DETECTION OF ACQUIRED ANTIBIOTIC RESISTANCE DETERMINANTS IN THE INTESTINAL MICROBIOTA OF FOOD PRODUCING ANIMALS IN HUNGARY

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INTRODUCTION

In June 2017, the European Union announced a new action plan to combat the spread of antimicrobial resistance (AMR): the European One Health Action Plan against Antimicrobial Resistance [1]. The objectives of this action plan include research to understand the epidemiology of AMR, closing knowledge gaps on AMR in the environment and on how to prevent transmission of AMR between animals, humans, and the environment. The aim of this work was the detection of acquired antibiotic resistance genes (ARGs) in the intestinal microbiota of selected food animal species in Hungary by culture-based and metagenomic approaches, with a One Health perspective [2].

MATERIALS AND METHODS

Altogether 26 domestic pig and wild boar (*Sus scrofa*), common carp (*Cyprinus carpio*) and broiler chicken (*Gallus gallus domesticus*) intestinal content samples collected in Hungary between 2016 and 2021 were examined. In addition, intestinal content sampling was performed by use of sterile swabs from the colon or caecum content of red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) at regular hunts in January 2023 at Vörösalma and Zsitfapuszta, in Somogy County (**Figure 1**).

Figure 1. Geographical locations of the sampling sites within Hungary. **(1)** Herceghalom, Pest County (domestic pig, broiler chicken) **(2)** Kaposvár, Somogy County (domestic pig) **(3)** Vörösalma, Zselic Hills (red deer) **(4)** Zsitfapuszta, Dráva Valley (red deer and fallow deer) **(5)** Füzérkomlós, Zemplén Mountains (wild boar) **(6)** Szarvas, Békés County (common carp).

Shotgun metagenomic sequencing of gDNA purified from intestinal content samples was performed on Illumina NextSeq 500 and NovaSeq 6000 platforms (Illumina Inc., San Diego, USA) using 2x150 bp paired-end chemistry by Xenovea Ltd. (Szeged, Hungary) and IMGM Laboratories GmbH (Martinsried, Germany), respectively. Metagenomic contigs were assembled by MEGAHIT. Sequencing data were analysed by the ABRicate Galaxy version 1.0.1 tool to detect acquired ARGs. Contigs of appropriate size were also annotated using Prokka and visualized by SnapGene Viewer [2].

Selected intestinal content samples were screened for the presence of antibiotic-resistant strains of *E. coli* and other intestinal bacteria. The cultured antibiotic-resistant *E. coli* isolates were further characterized by molecular and classical microbiological methods (**Figure 2**). *E. coli* draft genome sequencing was performed by Xenovea Ltd. (Szeged, Hungary) on Illumina platform.

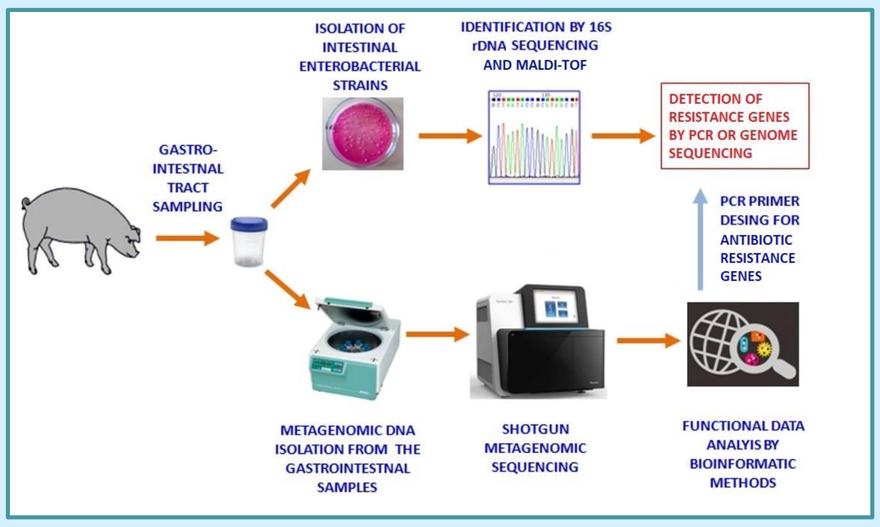

Figure 2. Summary diagram of the main steps of the workflow applied in this study

