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Meningitis Caused by *Escherichia coli* Producing TEM-52 Extended-Spectrum β-Lactamase within an Extensive Outbreak in a Neonatal Ward: Epidemiological Investigation and Characterization of the Strain▽

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Outbreaks caused by *Enterobacteriaceae* isolates producing extended-spectrum β-lactamases (ESBL) in neonatal wards can be difficult to control. We report here an extensive outbreak in a neonatal ward with a case of meningitis caused by an ESBL-producing *Escherichia coli* strain. Between 24 March and 29 April 2009, among the 59 neonates present in the ward, 26 neonates with ESBL-producing *E. coli* rectal colonization were detected (44%). One of the colonized neonates developed meningitis with a favorable outcome after treatment combining imipenem, gentamicin, and ciprofloxacin. Despite strict intensification of hygiene and isolation procedures for more than 1 month, ward closure to new admissions was necessary to control the outbreak. Randomly amplified polymorphic DNA and pulsed-field gel electrophoresis analysis performed on 31 isolates recovered from 26 neonates and two mother's milk samples showed a clonal strain. ESBL PCR assays indicated that the strain harbored a TEM-52 ESBL encoded by an IncI1 replicon. Phylogenetic analysis by multilocus sequence typing showed that the strain belonged to rare phylogenetic group C, which is closely related to group B1 but appears as group A by the triplex PCR phylogrouping method. The strain harbored the virulence genes *fuyA*, *aer*, and *iroN* and was virulent in a mouse model of septicemia. This work indicates the high potential of colonization, transmission, and virulence of some ESBL-producing *E. coli* clones.

With *Streptococcus agalactiae*, *Escherichia coli* is one of the two most common bacterial species frequently responsible of neonatal infections (34). *E. coli* is the second leading cause of neonatal bacterial meningitis in industrial countries, and recent studies suggest that extraintestinal pathogenic *E. coli* isolates belong mainly to phylogenetic group B2 (2, 6). An increased rate of *E. coli* ampicillin resistance after the initiation of intrapartum antimicrobial prophylaxis was previously reported (5, 34). However, most of the *E. coli* isolates remained susceptible to extended-spectrum cephalosporins (ESC), but currently, extended-spectrum β-lactamases (ESBL) are becoming an increasingly important cause of resistance to ESC in *E. coli*, frequently involving the CTX-M-type enzymes (29). In 2008, Boyer-Mariotte et al. reported one case of fatal neonatal meningitis caused by a CTX-M-15-producing *E. coli* strain (7). Emergence of ESBL-producing *Enterobacteriaceae* is a major problem, since the choice of drugs for antimicrobial treatment is limited; moreover, such strains have been increasingly implicated in nosocomial outbreaks in neonatal intensive care units (11, 23, 33, 35). While the spread of CTX-M-type ESBL in the family *Enterobacteriaceae*, especially in *E. coli*, has been described worldwide (11, 13, 15, 17), the spread of TEM-type ESBL has been less frequently reported (8, 9). We report here a case of neonatal meningitis caused by a TEM-52 ESBL-producing *E. coli* strain within a serious outbreak among neonates with rectal colonization and the characteristics of the strain.

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MATERIALS AND METHODS

Hospital setting. The Armand Trousseau children's hospital is a 350-bed, university-affiliated hospital with a 30-bed neonatal ward including two areas, an intensive care unit (6 rooms, 12 beds) and an intermediate care unit (9 rooms, 18 beds). These two units are located on the same floor, with a dedicated medical and nursing staff for each unit, but some staff members can be occasionally requested for one or the other unit. The outbreak was observed within the two units.

Patients. From 24 March to 29 April 2009, 59 babies (gestational age, 26 to 42 weeks; weight, 935 to 3,405 g) were hospitalized in the neonatal ward. Since 2004, the surveillance protocol in the ward has included rectal swabs for all patients at admission and then at weekly intervals. The infants were just screened for ESBL producers. Rectal swabs were inoculated onto a chromogenic cefpodoxime-containing medium (chromIDESBL; bioMérieux, Marcy-l'Etoile, France) (32). Neonates with a positive screening test were immediately subjected to contact precaution measures.

