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Risk determinants for the development of typical haemolytic uremic syndrome in Belgium and proposition of a new virulence typing algorithm for Shiga toxin-producing *Escherichia coli*

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Abstract

In Belgium, it is mandatory to report Shiga toxin-producing *Escherichia coli* (STEC) infections to the health inspection authorities. To facilitate the decision making regarding infection control measures, information about the risk factors for the development of the haemolytic uremic syndrome (HUS) can be helpful. We performed statistical analyses on a dataset of 411 Belgian STEC strains. Demographic and clinical patient characteristics as well as phenotypical and genotypical STEC strain characteristics were taken into account. Multivariate logistic regression models indicated that age categories ⩽5, 6–12 and ⩾75; the *stx2* gene; and the *eae* gene were significant HUS development risk determinants. The *stx2a* subtype had the highest risk (OR 29.6, 95% CI 7.0–125.1), while all *stx1* subtypes encompassed a significant lower risk (OR 0.3, 95% CI 0.1–0.5). Presence of the *stx1* gene without *stx2* encompassed a lower risk than the combined presence of *stx1* and *stx2*, or *stx2* solely. Based on these results, we propose a new virulence typing algorithm that will enable the National Reference Centre to provide the physicians and health inspection authorities with a risk classification for the development of HUS. We believe this will contribute to a more efficient STEC infection control management in Belgium.

Introduction

As the most frequent cause of the haemolytic uremic syndrome (HUS), Shiga toxin-producing *Escherichia coli* (STEC) (also called verocytotoxin-producing *E. coli* – VTEC) are the most feared diarrhoeagenic *E. coli*. HUS is characterised by the sudden occurrence of microangiopathic haemolytic anaemia, thrombocytopenia and renal insufficiency. Most cases are preceded by a prodromal episode of diarrhoea caused by the STEC infection, missing in the so-called atypical HUS cases, due to complement dysfunction [1]. In Belgium it is mandatory to report STEC infections to the regional health inspection authorities. These authorities investigate each notified case separately and decide which measures have to be taken to prevent further spread of the infection and perform outbreak screening and investigation if necessary. The criteria used for STEC case definition are those provided by the European Centre for Disease Control (ECDC). All Belgian clinical laboratories can send stool samples, rectal swabs from HUS patients, faecal cultures on agar and strains suspicious for STEC to the National Reference Centre (NRC) for diagnosis and strain typing free of charge. Referral of specimens to the NRC is voluntarily, but is highly recommended [2].

It can be difficult for the health inspection authorities to determine whether a case of STEC infection has to be excluded from a group (e.g. children's day care centres) or an occupation (e.g. food handlers) and whether it is necessary to screen for asymptomatic carriers. To facilitate the decision making process regarding the management of STEC infections, information about the risk factors for the development of HUS can be helpful. Previous studies identified the presence of the STEC virulence genes *stx2* in general, subtypes *stx2a* and *stx2d* more specifically, and *eae*, as well as young and older ages of the patient as risk determinants for HUS development. Since the large-scale STEC O104:H4 outbreak in 2011, the rare combination of enteroaggregative virulence genes and *stx*2 is also considered as high risk [3–6].

In order to provide insight in the risk determinants for typical HUS development in Belgium, we performed statistical multivariate analyses on a dataset of STEC strains isolated at the Belgian NRC STEC between 2011 and 2016. Based on the results, we propose an adapted

Table 1. Statistical significant results of the univariate logistic regression analyses

