Molecular characterization of *Escherichia coli* isolated from hemp seeds

ZUZANA REŠKOVÁ – MICHAL ANDREZÁL – HANA DRAHOVSKÁ – TOMÁŠ SZEMES – TOMÁŠ KUCHTA

Summary

Hemp seeds are increasingly produced on small farms in Slovakia and used for production of food. We detected contamination of 1.4×10^7 CFU·g^{-1} coliform bacteria in hemp seeds from a small farm and isolated three strains of *Escherichia coli*. These were characterized by GTG-typing and by whole genome sequencing. The three strains were indistinguishable from each other by GTG-typing but different from a reference strain *E. coli* CCM 2024. They were not distinguished either by multi-locus sequence type (MLST) deduced from whole genome sequences, as all strains were of sequence type 58, or by core genome-based MLST (cgMLST), as all strains belonged to type 148521. All strains were found to be fumC4/fimH32 type and serotype H21, O-antigen was not identified. No plasmid origin was detected. No gene encoding for shiga toxin was detected. Several virulence-associated genes were detected in all three strains at high identities with sequences in the database, namely, *fimH, lpfA, terC, csgA, hlyE, nlpI, yehB, yehC* and *yehD*. Further virulence-associated genes were detected at shorter lengths, namely, *fdeC, gad* and *yehA*. Several antibiotic resistance genes were also identified. The strains were found to be virtually identical. With a probability of being a human pathogen, they need to be addressed for on-farm food production safety.

Keywords

hemp; *Escherichia coli*; high-throughput sequencing; DNA; virulence; safety

Industrial cultivars of hemp (*Cannabis sativa*), which are characterized by low levels of psychoactive metabolites, are attractive crops for production on small farms. The hemp seeds produced are processed mainly to oil or to other food products such as "milk" or protein concentrates. When entering the food chain, it is important that they are microbiologically safe [1–3].

Together with the whole plant, seeds are susceptible to microbiological contamination in the field and during handling. The contamination may come from air, water, soil, animals, insects and humans. Due to the low activity of water on the surface of the seeds, the growth of most microorganisms is prevented but presence of live bacteria at potentially dangerous levels cannot be excluded. Among these, *Escherichia coli* belongs to the important ones due to its high prevalence in the field environment as well as in insects, animals and humans [4–6].

E. coli strains are usually commensal and co-exist with humans in good health and with mutual benefit. However, there are several highly adapted *E. coli* clones that have acquired specific virulence attributes and cause a broad spectrum of diseases. These pathotypes cause enteric/diarrheal disease, urinary tract infections and sepsis/meningitis. Therefore, it is necessary to trace them in food and food raw materials with high resolution in order to clearly distinguish the strains that may cause a threat to food safety [7, 8].

In our previous study on microbial contaminants of oil seeds [9], we encountered *E. coli* that contaminated hemp seeds at comparatively high

Zuzana Rešková, Tomáš Kuchta, Department of Microbiology, Molecular Biology and Biotechnology, Food Research Institute, National Agricultural and Food Centre, Priemyselná 4, P. O. Box 31, SK-82475 Bratislava 25, Slovakia.
Michal Andrezál, Hana Drahovská, Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, SK-84215 Bratislava, Slovakia.
Tomáš Szemes, Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Ilkovičova 6, SK-84215 Bratislava, Slovakia; Science Park, Comenius University, Ilkovičova 8, SK-84104 Bratislava, Slovakia; Geneton Ltd., Ilkovičova 8, SK-84104 Bratislava, Slovakia.

Correspondence author:
Zuzana Rešková, e-mail: zuzana.reskova@nppc.sk

levels. The seeds were not intended for use as food but were processed to oil, which would devitalize the bacteria. However, it seemed important to know if the strains were pathogenic as the presence of pathogens could pose a threat to safety because storage and production areas are not hygienically separated in small farms and cross-contamination of finished products could occur. Therefore, in the present study we isolated three *E. coli* strains from hemp seeds and characterized them using molecular methods. First, they were characterized by GTG-typing, which is a kind of repetitive sequence-oriented amplification-based method that provides moderate discrimination at low workload [10]. Then, whole genome sequences were obtained by high-throughput sequencing and the strains were genetically characterized by bioinformatic tools.

