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ECOLOGICAL SOURCES OF WILD-TYPE SHIGA-TOXIN AND INTIMIN-PRODUCING *Escherichia coli* ISOLATES IN BULGARIA: ANTIMICROBIAL SUSCEPTIBILITY AND MULTILOCUS SEQUENCE TYPING

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Abstract. Ruminant animals bread freely in Bulgarian national and natural reserves in the recent years serve as the main reservoir of Shiga-toxin and intimin-producing *Escherichia coli* strains, which pose risks for the members of these ecosystems, including humans. The ability of *E. coli* to maintain viability in the environment for months provides it with opportunities to circulate throughout the biotope and the various members of the respective biocenosis. The process is often facilitated by the run-off water resulting from heavy rains. The possibility of horizontal transfer between different strains of genes coding for pathogenicity factors increases the risk of their dispersal in the environment. The epidemiological typing of the pathogenic *E. coli* strains impacting biosafety, human and animal health is of paramount importance to control their spread. The present work aims to study Bulgarian Shiga-toxin and intimin-positive *E. coli* isolates from different sources, including their antimicrobial susceptibility and the intimin toxin type, as well as to perform Multilocus Sequence Typing analysis. The results showed that the isolates had wild-type susceptibility to the antibiotics tested, intimin (type beta 1, theta and omicron) and belonged to various sequence types and clonal complexes. The information obtained will compleent the profiles of circulating pathogenic strains of *E.coli* which will facilitate risk assessment and adoption of adequate measures for limiting contamination of biocenoses with pathogenic strains-infected faeces from agricultural animals.

Keywords: *Escherichia coli*, MLST, Shiga-toxin, intimin, sequence types, Clonal complex.

AIMS AND BACKGROUND

Escherichia coli is a facultative anaerobe species found in the gut of mammals, birds and reptiles. Studies on 5S and 16S ribosomal RNA (rRNA) suggest that *Escherichia* emerged 120–160 million years ago, evolving along mammals to the relationships we know today. Most *E. coli* strains play key beneficial roles in the bodies of animals and humans; however, there are pathogenic strains that cause

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urinary tract infections, neonatal meningitis, septicaemia and surgical infections. Infections of importance are caused by Shiga toxin (Stx) producing *E. coli* or diarrheagenic group (STEC/EHEC) strains (i.e. gastroenteritis and haemolytic uraemic syndrome (HUS) (Ref. 1)), and by diarrheagenic enteropathogenic *E. coli* (EPEC) as a result of consumption of contaminated sources such as water[2], sprouts[3], meat and dairy products[4]. STEC have their main reservoir in ruminants, but were also identified in pigs, horses, dogs, cats and wild animals like birds, deer, elk, foxes, raccoons, boars, coyotes, rabbits, and rodents. Even frogs and insects can act as physical carriers – i.e. houseflies (*Musca domestica*), dump flies (*Hydrotaea aenescens*), and dung beetles (*Catharsius molossus*)[5], which can spread in the environment and vector these pathogens to other animals, humans, fruits and vegetables. This is possible due to the low infectious dose and the potential that these pathogens have to survive in such sources[6] and the environment. The ability of *E. coli* to maintain viability in the environment for months provides it with opportunities to circulate throughout the biotope and the various members of the respective biocenosis.

Different Stx strains have different biological activity, some showing lower affinity to the glycolipid receptor Gb3 as a result of amino-acid substitutions in the protein B-subunit[7]. Another virulence factor involved in human diseases caused by STEC is intimin, which is implicated in attaching and effacing lesions (AELs) in enterocytes[8]. The gene encoding intimin (*eae*) is mainly detectable in diarrheagenic EPEC that form AELs (A/E) but do not produce Shiga toxins[9].

Extensive research in microbial resistance to antibiotics is available for risk assessment in many countries[10]. It is suggested that the antibiotic resistance in *E. coli* in humans is often due to the use of antimicrobial agents in agriculture[11]. As a result, there is an increase in resistance detections, as the resistance genes can spread through transposable genetic elements[12].

