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# Improved Molecular Diagnosis and Culture of the Emerging Heteropathotype Enterohemorrhagic *Escherichia coli* O80:H2 Using Its Non-Melibiose-Fermenting and Antibiotic-Resistance Properties

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**ABSTRACT** Enterohemorrhagic *Escherichia coli* (EHEC) O80:H2, belonging to sequence type ST301, is among the main causes of hemolytic and uremic syndrome in Europe, a major concern in young children. Aside from the usual intimin and Shiga toxin virulence factors (VFs), this emerging serotype possesses a mosaic plasmid combining extra-intestinal VF- and antibiotic resistance-encoding genes. This hybrid pathotype can be involved in invasive infections, a rare occurrence in EHEC infections. Here, we aimed to optimize its detection, improve its clinical diagnosis, and identify its currently unknown reservoir. O80:H2 EHEC strains isolated in France between 2010 and 2018 were phenotypically and genetically analyzed and compared with non-O80 strains. The specificity and sensitivity of a PCR test and a culture medium designed, based on the molecular and phenotypic signatures of O80:H2 EHEC, were assessed on a collection of strains and stool samples. O80:H2 biotype analysis showed that none of the strains ( $n = 137$ ) fermented melibiose versus 5% of non-O80 EHEC ( $n = 19/352$ ). This loss of metabolic function is due to deletion of the entire melibiose operon associated with the insertion of a 70-pb sequence (70mel), a genetic scar shared by all ST301 strains. This metabolic hallmark was used to develop a real-time PCR test (100% sensitivity, 98.3% specificity) and a melibiose-based culture medium including antibiotics, characterized by 85% specificity and sensitivity for clinical specimens. These new tools may facilitate the diagnosis of this atypical clone, help the food industry to identify the reservoir and improve our epidemiological knowledge of this threatening and emerging clone.

**KEYWORDS** culture medium, heteropathotype, PCR, Shiga toxin, diagnostic tools, enterohemorrhagic *Escherichia coli*, patient management

Enterohemorrhagic *Escherichia coli* (EHEC), also known as Shiga toxin-producing *E. coli* (STEC), are major foodborne pathogens responsible for outbreaks and sporadic cases of gastrointestinal diseases, ranging from simple diarrhea to hemolytic and uremic syndrome (HUS). HUS, defined by a triad including hemolytic anemia, thrombocytopenia, and acute renal failure, is the most severe complication in young children and the primary cause of acute renal failure in children under 3 years of age in France (1).

The historical O157:H7 serotype has long been the most frequent serotype to be

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involved in HUS worldwide. However, new molecular diagnosis methods and epidemiological evolution have revealed a change in the distribution of serogroups of EHEC associated with HUS in Europe (2), as well as in the United States (3). In France, EHEC O80:H2 emerged in 2010 and currently represents, along with O26 and O157 EHEC, the three most frequent serogroups found in HUS in children under 15 years of age (1). Initially described in France (4), this clone was then reported in other European countries, such as Belgium, the Netherlands, Switzerland, Spain, and Italy (5–9), and was the third most common EHEC serotype isolated among HUS cases in Europe in 2019, responsible for 9% of European HUS (2).

Belonging to sequence type (ST)-301 within clonal complex (CC)-165, *E. coli* O80:H2 is a particular EHEC, as, aside from carrying classical intestinal virulence factors (VFs) (intimin, *eae-ξ*, and Shiga toxin, *stx*), it also carries an additional plasmid (pR444\_A) that combines extra-intestinal VFs and an antibiotic resistance cassette (10). These attributes are shared by all O80:H2 strains, leading to antibiotic multi-resistance, especially to penicillin. The combination of intestinal and extra-intestinal virulence, here designated as a heteropathotype, leads to HUS associated with invasive infections, such as bacteremia (4, 5), a rare occurrence in EHEC infections. In contrast to other major serogroups, no *E. coli* O80:H2 outbreaks have occurred thus far, and potential sources of contamination for sporadic HUS due to O80:H2 EHEC are still unclear (11).