Bacterial strains and routine characterization. A total of 34 *E. coli* isolates were studied. Three isolates were recovered from a patient with meningitis (cerebrospinal fluid [CSF], blood, and rectal swab), 2 isolates were recovered from another patient with conjunctivitis (rectal swab, conjunctiva), and 24 rectal isolates were recovered from the other 24 patients present in the ward during the 5-week outbreak period.

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TABLE 1. Characteristics of 34 *E. coli* isolates shown in chronological order[a]

Strain no., origin	Site of isolation	Date of isolation (day/mo/yr)	PFGE type	RAPD type	Phylogenetic group
1, colonized neonate	Rectal swab	24.03.09	I	α	A
2, colonized neonate	Rectal swab	25.03.09	I	α	A
3, colonized neonate	Rectal swab	31.03.09	I	α	A
4, colonized neonate	Rectal swab	31.03.09	I	α	A
5, colonized neonate	Rectal swab	7.04.09	I	α	A
6, neonate with meningitis	Rectal swab	7.04.09	I	α	A
7, colonized neonate	Rectal swab	7.04.09	I	α	A
8, colonized neonate	Rectal swab	7.04.09	I	α	A
9, colonized neonate	Rectal swab	7.04.09	I	α	A
10, colonized neonate	Rectal swab	7.04.09	I	α	A
11, control patient 1	Rectal swab	7.04.09	II	ß	D
12, colonized neonate	Rectal swab	7.04.09	I	α	A
13, neonate with meningitis	CSF	12.04.09	I	α	A
14, neonate with meningitis	Blood culture	12.04.09	I	α	A
15, colonized neonate	Rectal swab	14.04.09	I	α	A
16, colonized neonate	Rectal swab	14.04.09	I	α	A
17, colonized neonate	Rectal swab	14.04.09	I	α	A
18, colonized neonate	Rectal swab	14.04.09	I	α	A
19, colonized neonate	Rectal swab	14.04.09	I	α	A
20, colonized neonate	Rectal swab	14.04.09	I	α	A
21, colonized neonate	Rectal swab	14.04.09	I	α	A
22, colonized neonate	Rectal swab	21.04.09	I	α	A
23, colonized neonate	Rectal swab	21.04.09	I	α	A
24, colonized neonate	Rectal swab	21.04.09	I	α	A
25, colonized neonate	Rectal swab	21.04.09	I	α	A
26, colonized neonate	Rectal swab	21.04.09	I	α	A
27, colonized neonate	Rectal swab	21.04.09	I	α	A
28, colonized neonate	Rectal swab	27.04.09	I	α	A
29, colonized neonate	Rectal swab	27.04.09	I	α	A
30, mother's milk 1	Milk sample	28.04.09	I	α	A
31, colonized neonate	Eye	29.04.09	I	α	A
32, mother's milk 2	Milk sample	11.05.09	I	α	A
33, control patient 2	Rectal swab	13.05.09	III	γ	B2
34, control patient 3	Rectal swab	13.05.09	IV	δ	B2

[a] All isolates except the control strains (no. 11, 33, and 34) showed identical PFGE and RAPD types and belong to phylogenetic group A, as determined by the triplex PCR method.

Two isolates were recovered from two mother's milk samples obtained through a breast pump. Three additional *E. coli* strains from patients hospitalized in other wards were also tested as controls (Table 1). Bacterial isolates were identified using the Rapid ID 32E system (bioMérieux), and K1 capsular antigen determination was done with an antiserum to *Neisseria meningitidis* group B (Bio-Rad, Marnes-La-Coquette, France) (14). ESBL-producing isolates were screened by the double-disk test of synergy between cefotaxime and amoxicillin-clavulanate (20). Susceptibility testing was performed by the disk diffusion method according to the Comité de l'Antibiogramme, Société Française de Microbiologie (http://sfm.asso.fr/). MICs were determined by the Etest method.