The free-range grazing of ruminants has become a common practice in their breeding in mountainous regions and natural parks in Bulgaria. In addition to the mechanical damage to the ecosystems these practices serve also as source of faecal contamination with *E. coli*. Such contamination was detected in various natural water sources in Pirin park[13]. The possibility of horizontal transfer between different strains of genes coding for pathogenicity and resistance factors increases the risks both for humans and for the wild members of these ecosystems. That makes it important to study and characterise potentially dangerous *E. coli* isolates, circulating in the country. In Bulgaria, Shiga-toxin and intimin-producing *E. coli* isolates from different sources have been obtained and studied; however, there are still no reports on Multilocus Sequence Typing (MLST) analysis of the intimin type in isolates that carry this gene, or their antibiotic susceptibility. In this context, present study aimed to determine the MLST profile of the *stx1, stx2* and

eae (intimin)-positive *E. coli* food isolates in Bulgaria, as well as to detect their intimin type and susceptibility to antimicrobial drugs (AMDs).

EXPERIMENTAL

Samples, bacterial isolation and identification. In this study we used seven shiga toxin- or intimin-producing *E. coli* isolates from different sources – chickens, cow milk and pork meat. Cultures were grown in Buffered Peptone Water and on solid nutrient media: Tryptone Bile X-glucuronide agar (TBX), MacConkey agar and Endo agar. Solid media were used for strain purification, whereas liquid media – for all subsequent experiments, i.e. biochemical assays, antimicrobial susceptibility assays and DNA extraction for polymerase chain reaction (PCR) and sequencing. The biochemical identification of the isolates was performed using the MICRONAUT-E system (Merlin).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing of the *E. coli* isolates studied was performed by the Kirby–Bauer disc diffusion assay, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. A total of 18 AMDs were tested: ampicillin, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cefalexin, cefuroxime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, amikacin, gentamicin, tobramycin, tigecycline, chloramphenicol and trimethoprim-sulphamethoxazole.

The minimum inhibitory concentration (MIC; μg/ml) against the *E. coli* isolates was determined using the agar dilution method according to EUCAST-2016. The Sensititre panel was used in order to detect the antimicrobial susceptibility of *Enterobacteriaceae* (EUVSEC).

PCR for intimin production, housekeeping genes and MLST. The primers used for intimin typing by PCR and sequencing are listed in Table 1. DNA was extracted from bacterial suspensions by Isolate II Genomic DNA Kit (Bioline, UK). PCR was carried out in 50-μl reaction volumes and each reaction mix included: 1 μl (10 pmol) of each primer, 3.5 μl of DNA (50–200 ng), 17.5 μl PCR water and 25 μl MyTaq PCR master mix 2x (Bioline, UK).

Table 1. Primers used for amplification of different *eae* gene variants, the reference sequences used and the annealing temperatures

Primer name	Reference sequences	Sequence 5′ to 3′	Position of primers	Size product bp	Ann. T (℃)
F – universal	all sequences	ATTACTCATGGTTKTTATDCCCGG	4–28	–	–
R1 alfa 1, 2, 8, nu	AF022236 α1 DQ523600 α2 FJ609835 α8	CCGTTACCTCCGCTRGCTTTC	2372–2352	2369	61.0
R2 mu+*	DQ523607 μ	GCCTTAATCTCAGTAATRCTGGCC	1994–1971	1991	61.0
R zeta, zeta 3, tau, nu	AF449417 ζ FM872423 ζ3 AY696839 τ DQ523615 ν	CGGTAATAGTTGTASTCCCCT	2467–2447 2470–2450	2464 2467	58.5
R rho, ypsilon	DQ523613 ρ AM116755 ϒ	GGCCATATTGCAACCAGACATCA	2350–2328	2347	60.5
R iota2	AY696842 ι 2	GCATTTAACTTAACCTGACCA	2354–2334	2351	56.5
R omicron	AY696838 o	GCAATTGCCGGATTTGCTGAG	2408–2388	2405	59.5
R theta, gamma2	AF449418 ϴ AF025311 γ2	GCCATGAATATGTACCATTACCACCG	2392–2367	2389	62.0
R internal – seq	all sequences	GTAAAGCGGGAGTCAATGTAACG	767–745	–	–
F2 internal – seq	all sequences	GAATACTGGCGAGACTAT	880–897	–	–