Contamination of food products (at the production and distribution levels) is monitored by national monitoring programs supervised by the French General Directorate of Food (DGAL). Such monitoring is carried out by official laboratories and the French National Reference Laboratory of *E. coli* by screening for the presence of the major STEC serogroups, including the O80:H2 serotype since 2017. No O80 STEC was detected among 2,275 bovine products (raw meat or raw-milk cheese) tested from 2017 to 2019 (<https://agriculture.gouv.fr/plans-de-surveillance-et-de-contrôle>).

Although O80:H2 strains have been isolated in symptomatic or asymptomatic cattle in Belgium and France (6, 10, 12, 13), further studies are still required to elucidate the reservoir of these strains. Accurate and sensitive culture media are warranted to facilitate the identification of its reservoir, as well as its isolation from stool samples of patients with HUS.

Here, we aimed to enhance our knowledge of O80:H2 EHEC by analyzing its phenotypic characteristics and developing specific diagnostic tools to improve the detection and prevention of infections caused by this emerging serotype.

## MATERIALS AND METHODS

**Bacterial strains and DNA samples.** The bacterial collection of O80:H2 strains included human EHEC strains isolated between January 2010 and September 2018 ( $n = 137$ ) by the French *E. coli* National Reference Center (NRC) and 10 strains from various sources (humans:  $n = 4$ ; animals:  $n = 1$  pork,  $n = 4$  cattle), the environment ( $n = 1$  water) and countries, as previously described (10). The biochemical traits of this serotype were compared with those of other EHEC serogroups by including all non-O80 EHEC isolated by the NRC between January 2017 and September 2018 ( $n = 352$ ), resulting in 92 O157, 95 O26, 6 O111, 14 O91, 14 O103, 20 O145, 10 O121, 11 O55, and 90 non-major serogroup strains.

The genomic sequences of non-French O80:H2 strains ( $n = 73$ ), as well as all non-O80 ST301 strains ( $n = 54$ ) available on the Enterobase website (<http://enterobase.warwick.ac.uk/>) on August 5, 2020, were also included. A total of 301 sequences belonging to ST301 were available and could be used at this date on Enterobase. RDEx444 (accession number [SAMN08915508](https://enterobase.warwick.ac.uk/entry/SAMN08915508)), a strain representative of an O80:H2 clone (10), was used for *in silico* analysis. *Escherichia coli* K12 (accession number [SAMN13412807](https://enterobase.warwick.ac.uk/entry/SAMN13412807)) and *Escherichia albertii* TW07627 (accession number [SAMN02436276](https://enterobase.warwick.ac.uk/entry/SAMN02436276)) were also used for genetic comparisons.

The analysis of the medium specificity was completed by testing wild-type melibiose-negative species other than *E. coli* (*Pseudomonas aeruginosa*,  $n = 2$ ; *Stenotrophomonas maltophilia*,  $n = 2$ ; *Proteus mirabilis*,  $n = 2$ ; *Morganella morganii*,  $n = 2$ ; and *Citrobacter koseri*,  $n = 2$ ).

**Clinical samples.** Several clinical populations were used to validate the developed diagnostic tools. First, 60 fecal samples were prospectively collected from diarrheic children hospitalized in the Robert Debré Hospital, Paris, France. Second, rectal swabs ( $n = 959$ ), obtained from children aged 6 to 24 months, either during routine check-ups with normal findings or when they presented with acute otitis media, as previously described (14), were also included. The study was approved by the Saint-Germain-en-Laye Hospital Ethics Committee (10/10/2010-CPP06063). Third, stool samples obtained from the NRC were tested according to standard EHEC diagnostic procedures, including peptone water enrichment. O80 EHEC-positive stool samples ( $n = 20$ ), non-O80 EHEC-positive stool samples ( $n = 20$ ) and EHEC-negative stool samples ( $n = 20$ ) were therefore prospectively tested to complete the

evaluation of the sensitivity and specificity of the PCR test and culture medium. To check for the presence of *E. coli*, all stool samples were also plated on non-selective media.

