Final report for research project K 140344

Shiga toxigenic and commensal *Escherichia coli* and bacteriophages in cattle

Escherichia coli, while being a dominant commensal member of the mammal intestinal microbiota, also contains several pathogenic strains capable of causing serious enteric or systemic diseases, and having a diverse array of virulence factors, including various cytotoxins which are often associated with mobile genetic elements. Special attention is given to Shiga toxin producing *E. coli* (STEC) strains, associated with food-borne zoonotic infections in humans, cattle being its main reservoir. *E. coli* strains belonging to other pathotypes are also frequently reported from cattle.

In the current project we sampled of cattle farms in Hungary. Our aim was to isolate STEC and related *E. coli* strains from the samples, and to characterise them in-depth, both pheno- and genotypically, with emphasis on mobile genetic elements, and especially on prophages. Comparative genomic studies were conducted using *E. coli* isolates of human origin. Another aim was to characterise the inducible prophages carried by the strains, as well as the free coliform-specific lytic phages isolated from the same samples. Determination of the phages' host specificity and lytic efficiency will help to further evaluate their role in horizontal gene transfer (HGT), as well as their possible use as therapeutic or biocontrol agents against pathogenic *E. coli* and other coliform bacteria.

1. Prevalence and characteristics of Shiga toxigenic, enteropathogenic and other cytotoxin-producing *E. coli* in cattle herds in Hungary

Samples were collected from cattle farms throughout Hungary, both from the faeces and milk of animals, as well as samples representing the environment, collected from the barn floor at selected farms. A total of 309 samples, comprising of faecal samples (n = 215), and milk samples (n = 81) from 215 healthy cattle, along with 13 samples from the farm environment were taken. Sampling was performed on 18 cattle farms representing different regions in Hungary between 2017 and 2018.

The *stx* genes were detected in 20 out of 309 samples (6.5%) and from the *stx*-positive samples a total of 47 STEC isolates were identified, as multiple colonies were stored from each sample. Out of these, 18 also carried the *eae* gene encoding the intimin adhesin, and they were classified as EHEC; these EHEC isolates represented nine samples altogether. A further 18 samples (5.8%) contained isolates carrying *eae* without *stx*, and altogether 44 such genotype isolates, classified as EPEC, were identified.

In addition to the animal samples, 184 *E. coli* strains collected in the National Food Chain Safety Office during 2017 and 2018, were included in the screening for key virulence genes of

STEC and EPEC. Out of this strain collection 5 strains proved to be EPEC and 11 strains harboured the *cdt* (cytolethal distendin toxin) genes. None of them carried *stx* or *eae* genes.

1.1 Genome analysis of STEC and aEPEC strains

The draft genomes of altogether 26 E. coli strains (12 bovine STEC, (out of them 9 EHEC), 4 bovine EPEC, 3 human EHEC, and 2 human EPEC strains, as well as 5 *stx-, eae- E. coli* strains labelled as 'commensal') were determined analysed using Illumina Miseq platform. The bovine strains represented strains isolated in the current study as well as those from the earlier collection by Tóth et al, 2009 (Tóth et al., 2009). The virulence, antimicrobial resistance and prophage gene pool of the strains was also determined using online platforms (VirulenceFinder, ResFinder, PHASTER). All EPEC strains lacked the gene encoding the bundle forming pilus (*bfp*), therefore they were categorized as atypical EPEC (aEPEC). The integration sites of their key virulence genes, as well as their phylogenetic relations have also been assessed. No substantial difference was found between the virulence gene array and the phylogenetic positions of bovine and human strains, pointing to the zoonotic potential of the former. The integration sites of key virulence genes corresponded to the typical ones associated with the sequence types and serotypes of the strains, albeit in one case the integration site of the of the Stx2 prophage could not be determined, suggesting a potentially new integration site for this mobile genetic element (Sváb et al., 2022a). Besides the publication, and the domestic congresses mentioned in the yearly report, we also presented the results as a poster at the international FEMS2022 hosted by the Federation of European Microbiological Societies in Belgrade, Serbia, held between 2022-06-29--07-02. Genomes were deposited in GenBank under accession numbers PRJNA764596, SAMN21509413-SAMN21509438, and SRR15970245-SRR15970246.

