE_07	*mex*K	81.3	AE004091.2:4119265-4116187	MexK is the inner membrane resistance-nodulation-cell division (RND) transporter in the MexJK multidrug efflux protein.
10	NODE_104	*mex*W	82.6	NC_002516.2:4904646-4907703	MexW is the RND-type membrane protein of the efflux complex MexVW-OprM.
11	NODE_115	*opm*H	77.58	AE004091.2:5584100-5585549	OpmH is an outer membrane efflux protein required for triclosan-specific efflux pump function.
12	NODE_116	*rpo*B2	76.19	AP006618.1:4835199-4838688	Due to gene duplication the genomes of Nocardia species include both rifampin-sensitive beta-subunit of RNA polymerase (rpoB) and rifampin-resistant beta-subunit of RNA polymerase (rpoB2) genes with ~88% similarity between the two gene products. Expression of the rpoB2 variant results in replacement of rifampin sensitivity with rifampin resistance.
13	NODE_117	*emr*R	86.25	U00096.3:2810769-2811300	EmrR is a negative regulator for the EmrAB-TolC multidrug efflux pump in *E. coli*. Mutations in this gene lead to EmrAB-TolC overexpression.
14	NODE_117	*emr*A	81.6	AP009048:2810082-2811255	EmrA is a membrane fusion protein providing an efflux pathway with EmrB and TolC between the inner and outer membranes of *E. coli* a Gram-negative bacterium.
15	NODE_117	*emr*B	84.92	U00096:2812615-2814154	EmrB is a translocase in the EmrB-TolC efflux protein in *E. coli*. It recognizes substrates including carbonyl cyanide m-chlorophenylhydrazone (CCCP) nalidixic acid and thioloactomycin.

cont. Supplementary table 1. Antibiotic resistance genes identified in virus like particles (VLPs)*

	SEQUENCE	GENE	IDENTITY (%)	ACCESSION	PRODUCT
15	NODE_117	*emr*B	84.92	U00096:2812615-2814154	EmrB is a translocase in the EmrB-TolC efflux protein in *E. coli*. It recognizes substrates including carbonyl cyanide m-chlorophenylhydrazone (CCCP) nalidixic acid and thioloactomycin.
16	NODE_13	*sdi*A	76.14	AE006468.2:2040377-2039654	SdiA is a cell division regulator that is also a positive regulator of AcrAB only when it's expressed from a plasmid. When the sdiA gene is on the chromosome it has no effect on expression of acrAB.
17	NODE_13	*emr*E	76.42	Z11877.1:485-818	Member of the small MDR (multidrug resistance) family of transporters; in *E. coli* this protein provides resistance against several positively charged compounds including ethidium bromide and erythromycin; proton-dependent secondary transporter which exchanges protons for compound translocation
18	NODE_14	*bac*A	82.15	U00096.3:3204131-3203309	The bacA gene product (BacA) recycles undecaprenyl pyrophosphate during cell wall biosynthesis which confers resistance to bacitracin.
19	NODE_14	*tol*C	82.83	FJ768952:0-1488	TolC is a protein subunit of many multidrug efflux complexes in Gram negative bacteria. It is an outer membrane efflux protein and is constitutively open. Regulation of efflux activity is often at its periplasmic entrance by other components of the efflux complex.
20	NODE_142	*bla*CMY-104	85.78	KF150216:1-1146	BlaCMY-104 is a beta-lactamase
21	NODE_142	*bla*CMY-59	85.02	AB587082:0-1188	BlaCMY-59 is a beta-lactamase found in *Shigella* spp.
22	NODE_142	*bla*CMY-157	86.47	NG_055587.1	BlaCMY157 is a class C beta-lactamase
23	NODE_142	*bla*CMY-104-1	85.78	KF150216	BlaCMY-104 is a beta-lactamase
24	NODE_1420	*cat*B4	100	EU935739:59054-59602	CatB4 is a chloramphenicol acetyltransferase.
25	NODE_156	*mdt*G	77.38	CP000800.1:1192954-1191727	The MdtG protein also named YceE appears to be a member of the major facilitator superfamily of transporters, and it has been reported when overexpressed to increase fosfomycin and deoxycholate resistances. mdtG is a member of the marA-soxS-rob regulon.
26	NODE_156	*mdt*H	79.16	U00096:1125326-1124117	Multidrug resistance protein MdtH
27	NODE_18	(*Bla*) *amp*H	81.35	AP012030:395554-396711	(Bla) ampH is a beta-lactamase found in *E. coli.*
28	NODE_18	*amp*H	81.35	AP012030.1:396711-395553	AmpH is a class C ampC-like beta-lactamase and penicillin-binding protein identified in *E. coli*.
29	NODE_18	*acr*B	86.41	U00096.3:484403-481253	Protein subunit of AcrA-AcrB-TolC multidrug efflux complex. AcrB functions as a heterotrimer which forms the inner membrane component and is primarily responsible for substrate recognition and energy transduction by acting as a drug/proton antiporter.
30	NODE_18	*acr*A	83.33	U00096.3:485619-484425	AcrA is a subunit of the AcrAB-TolC multidrug efflux system that in *E. coli*.
31	NODE_182	*sme*R	76.71	AF173226.1:1041-351	SmeR is a component of a two-component signal transduction system that includes smeS and regulates many resistance genes.
32	NODE_19	*acr*D	81.05	AP009048.1:2586250-2589364	AcrD is an aminoglycoside efflux pump expressed in *E. coli*. Its expression can be induced by indole and is regulated by baeRS and cpxAR.
33	NODE_20	*crp*	89.89	AP009048.1:4154296-4153663	Crp is a global regulator that represses mdtEF multidrug efflux pump expression.
34	NODE_2146	*mex*B	76.94	L11616:1569-4710	MexB is the inner membrane multidrug exporter of the efflux complex MexAB-OprM.

