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Abstract

Background. Shigatoxin (Stx)-producing Escherichia coli (STEC) are the most common causes of hemolytic uremic syndrome (STEC-HUS). The aim of our study is to compare the risk of developing STEC-HUS in relation to the type of Stx genes (Stx1, 2 or both).

Methods. This is a prospective, observational, multicenter study involving 63 pediatric units in Northern Italy (ItalKid-HUS Network). STEC-infected children were identified within a screening program for bloody diarrhea during a 10-year period (2010-2019). *Stx* genes were detected by Reverse Dot Blot or Real-time-PCR. Following the identification of STEC infection children were followed until diarrhea complete recovery for the possible development of STEC-HUS.

Results. Of the 214 Stx positive patients, 34 (15.9%) developed STEC-HUS. The risk of HUS in STEC-infected children with Stx1 (n: 62; 29.0%) and Stx2 (n: 97; 45.3%) was respectively of 0% and 23.7%, while in patients carrying both Stx1 and Stx2 (n: 55; 25.7%) the risk was 12.7% (p: 0.001).

Conclusions. Our data confirm that Stx1 is a very rare cause of STEC-HUS and demonstrate that the risk halves in case of Stx1+2-producing *Escherichia Coli* infection compared with infections where Stx2 is present alone. This observation is helpful in assessing the risk of individual STEC-infected patients for the development of eHUS and suggests that Stx1, in the presence of Stx2, might exert a protective role possibly by receptor competition.

Keyword: shiga toxin, hemolytic uremic syndrome, diarrhea, children.

Abbreviation: HUS-Hemolytic uremic syndrome, STEC-Shigatoxin (Stx)-producing Escherichia coli, RRT-renal replacement therapy

Introduction

Hemolytic uremic syndrome (HUS) is a life-threatening disease defined by the simultaneous occurrence of platelets consumption, microangiopathic hemolysis and acute kidney injury with or without multiorgan damage. In Western countries, the disease remains among the main causes of acute kidney failure in children [1]. The most common causes of HUS are Shiga toxins (Stx)-producing Escherichia coli (STEC), following a gastrointestinal infections. It has been reported that STEC-HUS complicates 6 to 9% of STEC infections and usually begins 3 to 10 days after the onset of diarrhea [2]. STEC-HUS still has no specific treatment, so children can only be given supportive care: transfusions, correction of acidosis and electrolyte abnormalities, appropriate fluid management, antihypertensive treatment, nutrition and renal replacement therapy (RRT) for fluid removal and correction of metabolic abnormalities [3-6]. Several methods for the diagnosis of STEC infection are becoming widely available through the examination of fecal material or sera: stool culture, Shiga toxins immunoassays, and/or polymerase chain reaction for the detection of Stx genes being the latter the most sensitive [7]. As a consequence of the increasing availability of the mentioned diagnostic tools, STEC infection is being increasingly diagnosed but once a patient is diagnosed with STEC infections, there are no ways to predict if and when will eventually develop the systemic complication.

Several reports document that STEC strains producing Stx2 are more commonly associated with STEC-HUS than both Stx1+2 [8,9], it is not clear if this is just related to the fact that the STEC responsible of the primary infection more frequently produce Stx2 or to other factors including the association of Stx2 with more virulent serotypes.

The present brief report is aimed at estimating the risk of developing STEC-HUS in relation to the type of Stx genes (1. 2 or both) towards improving the risk assessment of children being diagnosed with STEC infection.

Patients and methods

From 2010 to 2019, a screening program of bloody diarrhea (BD) for STEC infection in children (<20 years old) has been developed. It involves 63 pediatric units in Northern Italy (ItalKid-HUS Network), with a referral pediatric population of 2.3 millions (organizational and methodological details are given elsewhere) [1]. The program is devoted to the early diagnosis of STEC infection and the present analysis considers the subset of patients in which the screening was started before the diagnosis of HUS was established and who turned to be positive for Stx. Stx-infected children, once identified were closely monitored for the eventual development of STEC-HUS until complete recovery of diarrhea. In addition, during the same period of time, all HUS being referred to our Center, have been also screened for Stx and those who were positive were separately analyzed for the relative distribution of identified Stxs. Reverse Dot Blot analysis (until 2018) and real-time-PCR were performed on biological material (stools) using commercial kits (Genotype EHEC-Arnika and RIDA Gene-EHEC/EPEC, R-biopharm) which identifies Stx1, Stx2, eae and ipaH genes. Stx positive samples were tested by Real-time PCR assay to detect the presence of O157, O26, O111, O145, O103

STEC serogroup-associated genes using the primers and the conditions indicated in the EU-RL VTEC Method [10]. The study received the approval by the Ethical Committee of our Institution.