1.2 Presence of P2-like prophage regions in *cdt*+ and *cdt*- strains
Using targeted PCR reactions, we screened for the presence of characteristic P2-like phage genes among 27 freshly isolated CDT+ as well as 16 CDT-negative bovine *E. coli* strains. Thirty-nine out of 43 strains carried at least one P2-like prophage gene, and 8 prophage regions were exclusively associated with the presence of *cdt* genes. Twenty strains carried at least one variant of the regulatory gene C, with 5 strains carrying two variants at the same time. In 5 out of the 27 cdt-positive strains the direct association of the *cdt* gene cluster to the T/O integration hotspot of the P2-like prophage was shown. Interestingly, there were also three cdt+ strains that did not carry any P2-like prophage genes, suggesting an alternative genetic vector for the cdt gene cluster in bovine *E. coli* strains (Kotogán, 2020).

The genome sequence of five *cdt*-positive bovine *E. coli* strains isolated in the current project was also determined to the draft level. It was found that besides the *cdt* gene cluster, they harboured 2-4 additional virulence genes. All of them carried the *gad* with a putative role in

glutamate-dependent acid resistance, and the *iss* associated with serum resistance. All strains harboured at least two plasmids, with the IncFB plasmid carried by all of them.

Nanopore sequencing aimed to close the genomes of the STEC and EPEC strains, as well as the CDT-positive *E. coli* strains is currently underway, which will further help in elucidating the presence and role of mobile genetic elements in the genome evolution and pathogenicity of these strains.

1.3 Complete genome sequence of a historical *Shigella dysenteriae* serotype 1 isolate and comparative analysis

In order to gain more data about the evolutionary history of Stx genes and prophages, using Illumina and Nanopore platforms, we determined the complete genome sequence of the historical Hungarian *Shigella dysenteriae* type 1 isolate, HNCMB 20080, originally isolated in 1954. In addition to the 4,393,622 bp long chromosome, HNCMB20080 carries two small plasmids (8,953 and 3,069 bp) as well. The conserved nature of SD1 genomes was confirmed, although when compared to available complete SD1 genomes, HNCMB20080 had 42 chromosomal inversions. The PHASTER search identified altogether 27 prophage-like regions amounting to a total length of more than 370 kbp. Eight regions were considered complete prophages, with the Stx1-carrying prophage among them. ResFinder indicated the presence of the mdr macrolide-resistance encoding gene. Phenotypic tests showed that HNCMB20080 has a low-level resistance to erythromycin with a minimum inhibitory concentration (MIC) of 32 μg/ml. Data search analysis of the available complete *S. dysenteriae* genomes (n=20) in GenBank and the failure of the Stx prophage induction indicate the notion that Stx-encoding phages of STEC did not originate from *S. dysenteriae* serotype 1 strains. We deposited the chromosome and plasmid sequences to GenBank with accession numbers CP061527-CP061529 and published our detailed findings (Sváb et al., 2021b).

1.4 Characterisation of aEPEC strains isolated from broiler chicken in Hungary

In addition to aEPEC isolated from bovine and human sources, we wished to assess the prevalence and characteristics of these pathogens from poultry as well. By investigating a total of 288 *E. coli* strains isolated from poultry no STEC were identified but 83 aEPEC strains were identified and characterized. Thirty-five aEPEC strains from the slaughterhouse and 48 aEPEC strains from the National Reference Laboratory (NRL) collection were found. The aEPEC isolates belonged to serogroups O14, O108, and O45. Importantly multidrug resistance (MDR) with several antibiotic groups abundant in aEPEC strains (80 out of 83 aEPEC) with a diverse resistance pattern (n=56). Regarding the phylogenetic groups of aEPEC, all four main groups

were represented but there was a shift toward the B2 group (25%) as compared with the non-EPEC isolates (3%).

