Biochemical characteristics of *E. coli* isolated from food of animal origin

*D. Tomašković, L. Hlebić, L. Peinović and A. Humski**

Abstract

Food of animal origin is a potential source of pathogenic *E. coli* that are dangerous to humans. While most strains are intestinal commensals, some can cause intestinal and extraintestinal infections. Their pathogenicity is associated with the presence of virulence genes, phylo-group and, in some strains, biochemical characteristics. In this study, a total of 61 *E. coli* isolates from meat, minced meat, meat preparations and carcass swabs from different animal species were analysed. The biochemical properties of the strains were determined using VITEK2 system and the data was compared with the presence of virulence genes from previous studies. The results showed a correlation between the presence of the *eae* virulence gene and the alkalinization of succinate. In addition, the presence of the virulence gene *cnf1* was correlated with the enzyme tyrosine arylamidase, though the correlation between biochemical characteristics and the presence of virulence genes is questionable due to the small sample size and more strains need to be tested for further conclusions. Although no significant correlations were found between biochemical characteristics and the presence of virulence genes, this study describes the diversity of *E. coli* in the Republic of Croatia. The collected data also reveal new insights into biochemical characteristics of *E. coli* isolated from domestic and wild animals in the Republic of Croatia.

Key words: *Escherichia coli; biochemical characteristics; different animal species*

Introduction

The bacterium *Escherichia coli* (*E. coli*) is a Gram-negative, facultatively anaerobic, rod-shaped bacterium that belongs to the genus *Escherichia* and the family *Enterobacteriaceae* (Bettelheim, 1994). Although most strains are intestinal commensals in numerous animal species and humans (Tenaillon et al., 2010), some can cause intestinal and extraintestinal infections (Kaper et al., 2004; Denamur et al., 2021). The pathogenicity of strains is primarily related to the presence of virulence genes (Kaper et al., 2004; Croxen and Finlay, 2010), and for certain serovars, also to biochemical properties.

To distinguish *E. coli* from other members of the *Enterobacteriaceae* family, individual biochemical tests or ready-made biochemical arrays with a larger number of biochemical properties are used, such

Dora TOMAŠKOVIĆ, DMV, PhD, Expert Advisor in Science, Lucija HLEBIĆ, DMV, Expert Associate in Science, Lovran PEINOVIĆ, Expert Associate in Science, Andrea HUMSKI*, DMV, PhD, Scientific Advisor in Tenure, Assistant Proffesor (Corresponding author: e-mail: humski@veinst.hr), Croatian Veterinary Institute, Zagreb, Croatia

as API 20E, API 32, VITEK2 (Habrun, 2014). Other methods such as MALDI-TOF MS, should be mentioned as it has been shown to be a rapid and reliable technique for the identification of bacteria (Singhal et al., 2015). The differentiation of *E. coli* from other members of the family can be demonstrated by individual biochemical tests. The most important are a positive reaction to methyl red and indole production, a negative reaction to the Voges-Proskauer (VP) test and the inability to utilise citrate and urea. The use of individual biochemical tests has largely been abandoned due to the development of new technologies. The above mentioned commercially available biochemical tests are predominantly used for the detection of bacterial species due to the simple application, specificity and accuracy of the results (Bettelheim, 1994; Quin et al., 1994).

Selective chromogenic media are used to facilitate the isolation and differentiation of *E. coli* from other bacteria of the *Enterobacteriaceae* family. The growth of the individual microorganisms can be distinguished by utilisation of carbohydrates or other compounds. One of the most important characteristic in about 97% of *E. coli* strains is the ability to produce β-glucuronidase (Killian and Bülow, 1976, Rice et al., 1990). This characteristic enabled the development of the chromogenic selective medium Tryptone Bile X-glucuronide (TBX). It should be emphasised that certain *E. coli*, belonging to the enterohemorrhagic *E. coli* (EHEC), do not have the ability to produce β-glucuronidase. This pathogroup is important for its ability to produce verotoxins (Verotoxigenic *E. coli* – VTEC), with serotype O157:H7 its most important representative. The latter is often associated with foodborne outbreaks and haemolytic uremic syndrome (HUS). Along with the inability to produce β-glucuronidase, it is also characterised by the inability to ferment sorbitol (March and Ratnam, 1986). Therefore, the inability to ferment sorbitol is used as a routine "screening" method to easily distinguish *E. coli* O157 from others (Galar et al., 2013) by using Sorbitol-MacConkey medium (SMAC) as a chromogenic selective medium (Thomson-Carter, 2001). This medium is not satisfactory for isolating other VTEC and is not reliable for isolating all O157, because some strains are sorbitol and β-glucuronidase positive (Thomson-Carter, 2001). It is also possible to isolate sorbitol-negative colonies that do not belong to the group of enterohaemorrhagic *E. coli* (EHEC), but to the enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) or enteroinvasive *E. coli* (EIEC) (Ojeda et al., 1995). Unlike O157, other VTEC strains differ greatly in terms of genotype, serotype and other phenotypic characteristics. According to their biochemical properties and growth on common culture media, they have similar characteristics to non-toxigenic strains, and their identification from faeces, food or other sources is very complex. An example of the biochemical diversity of VTEC strains is presented by Souza et al. (2010), who classified the strains into 14 biotypes by analysing 38 strains with 28 biochemical tests. They highlighted the wide diversity of metabolic characteristics of VTEC strains, which do not differ from non-pathogenic *E. coli* according to biochemical and phenotypic characteristics. In addition, the ability to produce β-glucuronidase was investigated by Verhaegen et al. (2015) as a part of the developmental research of selective chromogenic media for the detection of VTEC serogroups. According to their research, all VTEC strains tested were β-glucuronidase positive. In regards to other pathogroups, it is important to mention