Characteristic	Total number (% of total)[a]	Number of HUS (% of total)	*P* value	Odds ratio (95% CI)[b]	PPV (%)[c]	NPV (%)[d]
Higher risk for HUS development						
Patient age ⩽5 years	164 (40.1)	37 (22.6)	0.03	9.6 (1.3–72.7)	22.6	83.7
Patient age 6–12 years	81 (19.8)	21 (25.9)	0.02	11.5 (1.5–89.8)	25.9	82.9
Patient age ⩾75 years	29 (7.1)	8 (27.6)	0.02	12.6 (1.5–107.9)	27.6	81.8
Serogroup O157	205 (49.9)	50 (24.4)	0.00	3.2 (1.6–6.5)	24.4	86.4
Serogroup O145	17 (4.1)	8 (47.1)	0.00	8.9 (2.9–27.7)	47.1	82.2
stx2 gene (with or without *stx2*)	315 (76.6)	76 (24.1)	0.00	14.9 (3.6–62.1)	24.1	97.9
stx2 gene alone (not *stx1*)	189 (46.0)	63 (33.3)	0.00	4.3 (2.3–8.3)	33.3	93.2
stx2a subtype	173 (42.1)	67 (38.7)	0.00	29.7 (7.1–124.6)	38.7	95.4
eae gene	326 (79.3)	72 (22.1)	0.00	3.7 (1.6–8.9)	22.1	92.9
Lower risk for HUS development						
Sorbitol fermentation	182 (44.3)	25 (13.7)	0.02	0.5 (0.3–0.9)	13.7	76.9
β glucuronidase production	178 (44.2)	24 (13.5)	0.02	0.5 (0.3–0.9)	13.5	76.9
stx1 gene (with or without *stx2*)	222 (54.0)	15 (6.8)	0.00	0.1 (0.1–0.3)	6.8	66.7
stx1 gene alone (not *stx2*)	96 (23.4)	2 (2.1)	0.03	0.2 (0.0–0.08)	2.1	75.9
stx1a subtype	179 (43.6)	14 (7.8)	0.00	0.2 (0.1–0.3)	7.8	72.4
stx1c subtype	43 (10.5)	1 (2.3)	0.00	0.0 (0.0–0.4)	2.3	79.1

[a]The total number of patients with known age was 409; the total number of strains was 411 for all characteristics except for *β* glucuronidase production which was known for only 403 strains.
[b]CI, confidence interval.
[c]PPV, positive predictive value.
[d]NPV, negative predictive value.

STEC typing algorithm and risk classification of STEC strains to provide our local public health authorities with the most useful information.

Materials and methods

Data collection

Laboratories sending samples to the NRC STEC for diagnosis and strain typing are asked to fill out a request form. This form contains questions about the sample (type of sample, sample date) and the patient (age, sex, clinical manifestations, date of onset, recent travel, suspected vehicle, cluster or sporadic case). At the NRC, attempts are made to isolate a STEC strain from each *stx*-positive sample by screening up to 20 single colonies for the presence of *stx* genes. Typing of the *stx*-positive strains is performed in different phases. Biochemical characterisation, motility testing, detection of the top six O-serogroups (O26, O103, O111, O121, O145, O157); antibiotic resistance testing; and PCR for *stx1*, *stx2*, *eae*, *hlyA*, *aaiC* and *aggR* virulence genes are performed immediately as described previously [7, 8]. In order to quickly identify outbreaks of STEC O157, the most common O-serogroup associated with outbreaks in Belgium, IS629-typing of STEC O157 is also performed as soon as possible [9]. Additional serotyping using sequence-based typing of the *gnd*-gene locus and *stx*-subtyping is performed in batch every 3 months [10, 11]. Suspicion of outbreaks by other serotypes is confirmed by pulsed field gel electrophoresis or whole genome sequencing analysis. The typing results of each strain, as well as the according sample and patient information, are collected in an anonymised database. Only one strain per patient per infection episode is stored.

Strains selection

Only STEC from Belgian patients with known HUS status (HUS or non-HUS) isolated between 2011 and 2016 were included. In case of known clusters of infection or outbreaks, only one strain per outbreak, derived from the patient with the worst outcome, was selected. Using these criteria, 411 strains were selected for statistical analysis.

