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Molecular Characterization of Shiga Toxin-Producing *Escherichia coli* Strains Isolated in Poland

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) strains also called verotoxin-producing *E. coli* (VTEC) represent one of the most important groups of food-borne pathogens that can cause several human diseases such as hemorrhagic colitis (HC) and hemolytic – uremic syndrome (HUS) worldwide. The ability of STEC strains to cause disease is associated with the presence of wide range of identified and putative virulence factors including those encoding Shiga toxin. In this study, we examined the distribution of various virulence determinants among STEC strains isolated in Poland from different sources. A total of 71 Shiga toxin-producing *E. coli* strains isolated from human, cattle and food over the years 1996–2010 were characterized by microarray and PCR detection of virulence genes. As *stx1a* subtype was present in all of the tested Shiga toxin 1 producing *E. coli* strains, a greater diversity of subtypes was found in the gene *stx2*, which occurred in five subtypes: *stx2a, stx2b, stx2c, stx2d, stx2g*. Among STEC O157 strains we observed conserved core set of 14 virulence factors, stable in bacteria genome at long intervals of time. There was one cattle STEC isolate which possessed verotoxin gene as well as *sta1* gene encoded heat-stable enterotoxin STIa characteristic for enterotoxigenic *E. coli*. To the best of our knowledge, this is the first comprehensive analysis of virulence gene profiles identified in STEC strains isolated from human, cattle and food in Poland. The results obtained using microarrays technology confirmed high effectiveness of this method in determining STEC virulotypes which provides data suitable for molecular risk assessment of the potential virulence of this bacteria.

Key words: *Escherichia coli*, microarray detection, Shiga toxin-producing, virulence gene

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains also called verotoxin-producing *E. coli* (VTEC) represent one of the most important groups of food-borne pathogens that can cause several human diseases such as hemorrhagic colitis (HC) and hemolytic – uremic syndrome (HUS) (EFSA, 2010; Nataro and Kaper, 1998). Over 400 serotypes of STEC have been isolated from human. However, the majority of clinical STEC infections, particularly those associated with outbreaks and serious patient outcomes, are attributable to a subset of serogroups including *E. coli* O157:H7 (the prototype bacterium for enterohaemorrhagic *E. coli* – EHEC), O26: H11, O103:H2, O111:H8, O121:H19 and O145:H28 (Nataro and Kaper, 1998). In recent years, new serotypes of EHEC have also emerged (Bielaszewska *et al*., 2011).

The ability of STEC strains to cause disease is associated with the presence of a wide range of identified and putative virulence factors including those encoding Shiga toxin (Nataro and Kaper, 1998). The Stx family consists of 2 major groups, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), which are well distinguished by their amino acid sequences. Several other virulence factor are known, including genes conferring the ability to cause attaching-effacing lesions located on LEE pathogenicity island. The LEE encodes intimin (Eae), translocated intymin receptor (Tir), a type III secretion system (EspA, EspB, EspD) and effector proteins translocated by the secretion system. Beyond Stx and LEE genes, "typical STEC" associated with human disease usually carry virulence factors encoded by 60-MDa plasmid as enterohemolysin (Ehx), serine protease (EspP), catalase peroxidase (KatP) and a type II secretion system (EtpD) (Nataro and Kaper, 1998).

As more atypical STEC strains have been reported, several proteins have been proposed as potential virulence determinants as autoagglutinating adhesion (Saa), subtilase cytotoxin (SubA), non-LEE encoded effectors (Nle) (Coombes *et al*., 2008; Bugarel *et al.*, 2010; Paton and Paton, 2010). Moreover, recently there have been many scientific reports describing the presence of STEC isolated from humans, animals and the environment,

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carrying specific virulence determinants characteristic for different *E. coli* pathotypes as intermediate STEC/ETEC or EAEC/STEC virulence factor profiles (Bielaszewska *et al*., 2011; Prager *et al*., 2011). Therefore determining the virulence potential of STEC relies upon the determination of somatic and flagellar antigens, together with the identification of virulence genes what can be used to make a "molecular risk assessment" of the predict potential virulence of strains (Coombes *et al*., 2008).