MATERIALS AND METHODS

Samples

Hemp seeds were collected from a farm in Slovakia immediately after harvesting. Based on microbiological analysis, it was specified that they contained 1.5×10^7 CFU·g^{-1} total mesophilic aerobes, 1.4×10^7 CFU·g^{-1} coliform bacteria, 4.5×10^5 CFU·g^{-1} moulds, less than 10^5 CFU·g^{-1} yeasts, less than 5.0×10^3 CFU·g^{-1} *Bacillus cereus*, while no coagulase-positive staphylococci, *Salmonella enterica* and *Listeria monocytogenes* were detected [9]. The water activity was 0.48. From plates of Chromocult coliform agar (Merck, Darmstadt, Germany) used for quantification of coliform bacteria, three characteristic colonies were picked and designated Strain A, Strain B and Strain C. The strains were subcultured in tryptic soy broth (Merck), purified on tryptic soy agar (Merck) and stored freeze-dried. The reference strain *Escherichia coli* CCM 2024 was obtained from Czech Collection of Microorganisms (Brno, Czech Republic). For GTG-typing, DNA was isolated from bacterial cultures by DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For high-throughput sequencing, DNA was isolated by High purity bacterial genomic DNA isolation kit (Canvax, Córdoba, Spain).

GTG-typing

For GTG-typing, primer GTG5 (5'-GTG GTG GTG GTG GTG-3') was used. Polymerase chain reaction (PCR) was performed in a volume of 25 μl with 1.5 U Kapa Taq HotStart PCR (Kapa Biosystems, Potters Bar, United Kingdom), 2 μmol·l^{-1} of the primer (synthesized by Eurofins, Ebersberg, Germany), 10 mmol·l^{-1} of dNTP mixture (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 3 μl of the isolated DNA solution in Veriti thermocycler (Thermo Fisher Scientific). The PCR programme consisted of the initial denaturation of 3 min at 95 °C and 35 cycles of 30 s at 95 °C, 30 s at 54 °C and 60 s at 72 °C. The reaction was ended by final polymerization of 7 min at 72 °C. PCR products were separated and visualized by flow-through gel electrophoresis in Qiaxcel Advanced (Qiagen, Hilden, Germany) using a high resolution cartridge, OM1200 method, QX DNA size marker FX174/HaeIII and QX alignment marker 5 bp/3 kb. Electrophoresis results were analysed by Qiaxcel ScreenGel software (Qiagen).

Library preparation and whole genome sequencing

Nextera XT DNA library preparation kit (Illumina, San Diego, California, USA) was used to prepare libraries for whole genome sequencing. Libraries were purified by AMPure XP magnetic beads, their concentration was determined with Qubit dsDNA HS assay kit (Thermo Fisher Scientific) and consistency by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Libraries were normalized to 4 nmol·l^{-1}, samples for individual strains were pooled, denatured by addition of 5 μl of 0.2 mol·l^{-1} NaOH to 5 μl samples and loaded to MiSeq cartridge (Illumina). Sequencing was carried out in a MiSeq instrument (Illumina) and 300 bp paired-end reads were obtained.

Whole genome sequence assembly and analysis

Reads were de novo assembled into contigs using SPAdes 3.10.1 genome assembler (Center for Algorithmic Biotechnology, St. Petersburg, Russia), reads shorter than 500 bp and reads with coverage lower than 20 being discarded. Contigs were annotated and analysed using the online platform BV-BRC (Bacterial and Viral Bioinformatics Resource Center, Chicago, Illinois, USA). The obtained whole genome sequences were analysed to determine the multi-locus sequence type (MLST) of the strains and presence of genes connected to pathogenicity or virulence [11] using MLST 2.0.9 software (Center of Genomic Epidemiology, DTU National Food Institute, Kongens Lyngby, Denmark), using MLST allele sequence and profile data from PubMLST (University of Oxford, Oxford, United Kingdom; database version of 14 November 2022) [12]. Core genome-based MLST (cgMLST) was determined by cgMLSTfinder 1.2 version 1.0.1