**Biotype analysis.** Biochemical analysis was performed using the API 20E bacterial identification system (bioMérieux, Marcy l'Étoile, France), including a rapid test for melibiose fermentation. Strip analysis was made after an incubation of 24 h at 37°C.

**Genetic analysis of the melibiose operon.** *In silico* analysis of the melibiose operon and its flanking regions was performed using the MicroScope web interface (<https://mage.genoscope.cns.fr/microscope/home/index.php>) and the genomes of RDEx444, *E. coli* K12 and *E. albertii* TW07627. Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was also used for genetic comparisons.

**Real-time PCR 70mel.** Bacterial DNA was obtained using a thermal procedure (boiling) for strains or by extraction with a DNA tissue kit on an EZ1 XL extractor (Qiagen; Germantown, Maryland, USA) for clinical samples. Real-time PCR was performed using the SYBR green I Master kit on a LightCycler 480 system (Roche Life Science, Bale, Switzerland). Primers ( $\Delta$ MEL-F: 5'-AGC GCC AGA AGT GTT CAA TCT-3',  $\Delta$ MEL-R: 5'-TCA GGC TGT AAA GCG CAT GA-3') were designed from the RDEx444 sequence to specifically amplify the 70mel sequence (amplicon of 209 bp). The primers were obtained from Eurogentec (Liège, Belgium). The PCR mixture was adjusted to 50  $\mu$ l with 25  $\mu$ l SYBR green I Master Mix, 1  $\mu$ l of each primer (1 mM), 18  $\mu$ l sterile water, and 5  $\mu$ l DNA extract. Amplification was carried out with an initial pre-incubation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, and then a final elongation step at 72°C for 7 min.

We excluded the presence of PCR inhibitors by performing amplification of *uidA* ( $\beta$ -d-glucuronidase), a conserved *E. coli* marker, for the 60 diarrheic samples and repeating the PCR test after adding O80 DNA extracts to the 959 fecal swabs.

**Melibiose MacConkey (M-MAC) culture medium.** D-(+)-melibiose (Sigma-Aldrich, Merck, St. Louis, Missouri, USA) and piperacillin (Panpharma, Luitré, France) were added to a MacConkey agar base powder without lactose (BD-Difco), to develop a specific culture medium.

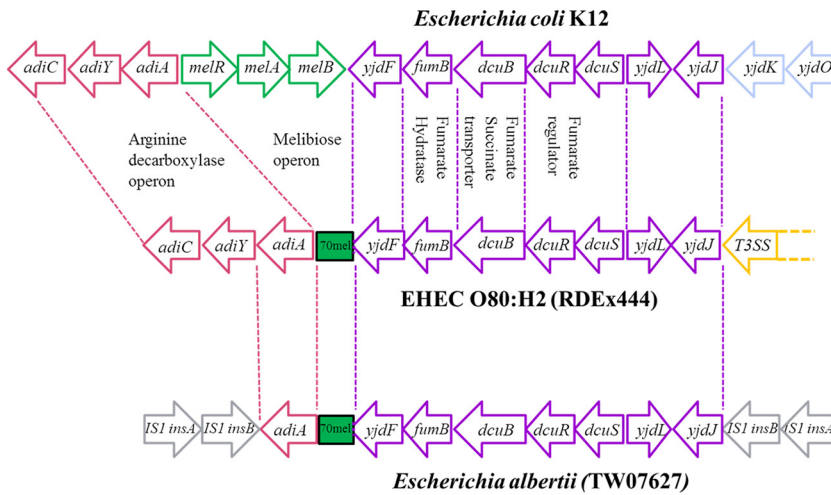
## RESULTS

**Biochemical analysis.** We first assessed the biochemical characteristics of *E. coli* O80:H2 by retrospectively analyzing the API 20E strip results of all O80:H2 EHEC strains isolated between January 2010 and September 2018 ( $n = 137$ ) at the NRC. Strip analysis showed none of these human strains to ferment melibiose. This metabolic trait was shared with non-human O80:H2 strains originating from various sources and countries ( $n = 10$ ) (10). This particular characteristic is infrequent among *E. coli* species, as 75% of the strains classically ferment melibiose according to the API 20E strip-reading table (bioMérieux, Marcy-l'Étoile, France).

