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Enterohemorrhagic *Escherichia coli* (EHEC) in water from karst springs: detection with real-time PCR and isolation of strains

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Abstract Within 2 months, two water sources in a karst area in Switzerland were sampled 9 times each, and analyzed by real-time PCR for 6 EHEC O-types, Shiga-like-toxin (*stx*1 and *stx*2) and intimin (*eae*) genes. With the exception of O111, 5 O-types were recorded regularly and at high frequencies (O26: 33.3 %; O157: 33.3 %; O104: 66.6 %; O103: 72.2 %; O145: 94.4 %). Genes for Shiga-like-toxins and intimin were almost omnipresent (*stx*1: 77.8 %; *stx*2: 83.3 %; *eae*: 77.8 %). Strain isolation was undertaken for O-groups 26, 103, 104, 145 and 157. Sample selection for strain isolation was based on Cq-values for the O-groups and *stx*1, *stx*2 and *eae*. From selected samples, frozen enrichment cultures were cultivated on EHLY-agar and 50 typical colonies screened for the O-type and genes encoding for *stx*1, *stx*2 and *eae*. With this approach, only one virulent EHEC-strain could be isolated (*Escherichia coli* O103, *stx*1 +; *stx*2 −; *eae* +). We carried out one extensive testing with 800 colonies of O-group O145, and no virulent strain was isolated. Our findings showed that PCR-results are not sufficient to formulate epidemiological conclusions and that the isolation of strains is necessary. However, as the detection procedure of EHEC in foods is cumbersome and expensive, the appropriateness of such an approach in official food control is a matter of debate.

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1 Introduction

In 1992, Enterohemorrhagic *Escherichia coli* (EHEC) was described for first time when cases of bloody diarrhea were observed after the consumption of undercooked hamburgers in the U.S. The causative agent in this first EHEC-outbreak was the strain O157:H7 EDL933 (O'Brien et al. 1993). In Switzerland, EHEC infections have been registered since 1999 with the annual number of cases being low (between 30 and 70). Since the initiation of mandatory registration, noteworthy EHEC outbreaks have not been reported; only a small outbreak in 2005 was observed owing to contamination of drinking water with fecal material of bovine origin after a heavy rainfall event (Bundesamt für Gesundheit 2008). However, in 2015 a significant increase of cases was reported. The reason behind this increase is not fully elucidated, but there is evidence that suggest that the introduction of multiplex PCR assays for gastrointestinal agents in diagnostic laboratories may be responsible (Bundesamt für Gesundheit 2015).

For the transmission of EHEC to humans, meat and meat-products are the main risk factors. However, there are also outbreaks known due to contaminated foods of plant origin such as sprouts. A spectacular outbreak with thousands of infected persons occurred 1996 in Japan with epidemiological evidence showing that radish sprouts were the most probable origin of infection (Michino et al. 1999). In 2011, again

sprouts were responsible in an outbreak in Germany. After consumption of contaminated fenugreek, 3842 infections due to an EHEC-strain of serotype O104:H4 were reported (2897 cases of gastroenteritis; 855 cases of hemolytic uremic syndrome, HUS with 53 fatalities) (Robert Koch Institute 2011). Consequently, the responsible authorities of the EU decreed a microbiological criterion for EHEC in sprouts, which Switzerland has adopted in the Ordinance on Hygiene (Eidgenössisches Departement des Innern 2014). This criterion states that EHEC serotypes O26, O103, O104, O111, O145 and O157 should not be detectable in 25 grams of sample after enrichment. According to the EU, testing must be done using the reference method ISO/TS 13136:2011 and a method of the European reference laboratory for *E. coli*. PCR is used to screen for the target serotypes and virulence factors. If positive, EHEC-strains must also be isolated. Isolation is needed to confirm PCR result and also to have strains for epidemiological typing. In the present study, raw water samples from microbiologically vulnerable sources were tested for the presence of EHEC using the recommended EU method. Difficulties and challenges in connection with the screening and with the isolation of strains are also presented and discussed here.