Molecular typing. Two techniques were used for molecular typing, i.e., rapid screening by randomly amplified polymorphic DNA (RAPD) analysis and control genotyping by pulsed-field gel electrophoresis (PFGE). RAPD analysis was performed on all *E. coli* isolates as described elsewhere (28). Primer P2 (5′-GCCCCC AGGGGCACAGT-3′) was used. In brief, DNA was amplified in 50 μl of a solution containing 50 ng of DNA, 3 μM primer, 2.5 U of AmpliTaq DNA polymerase, the four deoxynucleoside triphosphates (400 μM each), 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl. Analysis and comparison of isolate patterns were performed easily by visual observation. PFGE was performed on all *E. coli* isolates as reported elsewhere (4). Briefly, bacterial cells were embedded in agarose and lysed with detergent and proteinase K. DNA was digested with NotI (Roche Diagnostics, France) and subjected to PFGE in 1% agarose gel at 6 V/cm for 27 h with pulse times varying linearly between 2 and 49 s. Analysis and comparison of isolates patterns were also easily performed by visual observation.

PCR phylogrouping and virulence factor detection. The phylogenetic groups to which all of our *E. coli* isolates belonged were determined by the triplex PCR phylogrouping method as previously reported (2, 12). The presence of virulence factors was determined as previously reported (3), with a multiplex PCR-based technique that detects the *fuyA*, *hly*, *sfac/foc*, *papC*, *papGIII*, and *papGII* alleles, *aer*, *cnf1*, and *iroN*.

Multilocus sequence typing (MLST). MLST was performed using *dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA* partial sequences on the meningitis strain (21). Further details on this MLST schema can be found at http://www.pasteur.fr/mlst. Phylogenetic analysis was performed with the concatenated sequences of the eight genes from the meningitis strain, the *E. coli* reference (ECOR) strains, and other bacteremic strains (18, 21) by the maximum-likelihood method in the PHYML program (19) with *E. fergusonii* as the outgroup.

Mouse lethality assay. A mouse model of systemic infection was used to assess the intrinsic virulence of the meningitis strain (30). Ten outbred female Swiss OF1 mice (3 to 4 weeks old, 14 to 18 g) received a subcutaneous injection of 2×10^8 CFU/ml log-phase bacteria. The mice were monitored for death for 7 days. As a control, the highly virulent CFT073 strain and the nonvirulent K-12 MG1655 strain were also tested. Animals were maintained and handled according to the guidelines of the French Ministry of Agriculture (approval A75-18-05).

Characterization of β-lactamase-encoding gene (*bla*) and isoelectric focusing (IEF) of β-lactamase. PCR assays for detection of bla_{TEM}, bla_{CTX-M}, and bla_{SHV} were performed on the meningitis strain. The PCR product was subjected to direct sequencing with an Applied Biosystems sequencer (model ABI 377), and the nucleotide sequence was analyzed with the BLAST and ClustalW programs (15). IEF of the β-lactamase was performed as described elsewhere (15). Briefly, bacterial broth cultures were pelleted and sonicated and IEF was performed on a pH 3.5 to 10 ampholine polyacrylamide gel. The pI values used as standards were those of TEM-1 (pI 5.4), TEM-2 (pI 5.6), and TEM-3 (pI 6.3).

Plasmid replicon type determination. *E. coli* strain J53-2 Rif^r (Stratagene, La Jolla, CA) was used for transfer assays in plasmid replicon type determination. After transfer of the plasmid conferring ESC resistance by conjugation of the meningitis

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strain to *E. coli* J53-2 Rif^r, selection of transconjugants was made on a Mueller-Hinton agar plate containing rifampin (250 mg/liter) and ceftazidime (2 mg/liter). Plasmid replicons were determined in the parental and recipient strains by using the PCR-based replicon typing scheme described by Carattoli et al. (10).

RESULTS

Description of the outbreak. Between 24 and 31 March 2009, five neonates were first found to be colonized (rectal swabs) by ESBL-producing *E. coli*. Three of them were present in the ward for more than 10 days. On 7 April, seven additional neonates tested positive and one of them developed meningitis. The colonized neonates were isolated in single rooms or gathered together with up to three in the same room. Contact precautions were implemented according to the institutional protocol, i.e., use of an alcoholic hand hygiene rub, use of gloves and individual gowns for any contact with the patients, individualization of instruments (stethoscope, height gauge), and disinfection of any apparatus shared by several patients (breast pump, baby scales, etc.).