the characteristics of EIEC strains, which are often biochemically different from other *E. coli*. For example, 70% of EIEC strains are lactose negative, mostly lysine decarboxylase-negative and non-motile (Doyle and Padhye, 1989). In addition to the listed biochemical characteristics, Kameyama et al. (2015) reported that the negative reaction of sorbose fermentation could be useful for the identification of non-O157/O26 EHEC strains, while Lehmacher and Bockemühl (2007) stated that most EIEC and ETEC isolates do not ferment sorbose and that culture media containing L-sorbose could be used for their isolation. In addition to intestinal *E. coli*, it is important to mention the extraintestinal pathogenic *E. coli* (ExPEC) described in the studies of Davies (1976). The results show the biotypes of *E. coli* isolated from samples of patients with urinary tract infections, using tests for lysine and ornithine decarboxylase production (69% and 71% of strains) and sucrose fermentation (41% of strains) to differentiate the biotypes. Based on the results obtained, 574 isolates were categorised into biotypes, 42% of which were classified into two biotypes.

Biochemical characterisation of bacterial species involves the response of isolates to a broad range of biochemical tests, from sugar fermentation to determination of enzyme activity. Although *E. coli* has characteristic biochemical properties, the isolates are often biochemically variable. According to the stated research, it has been observed that biochemical characterisation is not sufficient to determine strain pathogenicity. In order to determine the pathogenicity of the strain, molecular methods are used to detect the presence of virulence genes.

The aim of this study was to determine the biochemical characteristics of *E. coli* strains isolated from food of animal origin. Furthermore, to compare with the results of previous studies and determine whether there is a mutual connection between the biochemical characteristics and the presence of virulence genes and whether the biochemical characteristics can be used in daily laboratory work to address suspicions about the potential pathogenicity of *E. coli* isolates.

Material and methods

For this study, 61 strains of *E. coli* were isolated from samples originating from: a) poultry meat (*n*=20), b) cattle meat, minced meat, meat preparations and cattle carcass swabs (*n*=16), c) pig meat, minced meat and pig carcass swabs (*n*=15), and d) game meat (*n*=10) obtained for the purpose of regular microbiological examinations. For the isolation of bacterial strains and statistical data processing, the procedures described in a previous study were used (Stojević et al., 2022).

Biochemical analysis of bacterial isolates

Biochemical analysis of bacterial isolates was performed using the VITEK2 system (Biomerieux, Marcy-l'Étoile, France). VITEK2 GN cards were used to determine the bacterial species and the biochemical profile of the isolated microorganism.

Results

Biochemical characteristics

The results show identical biochemical reactions for most isolates, while some were variable. The isolates analysed showed the same biochemical properties according to the reactions: Ala-Phe-Pro-Arylamidase (APPA), Adonitol (ADO), L-Pyrrolidoline (PyrA), L-Arabitol (lARL), D-Cellobiose (dCEL), Beta-Galactosidase (BGAL), Hydrogen Sulfide (H2S)

production, Beta-N-Acetyl-Glucosaminidase (BNAG), glumamyl-arylamidase pNA (AGLTp), D-glucose (dGLU), glucose fermentation (OFF), β-glucosidase (BGLU), D-maltose (dMAL), D-mannitol (dMAN), D-mannose (dMNE), β-oxylosodase (BXYL), β-alanine arylamidase pNA (Balap), Lipase (LIP), palatinose (PLE), urease (URE), D-sorbitol (dSOR), D-tagatose (dTAG), D-trehalose (dTRE), sodium citrate (CIT), malonate (MNT), α-glucosidase (AGLU), β-N-acetyl-galactosaminidase (NAGA), phosphatase (PHOS), glycine arylamidase (GlyA), lysine decarboxylase (LDC), utilisation of L-histidine (IHISa), coumarate (CMT), glu-gly-arg-arylamidase (GGAA), utilisation of L-malate (IMLTa), Ellmann's reagent (ELLM) and lactate utilization (ILATa). The isolates showed variable biochemical reactions for: gamma-glutamyl transferase (GGT), L-proline arylamidase (ProA), tyrosine arylamidase (TyrA), sucrose (SAC), 5-keto-D-gluconate (5KG), alkalinisation of L-lactate (ILATk), succinate alkalinisation (SUCT), alpha-galactosidase (AGAL), ornithine decarboxylase (ODC), beta-glucuronidase (BGUR) and resistance to O/129 (O129R).