Although the number of STEC isolates reported yearly is relatively low in Poland (few cases of human STEC infections), they are often responsible for serious illnesses or HUS complications (Jakubczak *et al.*, 2008; EFSA, 2010; Januszkiewicz *et al.*, 2010; 2012). What is interesting one outbreak of STEC infection related to international outbreak of STEC O104:H4 in 2011 was reported in Poland (Januszkiewicz *et al.*, 2012). There is no data containing molecular characterization of Shiga toxin-producing *E. coli* strains isolated in our country both from human infections as well as STEC strains isolated from animal and food. The aim of our study was to investigate the distribution of various virulence determinants among STEC strains isolated in Poland from different sources. To achieve our goal we used commercially available microtube array system designed to detect virulence genes in *E. coli* of various pathotypes.

Experimental

Material and Methods

Bacterial strains. A total of 71 Shiga toxin-producing *E. coli* (STEC) strains were analyzed (Table I). Those isolates were from collection of Department of Bacteriology (n = 38), Department of Food and Consumer Articles Research (n = 4) of National Institute of Public Health – National Institute of Hygiene (NIPH – NIH) and Department of Hygiene of Food of Animal Origin in National Veterinary Research Institute (NVRI) in Puławy (n = 29). STEC strains collected during 1996–2010 were isolated from human (n = 45), cattle (n = 16) and food (n = 10) and were classified into following groups: serotype O157 (n = 45), serotype O26 (n = 10), serotype O111 (n = 6) and NT (not typable using available Biomex latex assay) (n = 10). More than 90% of tested human STEC O157 isolates were from reported human cases in our country. All of them were from sporadic cases. Tested STEC strains presented different band patterns when typed with pulsed-field gel electrophoresis (PFGE) using XbaI enzyme (data not showed) which indicates that they had no epidemiological link.

Identification and virulence analysis of STEC strains. Classical tube test was used for *E. coli* re-identification. Serotyping was performing using latex agglutination test (Biomex). Stx production was confirmed by VTEC-RPLA assay (Oxoid). The cytotoxity assay with Vero cell monolayers (VCA) in 96 – well plates was performed as described previously (Januszkiewicz *et al*., 2010). The presence of *stx1*, *stx2* and *eae* genes were examined by PCR based methods according to protocols described previously (Januszkiewicz *et al*., 2010). Detection of Stx gene subtypes was performed by PCR based method according to WHO protocol (Scheutz *et al*., 2012). Presence of H7 antigen (*fli*C gene) in STEC O157 isolates was performed by PCR-RFLP (Januszkiewicz *et al*., 2010). A DNA microarray (Identibac Ec v. 03, Alere Technologies GmbH) was used to determine the presence of virulence genes among STEC strains. The microarrays were used according to the manufacturer's instructions. Array images were processed in IconoClust 3.0 (Clondiag, Germany) and signals were analyzed using the *gap*A-positive control gene for normalization and with cut-offs as recommended by manufacturer (> 0.4 = present, 0.4 to 0.3 = ambiguous, < 0.3 = absent, relative to the *gap*A signal).

Results

Phenotypic and genotypic assays of STEC identification. For a summary of results, see Table I and II. All of the STEC isolates produced a cytopathic effect on Vero cell monolayers, confirming their ability to express verotoxins. The Stx-cytotoxic activity was neutralized with antiserum. Production of appropriate verotoxins: Stx1, Stx2 or both were confirmed in tested strains by the RPLA assay. Genetic hallmarks of a appropriate toxins (*stx1* and *stx2*) and intimin (*eae*) were detected by PCR (Table I). All STEC O157 strains were positive for *fli*C (H7) gene. PCR subtyping of the *stx1* gene revealed subtype *stx1a* in all isolates which produced Stx1 toxin (Table II). STEC isolates produced Stx2 toxin possessed various subtypes of *stx2* gene: *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2g* (Table II). Stx2c subtype was produced by STEC O157 only.

Microarray analysis. All tested STEC isolates were positive for control, species-specific genes *gad*, encoding glutamate decarboxylase and *ihf*A, encoding integration host factor subunit alpha. Genes known to be present in control strain STEC O157 EDL933 and which were represented on the array were successfully detected. There were genes encoding: Shiga toxins (*stx1, stx2*); adhesins: an outer membrane protein important for the attachment to host cells – intimin (*eae*A) and adherence-conferring protein (*iha*); toxins: heat stable enterotoxin (*ast*A), enterohemolysin (*ehx*), cytotoxin B (*tox*B); type III secretion system: non-LEE-encoded effector protein A (*nle*A), non-LEE-encoded effector protein B (*nle*B), non-LEE-encoded effector

Table I

Virulence genes in STEC isolated in Poland. Definition of virulence types (virulotypes) based on present genes/gene variants (●) as determined by Identibac Ec v. 3 microarray assay. Only genes positive for at least one STEC strain are showed. The genes present in at least 90% of STEC strains are highlighted: for STEC O157 in dark grey, for STEC O26 in grey, for STEC O111 in bright grey color. Black color marks the genetic determinants present only in single isolates. NT – isolates which were not typable using commercially available latex (Biomex Sp. z o.o.). The *stx* subtypes (bold black frame) were determined by PCR according to WHO protocol (WHO protocol).