(Center of Genomic Epidemiology) [13]. In silico serotyping was performed by SerotypeFinder 2.0 (BioTools, Copenhagen, Denmark; software version 2.0.1, database version of 14 November 2022) [14]. Probability of potential human pathogenicity of strains was determined by PathogenFinder (Center of Genomic Epidemiology) [15]. Virulence-associated genes were identified using VirulanceFinder (Center of Genomic Epidemiology, version 2.0.3 with a database version of 2 February 2022) [16]. For further subtyping, CHtyper software tool (Center of Genomic Epidemiology) was used to identify *fumC* and *fimH* allele types of strains [17]. RestrictionModificationFinder software (Center of Genomic Epidemiology, version 1.1 of June 2015) was used to detect the genes of restriction-modification systems [18]. ResFinder FG 1.0 (Center of Genomic Epidemiology) was used to identify antibiotic resistance genes [19]. PlasmidFinder 2.1 software (Center of Genomic Epidemiology, version 2.0.1, database version of 18 January 2023) was used to analyse the genomes for plasmids [20]. Comparative analysis of genomes based on 2 513 loci of cgMLST by unweighted pair group method with arithmetic mean (UPGMA) and dendrogram construction was performed by Mega X (MEGA Software Development Team, Temple University, Philadelphia, USA) [21]. The sequences were deposited in BioProject database (National Center for Biotechnology Information, Bethesda, Maryland, USA) under accession number PRJNA936714 and in EnteroBase (University of Warwick, Coventry, United Kingdom) under accession numbers KMB-1146, KMB-1147 and KMB-1148.

RESULTS AND DISCUSSION

The three *E. coli* strains A, B, C were indistinguishable from each other by GTG-typing, while the reference *E. coli* strain was distinguished by the absence of a remarkable DNA fragment of 520 bp and by some further minor differences (Fig. 1).

Whole genome sequencing produced 160–210 contigs with the average coverage of 32–54 and a total genome size of 4.46–4.62 Mbp. The strains A, B, C were identical by MLST, as all strains were of sequence type (ST) 58. By C-H typing scheme based on sequencing of *fumC* and *fimH* genes, the strains belonged to the fumC4/fimH32 type. Virtually no differences were also found by cgMLST as all strains belonged to type 148521 differing by 7–38 missing alleles. These differences were probably caused by sequencing inaccuracies.

By comparing with other *E. coli* ST58 strains described previously [22, 23], *E. coli* strains of this study created a separate cluster and belonged to BAP3 group of strains (Fig. 2). All three strains were found to be flagellar serotype H21, but the O-antigen gene cluster did not match with any described O serotype. We also did not detect any plasmid origin by PlasmidFinder.

All three strains contained a very similar set of virulence genes (Tab. 1). While no gene encoding for shiga toxin was detected, several virulence-associated genes were detected in all three strains at high identities with sequences in the database or at shorter lengths of identical sequence. Strains contained genes for synthesis of enterobactin si-

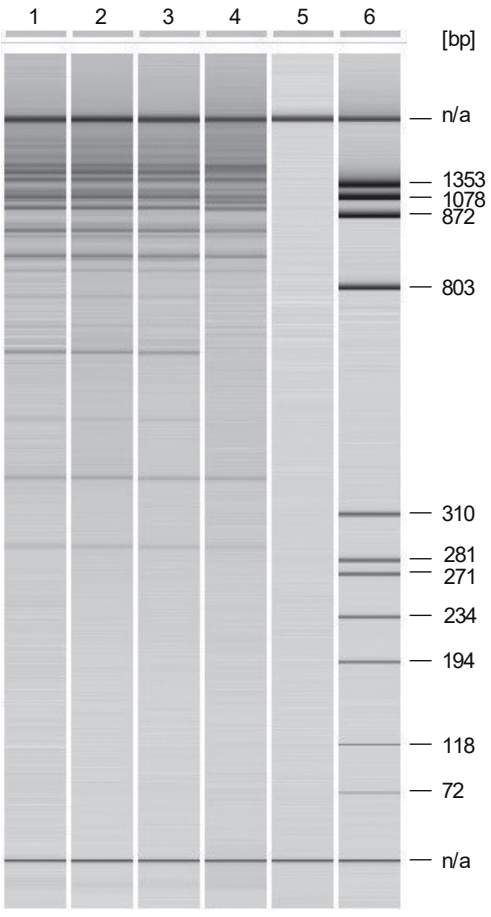

Fig. 1. Electrophoretic separation of products of GTG-PCR.