We next assessed whether this atypical non-melibiose fermenting (Mel (–)) characteristic is shared by other EHEC by retrospectively analyzing all non-O80 serogroup strains isolated by the NRC between January 2017 and September 2018 ( $n = 352$ ). Among them, only 5% (19/352) were Mel (–) and were distributed among three serotypes: O55:H9 ( $n = 11$ ), O177:H25 ( $n = 7$ ), and O117:H7 ( $n = 1$ ). Of particular interest, EHEC O55:H9 belongs to ST301 and is closely related to the O80:H2 clone (15). The eight remaining strains (2% of the total isolated EHEC) do not belong to ST301. According to these initial results, we hypothesized that the Mel (–) phenotype may be a suitable marker for ST301.

**Genetic analysis of the melibiose operon.** We analyzed RDEx444 (O80:H2) and *E. coli* K12 sequences on the MaGe website to explore the molecular mechanism behind the non-fermentation of melibiose. This comparison revealed that the complete melibiose operon (*melA*, *melB*, and *melR*) and 3' extremity of the *yjdF* gene were deleted in the RDEx444 strain. This deletion was accompanied by the insertion of a 70-bp sequence (further designated as 70mel) of unknown origin and function (Fig. 1). We examined whether this potential genetic hallmark is shared by all O80:H2 EHEC by extending the *in silico* analysis to all O80:H2 EHEC strains isolated by the French NRC from 2010 to 2018 ( $n = 137$ ), as well as all non-French O80:H2 sequences available on the Enterobase database ( $n = 73$ ). All O80:H2 strains showed loss of the melibiose operon, with incorporation of the specific 70mel sequence, suggesting that this genomic scar is well conserved in this serotype.

Then, we used NCBI BLAST to search for the 70mel sequence among two to five representative strains of major serogroups of EHEC in France (O157, O26, O55, O103, O91, O111, O145, and O121) sequenced between January 2017 and September 2018. All except the O55 strains carried a full melibiose operon without the 70mel sequence,



**FIG 1** Comparison of the genetic organization of the melibiose operon and its flanking regions between *Escherichia coli* K12, a representative O80:H2 EHEC clone (RDEx444), and *Escherichia albertii*. Schematic representation of the molecular mechanism of the non-melibiose fermentation observed with the clone EHEC O80:H2. This metabolic trait is due to deletion of the entire melibiose operon and the insertion of a specific 70-bp sequence of unknown function and origin (70mel). A similar genetic scar is present in *Escherichia albertii*. IS, insertion sequence, T3SS, type three secretion system.

consistent with the melibiose positive (Mel (+)) phenotype reported above. *In silico* analysis of the eight Mel (-) strains of serotype O177:H25 and O117:H7, already described, showed that they do not possess the 70mel sequence and harbor the complete melibiose operon or a unique melB gene deletion. The reason for the Mel (-) phenotype for these eight strains remains unknown.