Virulotype	Serotype	Source	No of strain	*sstx1* subtype	*sstx2* subtype	*stx1AB*	*stx2AB*	*eae*	*tir*	*nleA*	*espJ*	*iha*	*espF*	*ehx*	*tccP*	*etpD*	*nleC*	*espP*	*katP*	*toxB*	*nleB*	*cif*	*lpfA*	*espA*	*efa1*	*iss*	*espB*	*astA*	*cba*	*cdtB*	*celB*	*espI*	*mchA*	*mchB*	*mchF*	*mchC*	*saa*	*subA*	*st1a*	*epeA*	*iroN*
V12	O157	human	19/99	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	443/07	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	474/07	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	568/99	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	4/10	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	199/01	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
V2	O157	human	44/02	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	247/02	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	225/96	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	598/03	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	49/04	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	214/04	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	365/05	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
	O157	human	206/06	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●														
V3	O157	human	171/09		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	441		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	cattle	420		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	cattle	418		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	food	345/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	food	346/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
	O157	human	440/98		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●														
V4	O157	human	98/08	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																					
	O157	human	251/10	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																					
	O157	human	448	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																					

Table I
Continued

Virulotype	Serotype	Source	No of strain	*sstx1* subtype	*sstx2* subtype	*stx1AB*	*stx2AB*	*eae*	*tir*	*nleA*	*espJ*	*iha*	*espF*	*ehx*	*tccP*	*etpD*	*nleC*	*espP*	*katP*	*toxB*	*nleB*	*cif*	*lpfA*	*espA*	*efa1*	*iss*	*espB*	*astA*	*cba*	*cdtB*	*celB*	*espI*	*mchA*	*mchB*	*mchF*	*mchC*	*saa*	*subA*	*st1a*	*epeA*	*iroN*
V5	O157	food	349/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																					
	O157	food	445		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●																					
V6	O157	food	1124	a		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●			●						●											
	O157	food	1128	a		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●			●						●											
VV7	O157	food	1125	a		●		●	●		●	●	●	●	●	●	●	●	●	●	●			●						●											
	O157	food	1126	a		●		●	●		●	●	●	●	●	●	●	●	●	●	●			●						●											
V8	O157	human	442		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●									●											
V9	O157	food	348/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●						●											
V10	O157	human	174/03		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●													
V11	O157	human	443	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●	●											
	O157	human	221/08	a	a	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●				●	●											
V12	O157	human	286/00		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●																
V13	O157	human	577/01	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●																
V14	O157	human	175/06	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●	●											
V15	O157	cattle	447	a	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●								●											
V16	O157	Food	347/96		a, c		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●							●		●										
V17	O157	human	74/10		C		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●		●		●												
V18	O157	human	535/97		C		●	●	●	●	●	●	●	●	●	●		●	●	●	●																			
V19	O157	human	440	a		●		●	●	●		●	●	●	●	●	●	●	●	●	●			●				●												
V20	O157	human	9/08		a		●	●	●	●	●		●	●	●	●	●				●				●			●		●											
V21	O157	human	371/08		a		●	●	●	●		●	●	●	●	●	●				●				●			●													
V22	O26	human	319/01	a		●		●	●	●	●	●	●	●	●		●	●	●			●	●	●	●	●	●				●										
V23	O26	human	706/98	a		●		●	●	●	●	●	●	●	●			●	●	●		●	●	●	●	●	●	●													
V24	O26	human	50/97	a		●		●	●	●	●	●	●	●	●			●	●		●	●	●	●	●	●	●														