2 Methods and materials

2.1 Collection and processing of water samples

Two karst sources with reported elevated bacterial counts in the St. Imier valley (Canton Bern, Switzerland) were sampled 9 times each between September and October 2014. Samples were collected in sterile 1 l glass Schott bottles, placed in a cool-box and transported to the FSVO laboratories within 2 h of collection. Depending on filterability of the sample, either 500 ml (source A) or 1000 ml (source B) was filtered through a 0.45 μm pore size membrane (Millipore, HAWG047S6).

2.2 Enrichment of EHEC

The filters were transferred into 15 ml Falcon tubes, 10 ml of buffered peptone water, BPW (Oxoid, CM0509) was added and the tubes incubated at 100 rpm for 37 °C for enrichment. Subsequently, 1 ml was centrifuged for 10 min at 5000×*g* at room temperature. The supernatant was discarded and the pellet re-suspended in 1 ml peptone-saline-solution, PSP (8.5 g NaCl and 1 g peptone/1 l H_2O). After a further centrifugation step as previously described, the pellets were frozen at −20 °C until used for DNA-isolation. Furthermore, 1 ml of the enrichment broth was centrifuged for 10 min at 5000×*g*, the pellet re-suspended in 100 μl PSP and transferred to microbank cryovials (Pro-Lab Diagnostics, REF Pl. 170/R). The prepared vials were stored at −70 °C until used for the isolation of viable EHEC-strains.

2.3 Isolation of DNA

DNA-isolation was performed using 1 ml of the enrichment broth (see Sect. 2.2). The cells were processed on a QIAcube extraction robot (Qiagen, 9001293) using the DNeasy blood and tissue kit (Qiagen, 69504). The eluate of 150 μl was kept at −20 °C until qPCR was performed without further dilution.

2.4 Real-time PCR

Real-time PCR was performed following both ISO protocol (International Organization for Standardization 2011) and a European reference method (European Union Reference Laboratory for *E. coli* 2013) with the following modification: Probe wzx (O103) is listed in the ISO-method without any Tm-modifier but according to the original literature (Perelle et al. 2005), labelled with a minor groove binder, was used with the alternative Tm-modifiers propynyldeoxy (pd) C and pd U (Microsynth AG, Balgach, Switzerland). The wzx (O103) probe sequence was as follows: (FAM)-pdCApdU AGpdC pdCpdUG pdUpdUG TpdUpdU pdUAT-(BHQ1). Tests were carried out in duplicates and results with Cq-values of >30 considered to be negative.

2.5 Isolation of EHEC-strains

Based on the Cq-values generated in the screening of water samples we decided whether attempted isolation of EHEC-strains was advisable (i.e. where Cq-values were <30, isolation was conducted). For each O-group, the test which revealed the lowest Cq-value (i.e. the highest target copy number) was identified and if the signals for the tested virulence factors (*stx*1, *stx*1 and *eae*) were clearly positive, isolation of EHEC-strains was attempted. For that purpose, one microbank cryovial bead (see Sect. 2.2) was transferred into 3 ml of BPW and incubated for 4 h at 37 °C in an incubation shaker at 100 rpm. Subsequently, 10 plates of enterohemolysine agar, EHLY (Oxoid, PB5105A) were inoculated with 10 μl each of BPW-culture and incubated aerobically at 37 °C overnight. 50 typical

colonies were separated into 5 pools and further analyzed as follows: cell material from a typical colony was taken with a plastic tip, first spotted on a defined area of a TBX plate (Oxoid, CM0945) and then washed out in 50 µl of H_2O. A complete pool with cell material of 10 typical colonies was diluted 1:10 in 0.2× TE buffer and the suspension heated at 95 °C for 5 min. Finally, cell debris were sedimented by centrifugation at 20,000×*g* for 1 min and 70 µl of supernatant taken for PCR analysis. From pools where fully virulent strains were suspected according to the PCR-result, all the isolates belonging to the pool were taken from the TBX plate and individually analyzed by PCR. This screening utilizing 50 colonies was performed for the O-groups 26, 103, 104, 111, 145 and 157. In one additional trial (O-group 145), 800 colonies were screened to increase the probability of a successful isolation of fully virulent EHEC-strains.