Finally, we assessed the specificity of the 70mel sequence by performing a general BLAST of the NCBI database and found hits for 139 strains of *E. coli* but no other species, except *E. albertii*. Among these 139 *E. coli* strains, 77% were of the O80:H2 serotype and 94% belonged to ST301, showing that this sequence is mainly found among this ST. The remaining 6% consisted of strains of various unrelated serotypes and STs. Interestingly, *in silico* analysis of *E. albertii* TW07627 revealed that this species probably acquired a 10-kb sequence, including the 70mel sequence, likely by transposition from a precursor shared with ST301 *E. coli* strains, as this sequence in *E. albertii* TW07627 is surrounded by two insertion sequences (IS) (Fig. 1). We also screened all ST301 strains available on the EnteroBase database on August 5, 2020 (n = 301) for the 70mel sequence. All ST301 strains contained the 70mel sequence and none carried the melibiose operon, making this genetic trait a signature of this ST. Since 2010, O80 EHEC and, more rarely O55 EHEC, have been the only ST301-related serogroups isolated in France. Thus, the 70mel sequence could be considered to be a good marker of O80/O55 in EHEC infections and could be used for developing new detection methods.

**Real-time PCR of 70mel.** We developed a specific real-time PCR test based on the 70mel sequence, as it appears to be highly characteristic of ST301 and thus appropriate for the detection of O80/O55 strains.

We first performed the PCR on 18 Mel (-) strains carrying the 70mel sequence (11 O80:H2 and 7 O55:H9) to validate the primer choice. An amplification curve was detected for all strains, proving that the designed primers were able to amplify this sequence. Subsequently, the PCR was tested on 20 EHEC Mel (+) strains of diverse serotypes and the eight phenotypically Mel (-) strains already described (which did not carry the 70mel sequence). No amplification was obtained.

We tested three populations to confirm the specificity and evaluate the sensitivity of our PCR on clinical samples. First, no amplification was obtained for 60 fecal samples obtained from diarrheic children, despite the presence of *E. coli*, confirmed by amplification of the  $\beta$ -glucuronidase gene. Second, fecal swabs of asymptomatic community-

**TABLE 1** Performances of the two diagnosis methods developed

| Clinical sample types (no. of tests)                                   | Real time 70mel PCR |                  |                              |                 | Method performances           |
|--|---------------------|------------------|------------------------------|-----------------|-------------------------------|
|  | PCR results         |                  |                              |                 |                               |
|  | Positive (total)    | Negative (total) | False positives <sup>a</sup> | False negatives |                               |
| O80:H2 EHEC positive stools ( <i>n</i> = 20)                           | 20                  | 0                | 0                            | 0               | Sensitivity : 100% (20/20)    |
| Fecal swabs of asymptomatic community-based children ( <i>n</i> = 959) | 17                  | 942              | 16                           | 0               | Specificity : 98.3% (943/959) |

| Clinical sample types (no. of tests)  | M-MAC                       |                  |                              |                              | Method performances       |
|---|-----------------------------|------------------|------------------------------|------------------------------|---------------------------|
|   | Presence of Mel (–) strains |                  |                              |                              |                           |
|   | Positive (total)            | Negative (total) | False positives <sup>b</sup> | False negatives <sup>c</sup> |                           |
| O80:H2 EHEC positive stools ( <i>n</i> = 20)  | 17                          | 3                | 0                            | 3                            | Sensitivity : 85% (17/20) |
| EHEC other than O80:H2 positive stools ( <i>n</i> = 20) and EHEC negative stools ( <i>n</i> = 20) | 6                           | 34               | 6                            | 0                            | Specificity : 85% (34/40) |

<sup>a</sup>Non-O80:H2/O55:H9-signal corresponding to false positives.

<sup>b</sup>Growth of MEL (–) colonies which not corresponding to O80:H2/O55:H9 EHEC strains but to *Citrobacter freundii* (*n* = 1), *Pseudomonas alcaligenes* (*n* = 1), *Hafnia alvei* (*n* = 1), *Pseudomonas putida* (*n* = 1), *Stenotrophomonas maltophilia* (*n* = 1), and *Escherichia coli* belonging to a serotype other than O80:H2 or O55:H9 (*n* = 1) strains.