Table I
Continued

Virulotype	Serotype	Source	No of strain	*stx1* subtype	*stx2* subtype	*stx1AB*	*stx2AB*	*eae*	*tir*	*nleA*	*espJ*	*iha*	*espF*	*ehx*	*tccP*	*etpD*	*nleC*	*espP*	*katP*	*toxB*	*nleB*	*cif*	*lpfA*	*espA*	*efa1*	*iss*	*espB*	*astA*	*cba*	*cdtB*	*celB*	*espI*	*mchA*	*mchB*	*mchF*	*mchC*	*saa*	*subA*	*st1a*	*epeA*	*iroN*
V25	O26	human	169/97	a		•	•	•	•	•	•	•	•	•	•			•	•	•			•	•	•	•	•	•					•							
V26	O26	human	201/01		a		•	•	•	•	•	•	•	•	•		•	•	•		•	•	•	•	•	•	•		•											
V27	O26	human	368/01		a		•	•	•	•	•	•	•	•	•			•	•	•	•	•	•	•	•	•	•													
V28	O26	human	6/01		a			•	•	•	•		•	•				•	•		•	•	•	•	•	•														
V29	O26	human	317/01		B		•	•	•	•	•	•	•	•	•						•	•	•	•	•	•														
V30	O26	human	318/01		B		•	•	•	•	•	•	•	•	•		•				•	•	•	•	•	•														
V31	O26	human	237/05	a		•		•	•	•	•		•	•	•		•	•	•	•		•	•	•	•	•	•													
V32	O111	human	479	a		•		•	•	•	•	•	•		•			•			•	•	•	•	•			•	•											
	O111	cattle	477	a		•		•	•	•	•	•	•		•						•	•	•	•	•				•											
V33	O111	human	480	a		•		•	•	•	•	•	•	•	•						•	•	•	•	•															
V34	O111	human	481	a		•		•	•	•	•	•	•	•	•						•	•	•	•	•															
V35	O111	human	245/10		a		•	•	•	•	•	•	•	•	•						•	•	•	•	•				•											
V36	O111	cattle	483	a		•		•	•	•	•	•	•	•			•	•	•		•	•	•	•	•															
V37	NT	cattle	100/00	a		•		•	•	•	•		•	•	•		•	•			•	•	•	•	•															
V38	NT	cattle	102/00	a		•		•	•	•	•		•	•	•				•		•	•	•	•	•															
V39	NT	cattle	476	a		•		•		•	•		•	•	•								•	•	•	•	•					•								
V40	NT	cattle	474	a		•		•					•	•		•								•	•	•	•					•								
V41	NT	cattle	473	a		•		•					•	•										•	•	•	•					•								
V42	NT	cattle	475	a		•		•		•	•			•	•	•		•																						
V43	NT	cattle	101/00	a		•																	•				•													
V44	NT	cattle	489		g		•							•					•									•												
V45	NT	cattle	449	a		•						•						•												•			•	•						•
V46	NT	cattle	478		d		•					•		•				•					•							•	•									

Table II
Prevalence of *stx1* and *stx2* subtypes in the genome of 71 STEC strains

STEC serotype (number of strains)	Number of STEC strains with the *stx* gene subtype:								
	stx1	*stx2*						*stx1* + *stx2*	
	stx1a	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2g*	*stx2a* + *stx2c*	*stx1a* + *stx2a*	*stx1a* + *stx2c*
O157(n = 45)	5	2	0	11	0	0	5	1	21
Non-O157 (n = 26)	18	4	2	0	1	1	0	0	0
Total	22	7	2	11	1	1	5	1	21

protein C (*nle*C), EspA protein (*esp*A), EspF effector protein (*esp*F), non-LEE-encoded EspJ effector protein (*esp*J), Tir – cytoskeleton coupling protein (*tcc*P); type II secretion pathway related protein (*etp*D); serin protease autotransporters – SPATE (*esp*P); peroxidase-katalase (*kat*P) and translocated intimin receptor (*tir*).

All STEC O157: H7/H-isolates (n = 45) tested positive for markers encoding adhesins (*eae*A, *iha*), toxins (*ehx*, *tox*B), secretion systems proteins (*nle*A, *nle*B, *nle*C, *esp*F, *esp*J, *etp*D, *tcc*P), serin protease autotransporters – SPATE (*esp*P), peroxidase-katalase (*kat*P) and translocated intimin receptor (*tir*), while genes encoding heat stable enterotoxin AstA, colicin B activity protein Cba and cytolethal distending toxin subunit B CdtB, secretion III system protein EspA, Efa adhesin and assiociated with increased serum survival marker Iss were variable absent or present (Table I).