RESULTS

Overall, 59 acquired ARG types were identified by metagenomic analyses from domestic pig and common carp intestinal content samples. The detected ARG types belonged to the antibiotic classes aminoglycosides (27.1%), tetracyclines (25.4%), β-lactams (16.9%), and others, where *tet*(E), a bla_{OXA-48}-type β-lactamase gene, as well as *cphA4, ampS* and *qnrS2* were identified only in the examined carp intestinal sample (**Table 1**). Metagenomic sequencing and PCR demonstrated *tet*(Q), *tet*(W), *tet*(O) and *mef*(A) genes in the gut microbiota of free-living wild boars from the Zemplén Mountains [2, 3].

A broad variety of mobile genetic elements involving various plasmids, transposons, and/or integrons was associated with the acquired ARGs discussed in this study, as indicated by their immediate genetic context (for examples, see **Figure 3**), suggesting their potential for horizontal transfer into other bacterial strains or species [2, 3].

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	Aminoglycoside	Tetracycline	β-Lactam	Macrolide	Others
Domestic pig	*aac(6')-Im*	*tet*(40)	bla_{ACI-1}	*lnu*(B)	*catP*
	aac(6')-aph(2")	*tet*(44)	OXA-61 family β-lactamase	*lnu*(C)	*cfr*(C)
	ant(3")-Ia (aadA1)	*tet*(A)	bla_{ROB-1}	*lnu*(P)	*nimJ*
	ant(6)-Ia (aadE)	*tet*(B)	*cfxA3*	*lsa*(E)	*sul2*
	ant(6)-Ib	*tet*(C)	*cfxA4*	*mef*(A)	
	ant(9)-Ia	*tet*(H)	*cfxA5*	*msr*(D)	
	aph(2")-Ib	*tet*(L)	*cfxA6*	*erm*(B)	
	aph(2")-Ic	*tet*(M)		*erm*(F)	
	aph(2")-If	*tet*(O)		*erm*(G)	
	aph(2")-Ih	*tet*(Q)		*erm*(Q)	
	aph(3')-Ia	*tet*(W)		*vatE*	
	aph(3")-Ib (strA)	*tet*(X)			
	aph(6)-Id (strB)	*tetA*(P)			
	aph(3')-III	*tetB*(P)			
	rmtF				
Common carp	*ant(3")-Ia (aadA2)*	*tet*(A)	*ampS*		*dfrA3*
	aph(3")-Ib (strA)	*tet*(B)	*cphA4*		*qnrS2*
		tet(E)	OXA-48-type carbapenemase		*sul1*
		tet(X)			

Table 1. Summary of the ARG types detected in domestic pig and common carp intestinal samples from Hungary by shotgun metagenomic sequencing and bioinformatic analyses [2]. The detected ARGs are grouped by the respective antibiotic classes.

A ROB-1 β-lactamase encoding gene was detected on a swine metagenomic contig that also carried an *aph(3")-Ib* (*strA*) aminoglycoside phosphotransferase gene and a *sul2* dihydropteroate synthase gene (**Figure 3A**), and showed 99.9% identity in a 6392 bp region with the *Actinobacillus porcitonsillarum* pKMA202 plasmid [4]. A *tet*(C) gene was identified on a metagenomic contig (**Figure 3B**) that shared 99.6% identity in a 5830 bp region with the *tet(C)* genomic island of the *Chlamydia suis* strain R27 [5]. An erythromycin resistance determinant *erm*(B) gene, encoding a ribosomal RNA methyltransferase, was harboured by a metagenomic contig of 2926 bp (**Figure 3C**), displaying 99.2% identity at 92% coverage with the corresponding segment of the *Lactobacillus crispatus* CHCC3692 strain transposon Tn*3692* [6].