cont. Supplementary table 1. Antibiotic resistance genes identified in virus like particles (VLPs)*

	SEQUENCE	GENE	IDENTITY (%)	ACCESSION	PRODUCT
35	NODE_22	*cpx*R	78.77	LT673656.1:1885022-1884344	CpxR is directly involved in activation of expression of RND efflux pump MexAB-OprM in *P. aeruginosa*. CpxR is required to enhance mexAB-oprM expression and drug resistance in the absence of repressor MexR.
36	NODE_23	*mdf*A	76.66	JQ394987:0-1233	Multidrug efflux pump in *E. coli*. This multidrug efflux system was originally identified as the Cmr/CmlA chloramphenicol exporter.
37	NODE_24	*acr*F	77.89	U00096:3415032-3418137	AcrF is a inner membrane transporter similar to AcrB.
38	NODE_24	*acr*E	76.81	U00096:3413863-3415021	AcrE is a membrane fusion protein similar to AcrA.
39	NODE_260	*opm*H	78.22	AE004091.2:5584100-5585549	OpmH is an outer membrane efflux protein required for triclosan-specific efflux pump function.
40	NODE_260	*emr*E	76.13	AE004091.2:5606102-5606435	EmrE is a small multidrug transporter that functions as a homodimer and that couples the efflux of small polyaromatic cations from the cell with the import of protons down an electrochemical gradient. Confers resistance to tetraphenylphosphonium methyl viologen gentamicin kanamycin and neomycin.
41	NODE_27	*mex*B	78.28	L11616:1569-4710	MexB is the inner membrane multidrug exporter of the efflux complex MexAB-OprM.
42	NODE_2739	*lmr*B	81.2	JYFL01000006.1:113503-112069	LmrB is a chromosomally-encoded efflux pump that confers resistance to lincosamides in *Bacillus subtilis*.
43	NODE_28	*mex*E	79.19	AE004091.2:2808742-2809987	MexE is the membrane fusion protein of the MexEF-OprN multidrug efflux complex.
44	NODE_28	*mex*F	86.23	AE004091.2:2810008-2813197	MexF is the multidrug inner membrane transporter of the MexEF-OprN complex. mexF corresponds to 2 loci in Pseudomonas aeruginosa PAO1 and 4 loci in *Pseudomonas aeruginosa* LESB58.
45	NODE_28	*opr*N	77.07	AE004091.2:2813193-2814612	OprN is the outer membrane channel component of the MexEF-OprN multidrug efflux complex.
46	NODE_28	*mux*B	76.3	AE004091.2:2854014-2850882	MuxB is one of the two necessary RND components in the *Pseudomonas aeruginosa* efflux pump system MuxABC-OpmB.
47	NODE_2975	*rpo*B2	76.88	AP006618.1:4835199-4838688	Due to gene duplication the genomes of *Nocardia* species include both rifampin-sensitive beta-subunit of RNA polymerase (rpoB) and rifampin-resistant beta-subunit of RNA polymerase (rpoB2) genes with 88% similarity between the two gene products. Expression of the rpoB2 variant results in replacement of rifampin sensitivity with rifampin resistance.
48	NODE_43	*kdp*E	79.94	U00096.3:721733-721055	KdpE is a transcriptional activator that is part of the two-component system KdpD/KdpE that is studied for its regulatory role in potassium transport and has been identified as an adaptive regulator involved in the virulence and intracellular survival of pathogenic bacteria. KdpE regulates a range of virulence loci through direct promoter binding.
49	NODE_47	*msb*A	83.13	U00096.3:966620-968369	MsbA is a multidrug resistance transporter homolog from *E. coli* and belongs to a superfamily of transporters that contain an adenosine triphosphate (ATP) binding cassette (ABC) which is also called a nucleotide-binding domain (NBD). MsbA is a member of the MDR-ABC transporter group by sequence homology. MsbA transports lipid A, a major component of the bacterial outer cell membrane and is the only bacterial ABC transporter that is essential for cell viability.