On 14 April, seven newly colonized neonates were identified. All staff members were repeatedly informed of the importance of the outbreak, hand hygiene protocols were emphasized, and environment hygiene procedures were intensified. The staff members allowed into the rooms were limited to those directly treating the neonates, excluding all students. Several educational sessions were organized for the staff, as well as for visitors, who were strictly restricted to the closest family members (mother and father). The decision was made to stop the admission of new neonates to the ward, except for those coming from the Armand Trousseau hospital maternity ward.

On 21 April, five new cases of rectal colonization were detected, leading to the decision to stop any new admissions to the ward, including those coming from the Armand Trousseau hospital maternity ward, on 24 April (1 month after the first case was detected). However, on 27 April, 2 negative neonates present in the ward became colonized, leading to a total of 26 colonized neonates. A new temporary neonatal unit was opened on the upper floor within a separate ward to receive eight new neonates from the Armand Trousseau hospital maternity ward.

Detection of the epidemic *E. coli* strain was not performed on the staff members or the environment but was performed on mother's milk samples obtained by breast pump. Of all of the milk samples tested since 25 April, only two samples were positive for the epidemic *E. coli* strain. Four breast pumps were shared between mothers and decontaminated between uses. However, the pump set (valve, membrane, adapter, tub, etc.) was single use. Breast pump use was then forbidden.

During the month of May, the colonized neonates were isolated in a separate area of the ward, apart from the "clean area" receiving the noncolonized neonates. Each area had its own nursing and medical staff. All of the colonized and noncolonized neonates left the ward in the following month, and comprehensive cleaning of surfaces, instruments, and apparatus was carried out after each one left. New admissions to the ward were allowed as of 15 June, and no new colonization was observed thereafter.

***E. coli* neonatal meningitis.** A baby weighing 2,370 g was delivered by emergency caesarean section for version failure on transverse position, at a gestational age of 34 weeks. The initial infection assessment was negative, and this premature infant did not receive any antibiotic. On day 5, the neonate tested positive for ESBL-producing *E. coli* by rectal swab. The infant's clinical development was satisfactory from birth to day 9. On day 10, the following symptoms were observed: hyperthermy and inflammatory syndrome (the C-reactive protein level increased to 32 mg/liter), as well as an increase in the procalcitonin level (3.15 ng/ml). After blood cultures and a lumbar puncture were performed, intravenous treatment with imipenem (60 mg/kg/day, 21 days) combined with gentamicin (3 mg/kg/day, 3 days) and ciprofloxacin (20 mg/kg/day, 6 days) was started. Microscopic examination of CSF showed 4,700 leukocytes/ml with 95% neutrophils and Gram-negative rods. The CSF protein and glucose levels were, respectively, 7.7 g/liter and <0.1 mmol/liter. CSF and blood cultures were positive for *E. coli*. The same susceptibility pattern was observed at the same time for the meningitis and epidemic isolates, the latter already colonizing 11 neonates in the ward. On day 12, a control lumbar puncture showed a CSF cell count of 1,430 leukocytes/ml with 73% neutrophils and the presence of some Gram-negative rods on microscopic examination. A CSF culture remained sterile. Rapid improvement of the baby was observed with a decrease in the CSF protein level (4.3 g/liter), a still low glucose level (<0.1 mmol/liter), and normalization of the C-reactive protein level. We did not detect any immunodeficiency in the patient.