Our results indicate that the high frequency of aEPEC in broilers and on their carcass surface, with frequent MDR to several antibiotic groups, raises the possibility that these strains pose a zoonotic risk to humans (Adorján et al., 2020).

2. Characterisation of lytic bacteriophages active against STEC and other pathogenic Enterobacteria

An important aim of the project was to characterize lytic bacteriophages capable of reducing or eliminating intestinal pathogenic *E. coli* from various environments, with a focus on eradicating O157 strains from beef. Therefore, we characterised several novel lytic phages, both of earlier isolation and those isolated in the current project.

2.1 Characterisation of T5- and rV5-like lytic bacteriophages effective against *E. coli* O157 and other Enterobacteria

We investigated the biocontrol potential of T5-like and rV5-like phages isolated earlier against food borne pathogens including EHEC O157, Shigella as well multi drug resistant *E. coli* strains. Their whole genome sequences and phylogenetic relations were also determined. Results of these studies were presented at the 10th International symposium of STEC/ VTEC (Florence, 2018) and were published in two full length scientific papers (Sváb et al., 2018a, 2018b).

2.2 Characterisation of a new type of Myovirus

Earlier we isolated a new bacteriophage from cheese on *E. coli* MG1655, which we named C130_2. In the current project we proceeded to its in-depth characterisation. The phage was morphologically a member of the family Myoviridae. Its whole genome sequence was determined, and we found that the 41,775-base-pair double-stranded DNA genome of C130_2 contains 59 ORFs but exhibits overall low sequence similarity to bacteriophage genomes for which sequences are publicly available. Phylogenetic analysis indicated that C130_2 represents a new phage genus. The phage could be propagated well on EHEC O157:H7 and other pathogenic *E. coli* strains, as well as on strains of various *Shigella* species (Sváb et al., 2019a).

2.3 Characterisation of a new P2-like phage capable of lysing *Citrobacter rodentium* and *Shigella sonnei*

We characterised a new bacteriophage, R18C of rabbit origin, which was capable of lysing *C. rodentium* strain ICC169 as well as multiple *Shigella sonnei* strains. Morphologically it belonged to the Myoviridae family of Caudovirales. Its whole genome sequence was determined, and it proved to be a representative of P2-like phages (Peduovirinae subfamily of Myoviridae), of which the majority are not lytic in lifestyle. An effective lytic phage of *C. rodentium*, a model organism of human EPEC in mice could be a valuable asset in experiments

aiming the control of these pathogens using phages (Sváb et al., 2019b). Besides the publication, results were also presented as a poster at the Phages 2019 conference in Oxford, United Kingdom between 2019-09-11—09-12.

2.4 We have determined the genome sequence of 11 new bacteriophages isolated in the current project, which showed lytic activity on *E. coli* strains of the O157 serogroup (GenBank accession numbers MT884006-MT884015 and MT951623; Sváb et al., 2021a). Based on sequence homologies the phages represented three different bacteriophage genera (Tequatrovirus, Vequintavirus, Dhillonvirus). We have submitted the genome sequences to GenBank, the accession numbers are MT884006-MT884015 and MT951623 (2). Applying three chosen bacteriophages representing the three genera we monitored the inhibition of the EHEC prototype strain O157:H7 Sakai on minced beef. An average of 3-fold reduction in the viable bacterial count was achieved under various conditions when compared to the phage-free controls (Papp, 2020; Sváb et al., 2022b).

The EOP and virulence index (Storms et al., 2020) of eight additional bovine lytic phages as well as the cocktail of the three characterised phages against the Sakai, *E. coli* K-12 MG1655 and *C. rodentium* ICC169 strains were determined (Deák, 2021), Table 1.