1 – strain A, 2 – strain B, 3 – strain C, 4 – strain CCM 2024, 5 – no-template control, 6 – standard FX174/HaeIII. Molecular weight of standards is given on the right. Front and rear of samples are marked as n/a.

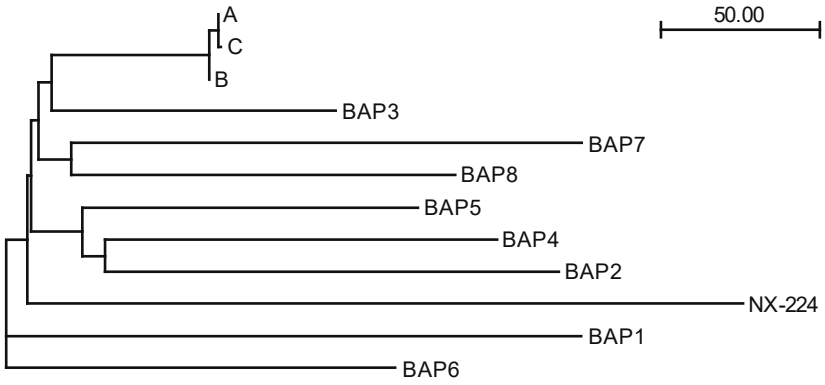

Fig. 2. Dendrogram of cgMLST relatedness of *Escherichia coli* strains.

A, B, C – *Escherichia coli* strains. BAP strains are representatives of major clusters of *E. coli* ST58 [22]. NX-224 is another related strain [24]. The abscissa indicates the number of different alleles.
Accession numbers of reference strains: BAP1 – DAFAUW000000000.1, BAP2 – DAFAVW010000001.1, BAP3 – DAFASU010000001.1, BAP4 – DAFAVM010000001.1, BAP5 – DAFATA010000001.1, BAP6 – DAFAVT010000001.1, BAP7 – AATACZ010000001.1, BAP8 – NOMP01000001.1, NX-224 – JANDCX010000000.

derophore, which enable them to accumulate iron from the environment. Genes encoding for several fimbrial adhesins were also detected, Fim, Sfm, Elf chaperone usher, type IV and curli fimbria were present together with FdeC non-fimbrial adhesin. The gene encoding for hemolysin E, an alpha pore-forming toxin expressed during anaerobic growth of *E. coli*, was also detected. Regarding restriction-modification systems, the genomes contained genes encoding for methyltrasferase type II enzymes M.Sso30807Dcm and M.EcoKII. Genomes of all three strains contained *ampC* EC-13 genes encoding for class C extended-spectrum β-lactamase conferring resistance to penicillins and cephalosporins.

E. coli ST58 strains are widely spread in environmental, animal and human samples worldwide, including wild birds [22]. This sequence type belongs to *E. coli* phylogroup B1, which contains mostly commensal strains but ST58 strains responsible for urinary tract infections or even sepsis recently emerged [22]. Pathogenic strains with the highest linkage to strains of this study were *E. coli* O39:H21, ST58, fumC4/fimH32 isolated in Italy from Bulldog puppies suffering from deadly diarrhea [23]. However, in contrast to our strains, whole genome sequencing revealed a complex resistome in those *E. coli* strains. Another genetically related *E. coli* O88:H21, ST58, fumC4/fimH32 was isolated from sheep in China, which carried a cefalosporin resistance gene [24]. Genomes of 752 *E. coli* ST58 isolates from diverse sources were recently sequenced and divided into six genome clusters [22]. Our *E. coli* ST58 strains showed highest similarity to the minor cluster BAP3, which contained mainly strains of bovine

Tab. 1. Virulence and antibiotic resistance genes detected in the genomes of *Escherichia coli* strains.

