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Diagnostic Value of Serological Tests Against Verotoxigenic *Escherichia coli* in Hemolytic Uremic Syndrome in Children

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Diarrhea-associated hemolytic uremic syndrome (HUS D+) caused by verotoxigenic *E. coli* strains (VTEC) is a major cause of acute kidney injury in children between 1 and 5 years of age. Because of the short presence of VTEC in the gastrointestinal tract as well as difficulties with the detection of the verotoxigenic strain, identification of HUS etiology might be challenging.
Objectives. The aim of the study was to assess the clinical and diagnostic value of serological tests for specific antibodies against verotoxigenic strains of *E. coli* in patients with HUS.
Material and Methods. Eight children aged 8 months – 7.1 years (mean 40 ± 29 months) with symptoms of acute kidney injury, hemolytic anemia and thrombocytopenia observed after hemorrhagic diarrhea were included to the study. VTEC presence was detected in a stool culture with subsequent analysis of the ability to produce verotoxin and the presence of VT1 and VT2 as well as intimin and enterohemolysin genes. In addition, the presence of specific IgA, IgM and IgG antibodies against *E. coli* serogroups O26, O103, O104, O111, O121, O145 and O157 was measured using ELISA.
Results. In 3 subjects, VTEC O26, O157 and O104 serogroups were cultured in the stool and the specific IgA, IgM and IgG antibodies were detected. In 4 subjects, no VTEC strains were cultured, however, high titers of IgA, IgM and IgG antibodies against *E. coli* O26, O157 and O111 were detected. In a single patient, the negative results of bacteriological and serological analyses excluded VTEC etiology of HUS.
Conclusions. A serological analysis of VTEC can confirm the result of stool culture for verotoxigenic *E. coli* strains and help to find the cause of HUS in case of negative results of a stool culture (**Adv Clin Exp Med 2015, 24, 6, 1031–1036**).

Key words: children, VTEC, hemolytic-uremic syndrome.

Hemolytic uremic syndrome (HUS) is the most common cause of acute kidney injury (AKI) in children in the course of primary kidney disease [1]. It is diagnosed on the basis of specific symptoms including hemolytic anemia, thrombocytopenia and AKI. Until now, diarrhea-associated HUS was classified as typical HUS (D+), with other forms as atypical HUS (D-) [2]. Classification of the type of HUS and determination of its etiology is extremely important for the patient because the treatment of both types is different. The highest incidence of HUS D (+) is observed in small children below 5 years of age [3, 4].

The treatment of HUS (D+) is symptomatic, while in the case of HUS (D-), depending on its etiology, the patient may require transfusion of fresh frozen plasma (FFP), therapeutic plasma exchange or immunosuppressive treatment [5].

The most common (in 90% of cases) cause of HUS is intestinal infection with Shiga-toxin (also called verotoxin) producing *E. coli* strains (STEC/ /VTEC). Verotoxigenic *E. coli* are zoonotic bacteria naturally found in the intestinal flora of farm as well as wild animals. The gastrointestinal tract of cattle, goats and sheep is the main reservoir of these bacteria. The ability to produce verotoxin has been observed in more than 100 serotypes of *E. coli*. The most commonly isolated VTEC is the O157 serologic group (41.1% of VTEC strains isolated from humans in the European Union) [6]. Epidemiological data shows that among non-O157 VTEC the most common cause of HUS are O26, O103, O111, O121 and O145 serotypes [7].

The course of VTEC infection is variable, from asymptomatic carrier-state through acute gastroenteritis with non-bloody diarrhea up to the most common (90% of cases), hemorrhagic colitis (HC). It is estimated that approx. 5–15% of patients (usually below 14 years old) with between 1 and 14 days of diarrhea may develop HUS [8].

The clinical course of infection is influenced by the type of verotoxin produced by the *E. coli* strain. The majority of VT1-producing strains cause HC or non-bloody diarrhea whereas an infection with VT2-producing *E. coli* strains (or, more rarely, both toxins) is more often complicated with HUS [7].

The basic diagnostic tool allowing for diagnosis of VTEC infection is stool culture and determination of the pathogenic features of the *E. coli* strain [9], including the ability to produce verotoxins. The presence of toxins might be detected in the strain as well as directly in stool samples using serological methods (serological and immunochromatographic tests), PCR-based methods (for the presence of VT1 and VT2 genes encoding toxins) or in a cytotoxic assay on the Vero cell line.

Literature data and our own observations suggest that a few days after diarrhea, especially if the patient was treated with antibiotics, a culture of VTEC strains might be impossible. In such cases, determination of HUS etiology might be facilitated with the assessment of antibodies against the *E. coli* serotypes responsible for HUS (D+) [10].

The aim of the study was to assess the diagnostic value of specific antibodies against selected *E. coli* serotypes in the determination of HUS etiology.