cont. Supplementary table 1. Antibiotic resistance genes identified in virus like particles (VLPs)*

	SEQUENCE	GENE	IDENTITY (%)	ACCESSION	PRODUCT
50	NODE_48	*cpx*A	84.42	BA000007.3:4905062-4903688	CpxA is a membrane-localized sensor kinase that is activated by envelope stress. It starts a kinase cascade that activates CpxR which promotes efflux complex expression.
51	NODE_515	*mex*B	79.33	L11616:1569-4710	MexB is the inner membrane multidrug exporter of the efflux complex MexAB-OprM.
52	NODE_62	*mdt*K	78.08	CP014358.1:2162750-2161325	A multidrug and toxic compound extrusions (MATE) transporter conferring resistance to norfloxacin doxorubicin and acriflavine.
53	NODE_64	H-NS	89.61	BA000007.3:1738104-1737690	H-NS is a histone-like protein involved in global gene regulation in Gram-negative bacteria. It is a repressor of the membrane fusion protein genes acrE mdtE and emrK as well as nearby genes of many RND-type multidrug exporters.
54	NODE_67	*ept*A	78.06	AP009048:4340268-4338624	PmrC mediates the modification of Lipid A by the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine resulting in a less negative cell membrane and decreased binding of polymyxin B.
55	NODE_73	*mar*A	86.79	AP009048.1:1621287-1621671	In the presence of antibiotic stress E. coli overexpresses the global activator protein MarA which besides inducing MDR efflux pump AcrAB also downregulates synthesis of the porin OmpF.
56	NODE_76	*rpo*B	78.43	NC_008618.1:1670624-1667063	*Bifidobacterium* are antibiotic resistant probiotics are prescribed to upkeep the population beneficial bacteria in the gut microbiome. However horizontal gene transfer among gut microbes could create harmful antibiotic-resistant pathogenic bacteria such as *Mycobacterium tuberculosis. B. animalis*, *B. longum* and *B. adolescentis* showed considerable resistance to pyrazinamide isoniazid and streptomycin for mutations in *rpo*B.
57	NODE_81	*cpx*R	75.27	LT673656.1:1885022-1884344	CpxR is directly involved in activation of expression of RND efflux pump MexAB-OprM in *P. aeruginosa*. CpxR is required to enhance mexAB-oprM expression and drug resistance in the absence of repressor MexR.
58	NODE_82	*ram*A	75.78	JQ727668:0-375	RamA (resistance antibiotic multiple) is a positive regulator of AcrAB-TolC and leads to high level multidrug resistance in *Klebsiella pneumoniae*, *Salmonella enterica* and *Enterobacter aerugenes* increasing the expression of both the *mar* operon as well as AcrAB. RamA also decreases OmpF expression.
59	NODE_92	*kpn*E	76.23	AP006725.1:2483889-2484252	KpnE subunit of KpnEF resembles EbrAB from *E. coli*. Mutation in KpnEF resulted in increased susceptibility to cefepime ceftriaxon colistin erythromycin rifampin tetracycline and streptomycin as well as enhanced sensitivity toward sodium dodecyl sulfate deoxycholate dyes benzalkonium chloride chlorhexidine and triclosan

* ARGs identification was completed used the most well-known ARGs databases (Papp et al. 2022)

Results and Discussion

A total of 36,071,420 Illumina sequencing reads were generated in paired files with a raw read length of 149 bp and an expected average insert size of 350 bp. Evaluation of DNA contamination by ViromeQC (Zolfo et al. 2019) showed a low abundance of genes encoding prokaryotic or eukaryotic ribosomal RNAs sequences and 27 microbial markers (0.2%, 0.3%, and 0.0001%, respectively). Although contamination with bacterial chromosomal DNA during VLPs purification is relatively frequent and variable, the results suggest minimal

contamination. Even the abundance of 16s rRNA sequences could be a consequence of generalized transduction events (Tian et al. 2015). A total of 5,783 contiguous sequences (nodes) were assembled. Sequence analysis of nodes showed 59 different ARGs (Table 1 and Supplementary table 1). For many of them, its mobilization by bacteriophages has already been described (Li et al. 2020). Remarkably, the sequence analysis revealed some nodes with several ARGs. For example, in node 142, there were sequences for three ß- lactamases: $bla_{\text{CMY-104}}$, $bla_{\text{CMY-59}}$, and bla_{CMY157} (Supplementary table 1). The results showed that ARGs can be mobilized by phages present in intestinal microbiota. Also highlight the importance of evaluating the presence of these genetic elements in free-range carnivores and open the possibility that wildlife animals could participate in its spreading in natural ecosystems. More studies are necessary to describe this phenomenon.

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