All STEC O26 (n = 11) and STEC O111 (n = 6) were positive for 13 and 11 markers presented on the array respectively (Table I). A positive spot signal in STEC O26 was detected for 13 genes encoding adhesins (*eae*, *iha*, type III secretion system EspB protein – *esp*B and lymphocyte inhibitory factor A-*efa1*), toxin (*ehx*), secretion systems proteins (*nle*A, *esp*A, *esp*F, *esp*J and cell cycle inhibiting factor – *cif*), major fimbrial subunit (*lpf*A), assiociated with increased serum survival marker *iss* and translocated intimin receptor (*tir*), while few strains were additionally present for genes encoding AstA, ToxB, CelB, Cba toxins, microcin M truncated protein MchcA, microcin H47 activity protein MchcB, putative microcin L transport protein MchcF, member of the microcin operon MchcC, secretion systems proteins (NleB, NleC, TccP) and EspP and KatP.

All STEC O111 (n = 6) were positive for 11 markers presented on the array encoding: adhesins (*eae*A, *iha*, *efa1*), secretion systems proteins (*nle*A, *nle*B, *esp*A, *esp*F, *esp*J, *cif*), major fimbrial subunit *lpf*A and translocated intimin receptor *tir* while adhesin *esp*B, toxins (*ehx*, *ast*A, *cel*B, *cba*), secretion systems proteins (*nle*C, *tcc*P) *esp*P, *kat*P and *iss* were variable present or absent.

Virulence gene profile. Based on presence or absence of virulence genes the tested STEC isolates were divided into 46 virulotypes (see Table I for details). Among 45 STEC O157 tested strains 21 virulotypes were distinguished (from V1 to V21) (Table I). STEC non-O157 strains isolated from cattle (n = 10), not typable using available latex assay, had different panel of virulence markers than tested isolates belonging to O157, O111 and O26 serotypes. Among STEC non-O157 strains isolated from cattle there were two strains which possessed virulence markers not present in any tested isolates. One of those strains (no. 478) possessed in *sub*A, *saa* and *epe*A genes (Table I) which are considered to encode subtilase toxin, autoagglutinating adhesion and serine protease autotransporters of *Enterobacteriaceae* (SPATE) respectively. The second strain (no. 489) had *st1a* gene encoded heat-stable enterotoxin ST1a and the third strain (no. 449) possessed *iro*N marker, encoded outer membrane protein receptor for siderophore.

Discussion

Besides Stx toxins, it is known that the STEC strains carry other virulence markers *E. coli* engagement in pathogenesis of STEC infection (Nataro and Kaper, 1998). Those virulence markers could be typical for STEC pathotype or acquire from other pathogenic *E. coli*. Therefore, in this study we conducted a comprehensive analysis of virulence gene profile in STEC strains from different origin isolated in Poland. To have the opportunity to detect a large number of target sequences present in genome of different *E. coli* pathotypes, the commercially available DNA microtube array was used. To the best of our knowledge, such a wide range of determinants of pathogenesis of STEC isolated in Poland have never been defined.

Using microarray data analysis the STEC strains isolated in Poland have been distinguished into 46 virulotypes. Interestingly, among STEC O157 strains, which constituted the most representative serological group in investigated strains collection, we observed conserved core set of virulence factors. Apart from the Stx toxin, all isolates possessed the LEE-associated genes for intimin (*eae*A), the translocated intimin receptor (*tir*) and the effector protein (*esp*F). Large virulence plasmid pO157 genes for enterohemolysin (*ehx*), toxin B (*tox*B),

type II secretion pathway related protein (*etp*D) were detected in all STEC O157 isolates. Moreover all isolates tested positive for the gene for adhesin Iha, *tcc*P and *esp*J markers and finally genetic markers for effector proteins NleA, NleB, NleC were present in all STEC O157 isolates assayed. Moreover, in the group of STEC O157 strains there were human, animal and food isolates which were isolated at long intervals of time. This data suggest that those panel of virulence factors might be essential for the survival of STEC O157 in a host organism and causing the infection.

These data are in agreement with the results of other researchers (Wu *et al.*, 2008; Söderlund *et al.*, 2010; Bugarel *et al.*, 2011). They confirmed that the panel of genetic determinants of virulence in the STEC O157 strains genomes are highly typical for this serotype. It is worth noting that only Söderlund *et al.* (2010) used commercially available EC 03 array to define virulence genetic determinants in STEC O157 strains so far. Results of the Swedish researchers clearly showed the presence of stable core of virulence markers in STEC O157 genome isolated from cattle (Söderlund *et al.*, 2010). The range of virulence determinants identified in STEC O157 strains using Array Tube Ec03 was the same as examined in the presented study.