Phage name	**Host**	**Virulence index**	**Reference**
vb_EcoM_9_1	EHEC O157:H7 Sakai	0,761	(Sváb et al., 2022b)
vb_EcoS_25_1D	EHEC O157:H7 Sakai	0,839	(Sváb et al., 2022b)
vb_EcoM_11CS3	EHEC O157:H7 Sakai	0,865	(Sváb et al., 2022b)
MAWA9	EHEC O157:H7 Sakai	0,666	not yet published
Emőd13	EHEC O157:H7 Sakai	0,118	not yet published
ML1/O	EHEC O157:H7 Sakai	0,591	not yet published
3-phage cocktail	EHEC O157:H7 Sakai	0,543	not yet published
EmődT7	EHEC O157:H7 Sakai	0,723	not yet published
ML9	EHEC O157:H7 Sakai	0,633	not yet published
ML10	*E. coli* K-12 MG1655	0,486	not yet published
MAWA3/6	*E. coli* K-12 MG1655	0,428	not yet published
MAWA9	*E. coli* K-12 MG1655	0,267	not yet published
HH14	*C. rodentium* ICC169	0,193	not yet published
HH13	*C. rodentium* ICC169	0,134	not yet published
R18C	*C. rodentium* ICC169	0,268	not yet published

3. Role of prophages in the genome evolution of STEC and related *E. coli* pathotypes

3.1 PCR typing scheme for STEC and other pathogenic *E. coli*

We published a typing scheme of 12 PCR reactions to be performed as 4 multiplex reactions, targeting prophage sequences present in O157 EHEC and STEC strains as well as pathogenic

E. coli of other pathotypes. The results of the reactions can be given as a 4-digit numeric code and can be used for the rapid typing and epidemiological tracing of intestinal pathogenic E. coli (Tóth et al., 2022).

3.2 Phage induction from bovine STEC strains

Phage induction was attempted with standard methods. Only in the case of an EHEC of human origin and of a bovine strain of earlier isolation, included in our comparative genomic study (Sváb et al., 2022a), did we manage to induce Stx1-carrying phages using ciprofloxacin, but these phages not proved to be stable enough. Nevertheless, their further characterisation is planned.

3.3 Transfer experiments using Stx phages

In the first experiments we successfully lysogenised the bovine commensal strain ML5/6D isolated in the current study with a Stx1 phage originating from a *Shigella sonnei* strain, described earlier (Tóth et al., 2016). Assessing the stability of lysogens and the genomic integration site of the Stx prophage is planned in further experiments.

We planned to publish our results in high-rated journals of the field with at least one open access publication, which we managed with eight full-length scientific papers, all of them in Q1 journals (of which one is D1) and a genome announcement. Five of the works were readily available upon publication as open access. Three university MSc students have helped in the work, as well as wrote and successfully defended their master's theses. Our results were frequently presented on conferences organised by the Hungarian Society for Microbiology and other congresses detailed in the yearly reports.

Our scientific aims were also achieved – we assessed the prevalence of STEC, aEPEC and other cytotoxic *E. coli* in Hungarian cattle herds, and determination of the whole genome sequences yielded valuable information on their pathogenic potential and phylogenetic relations, calling the attention to the need of frequent monitoring of these pathogens. From bovine and various other sources, we characterised several, hitherto undescribed lytic coliphages, the majority of which were capable of lysing *E. coli* O157 strains. Their further evaluation will help to assess their potential application as biocontrol against pathogenic *E. coli* in foodstuff and animals. We plan to publish our as-of-yet unpublished results in the near future as well.

Finally, we wish to thank all the participants and co-authors of the project, and to thank the NKFIH for the support. We respectfully ask for the acceptance of our report.

Domonkos Sváb

principal investigator

Papers and theses written and published with the support of the project

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Total impact factor: 43.495

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