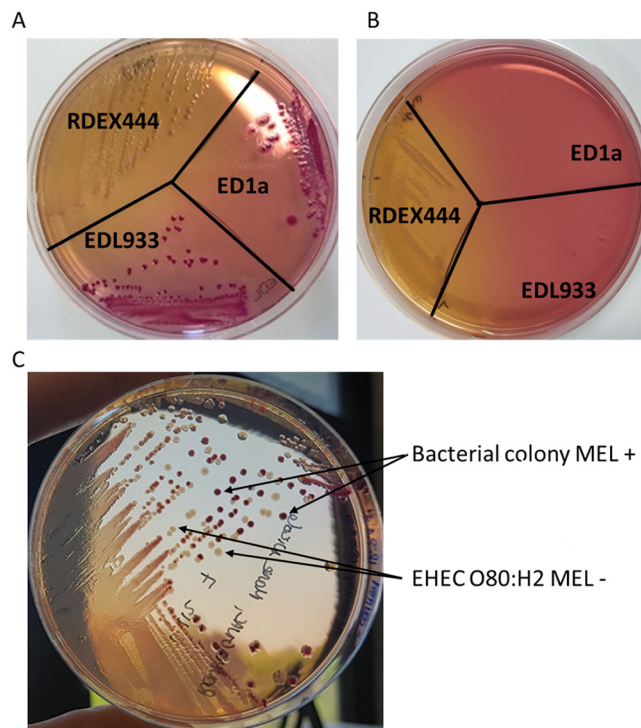
<sup>c</sup>No MEL (–) colony was present while stools were positive for O80:H2 EHEC in the PCR.

based children (*n* = 959) were analyzed. Among them, 17 samples (1.8%) were positive with this PCR. No PCR inhibitor was detected in any of the samples tested. Subsequently, Shiga toxin and O80/O55 serogroup specific PCRs detected one EHEC O80:H2 among the 17 positive samples, which was confirmed after isolation of the strain using our specific M-MAC culture medium. The remaining 16 positive signals were not due to *E. coli* O80 nor O55, and therefore could be due to ST301 *E. coli* belonging to other serogroups or *E. albertii* strains (no further subcultures were performed to identify such strains). Therefore, the specificity of the PCR test to detect O80/O55 EHEC was estimated to be 98.3% (943/959) in clinical samples. Third, 20 O80:H2 EHEC positive stools were prospectively tested, and a positive signal was obtained in all cases, resulting in 100% sensitivity (Table 1).

**Melibiose MacConkey (M-MAC) culture medium.** We developed a culture medium derived from MacConkey medium supplemented with melibiose as the sole source of carbon (M-MAC), at a concentration of 1%, to facilitate the isolation of O80:H2 EHEC strains as they do not ferment melibiose. In this medium, Mel (–) bacteria appear as pale yellow or uncolored colonies, whereas Mel (+) colonies are purple (Fig. 2).

Several Gram-negative bacilli do not ferment melibiose, such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Proteus mirabilis*, *Morganella morganii*, and *Citrobacter koseri*, which could reduce the specificity of the medium. We thus used the penicillin resistance (*bla*<sub>TEM</sub>) observed in almost all O80:H2 *E. coli* conferred by the pR444\_A plasmid (10) to improve M-MAC specificity and added piperacillin at a concentration of 6 mg/liter to the M-MAC media. This is the maximum concentration which inhibits most of other wild-type Mel (–) bacteria without alter the O80:H2 EHEC growth. Indeed, none of the tested *Pseudomonas aeruginosa* (*n* = 2), *Stenotrophomonas maltophilia* (*n* = 2), *Morganella morganii* (*n* = 2), or *Proteus mirabilis* (*n* = 2) strains were able to grow on our M-MAC culture media, although a weak positive culture was observed for *Citrobacter koseri* (*n* = 2), due to its natural penicillinase.