Interestingly, we observed the stability of virulence determinants composition in STEC O157 genome for long intervals of time. It was in fact the presence of the same virulotypes in strains from different sources isolated in Poland in different years (virulotypes: V1, V2, V3, V4) (Table I). This may indicate the correlation between this virulotype and serotype and suggest that the set of genes, observed in the majority of STEC O157 isolates in the world, might be essential for the survival of these organisms in the host organism and induce them to infection.

Next to Shiga toxins, intimin, encoded by LEE pathogenicity island, is the main virulence marker, whose presence is routinely determined in diagnostics of clinical STEC isolates. It is important factor in pathogenesis of EPEC and STEC isolates because it enables direct contact between the bacterial cell surface and epithelial cells of the intestine. Our results revealed that almost all STEC isolates possessed *eae* gene. Only four STEC non-O157 strains isolated from cattle were included in the LEE – negative group (not carry *eae* gene). LEE-negative STEC non-O157 strains have been isolated from human and animals from several years (Galli *et al.*, 2010; Irino *et al.*, 2010). Those isolates may carry other adhesion factors as LpfA, Iha and Saa which play role in pathogenesis of human infection of this bacteria (Galli *et al.*, 2010; Irino *et al.*, 2010). The results presented in the hearing, as well as the observations made by other authors, lead to the conclusion that the presence of LEE-negative STEC non-O157 strains is common and therefore there is a need to pay special attention in the diagnosis of infections caused by them.

Interestingly, there was one strain (no. 478) among LEE-negative STEC isolates which carried genetic determinant responsible for production of the other cytolysin – subtilase (SubAB). According to scientific reports, STEC strains producing subtilase were isolated from people with HC and HUS (Galli *et al.*, 2010; Irino *et al.*, 2010; Paton and Paton, 2010). Characteristic of genetic markers in subtilase – producing STEC isolates were described by other authors (Galli *et al.*, 2010; Irino *et al.*, 2010). The isolates no. 478 possessed *saa*, *epe*A, *iha*, *ehx*, *esp*P, *lpf*A, *cdt*B and *cel*B, but did not carry *eae* and *tir* markers. Study conducted in Argentina by Galli *et al.* (2010) have shown that among the VTEC strains isolated from humans with HC and HUS, there were isolates which had *sub*AB, *saa*, *lpf*A, *ehx*, *iha*, *cdt*B markers. Similar observations made Irino *et al.* (2010), who showed that the subtilase – producing *E. coli* strains which all produced enterohemolysin, they also had the *saa*, *lpf*A, *iha* genes responsible for the expression of adhesins. Understanding the profile of genetic determinants in the genome of LEE – negative STEC strains may determine their pathogenicity potential, resulting from the co-occurrence of rare genetic markers present.

Recently there have been many scientific reports describing the presence of *E. coli* isolated from humans, animals and the environment, carrying specific virulence determinants characteristic for different *E. coli* pathotypes (Bielaszewska *et al.*, 2011; Prager *et al.*, 2011). Using microarray technology it was possible to confirm the presence of a wide range of genetic markers present in different *E. coli* pathotypes in a single experiment. The results of our analysis showed that among 71 STEC strains there was one isolate (no. 489) which possessed Shiga toxin gene as well as *sta1* gene encoded heat-stable enterotoxin STIa characteristic for enterotoxigenic *E. coli*. Moreover this isolate as the only in the STEC collection in this study produced Stx2g subtypes. Our results indicate that in our country there may exist *E. coli* strains with specific genetic determinants for both Shiga toxin producing (STEC) and for enterotoxigenic (ETEC) *E. coli*. The occurrence of strains with similar properties were also found in Germany (Prager *et al.*, 2011). Molecular characterization of 24 Shiga-toxin producing isolates producing Stx2g, obtained from clinical material, animals and the environment, have found that all tested isolates, in addition to *stx2g* gene carry the *st1a* gene and did express STIa, which typically is associated with enterotoxin-producing *E. coli* (Prager *et al.*, 2011). The emergence of infections in humans strains with combination of virulence genes of different pathotypes of intestinal pathogenic *E. coli* may indicate the spread in the environment of a new, intermediate and emerging pathotype

(Bielaszewska *et al.*, 2011; Mellmann *et al.*, 2011; Prager *et al.*, 2011). This phenomenon was observed in the current year, during one of the biggest so far HUS outbreaks in Europe, which caused an epidemic strain of *E. coli* O104: H4. This strain exhibited features of two *E. coli* pathotypes: enteroaggregative (EAEC) and Shiga toxin producing (STEC) (Bielaszewska *et al.*, 2011; Mellmann *et al.*, 2011). Given a rising number of intermediate pathotypes becoming described among *E. coli,* a wider range of virulence markers should be included in the regular pathotype diagnostics.