Material and Methods

Eight children, aged 8 months – 7.1 years (mean 40 ± 29 months), admitted to the Department of Pediatric and Nephrology, Medical University of Warsaw in 2011–2013 from regional hospitals because of HUS symptoms developed after bloody diarrhea were included to the study (Table 1). In this group, 4 children were below 4 years old.

The treatment consisted of transfusions of packed red blood cells (pRBC) and FFP (10 mL/kg). In children who required renal replacement therapy, acute peritoneal dialysis (APD) or hemodiafiltration (HDF) were performed. After implantation of a Tenckhoff catheter under general anesthesia, peritoneal dialysis solutions containing 1.36–2.27% of glucose (Baxter) were used. Continuous veno-venous hemodiafiltration (CVVHDF) was performed on a PRISMA Flex machine (Gambro) with PRISMA ST 60 hemofilters. CVVHDF was performed through a double lumen dialysis catheter inserted into the right internal jugular vein.

Antibiotics were used in the treatment of hemorrhagic diarrhea or as a prophylaxis for surgical implantation of the catheter.

Laboratory Tests

A biochemical analysis of blood specimens (urea, creatinine, lactate dehydrogenase (LDH), AST – alanine transaminase, ALT – alanine transaminase, bilirubin) was performed using the dry chemistry method on a Vitros 500 analyzer (Ortho Clinical Diagnostics, Johnson & Johnson Company). Coagulation parameters were measured in citrated plasma on a CS-2100i analyzer (Sysmex) or BCS XP (Siemens). Complete blood count was measured in EDTA-plasma on a LH 750 analyzer (Beckman Coulter).

Glomerular filtration rate (GFR) was calculated by Schwartz formula [11]. The concentration of ADAMTS 13 as well as its activity was measured using ELISA (Imubind Adamts 13 Elisa).

Microbiological Analyses

Stool was cultured on MacConkey agar. Ten randomly selected colonies of *E. coli* were isolated and identified on the basis of their biochemical features, ability to produce β-D-glucuronidase and sorbitol fermentation. Identification was performed according to the protocol described previously [12]. The antigenic group of the respective isolates was analyzed with a latex test (Biomex) according to the manufacturer's protocol. In addition, the ability of the isolated strains to produce verotoxin was analyzed using a reversed passive latex agglutination test (VTEC-RPLA, Oxoid). The PCR method was utilized to detect fragments of the genes encoding verotoxins

Table 1. Basic clinical data and selected laboratory findings in the studied children. The lowest platelet number, hematocrit and hemoglobin level and minimum GFR value, as well as the highest concentrations of urea, creatinine, LDH, white blood count and serum bilirubin are given

	Patient							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sex	f	f	m	f	m	m	f	m
Age (months)	8	46	24	36	86	60	53	75
Diarrhea duration (days)	8	3	10	3	8	5	8	4
Onset HUS from starting diarrhea (day)	7	3	12	4	3	6	6	3
D-dimer (ug/L FEU) (normal range < 550)	20442	8345	4953	14482	21585	2832	5674	10598
PLT (G/L) (normal range > 150)	29	42	14	11	34	84	29	24
Hgb (g/L)/Hct (L/L)	47/0.139	76/0.226	72/0.203	63/0.181	72/0.214	62/0.177	63/0.186	56/0.162
WBC (G/L) (normal range 4–14)	22.8	16.8	16.6	13	11.1	17.2	27.2	22.8
AST/ALT U/L (normal range 15–50/10–24)	109/98	300/203	153/34	143/80	375/102	53/24	193/23	247/121
Urea (mmol/L) (normal range 2.49–6)	50	33.4	21.2	33.7	49.1	34.9	29.4	32.4
Creatinine (μmol/L) (normal range 17.7–61.9)	512.72	795.6	106.08	132.6	530.4	97.24	663	406.6
Min GFR (mL/min/1.73 m^2)	5	4.81	32.3	28	8	43.5	5.43	11.2
Bilirubin (μmol/L) (normal range 5.1–20.5)	32.5	46.17	41.04	59.85	66.7	30.78	12	54.72
LDH (U/L) (normal range 470–900)	10831	9772	8976	9570	15291	4257	8788	17373

AST – aspartate transaminase; ALT – alanine transaminase; LDH – lactate dehydrogenase; PLT – platelet count; WBC – white blood count; Hgb – hemoglobin (normal range 125–160 g/L); Hct – hematocrit (normal range 0.37–0.47).

(VT1 and VT2), intimin (*eae*) and enterohemolysin (*ehly*), according to the protocol described previously [12, 13].