Category	Locus	Function
Siderophores	*fep* operon	Ferric enterobactin transport system
	ent operon	Enterobactin siderophore biosynthesis
Adhesins	*fim* operon	Type-1 fimbrium
	sfm operon	Chaperone usher fimbrium
	elf operon	Chaperone usher fimbrium
	pil operon	Type IV fimbrium
	csg operon	Curli fimbriae
	fdeC	Adhesin
Hemolysins	*hlyE*	Hemolysin E
Antibiotic resistance genes	*ampC* EC-13	Class C extended-spectrum β-lactamase EC-13

origin and was characterized by low virulence as well as a low antibiotic resistance potential [22].

The fact that the three *E. coli* isolates characterized in this study were almost identical means that they were probably representative of the dominant strain in that environment. Based on the results of genome analysis, they were probably of animal origin. Although they did not contain many toxin genes, with a probability of being a human pathogen they should not be neglected. Possible reservoirs as well as routes of contamination on the farm at pre-harvest and harvest stages should be analysed [4, 25–27].

CONCLUSION

The three *E. coli* strains isolated from hemp seeds were found to be virtually identical, with minor differences attributable to experimental noise. With a probability of being a human pathogen, they need to be addressed for on-farm food production safety.

Acknowledgements

This work was supported by the Operational Program Integrated Infrastructure within the project Demand-driven research for the sustainable and innovative foods, Drive4SIFood, 313011V336, co-financed by the European Regional Development Fund. Authors thank Dr. Janka Koreňová for the preliminary microbiological analysis.

REFERENCES

1. Rupasinghe, H. P. V. – Davis, A. – Kumar, S. K. – Murray, B. – Zheljazkov, V. D.: Industrial hemp (*Cannabis sativa* subsp. *sativa*) as an emerging source for value-added functional food ingredients and nutraceuticals. Molecules, *25*, 2020, article 4078. DOI: 10.3390/molecules25184078.
2. Cerino, P. – Buonerba, C – Cannazza, G. – D'Auria, J. – Ottoni, E. – Fulgione, A. – Di Stasio, A. – Pierri, B. – Gallo, A.: A review of hemp as food and nutritional supplement. Cannabis and Cannabinoid Research, *6*, 2021, pp. 19–27. DOI: 10.1089/can.2020.0001.
3. Rehman, M. – Fahad, S. – Du, G. – Cheng, X. – Yang, Y. – Tang, K. – Liu, L. – Liu, F. H. – Deng, G.: Evaluation of hemp (*Cannabis sativa* L.) as an industrial crop: a review. Environmental Science and Pollution Research, *28*, 2021, pp. 52832–52843. DOI: 10.1007/s11356-021-16264-5.
4. Brar, P. K. – Danyluk, M. D.: Nuts and grains: microbiology and preharvest contamination risks. Microbiology Spectrum, *6*, 2018, article 6.2.25. DOI: 10.1128/microbiolspec.PFS-0023-2018.