*Common primer for types: α8; γ2; τ; ν; ε1 – DQ523606; ε2 – DQ523614; ε3 – AJ876649; ε4 – AJ876651; η – DQ523604; η2 – AJ876652; π – AJ705052; ξ – DQ523610; β1 – AJ277443; β2 – DQ523605; β3 – AJ876654; ι1 – DQ523601; κ – DQ523611; λ – DQ523609; ς – AJ781125.

We performed MLST analysis based on seven housekeeping genes: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), *icd* (isocitrate/isopropylmalatede hydrogenase), *recA* (ATP/GTP binding motif) (ID Genomics, USA). The primers and PCR conditions are previously described[14]. The amplified PCR products were purified by gel electrophoresis using Isolate II PCR and Gel Kit (Bioline, UK) and were sent for sequencing by Macrogen Europe (Amsterdam, Netherlands).

Qualitative and quantitative analysis of the extracted DNA and the PCR products was performed using 2% agarose electrophoresis (Lonza, USA) at 120 V, 80 mA for 40 min at room temperature. The DNA molecular size markers were 100 bp and 1 kb (Bioline, UK).

Sequence alignment and phylogenetic analysis were done using Mega7 software[15]. The aligned sequencing data of the housekeeping genes were analysed using the *E. coli* MLST Database PubMLST *E. coli* (Achtman) (University of Oxford, UK)[16].

RESULTS AND DISCUSSION

Identification of *stx* and *eae*-positive *E. coli* isolates from various agricultural animals and products thereof is just the 'peak of the iceberg' from the pathogen circulation in the country. Free-grazing ruminants seek natural water sources to drink and thus can quickly spread pathogenic and/or AMD resistant *E. coli* strains over large territories. The problem is aggravated by the use of some of these water sources by the inhabitants of nearby settlements. Furthermore the presence of agricultural animals and seasonal points of their presence in mountainous regions increases the number of mechanical carriers that come into contact with wild fauna. While in the dedicated breeding settings the environment is controlled by humans (partially valid for the seasonal ones) they also provide conditions for survival and dispersal of *E. coli* thus serving as anthropogenic nidus for infection. The *stx* genes are bacteriophage-borne genetic determinants (lysogenic lambdoid phage) and can be transferred horizontally[17]. The common localisation of the resistance genes is in the plasmids, which facilitates their transfer to other *E. coli* strains in the environment.

Our antimicrobial susceptibility assay showed that all isolates were susceptible to aminopenicillins, as well as to all other AMDs, corresponding to 'wild type susceptibility'. Due to its widespread presence *E. coli* could become a serious problem for humans as resistant strains can transfer antibiotic resistance determinants not only to other *E. coli* strains, but also to other species of bacteria in the gastrointestinal tract as well as acquire resistance from other microorganisms[18]. That is why

the acquisition of resistance genes by Shiga-toxin and intimin-producing *E. coli* could lead to difficulties in the treatment of infections in Bulgaria.

Based on the protocols developed for identification of intimin production, we determined that our isolates produced omicron, beta1 and theta type toxins. *E. coli* intimin beta 1 strains, which cause diarrhea in humans, are the most frequently isolated variant in humans (up to 61.1%), calves[19] and less often in pigs[20]. The second most common causative agent of diarrhea in children after beta 1 is theta toxin[21], which was also found in Bulgaria. The fact that our isolate was identified as a member of this group indicates that it poses health risks to both humans and animals. This also opens questions about its source, which could have been anthropogenic, in the process of food production. Intimin subtype omicron has been defined relatively recently and is reported in atypical EPEC (aEPEC) causing diarrhea in children[22]. In a previous study[23] Isolate 7 showed delayed cytotoxicity in Vero cells (at 72 h). In HeLa cells, aEBEC reportedly produced prolonged localised adherence (6 h) (LA6) (in typical EPEC, the LA production occurs after 3 h). Intimin subtype omicron mediates the formation of compact microcolonies (non-invasively), although the process involves another unknown invasive factor as well[22]. It could be speculated that the toxic effect of omicron subtype is not strong, which is why clinical symptoms occur in children in presence of additional invasive species.