The majority of isolates, regardless of their origin, showed a negative reaction to GGT (91.80%), ProA (93.44%) and 5KG (85.25%). The results showed positive biochemical reactions for TyrA (70.49%), SUCT (73.77%), AGAL (72.13%), O129R (73.77%) and BGUR (75.41%) in most strains, regardless of their origin. Among other variable characteristics, it was observed that all isolates reacted equally positively and negatively to SAC, ILATk and ODC. The results are shown in Table 1.

Regarding the origin of the isolates, all isolates reacted equally positive (49.18%) and negative (50.81%) to SAC, regardless of origin, while isolates from pigs (66.67%) and poultry (55%) reacted positively to ILATk, in contrast to the strains from cattle (68.75%), which mostly showed a negative reaction. A similar positive response to ODC was observed in isolates from game (70%) and cattle (75%), in contrast to isolates from poultry in which the majority (65%) reacted negatively (Table 2).

Comparison of biochemical characteristics and the presence of virulence genes

The results of biochemical tests were compared with the presence of virulence genes as described in Stojević et al. (2022). Briefly, most isolates showed no significance in terms of the biochemical characteristics detected and their association with the presence of genes encoding virulence factors. The exception were some isolates for which a statistical association was found. Statistical significance was found for isolates that detected the presence of the *eae* gene compared to those that did not detect the presence of this gene and their association with succinate alkalinisation (P=0.045) (Table 3).

Table 1. Biochemical characteristics of isolates

	Biochemical characteristics										
Strains (N)	GGT	ProA	TyrA	SAC	5KG	ILATk	SUCT	AGAL	ODC	BGUR	O129R
positive	5	4	43	30	9	32	45	44	33	46	45
negative	56	57	18	31	52	29	16	17	28	15	16

Table 2. Results of biochemical properties in relation to the origin of the isolate

Origin	GGT		ProA		TyrA		SAC		5KG		ILATk		SUCT		AGAL		ODC		BGUR		O129R	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Poultry	1	19	1	19	17	3	8	12	3	17	11	9	17	3	16	4	7	13	10	10	16	4
Game	0	10	0	10	5	5	5	5	2	8	6	4	7	3	7	3	7	3	9	1	6	4
Pig	2	13	2	13	12	3	8	7	3	12	10	5	12	3	10	5	7	8	13	2	12	3
Cattle	2	14	1	15	9	7	9	7	1	15	5	11	9	7	11	5	12	4	14	2	11	5
Total	5	56	4	57	43	18	30	31	9	52	32	29	45	16	44	17	33	28	46	15	45	16

Table 3. Correlation between isolates possessing the *eae* gene with respect to alkalinisation of succinate

Virulence gene		SUCT		*P*
		Absent	Present	
eae	Absent	11	41	0.045
	Present	5	4	
Total		16	45	61

Table 4. Correlation between isolates possessing the cnf1 gene in relation to tyrosine arylamidase

Virulence gene		TyrA		*p*
		Absent	Present	
cnf1	Absent	13	42	0.007
	Present	5	1	
Total		18	43	61

In addition, isolates with a detected *cnf1* gene were found to have an association and statistical significance in relation to the activity of the arylamidase enzyme towards tyrosine, compared to those in which the gene was not detected (Table 4).

Discussion

E. coli has numerous biochemical characteristics. In this study, isolates were biochemically characterised using the automated VITEK2 system. The re-

sults obtained from the comparison of the biochemical characteristics show that the studied *E. coli* isolates were biochemically very similar with no significant differences. For this reason, species identification was possible (via the VITEK2 System Product Information), while some isolates show deviations.