5. Derić, N. R. – Todorić, O. M. – Rošul, M. D. – Kocić-Tanackov, S. D. – Sikora, V. Š. – Kiprovski, B. M. – Mandić, A. I.: Influence of different chemical agents and storage conditions on the microbiological content of industrial hemp (*Cannabis sativa* L.) seeds. Food and Feed Research, *47*, 2020, pp. 159–168. DOI: 10.5937/ffr47-29058.
6. Guo, L. – Chen, X. – Li, Z. – Wang, M. – Che, Y. – Zhang, L. – Jiang, Z. – Jie, S.: Effects of continuous cropping on bacterial community and diversity in rhizosphere soil of industrial hemp: A five-year experiment. Diversity, *14*, 2022, article 250. DOI: 10.3390/d14040250.
7. Kaper, J. B. – Nataro, J. P. – Mobley, H. L. T.: Pathogenic *Escherichia coli*. Nature Reviews, *2*, 2004, pp. 123–140. DOI: 10.1038/nrmicro818.
8. Enciso-Martínez, Y. – González-Aguilar, G. A. – Martínez-Téllez, M. A. – González-Pérez, C. J. – Valencia-Rivera, D. E. – Barrios-Villa, E. – Ayala-Zavala, J. F.: Relevance of tracking the diversity of *Escherichia coli* pathotypes to reinforce food safety. International Journal of Food Microbiology, *374*, 2022, article 109736. DOI: 10.1016/j.ijfoodmicro.2022.109736.
9. Rešková, Z. – Andrezál, M. – Drahovská, H. – Koreňová, J. – Lopašovská, J. – Kuchta, T.: Bacterial and fungal communities associated with the surface of hemp seeds and poppy seeds. Journal of Food and Nutrition Research, *62*, 2023, pp. 63–69. ISSN: 1336-8672 (print), 1338-4260 (online). <https://www.vup.sk/download.php?bulID=2183>
10. Versalovic, J. – Schneider, M. – de Bruijn, F. J. – Lupski, J. R.: Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods in Molecular and Cellular Biology, *5*, 1994, pp. 25–40. ISSN: 0898-7750.
11. Larsen, M. V. – Cosentino, S. – Rasmussen, S. – Friis, C. – Hasman, H. – Marvig, R. L. – Jelsbak, L. – Sicheritz-Pontén, T. – Ussery, D. W. – Aarestrup, F. M. – Lund, O.: Multilocus sequence typing of total-genome-sequenced bacteria. Journal of Clinical Microbiology, *50*, 2012, pp. 1355–1361. DOI: 10.1128/JCM.06094-11.
12. Jolley, K. A. – Bray, J. E. – Maiden, M. C. J.: Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Research, *3*, 2018, article 124. DOI: 10.12688/wellcomeopenres.14826.1.
13. Zhou, Z. – Alikhan, N. F. – Mohamed, K. – Fan, Y. – Agama Study Group – Achtman, M: The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. Genome Research, *30*, 2020, pp. 138–152. DOI: 10.1101/gr.251678.119.
14. Joensen, K. G. – Tetzschner, A. M. – Iguchi, A. – Aarestrup, F. M. – Scheutz, F.: Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. Journal of Clinical Microbiology, *53*, 2015, pp. 2410–2426. DOI: 10.1128/JCM.00008-15.
15. Cosentino, S – Voldby Larsen, M. – Møller Aarestrup, F. – Lund, O.: PathogenFinder – Distinguishing friend from foe using bacterial whole genome sequence data. PLoS One, *8*, 2013, article