Statistical analysis

Data are provided in absolute number and percent or as median and interquartile range (IQR). The effect modification of Stx across serotypes was assessed by fitting a log-binomial regression model including as covariates Stx (dichotomous variable: Stx1+2 vs Stx2), serotypes (four categories), and three product terms between Stx and serotypes. The three product (interaction) terms were tested together using a global Wald test. Finally, we fitted a multivariable log-binomial regression model including only Stx and serotype main effects to calculate adjusted relative risks (RR) and 95% confidence intervals (CI) for Stx. Statistical analysis was performed with Stata 16 (StataCorp. 2019).

Results

Between 2010 and 2019, 214 children (58.4% males, median age of 4.0 years; IQR 1.8-7.5) with bloody diarrhea were found positive for one or more of the Stxs. The relative distribution of patients by Stx type was as follows: 62 (29.0%) Stx1, 97 (45.3%) Stx2 and 55 (25.7%) Stx1+2 without significant differences as to gender (p: 0.74) and age (p: 0.11) according to the type of identified Stx, although patients infected with both Stx1+2 were slightly older with a median of 5.1 years (IQR 2.2-9.1), compared to those carrying Stx1 (3.2 IQR 1.4-6.5) or Stx2 alone (3.3 IQR 2.1-6.8) (p: 0.21).

Of the 214 Stx positive, 34 (15.9%) developed STEC-HUS. None of the patients carrying Stx1 alone developed the disease, 23 out of 97 carrying Stx2 turned into HUS whereas among those with both Stx1+2, only 7 of the 55 did evolve into HUS. Thus the risk of Stx positive infection to turn into STEC HUS was significantly different according to the isolated Stx: 0% for Stx1, 23.7% for Stx2 and 12.7% for Stx1+2 (p: 0.001).

Stx2 alone was more commonly associated with STEC O26 infection with a relative frequency of 54.5% compared to the 37.2% observed with O157 or to 46.3% of other serotypes (p: 0.0001) and O26 was the single most common serotype identified in patients who developed HUS (34.5%). For this reason the possible role of Stx gene on the risk of HUS by serotype was explored. Stx1+2 positive was associated with a reduced HUS risk within each sertype (Table 1). The p-value for interaction was 0.83, indicating no effect modification of Stx effect across genotype. The crude RR for Stx1+2 was 0.54 with 95% CI: 0.25-1.17). The RR (Stx1+2 vs Stx2) adjusted for the involved serotype was 0.48 (95% CI: 0.22-1.07).
Finally, patients carrying Stx2 alone and O26 serotype showed a risk of developing HUS as high as 58.3% compared to that of other serogroups which was only 18.8% (p: 0.0002).

Table 1 – Risk of STEC-HUS in Stx2 and Stx1+2 positive patients by serotype.

Serotype	N°	Bloody Diarrhea	
		Stx2 Pos. HUS/Total (%)	Stx1+2 Pos. HUS/Total (%)
O26	17	7/12 (58.3)	**1/5 (20.0)**
O157	39	4/14 (28.6)	**4/25 (16.0)**
Other	54	9/41 (21.9)	**2/13 (15.4)**
Unknown	40	3/30 (10.0)	**0/12 (0.0)**
Total	**152**	**23/97 (23.7)**	**7/55 (12.7)**

Discussion

During the last 10 years, the management of STEC-HUS has considerably changed. The increased availability of diagnostic tools which are rapidly spreading in Western countries, have made the diagnosis of STEC infection widely accessibile. It is becoming increasingly common to diagnose STEC infection prior to the development of HUS whereas, in the past, the diagnosis was often ruled in after the clinical onset of the disease.