Statistical analyses

Statistical analyses were performed using the IBM SPSS Statistics software 24 and Microsoft Excel. Univariate logistic regression analyses were performed to assess the risk for HUS development of different patient and STEC strain characteristics. Variables with a P value < 0.05 were considered statistically significant. Positive predictive values (PPV) and negative predictive values (NPV) were computed for statistical significant variables in relation to the presence of HUS (Supplementary Tables S1–S3). Three different multivariate logistic regression models (A, B, C) were used on variables selected on the basis of their relevance and significance in univariate analyses. In model A, a distinction was made between *stx1* (with or without *stx2*) and *stx2* (with or without *stx1*) positives. In model B, *stx* was categorised in *stx1* (not *stx2*) positives, *stx1* + *stx2*

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positives and *stx2* (not *stx1*) positives. In model C, significant *stx1* and *stx2* subtypes were taken into account.

Results

Univariate analyses

Univariate logistic regression analyses showed the following characteristics to be statistically significantly correlated to the development of HUS: patient age categories ⩽5, 6–12 and ⩾75; STEC serotypes O157 and O145; and STEC genes *stx2*, subtype *stx2a* more specifically and *eae*. Presence of the *stx2a* gene had the best PPV and NPV, 38.7% and 95.4%, respectively. The following variables were significantly correlated to a reduced risk for HUS development: STEC fermentation of sorbitol and production of *β* glucuronidase; and presence of the *stx1* gene (Table 1, Supplementary Tables S1–S3).

Multivariate analyses

Analyses of the significant and relevant variables in multivariate logistic regression models excluded the STEC O serogroups O26, O145 and O157; sorbitol fermentation; and *β* glucuronidase production as significant risk predictors. Age categories ⩽5, 6–12 and ⩾75, and the *stx2* gene remained significant risk determinants in all three models (Table 2, Supplementary Tables S4–S6). Presence of the *eae* gene was significantly correlated to a higher risk in two out of the three models. Detection of the *stx2a* gene had the highest risk for HUS development (OR 29.6, 95% CI 7.0–125.1) (Table 2, Supplementary Table S6). Presence of the *stx1* gene, regardless of the subtype, had a lower risk for HUS development. Presence of the *stx1* gene without *stx2* appeared to encompass a lower risk than the combined presence of *stx1* and *stx2*, while the presence of *stx2* had a higher risk than both genes (Table 2, Supplementary Tables S3 and S5).

Table 2. Statistically significant results of the multivariate logistic regression models A (*stx1* (with or without *stx2*) *vs.* *stx2* (with or without *stx1*) positives), B (*stx1* (not *stx2*) positives, *stx1* + *stx2* positives, *vs.* *stx2* (not *stx1*) positives) and C (significant *stx1* and *stx2* subtypes)

Statistically significant variable	*P* value	Odds ratio (95% CI)[a]
Model A: *stx1* (with or without *stx2*) *vs.* *stx2* (with or without *stx1*) positives		
Higher risk for HUS development		
Patient age ⩽5 years	0.05	7.9 (1.0–62.2)
Patient age 6–12 years	0.02	13.1 (1.6–107.0)
Patient age ⩾75 years	0.04	10.7 (1.2–95.5)
stx2 gene (with or without *stx1*)	0.03	5.4 (1.2–25.1)
eae gene	0.04	2.8 (1.0–7.7)
Lower risk for HUS development		
stx1 gene (with or without *stx2*)	0.00	0.3 (0.1–0.5)
Model B: *stx1* (not *stx2*) positives, *stx1* + *stx2* positives, *vs.* *stx2* (not *stx1*) positives		
Higher risk for HUS development		
Patient age ⩽5 years	0.05	7.9 (1.0–62.2)
Patient age 6–12 years	0.02	13.1 (1.6–107.0)
Patient age ⩾75 years	0.04	10.7 (1.2–95.5)
stx2 alone (not *stx1*)	0.00	3.9 (1.9–7.7)
eae gene	0.04	2.8 (1.0–7.7)
Lower risk for HUS development		
stx1 alone (not *stx2*)	0.03	0.2 (0.0–0.8)
Model C: significant stx1 and stx2 subtypes		
Higher risk for HUS development		
Patient age ⩽5 years	0.03	9.6 (1.2–76.2)
Patient age 6–12 years	0.02	13.5 (1.6–111.5)
Patient age ⩾75 years	0.03	12.5 (1.3–115.6)
stx2a	0.00	29.6 (7.0–125.1)

[a]CI, confidence interval.