In the routine diagnosis of infections caused by STEC tests are also used based on the amplification of gene fragments encoding Shiga toxins. The Shiga toxin genes occur in many different nucleotide sequences-subtypes (Scheutz *et al.*, 2012). Therefore in the routine identification of the subtypes of Shiga toxins genes in the genomes of STEC strains is extremely important because it provides valuable data that are used in monitoring of the STEC infections in many countries (Leotta *et al.*, 2008). In addition, this method is applied in the routine diagnostics of STEC infections, especially in the situation of carrying out epidemiological investigations during outbreaks caused by these microorganisms (Bielaszewska *et al.*, 2011; Scheutz *et al.*, 2011).

It is worth noting that among the STEC producing Stx2, isolated in Poland (n = 49), the subtype *sxt2c* (n = 37) was dominated. Moreover, these subtype was present only in STEC from O157 serotype and performed alone (n = 11) or with *stx1a* (n = 21) or *stx2a* (n = 5). These results are accordance with the results obtained by other authors (Leotta *et al.*, 2008; Aspán and Eriksson, 2010; Käppeli *et al.*, 2011). In Switzerland all STEC O157 strains (n = 44) isolated from people from 2000 to 2009 years possessed *stx2a* or *stx2c* subtypes (Käppeli *et al.*, 2011). According to Eriksson and Aspan studies, STEC O157 strains isolated from cattle in Sweden carried *stx2a* or *stx2c* subtypes (Aspán and Eriksson, 2010). These subtypes of *stx2a* gene also predominated in STEC O157 strains isolated in Argentina, Australia and New Zealand (Leotta *et al.*, 2008).

It must be underline that the determination of the subtypes of Shiga toxin genes is extremely important because it provides information about the pathogenic potential of the STEC strain. Several studies have revealed that the individual *stx* subtypes differ in biological activity which were observed using various animal models and *in vivo* cytotoxicity assay (Fuller *et al.*, 2011). Moreover STEC strains possessed *stx2a* as their sole gene, or in combination with *stx2c* have been described as more closely associated with HUS, than STEC strains with other Stx gene combinations (Friedrich *et al.*, 2002). Further, purified Stx2, in contrast to purified Stx1 can elicit signs of HUS in baboons (Stearns-Kurosawa *et al.*, 2010). In addition, a recent study in mice model of disease (Fuller *et al.*, 2011) demonstrated that Stx2b and Stx2c had potencies similar to that of Stx1, while Stx2a, Stx2d, and elastase-cleaved Stx2d were 40 to 400 times more potent than Stx1. These findings may partly explain the high virulence of STEC O104:H4 strains, which is responsible for causing the outbreak of hemolytic uremic syndrome in May 2011 in Germany (Bielaszewska *et al.*, 2011; Prager *et al.*, 2011; Scheutz *et al.*, 2011). Epidemic *E. coli* O104: H4 strain possessed stx2a subtype, which could explain the high incidence of HUS in infected patients (one case of HUS in four cases of bloody diarrhea) (Bielaszewska *et al.*, 2011; Prager *et al.*, 2011; Scheutz *et al.*, 2011). Our results showed that STEC strains isolated in the country are diverse in terms of occurrence of *stx* subtypes, which according to scientific publications, are more potent (*stx2a*, *stx2d*) than other subtypes of the *stx* genes.

In conclusion, this is the first comprehensive analysis of virulence gene profiles identified in STEC strains isolated from human, cattle and food in Poland. Simultaneous detection of virulence markers provides data suitable for molecular risk assessment of the potential virulence of STEC isolates. The results obtained using microarrays technology confirmed high effectiveness of this method in determining STEC virulotypes, which certainly supports the implementation of this method for the diagnosis of STEC isolates in our country in selected provincial sanitary – epidemiological laboratories.

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