3 Results and discussion

3.1 Selection of isolation procedure

ISO/TS 13136:2011 (International Organization for Standardization 2011) defines that in the case of positive PCR-results, 50 colonies with *E. coli* morphology have to be screened in order to isolate EHEC strains, which might be present in a sample. Concerning the screening methodology, the ISO method allows for some variation between laboratories. We decided to use EHLY-agar developed by Beutin et al. (1996). Since typical colonies were not numerous, we trailed 3 methods to increase assay sensitivity:

1. Inoculating 10 EHLY-plates per sample
2. Combining commercial immunobeads with EHLY-agar and
3. Supplementing EHLY plates with vancomycin.

However, the yield of presumptive colonies did not increase using any of the aforementioned methods (data not shown).

3.2 Findings of qPCR-testing

Table 1 shows the finding of PCR-testing. Both karst sources were tested 9 times over a period of approximately 2 months. Although the sample volumes were not exactly the same (Source A: 500 ml and source B: 1000 ml), a qualitative comparison is passable for two reasons. Firstly, the sample volumes differed only by factor 2 and secondly, PCR testing was done after an enrichment step in a liquid medium overnight. A notable finding was the absence of O-group 111 in both sources. A malfunction of the applied test could be excluded since the control reactions were appropriate. Also, the O111 detection assay worked perfectly with samples from a waste water treatment plant (ARA Neubrück, Bern, approx. 380.000 population equivalent). The two investigated karst sources are located in rural areas with a considerable bovine population. A recent study in Switzerland investigating the occurrence of Shiga toxin-producing *E. coli* of the O-groups 26, 103, 111, 145 and 157 in slaughtered cattle showed that *E. coli* O111 occurred infrequently at only 0.8 % (Hofer et al. 2012). The five other O-groups were detected in both sources at approximately the same frequencies. The overall frequencies for the O-groups were as follows: 026: 6/18 (33.3 %); 0157: 6/18 (33.3 %); O104: 12/18 (66.6 %); O103: 13/18 (72.2 %) and O145: 17/18 (94.4 %). As shown in Table 1, the genes for *stx*1, *stx*2 and *eae* occurred regularly in both sources. The overall frequency for *stx*1 was 14/18 (77.8 %), for *stx*2 15/18 (83.3 %) and for *eae* 14/18 (77.8 %).

3.3 Isolation of EHEC-strains

For isolation purposes, those samples with the lowest Cq-values (i.e. the highest concentrations) for the O-groups were selected under the precondition that also *stx*1, *stx*2 and *eae* signals were detected. For O26, it was sample 3 of source A (Cq 21.4), for O103 sample 4 of source A (Cq 20.8), for O104 sample 4 of source B (Cq 21.6), for O145 sample 1 of source A (Cq 17.3) and for O157 sample 3 of source B (Cq 19.6). By screening 50 colonies as described in Sect. 2.5, we isolated on only one occasion virulent EHEC-strain O103 (*stx*1 +/ *stx*2 −/ *eae* +). This finding clearly demonstrated that qPCR-results alone have a very limited value regarding presence of viable EHEC-strains. The two karst sources studied here represent particularly difficult and complex hydrological situations. They collect water from rather large areas, with many potential contamination sources, which are available for quick transportation owing to the high hydraulic conductivity of karst systems. In another study, it was shown that both source A and B are affected by human and ruminant (bovine) fecal contamination (Diston et al. 2016 accepted for publication). Therefore, we intensively screened sample 1 from source A (Cq-value of 17.3, O group 145) with an intention of isolating viable EHEC-strains. Despite screening 800 colonies, no virulent EHEC-strain could be isolated.