Discussion

Univariate logistic regression analyses showed patient ages ⩽12 and ⩾75 years, STEC serotypes O157 and O145, the Shiga toxin *stx2* gene in general and the *stx2a* subtype more specifically, and the virulence gene *eae* to encompass a higher risk for HUS development. However, only patient age and the *stx2* gene remained significant risk determinants in all three multivariate models. The presence of the *eae* gene was significant in the multivariate models A and B, but not in model C. This indicated that the presence of *stx2a* was the most important risk marker of the included strain characteristics. Unfortunately, we were not able to make statistically significant risk predictions about other *stx2* subtypes based on our dataset.

Sorbitol fermentation and *β* glucuronidase production were protective markers for HUS development in the univariate analyses. This is in correlation with the fact that many non-O157 strains are sorbitol fermenters and *β* glucuronidase producers (178/204 and 175/198, respectively), while the majority of STEC O157 do not (203/205). After multivariate logistic regression analyses however only the *stx1* gene stayed statistically significantly correlated to a lower risk for the development of HUS. Because STEC strains can possess a combination of *stx1* and *stx2* genes, we conducted multivariate logistic regression analysis B. This revealed that strains positive for *stx2* but not for *stx1* had a higher risk for HUS than those possessing a combination of *stx1* and *stx2* genes, indicating *stx1* can tone down the effect of *stx2*.

The results of our study are not new; old and young age, and the genes *stx2a* and *eae* have been found to be statistical significant risk factors for HUS development in other studies before. However, previously published data often included a lower number of cases and were focussed on a specific country or region [12–14]. For this reason, they cannot be extrapolated to other countries or regions without further research. The more data from different countries get published, the more insight will be achieved in the possible risk factors for severe disease development associated with STEC infection. Furthermore, unlike previous studies, we have actually used the results of our study to turn around the STEC virulence typing scheme at the Belgian NRC. Upon the time of this study, all STEC isolated at the Belgian NRC were immediately characterised for the presence of the six most important STEC O serogroups (O26, O103, O111, O121, O145 and O157) and the virulence genes *stx1*, *stx2*, *eae*, *ehxA*, *aaiC* and *aggR*; while *stx* subtyping was only performed every

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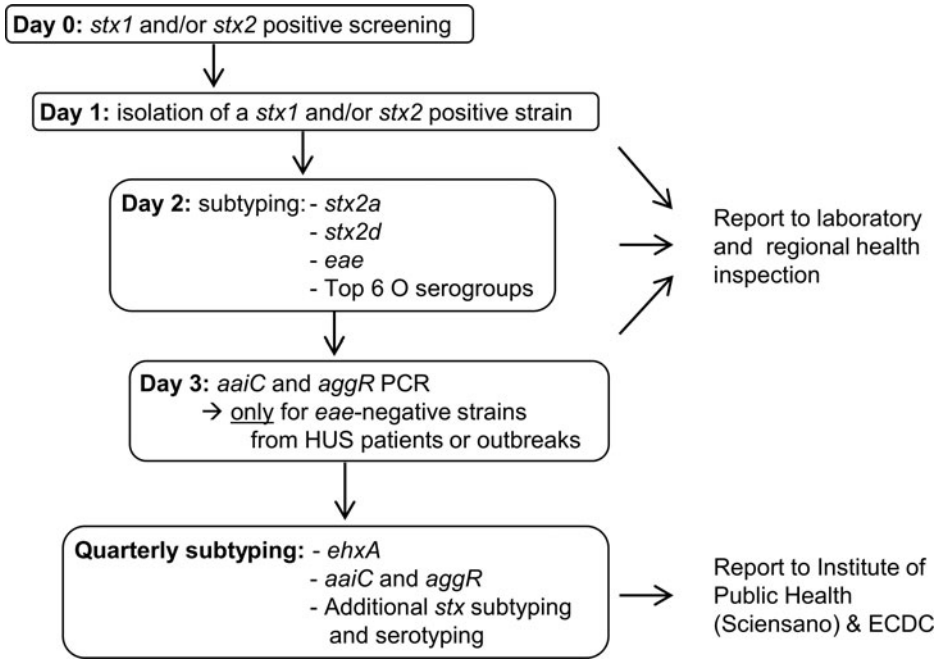