We evaluated the specificity and sensitivity of the medium on clinical samples by plating 60 stool samples (20 containing EHEC O80:H2, 20 containing EHEC other than O80:H2, and 20 negative for EHEC) from the NRC on M-MAC medium. The M-MAC medium allowed the recovery of O80:H2 EHEC-positive stool samples in 17/20 cases, resulting in 85% sensitivity. Twelve of the 20 stool samples were also plated on STEC culture medium (CHROMagar™ STEC, Mast, Bootle, UK) (16). Colony counts obtained on M-MAC media were similar or higher than those obtained on the STEC culture media. The use of STEC medium for the three stool samples with false negative results with M-MAC media allowed the isolation of O80:H2 EHEC in only one case, highlighting



**FIG 2** Melibiose MacConkey (M-MAC) medium derived from MacConkey medium. (A) M-MAC without piperacillin: melibiose negative (Mel  $-$ ) EHEC O80:H2 (RDEX444) appears pale or yellow, whereas melibiose positive (Mel  $+$ ) EHEC of serotype O157:H7 (EDL933) and commensal *E. coli* (ED1a) appear purple. (B) M-MAC with piperacillin at a concentration of 6 mg/liter: sensitive *E. coli* (EDL933 and ED1a) are inhibited, whereas the O80:H2 clone continued to grow on the agar. (C) Isolation of EHEC O80:H2 from a stool sample after enrichment in peptone water. EHEC O80:H2 appears pale and is easily distinguishable from the other colonies.

the inherent difficulty of isolating EHEC. For the 40 remaining stool samples devoid of O80:H2 EHEC, we observed Mel  $-$  colonies for only seven samples, resulting in 82.5% specificity. These Mel  $-$  strains were identified as *Citrobacter freundii* ( $n = 1$ ), *Pseudomonas alcaligenes* ( $n = 1$ ), *Hafnia alvei* ( $n = 1$ ), *Pseudomonas putida* ( $n = 1$ ), *Stenotrophomonas maltophilia* ( $n = 1$ ), and *Escherichia coli* ( $n = 2$ ). The two *E. coli* strains produced either an extended-spectrum beta-lactamase (ESBL) or a TEM type-penicillinase. Unlike the ESBL-producing *E. coli* strain, the other belonged to the O55:H9 serotype and ST301 and should therefore not be considered as a false positive, leading to a corrected sensitivity of 85% for the M-MAC medium (Table 1).

## DISCUSSION

Here, we identified a particular genetic and phenotypic trait of the heteropathotype EHEC O80:H2. The inability of this emerging clone and public health concern to ferment melibiose may improve its detection. Indeed, this metabolic trait is uncommon among *E. coli*, especially EHEC, as 98% of the 352 non-O80/O55 EHEC strains of the NRC collection were able to utilize this carbohydrate source.

Melibiose is a carbohydrate formed by an alpha-(1 $\rightarrow$ 6)-linkage between D-galactose and D-glucose. The inability of *E. coli* O80:H2 to ferment melibiose is associated with the deletion of the entire melibiose operon (*melA*, *melB*, *melR*), which has been replaced by a non-previously described 70-bp sequence. This is the first description of the complete deletion of the melibiose operon in *E. coli*. Only promoter mutations (17) or the inhibition of cosubstrate transport due to *melB* mutations (18) have been previously described in Mel  $-$  *E. coli*.

Although the melibiose-negative phenotype is rare among *E. coli* strains, it is common in several other Gram-negative species. Indeed, the non-fermenting melibiose phenotype

has been mainly described in 75%–90% of *Pseudomonas* spp., more than 96% of the *Shigella sonnei*, *Yersinia enterocolitica*, and *Citrobacter koseri* strains and 100% of the strains belonging to the Proteae family, *Hafnia alvei*, and *Stenotrophomonas maltophilia*. The chosen piperacillin concentration (6 mg/liter) inhibits most of these wild-type strains as 60% of *Pseudomonas aeruginosa* isolates, 80–96% of the Proteae isolates and 100% of the *Shigella sonnei* isolates have MICs  $\leq 4$  mg/liter (EUCAST data).