Serological Tests

Serum levels of IgA, IgG and IgM antibodies against *E. coli* from O26, O103, O104, O111, O121, O145 and O157 serological groups were measured in the studied patients. Lipopolysaccharide (LPS) antigens obtained with a modified Boivin method were used as antigens in ELISA assay [14]. Concentration of the working solution of all LPS preparations was established at 25 μg/mL using the chessboard titration method. In the classification of the result, a cut-off value calculated on the basis of the analysis of sera obtained from healthy blood donors (mean arithmetical value plus two standard deviations) was used.

Results

Mean hospitalization duration was 19.6 ± 8.4 days (range: 11–31 days). Diarrhea was observed in all the studied children, while bloody stools were present in 5 of them. In 5 children, HUS symptoms were observed during diarrhea (patients #1, 2, 5, 7 and 8). The course of HUS in these children was severe and they required renal replacement therapy. In the remaining 3 patients (patients #3, 4 and 6), HUS was observed 1–2 days after resolution of the gastrointestinal symptoms (Table 1). Mean duration of thrombocytopenia (< 150 G/L) was 9.2 ± 3.3 days and normalization of hemoglobin concentration was observed after 11.8 ± 6.5 days. Symptoms of intensive diathesis were not observed in any patients. Normalization of creatinine concentration was observed after 17.8 ± 8.5 days (range: 8–28 days) from the beginning of the

disease, while normalization of transaminase activity was after 9.2 ± 4.5 days. After the acute period of the disease, 6 children developed arterial hypertension grade I (95–99th percentile for age, sex and height) that required 1 antihypertensive drug (patients #1, 2, 3, 4, 5 and 8). After the acute period of the disease, abnormal results of urinalysis were observed in 6 children, including hematocyturia (patients #3, 4 and 6) and hematocyturia with non-nephrotic proteinuria (patients #1, 2 and 7). Only in 2 children (patients #5 and 8), were no abnormalities in urinalysis after the acute period of the disease observed.

In all the studied children, a medical history was taken to establish the potential source of infection. In 6 children no source of infection was found. One child (patient #2) was infected after contact with piglets with postweaning diarrhea, an animal disease caused by *E. coli*. In the case of patient #5, the disease was transmitted within the household (from an ill father) (Table 2).

Microbiological Test Results

E. coli was present in the stool culture in 4 out of 8 children with clinical symptoms of HUS. The cultured *E. coli* strains were later confirmed to produce verotoxin. These strains expressed the VT2 gene as confirmed with PCR and actively produced verotoxin VT2 as confirmed with VTEC-RPLA. Serological typing revealed that the isolates were from O157, O26 and O104 serologic groups. In the isolates obtained from patient #8, no VTEC strains were confirmed (Table 2). In the remaining 4 children, the stool culture was negative for *E. coli.*

Serological Test Results

In 7 of the studied children, IgM, IgG and IgA antibodies against the LPS of verotoxigenic strains of *E. coli* were detected (Table 2). The results of the serological assessments revealed the presence of

Table 2. The results of microbiological tests, characteristics of VTEC strains isolated from patients with HUS and applied treatment

		Patient							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
VTEC serotyping		O157:H-	O26:Hn	*E. coli* – no growth	*E. coli* – no growth	O104:H4	*E. coli* – no growth	*E. coli* – no growth	Among cultured *E. coli* no VTEC strains were observed
Result of RPLA-VTEC		VT2	VT2	nt	nt	VT2	nt	nt	–
Presence of antibodies against VTEC serotype*		O157	O26	O111	O26	O104	O157	O157	–
Course severity –PD length		PD – 7 day	PD – 19 days	–	–	PD – 12 days	–	PD – 10 days	HDF – 10 days
Antibiotic used		cefuroxime	cefuroxime	cefuroxime	–	cefuroxime, imipenem + cilastatin, xifaxan	ceftriaxone	cefuroxime, imipenem + cilastatin	cefotaxime
Source of infection		piglets	not found	not found	not found	household contact	not found	not found	not found
PCR results	VT1	–	–	nt	nt	–	nt	nt	–
	VT2	VT2a	VT2a	nt	nt	VT2a	nt	nt	–
	eae	+	+	nt	nt	–	nt	nt	–
	ehly	+	+	nt	nt	–	nt	nt	–

RPLA-VTEC – verotoxins VT1 and VT2 detection test; Hn – antigen not determined; H – immobile strain; VT1 – verotoxin 1 gene; VT2 – verotoxin 2 gene; *eae* – intimin gene; *ehly* – hemolysin gene; nt – not tested; PD – peritoneal dialysis; HDF – hemodiafiltration; "–" – negative, "+" – positive; * presence of antibodies against *E. coli* O26, O104, O111, O121, O145 and O157 LPS antigens in serum samples obtained from patients with hemolytic uremic syndrome.

a humoral response against *E. coli* serotype O157 in 3 patients, serotype O26 in 2 patients and serotype O111 and O104 in single cases. Serological assessment of the VTEC strain was concordant with the results of the bacteriological analysis of VTEC strains cultured from the patients (#1, 2 and 5). In the stool culture of patients #3, 4, 6 and 7, no *E. coli* growth was observed, whereas serological analysis showed the presence of antibodies against VTEC (Table 2). In the *E. coli* isolates obtained from patient #8, neither VTEC strains nor antibodies against the LPS of the studied VTEC serotypes of *E. coli* were detected. In this patient, a decreased level of ADAMTS 13 protease was observed. In patients #3, 4 and 6, the level of ADAMTS 13 protease was in the normal range.