Table 2. Results from the sequence analysis of seven housekeeping genes and their allelic profiles based on MLST Database PubMLST *E. coli* (Achtman)

Isolates/Source	*Escherichia coli* (Achtman), Housekeeping genes, allelic profile							ST	Clonal complex
	adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*		
22 *stx1* a, d/chicken	6	4	5	26	20	638	14	40*	40*
39 *stx1* a, d/chicken	6	4	5	26	20	8	14	40	40
348 *stx2*g/pork meat	6	65	4	18	24	8	14	1432**	N/A
7 *eae* omicron/cow milk	9	23	33	18	11	8	6	642	278
138 *eae* beta1/pork meat	10	11	4	18	8	8	2	3529**	N/A
342 *eae* theta/pork meat	9	23	33	18	9	8	6	795	N/A
343 *eae* theta/pork meat	9	23	33	18	9	8	6	795	N/A

*Close to ST and Clonal complex 40;**The isolates without connection with other ST and Clonal complex.

This is the first study based on MLST analysis in Bulgarian Shiga-toxin and intimin-producing *E. coli* isolates. The results from the allelic profiling of the housekeeping genes and the MLST analysis are presented in Table 2. Due

to the fact that the MLST database of *E.coli* profiles is still incomplete some of the sequence types identified in our study are not associated with specific clonal complex. Having in mind the high levels of conservation in housekeeping genes and their allelic redundancy within the studied isolates, a common origin can be inferred that (due to different biological circulation and evolution) gradually lead to the formation of diverse sequence types observed. ST40 and ST642 appeared as basal type that gave rise to new types (Fig. 1). Because of that we have to expect new MLST profiles. For example, the isolate 22 with one point mutation in *purA* gene is changed from allelic profile 8 to 638. The presence of further basal and undefined STs and Clonal complexes can later reveal new MLST types.

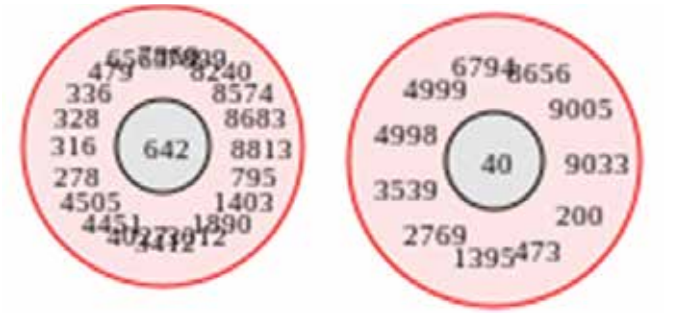

Fig. 1. E-BURST (pubmlst) analysis of five *E. coli* isolates (group definition: 5 or more matches; groups with basal ST are displayed as an image)

Free-grazing of ruminant agricultural animals in the mountainous regions increases the risk of surface water and groundwater sources' contamination, which (together with the food) is the main source of *E. coli* infections in children[24]. Therefore our results demonstrate that the *E. coli* isolates studied pose risk for children which can arise from poor hygiene during picnics and other outdoor activities in the mountains, from the food in the huts, as well as by mechanical contamination along the routes followed by agricultural animals all of which can serve as a potential source of pathogenic (STEC, EPEC) *E. coli*.

CONCLUSIONS

The results obtained in this study clearly demonstrate the importance of continuing the screening for other Shiga-toxin and intimin-producing *E. coli* isolates from food products, animals and humans, to build up a database of their MLST profiles and the characteristics of their toxigenic, virulence and antimicrobial susceptibility genes. The information obtained will complement the profile of circulating pathogenic *E. coli* strains that is needed for assessing the risks and taking countermeasures for limiting agricultural faecal contamination of surface and ground waters. The

presence of basal and undefined STs and Clonal complexes indicates that new MLST types should be also identified in further studies.

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