Fig. 1. Proposal of a new STEC virulence typing algorithm at the Belgian NRC STEC.

3 months. Based on the presence of the *eae* and *ehxA* genes, strains were reported as being 'typical EHEC' when both genes were present, while they were reported as 'atypical EHEC' when they lacked one or both genes. Studies showed this classification was not clearly correlated to the outcome of the infection, especially with the risk for HUS development [15]. Because the results of our statistical analysis confirmed that *stx* subtype is the most important indicator of HUS, we have proposed a new typing algorithm that requires an equal amount of labour as our present one. Our analysis showed *stx2a* to be the only *stx* subtype with a statistical significant higher risk for HUS development and this will be detected and reported immediately. Because *stx2d* is a rare subtype in Belgium (only 8/411 strains (Supplementary Table S3)) we were not able to tell something with statistical significance about this subtype. However, *stx2d* is considered as a high risk subtype in other studies, and for this reason, we decided to also include this subtype in our first-line typing [3]. Because the role of *ehxA* in HUS development was not significant in our statistics, this gene will only be detected quarterly on groups of isolates. Detection of the rare enteroaggregative genes *aaiC* and *aggR* will only be performed promptly for *eae*-negative strains from HUS patients or outbreaks (Fig. 1). Although O serogroups do not appear to be important HUS risk factors, we will still report them, as they are informative and easy to obtain by agglutination of colonies. Biochemical identification of the strains, including sorbitol fermentation testing, will still be performed immediately as well. These tests are easy and cheap and allow us to detect sorbitol-fermenting STEC O157, which are rare in Belgium, but have been the cause of severe disease and outbreaks in Germany and other European countries [4, 16, 17]. By implementing this new virulence typing algorithm, we will be able to provide the physicians and health inspection authorities with a risk classification for the development of HUS which is primarily based on the *stx* (sub)type (Table 3). We believe this classification will contribute to optimal infection control management, as for instance during the decision making regarding children's day care centres exclusion policies. Nevertheless, we are aware of the fact that only a limited number of patient and strain characteristics were studied here and that a classification based on the *stx* subtype only also has its limitations. A recent study in Norway identified the non-LEE effector protein *nleH1-2* as an additional potential independent risk factor for HUS development [18]. In the future, more extended virulence profiling could be done by using whole genome sequencing of STEC. We also acknowledge the fact that there is a possible bias regarding the cases that could be included in this study. As the NRC, we are dependent on the external laboratories and practitioners who refer their samples to provide us with the clinical information of the patient. Unfortunately, we do not always receive information about the disease of a patient diagnosed with STEC, and these strains could not be included in this study. Finally, it should be noted that every STEC regardless of its profile has the potential to cause severe disease as several host factors including the patient's genetic background already have shown to play a role in the susceptibility to and severity of the disease [19].

Table 3. Proposal of a new risk classification of STEC strains for the development of HUS

Risk for HUS development	*stx* (sub)types
High	*stx2a* or *stx2d*-positive strains
Medium	Other *stx2*-positive strains (with or without *stx1*)
Low	*stx1* only positive strains

The presence of *eae* or *aaiC/aggR* is also associated with a higher risk for HUS development.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0950268818002546.

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Conflict of interest. None.

Ethical standards. Epidemiological data were collected anonymously in the frame of Decision No 2119/98/EC of the European Parliament and of the Council, concerning the epidemiological surveillance and control of communicable diseases in the Community, as completed by Decision No 1082/2013/EU.

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