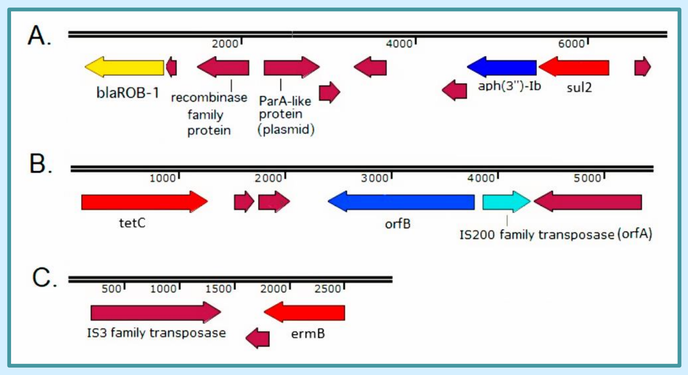

Figure 3. The immediate genetic environment of acquired ARGs on selected swine gut metagenomics contigs from Kaposvár [2].

By the culture-based approach *E. coli* strains resistant to tetracycline, ampicillin, streptomycin and trimethoprim/sulfamethoxazole and harbouring *tet*(A) or *tet(B)* and *aadA* ARGs were isolated from domestic pig faecal samples, while an *E. coli* strain carrying *aac(3)-VIa*, *aadA1*, *aph(3")-Ib*, *aph(6)-Id*, *sul1*, *sul2*, bla_{TEM-1B}, bla_{CMY-2} and *tet*(A) determinants was identified from chicken faeces (**Table 2**). Furthermore, *E. coli* strains resistant to ampicillin, streptomycin and tetracycline, and harbouring various acquired ARGs were cultured from the colon or caecum contents of red deer and fallow deer during regular hunts held at Vörösalma and Zsitfapuszta in Somogy County (**Figure 1** and **Table 2**).

Sampling location	Sampled animal species	Sample type	Cultured isolate	Method of identification	ARGs and other genes detected	Method of ARG detection
Herceghalom	domestic pig *(Sus scrofa)*	faeces	*E. coli*	MALDI-TOF, 16S rDNA sequencing	*tet(A) , aadA , integrase*	PCR
Herceghalom	domestic pig *(Sus scrofa)*	faeces	*E. coli*	16S rDNA sequencing	*tet(B) , aadA*	PCR
Herceghalom	chicken *(Gallus gallus domesticus)*	rectum content	*E. coli*	MALDI-TOF, 16S rDNA sequencing	*aac(3)-VIa, aadA1, sul1, sul2, strA, strB,* bla_{TEM-1B}, bla_{CMY-2}, *tet(A)*	draft genome sequencing
Zsitfapuszta	fallow deer (*Dama dama*)	caecum content	*E. coli*	16S rDNA sequencing	*tet(B)*	PCR
Zsitfapuszta	red deer *(Cervus elaphus)*	caecum content	*E. coli*	16S rDNA sequencing	*tet(B), strA, strB*	PCR
Zsitfapuszta	red deer *(Cervus elaphus)*	caecum content	*E. coli*	16S rDNA sequencing	*tet(A)*	PCR
Vörösalma	red deer *(Cervus elaphus)*	colon content	*E. coli*	16S rDNA sequencing	*tet(B), strA, strB*	PCR

Table 2. Selected *E. coli* strains carrying acquired ARGs and cultured from intestinal content samples of food producing animals in Hungary during this study.

In conclusion, by use of an up-to-date metagenomic and culture-based approach a broad variety of acquired antimicrobial resistance determinants were detected in the intestinal microbiota of food-producing animals in Hungary, including ARGs for two critically important antimicrobial classes, the quinolones (*qnrS2*) and the carbapenems (bla_{OXA-48}).

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