Table 1 Real-time PCR screening of samples from two karst water sources for presence of enterohemorrhagic *E. coli* (EHEC) of serotypes O26, O103, O104, O111, O145 and O157

Source	Week	qPCR-Findings (Cq-values)[a]								
		*stx*1	*stx*2	*eae*	O26	O103	O104	O111	O145	0157
A	1	24.9	22.4	23.8	nd	23.5	27.6	nd	17.3	29.7
A	2	26.0	23.4	23.0	27.1	24.7	26.7	nd	20.3	nd
A	3	22.9	24.0	20.5	21.4	nd	27.8	nd	20.7	28.0
A	4	21.0	21.8	19.8	nd	20.8	nd	nd	21.5	nd
A	5	25.8	nd	21.5	23.8	20.9	nd	nd	19.9	nd
A	6	21.7	20.0	23.3	nd	nd	23.2	nd	19.9	nd
A	7	25.1	20.7	24.2	nd	22.0	26.8	nd	21.1	nd
A	8	21.2	18.5	nd	nd	nd	nd	nd	21.3	24.3
A	9	nd	22.2	nd	nd	nd	nd	nd	20.9	nd
B	1	24.0	25.1	20.2	28.1	22.0	23.6	nd	22.2	nd
B	2	25.2	23.6	20.2	25.7	22.5	19.4	nd	21.2	nd
B	3	25.5	23.6	20.4	25.9	19.4	23.1	nd	22.6	19.6
B	4	28.6	24.6	20.3	21.9	24.2	21.6	nd	23.0	25.2
B	5	26.7	26.7	20.0	nd	20.9	23.7	nd	22.4	nd
B	6	nd	nd	20.4	nd	20.2	24.4	nd	22.2	23.1
B	7	25.5	23.4	23.7	nd	22.4	25.3	nd	22.6	nd
B	8	nd	nd	nd	nd	nd	nd	nd	nd	nd
B	9	nd	27.4	nd	nd	25.8	nd	nd	21.0	nd

nd not detectable (Cq-value >30)

[a] Mean value of double measurement

4 Conclusions

In the EU, official microbiological criteria (Food safety criteria) for certain EHEC serotypes in sprouts has been decreed (Eidgenössisches Departement des Innern 2014). National authorities for food control also test for EHEC in other foods than sprouts as indicated by RASFF alerts (European Commission 2014). However, the application of a microbiological criterion for EHEC can be problematic. As shown in our study, one difficulty is that the analytics are based on PCR, which limits the epidemiological explanatory power considerably. In the present study, PCR data suggested at a first glance that the examined sources are regularly contaminated with diverse serotypes of EHEC. In fact, the detected O-groups and the genes for *stx*1, *stx*2 and *eae* probably derived from a broad variety of *E. coli* strains harboring single, but not all, traits necessary for a virulent EHEC. Even with promising screening data (low Cq-values for O-group), the screening of 50 colonies from EHLY-agar, and extensive testing of 800 colonies, only once resulted in a successful isolation of a virulent EHEC-strain. This finding reveals that PCR results alone allow only limited conclusions to be drawn and that the isolation of viable EHEC strains is essential. Altogether, the examination of foods for EHEC is a complicated and expensive undertaking. However, official microbiological criteria should be testable whenever possible with simple, unambiguous and cost-efficient standard methods. The latter is also important because food-producers have to carry out microbiological tests in the context of self-responsibility and self-control in order to prove that legal requirements are fulfilled. Therefore, the question is whether complicated and costly analytics such as EHEC-detection in foods should not be limited to epidemiological questions (for example outbreak investigations) and whether safety of EHEC risk products could not be pursued with simple criteria for apathogenic *E. coli* serving as hygiene indicators?

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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