E. coli most often associated with foodborne outbreak is the verotoxigenic O157:H7. This serotype has specific biochemical reactions such as the inability to ferment sorbitol and produce β-glucuronidase. When β-glucuronidase-negative strains are isolated, the pathogenicity of the isolate and the presence of VTEC serogroup O157 are suspected, as the absence of β-glucuronidase activity is characteristic to this group. Accordingly, the presence of O157:H7 could be excluded in this study for all isolates positive for sorbitol (n=61, 100%) and β-glucuronidase (n=46, 75.41%), and suspected for 15 (24.59%) of the β-glucuronidase-negative *E. coli* isolates. Results from previous studies have confirmed the negative biochemical reactions to sorbitol and β-glucuronidase (Leclercq et al., 2001), according to which *E. coli* O157:H7 and O157:H(-) differed biochemically from the other strains examined. In contrast, King et al. (2014) described a case of foodborne outbreak associated with sorbitol-fermenting *E. coli* O157:[H7] and Verhagen et al. (2015) detected β-glucuronidase activity in 39 VTEC isolates, two EPEC isolates and four commensal *E. coli* isolates isolated from cow's milk, cattle and human faeces, and cattle carcass swabs. Since biochemical characteristics are not sufficient for the identification of pathogenic strains, the results of this study were compared with the results from previous research (Stojević et al., 2022). Accordingly, the presence of VTEC was not detected in β-glucuronidase-negative isolates and most virulence factor genes were detected in β-glucuronidase-positive strains. In addition, the absence of β-glucuronidase enzyme activity and the inability to ferment sorbitol is not considered a guideline for determining the pathogenicity of the isolate.

In this study, the biochemical properties of *E. coli* in foods of animal origin were determined and compared with previous studies. Although no significant correlation was found between the biochemical properties and the presence of virulence genes, this study described the diversity of *E. coli* isolated in the Republic of Croatia, as by studying their diversity, we can also study their impact on public health. Previous research has shown that certain strains are specific to a particular location and that the diversity of the *E. coli* population has a direct impact on public health (Janezic et al., 2013). Further research can focus on monitoring the population of *E. coli* isolated from food and comparing it with isolates of human origin and isolates from the environment.

Conclusions

The results of this study describe the biochemical characteristics of *E. coli* in foods of animal origin. The biochemical characterisation showed that they are very similar and that there were no significant differences. By comparison with the presence of virulence genes, a connection was found between the *eae* gene and succinate alkalinisation and the *cnf1* gene and the activity of the enzyme arylamidase towards tyrosine. For the reliability of the observed connection, a larger number of strains with these characteristics should be analysed.

Future studies on a larger samples size could help to better understand the relationship between biochemical charac-

teristics and virulence factors. This would facilitate identification for the purpose of monitoring *E. coli* isolated from food and thus protecting human health.

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Biokemijske karakteristike E. coli izdvojene iz hrane životinjskog podrijetla

Dr. sc. Dora TOMAŠKOVIĆ, dr. med. vet., stručni savjetnica u sustavu znanosti, Lucija HLEBIĆ, dr. med. vet, stručna suradnica u sustavu znanosti, Lovran PEINOVIĆ, stručni suradnik u sustavu znanosti, dr. sc. Andrea Humski, dr. med. vet., znanstvena savjetnica u trajnom zvanju, docentica, Hrvatski veterinarski institut, Zagreb, Hrvatska

Hrana životinjskog podrijetla predstavlja čest izvor patogenih sojeva *Escherichia coli* opasnih za ljude. Iako su većina sojeva crijevni komenzali, neki od njih mogu prouzročiti crijevne (intestinalne) i izvancrijevne (ekstraintestinalne) infekcije. Njihova je patogenost kod pojedinih serovarova povezana s prisutnošću gena za čimbenike virulencije, pripadnošću filogrupi te s biokemijskim svojstvima. U ovom istraživanju pretražen je 61 izolat bakterije *E. coli* izdvojenih iz uzoraka mesa: mljevenog mesa, mesnih pripravaka i obrisaka trupova različitih vrsta životinja. Izolatima su određene biokemijske karakteristike VITEK2 sustavom, a podatci su uspoređeni s prisutnošću gena za čimbenike virulencije obrađenim u prijašnjem istraživanju. Rezultati prikazuju povezanost između prisustva *eae* gena i aktivnosti alkalinizacije sukcinata. Osim navedenog, uočena je povezanost između prisustva *cnf1* gena i aktivnosti enzima arilamidaze prema tirozinu. S obzirom na mali broj ostvarenih rezultata, nejasna je povezanost između biokemijskih karakteristika i prisutnosti gena za čimbenike virulencije. Stoga su potrebna daljnja istraživanja s većim brojem izolata. Iako nisu uočene značajne poveznice između biokemijskih svojstava i prisutnosti gena za čimbenike virulencije, ovim radom prikazane su raznolikosti *E. coli* u prikupljene na području Republike Hrvatske, a prikupljeni podatci prikazuju nova saznanja o biokemijskim karakateristikama *E. coli* u domaćih i divljih životinja na području Republike Hrvatske.

Ključne riječi: *Escherichia coli, biokemijske karakteristike, različite vrste životinja*