e77302. DOI: 10.1371/journal.pone.0077302.
16. Malberg Tetzschner, A. M. – Johnson, J. R. – Johnston, B. D. – Lund, O. – Scheutz, F.: *In silico* genotyping of *Escherichia coli* isolates for extraintestinal virulence genes by use of whole-genome sequencing data. Journal of Clinical Microbiology, *58*, 2020, article e01269-20. DOI: 10.1128/JCM.01269-20.
17. Roer, L. – Johannesen, T. B. – Hansen, F. – Stegger, M. – Tchesnokova, V. – Sokurenko, E. – Garibay, N. – Allesøe, R. – Thomsen, M. C. F. – Lund, O. – Hasman, H. – Hammerum, A. M.: CHtyper, a web tool for subtyping of extraintestinal pathogenic *Escherichia coli* based on the *fumC* and *fimH* alleles. Journal of Clinical Microbiology, *56*, 2018, article e00063-18. DOI: 10.1128/JCM.00063-18.
18. Roer, L. – Hendriksen, R. S. – Leekitcharoenphon, P. – Lukjancenko, O. – Kaas, R. S. – Hasman, H. – Aarestrup, F. M.: Is the evolution of *Salmonella enterica* subsp. *enterica* linked to restriction-modification systems? mSystems, *1*, 2016, article e00009-16. DOI: 10.1128/mSystems.00009-16.
19. Pehrsson, E. C. – Tsukayama, P. – Patel, S. – Mejía-Bautista, M. – Sosa-Soto, G. – Navarette, K. M. – Calderon, L. – Cabrera, L. – Hoyos-Arango, W. – Bertoli, M. T. – Berg, D. E. – Gilman, R. H. – Dantas, G.: Interconnected microbiomes and resistomes in low-income human habitats. Nature, *533*, 2016, pp. 212–216. DOI: 10.1038/nature17672.
20. Carattoli, A. – Zankari, E. – Garcia-Fernandez, A. – Voldby Larsen, M. – Lund, O. – Villa, L. – Møller Aarestrup, F. – Hasman, H.: PlasmidFinder and pMLST: in silico detection and typing of plasmids. Antimicrobial Agents and Chemotherapy, *58*, 2014, pp. 3895–3903. DOI: 10.1128/AAC.02412-14.
21. Kumar, S. – Stecher, G. – Li, M. – Knyaz, C. – Tamura, K.: MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, *35*, 2018, pp. 1547–1549. DOI: 10.1093/molbev/msy096.
22. Reid, C. J. – Cummins, M. L. – Börjesson, S. – Brouwer, M. S. M. – Hasman, H. – Hammerum, A. M. – Roer, L. – Hess, S. – Berendonk, T. – Nešporová, K. – Haenni, M. – Madec, J. Y. – Bethe, A. – Michael, G. B. – Schink, A. K. – Schwarz, S. – Dolejska, M. – Djordjevic, S. P.: A role for ColV plasmids in the evolution of pathogenic *Escherichia coli* ST58. Nature Communications, *13*, 2022, article 683. DOI: 10.1038/s41467-022-28342-4.
23. Mattioni Marchetti, V. – Bitar, I. – Mercato, A. – Nucleo, E. – Marchesini, F. – Mancinelli, M. – Prati, P. – Scarsi, G. S. – Hrabak, J. – Pagani, L. – Fabbi, M. – Migliavacca, R.: Deadly puppy infection caused by an MDR *Escherichia coli* O39 $bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-2}}$, $bla_{\text{DHA-1}}$, and *aac(6)-lb-cr* – positive in a breeding kennel in central Italy. Frontiers in Microbiology, *11*, 2020, article 584. DOI: 10.3389/fmicb.2020.00584.
24. Zhou, X. – Zhao, H. – Zhou, Z. – Miao, Y. – Li, R. – Yang, B. – Cao, C. – Xiao, S. – Wang, X. – Liu, H. – Wang, J. – Yang, Z.: Characterization of extended-spectrum β-lactamase-producing *Escherichia coli* isolates that cause diarrhea in sheep in northwest China. Microbiology Spectrum, *10*, 2022, article e0159522. DOI: 10.1128/spectrum.01595-22.
25. Mukherjee, A. – Speh, D. – Diez-Gonzalez, F.: Association of farm management practices with risk of *Escherichia coli* contamination in pre-harvest produce grown in Minnesota and Wisconsin. International Journal of Food Microbiology, *120*, 2007, pp. 296–302. DOI: 10.1016/j.ijfoodmicro.2007.09.007.
26. Park, S. – Navratil, S. – Gregory, A. – Bauer, A. – Srinath, I. – Szonyi, B. – Nightingale, K. – Anciso, J. – Jun, M. – Han, D. – Lawhon, S. – Ivanek, R.: Multifactorial effects of ambient temperature, precipitation, farm management, and environmental factors determine the level of generic *Escherichia coli* contamination on preharvested spinach. Applied and Environmental Microbiology, *81*, 2015, pp. 2635–2650. DOI: 10.1128/AEM.03793-14.
27. Fuentes-Castillo, D. – Navas-Suárez, P. E. – Gondim, M. F. – Esposito, F. – Sacristán, C. – Fontana, H. – Fuga, B. – Piovani, C. – Kooij, R. – Lincopan, N. – Catao-Dias, J. L.: Genomic characterization of multidrug-resistant ESBL-producing *Escherichia coli* ST58 causing fatal colibacillosis in critically endangered Brazilian merganser (*Mergus octosetaceus*). Transboundary and Emerging Diseases, 68, 2021, pp. 258–266. DOI: 10.1111/tbed.13686.

Received 28 February 2023; 1st revised 9 May 2023; accepted 9 May 2023; published online 17 May 2023.