Discussion

According to epidemiological data, 222 cases of HUS (D+) were registered in European countries in 2010, which accounts for 5.5% of all laboratory confirmed VTEC infections. Infection with VTEC O157 was the most common cause of HUS (identified in 42.5%). Less common serotypes were VTEC O26 (19.2% of patients), VTEC O111 and VTEC classified to other antigen groups [6]. Although the studied group was small, our results are concordant with this data. In the largest number of cases (3/8), infection with VTEC O157 was observed. The second-most common cause of HUS was the O26 strain (2/8), whereas O111 and O104 serotypes were found in single patients (1/8 each).

The genes encoding verotoxin (VT1 and VT2) are transferred *via* prophages integrated with a bacterial chromosome and replicated simultaneously. Because of this, effective expression of these genes and the production of toxins is observed only after induction of the prophage, initiation of the lytic cycle and lysis of the bacteria [15]. The induction of prophages might be spontaneous or induced by external triggers. One of the most important factors inducing prophages are antibiotics, especially fluoroquinolones, co-trimoxasole, trimethoprim, furazolidone or polymyxin. It has been shown that antibiotic therapy in children with VTEC-mediated diarrhea may result in a severalfold increase in the risk of HUS [16]. The effect is caused not only by the induction of prophages but also by the large amount of the toxin released into the intestine from the bacteria killed by the drug and the consequently increased absorption of the toxin into the blood. Moreover, antibiotics increase the risk of a bacteriophage infection of other sensitive *E. coli* strains (e.g. physiological flora) that may result in increased production of VT. In our material, the children who developed HUS symptoms during diarrhea had a more severe course of the disease. All of these children were treated with antibiotics (2^{nd} or 3^{rd} generation cephalosporin) since the 2^{nd}–4^{th} day of diarrhea and that could influence the severity of the disease. In two patients (#3 and 6) who were treated with antibiotics, HUS symptoms were observed after cessation of the diarrhea. In these cases, the HUS might have been initiated by the antibiotic-mediated induction of the phage. However, the course of HUS in these children was mild and they did not require renal replacement therapy. The patients (#1, 2, 5 and 8) with max LDH serum level ≥ 10000U/L had a more serious course of HUS. They required renal replacement therapy despite antibiotic usage.

In 3 of the studied patients (#1, 2 and 5), the VTEC strains were cultured in the stool and the presence of VT2 was confirmed. In the serum obtained from these children, antibodies against the LPS antigens of the same serotypes of VTEC were detected. In the other 4 patients (#3, 4, 6 and 7), the lack of bacteriological confirmation of the etiology could indicate atypical HUS, however, the presence of IgA, IgG and IgM antibodies against VTEC O111, O26 and O157 suggested HUS (D+). In patient #8, with bloody diarrhea before HUS, the VTEC strain was not cultured and no specific antibodies against the selected antigenic groups of VTEC were detected. In this patient, atypical HUS not associated with intestinal infection was confirmed with the low level of ADAMTS 13 protease.

In the majority of our patients, serological analysis helped to diagnose or confirm the infection with verotoxigenic strains of *E. coli* and qualified the patient to HUS (D+) group. In the case of negative results of the bacteriological assays, serological analysis of the immune response to VTEC was of great complementary value. If both bacteriological and serological analyses for VTEC in a patient with diarrhea are negative, other causes of HUS should be considered (as in patient #8).

To perform a full serological analysis of HUS (D+), 2 sequential samples of serum should be analyzed to assess the dynamics of the antibody titers and increase the specificity of the antigen by using LPS fragments characteristic for specific serotypes. This aim will be achieved in future studies.

Because fast identification of HUS etiology determines the treatment and is extremely important for a patient's fate [23], the diagnosis of HUS etiology should be considered a routine bacteriological assessment. In this context, the availability of

commercial, chromogenic culture media, which is easy to use by any microbiological laboratory, for selective isolation of verotoxigenic *E. coli* strains (the most common serotypes including O157, O26 and O111) directly from stool are very promising.

The authors have concluded that a serological analysis of VTEC can confirm the result of a stool culture for verotoxigenic *E. coli* strains or may help to find the cause of HUS in the case of negative results of the stool culture.

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