Fortunately, only a minority of patients infected by STEC will develop HUS and this poses the problem of identifying, as much as possible, which patients need special attention to avoid useless and expensive medical procedures on many patients, for the few who will really benefit. Several studies have demonstrated the importance of fluid infusion in patients with STEC infection or early in the course of HUS to prevent the development of HUS and/or to mitigate the severity of the disease [5, 6]. These relatively new therapeutic approaches require that the disease is diagnosed as early as possible since the window for therapeutic opportunities is narrowed by the typically rapid transition from gastrointestinal infection to HUS. These concepts bring back the issue of correct risk assessment to avoid unnecessary procedures but at the same time to make sure that all subjects at risk are captured. Unfortunately, so far very few risk factors have been identified, most of which are either not easily or timely available at the bedside or they lack specificity.

Perhaps the individual risk assessment will require multiple approaches or biomarkers which are not available yet. In the meantime, physicians taking care of children infected with STEC need some clues as to whether a specific subjet is at high risk of turning into HUS in order to modulate the level of care and intervention.

The present analysis clearly confirms that Stx1 is a very rare cause of STEC HUS. We also confirm that Stx2-producing STEC, are definitly a more common cause of infection than Stx-1 and Stx1+2-producing STEC (45.3% vs 29.0 and 25.7%, respectively); thus the previously and repeatedly reported higher frequency of STEC-HUS related to Stx2 is not surprising. However, the higher frequency of Stx2-related HUS per se, does not necessarely mean a higher relative risk of HUS in Stx2-infected patients which instead is clearly demonstrated by the present analysis: the relative risk of HUS in patients positive to Stx2 alone is double compared to that of patients carrying Stx2 in combination with Stx1. This observation stands in favour that the concomitant presence of Stx1 reduces the pathogenetic capacity of Stx2.

As previously reported, we also show that the combination of Stx2 alone with STEC O26 serotype further increases the already high risk of developing HUS: with this combination (Stx2-producing STEC O26), which has become increasingly common in isolates from Europe, the risk that STEC infection turnes into HUS rises to as high as >50% [11,12].

Given the milder pathogenetic effect of Stx1 compared to Stx2, we speculate that in the setting of Stx1+2-producing STEC infection, the Stx1 might exert a protective role via receptor agonism by occupying the binding site which becomes no longer available for Stx2. This might occur during early toxemia when Stx binds to circulating cells though Gb3 (monocytes and platelets) and TLR4 (neutrophiles, monocytes and platelets) allowing the formation of leukocytes/platelet aggregates and microvescicles but also during the intoxication of target cells through Gb3 receptor [13-15].
An alternative explanation for the lower risk of HUS in case Stx2 is associated with Stx1 is that, assuming that a given STEC strain is capable of synthesizing a fixed maximum amount of Stxs, the simultaneous production of Stx1 may lower the amount of Stx2 produced with a consequent reduced toxicity. This does not seem to be the case since the lower lethal dose of Stx2 (compared to Stx1 or Stx1+2) has been demonstrated, in animal models [7,8]. These studies already introduced the concept that Stx1 may reduce both the pathogenicity and cytotoxicity of Stx2. In addition, we did not observe lower concentrations of Stx2 in patients infected by *E. coli* producing both Stxs compared with those infected with Stx2 only [16].

To the best of our knowledge, our data are the first which support a possible protective role of Stx1 when in combination with Stx2, in vivo.

In conclusion, patients infected with Stx1-producing STEC, have a negligible risk of HUS, those with both Stx1+2 will have a risk slightly above 10%, unless O26 serotype is involved (in which case the risk rises close to 20%) whereas the highest risk (around 23%) is shown by patients with Stx2 alone. The risk of the latter group will increase above 50%, if

the Stx2 is produced by STEC O26. Although the reported differences might not be strong enough to make differential clinical decisions, they can be helpful to better define the risk of individual patients towards a better patient and/or parents' information. Moreover, the hypotesis that Stx1 plays a protective role when associated with Stx2 though receptor competition may have potentials for drug development.

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Author's contribution

All authors contributed to the study conception and design. Material preparation, data collection were performed by Gianluigi Ardissino and Valentina Capone. Laboratory test were performed by Chiara Vignati and Laura Daprai. Data analysis were performed by Gianluigi Ardissino, Dario Consonni, Maurizio Brigotti, and Mario Vittorio Luini. The first draft of the manuscript was written by Ilaria Possenti and Gianluigi Ardissino and all authors commented on the various versions of the manuscript. All authors read and approved the final manuscript.

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