Accordingly, the combination of melibiose, as the sole source of carbon, and piperacillin in a MacConkey base agar allowed us to obtain a specific (85%) and sensitive (85%) ST301 selective medium (M-MAC), which may facilitate the isolation of O80:H2 EHEC. The isolation of Shiga toxin-producing strains is still essential for diagnosis, epidemiological monitoring, and phylogenetic investigation especially in case of outbreaks. Nevertheless, isolation of EHEC strains among fecal microbiota remains difficult, despite the existence of selective media mainly developed for the O157:H7 serotype and based on its inability to ferment sorbitol (19). The current evolution of EHEC epidemiology requires the development of selective media allowing the isolation of non-O157 strains.

However, culture is time-consuming and a rapid screening method, such as PCR, may be useful. Commercial multiplex molecular panels are increasingly used in microbiological laboratories for detecting major gastrointestinal pathogens, including EHEC, and are mainly based on the detection of *stx* genes. To be sure of the EHEC implication and exclude a free Stx-phage, PCR of complementary EHEC VFs (*eae*, *ehxA*, ...), and even better isolation of the Stx-producing strain, should be performed but remains difficult for laboratories other than NRC. Here, we developed a specific real-time PCR based on 70mel sequence amplification, with a specificity of 98.3% on clinical samples for O80/O55 EHEC strains. Indeed, the genetic scar identified is a good marker for EHEC O80:H2, but it is present in all ST301 strains including O55:H9 EHEC. Utilization of the genetic and phenotypic characteristic is not only a powerful way to improve the diagnosis of O80:H2 EHEC infections, but it could also be used more broadly as an almost-specific marker of ST301 O80:H2/O55:H9 EHECs, which are both considered to be heteropathotypes (15). Thus, the tools that we have developed not only target one specific virulence gene but also a subgroup of EHEC capable of causing invasive infections and requiring a rapid diagnosis. These new diagnosis tools could be used alone or in association with the *stx* based-PCR to alert the clinician and thus can be an asset for the early adaptation of patient management.

Furthermore, these new diagnostic methods, consisting of culture and PCR, could offer a supplementary approach to systematically screen for this serogroup in animal samples and help identify its unknown reservoir. Indeed, this particular serogroup has not been systematically screened until recently as part of the control of food matrices in industry. These new tools could help to facilitate the screening of this serogroup of particular interest.

The recent emergence of EHEC O80:H2 in Europe (2, 5–10, 12) represents a major threat but is still poorly understood, in part because of its unknown reservoir. A complex interaction between host, environment, and bacterial factors is probably necessary to lead to the emergence of a serogroup. It is well established that the acquisition or loss of biochemical characters can increase bacterial fitness or confer additional virulence. For example, the loss of lysine decarboxylase (LDC) in *Shigella* spp. (20) has been shown to be necessary for the full expression of its virulence. Moreover, *Shigella* spp. are also characterized by the convergent degradation of the lactose operon, although this trait has not yet been related to the expression of its virulence (21).

Whether deletion of the melibiose operon and insertion of the 70mel sequence confers an additional advantage to EHEC O80:H2 is still unclear and providing a potential explanation for its recent emergence will require further studies. Of particular interest, we found that *E. albertii*, another enteric pathogen, harbors the same scar in place of the melibiose operon, probably acquired by horizontal transfer via IS. This reinforces the hypothesis that this particular genetic trait may provide an advantage to both enteric pathogens.



**Conclusion.** The non-melibiose fermenting phenotype and insertion of the 70mel sequence are characteristic markers of *E. coli* O80:H2 belonging to ST301. The culture medium and real-time PCR developed here are new tools that could potentially allow better and faster detection of O80:H2 strains, which may also improve the diagnosis and epidemiological knowledge of this threatening and emerging clone. Whether the deletion of melibiose and the insertion of the 70mel sequence provide an advantage to this hybrid pathotype is yet to be determined.

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