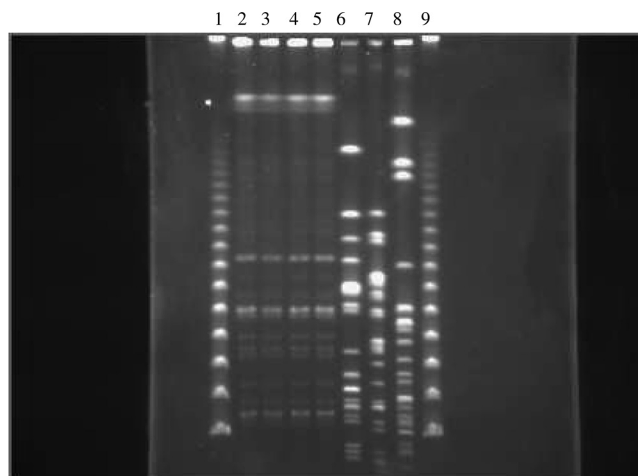

FIG. 1. PFGE patterns of some representative *E. coli* isolates. Lanes 1 and 9, molecular marker. Lanes 2 and 3, isolates from two mother's milk samples. Lane 4, isolate from CSF of the neonate with meningitis. Lane 5, isolate from a rectal swab of a representative colonized neonate. Lanes 6 to 8, unrelated *E. coli* isolates from control patients.

Characterization of the *E. coli* strain. All *E. coli* isolates showed the same antimicrobial susceptibility pattern, i.e., resistance to ESC and susceptibility to imipenem (MIC = 0.25 mg/liter), ciprofloxacin (MIC = 0.25 mg/liter), and gentamicin. These *E. coli* isolates also showed indistinguishable patterns obtained by the RAPD analysis and PFGE techniques (types α and I, respectively, as shown in Table 1). The PFGE patterns of four representative *E. coli* isolates are shown in Fig. 1 (type I). The control strains, from unrelated patients, showed RAPD

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patterns β, γ, and δ and PFGE types II, III, and IV, respectively (Table 1).

By the triplex PCR phylogrouping technique, our *E. coli* isolates belonged to phylogenetic group A, while the group of the control strain was D (Table 1). However, MLST analysis of the meningitis strain showed that it exhibited sequence type 7 and belonged to a phylogenetic group closely related to the B1 group that has been called the C group (16) or clonal complex 66 (21). Reference strain ECOR 70 belongs to this C group. The eight *E. coli* isolates tested harbored three virulence factors, all involved in iron capture, i.e., *fuyA*, *aer*, and *iroN*. This epidemic strain, also responsible for the case of meningitis found, was negative for K1 polysaccharide capsular antigen. The meningitis strain was virulent in the mouse model of septicemia, as it killed all 10 of the mice inoculated (22). As a comparison, nonvirulent commensal strain K-12 did not kill any of the 10 mice inoculated, whereas virulent urosepsis strain CFT073 killed all of the 10 mice inoculated.

PCR assays for detection of bla_{TEM}, $bla_{\mathrm{CTX\text{-}M}}$, and bla_{SHV} in the meningitis strain showed a positive result only for bla_{TEM}, and sequence analysis of the amplicon showed TEM-52. IEF assays showed a pI value of 6. With the PCR-based replicon typing technique, the recipient strain was positive for replicon I1, as well as the parental strain. The characteristics of *E. coli* isolates (site and date of isolation, PFGE and RAPD patterns, and phylogenetic group) are summarized in Table 1.

DISCUSSION

ESBL-producing, Gram-negative bacilli, mostly *Klebsiella pneumoniae* but also *E. coli*, have been increasingly implicated in nosocomial outbreaks in neonatal intensive care units (11, 13, 15, 17). Although many outbreaks have been resolved rapidly by reimplementation of hygiene control measures, there have been some instances where the outbreak was only resolved by temporary ward closure (26, 33, 35).

In our neonatal ward, the index case patient was not clearly identified among the five neonates who first tested positive by screening with rectal swabs. Some days later, one of these colonized neonates developed meningitis, fortunately with a favorable outcome. Among the initially colonized infants, 16/26 were treated with cefotaxime prior to detection on routine surveillance. The colonized neonates were probably the main reservoir of the epidemic strain, which was then hand transmitted. Despite strict implementation of hygiene and isolation measures, the outbreak lasted 5 weeks and was resolved only after the ward was closed to new admissions. Checking of staff practices did not show a major mistake in management; however, two positive milk samples led us to suspect transmission of the epidemic *E. coli* strain by milk samples provided by breast pump. This mode of transmission has been reported (26) following poor disinfection of a breast pump. Since the pump set was single use in our neonatal ward, hand contamination of milk samples was possible.

The pathogenesis of *E. coli* neonatal meningitis (ECNM) is characterized by high-level bacteremia, followed by penetration of the blood-brain barrier. The K1 capsular polysaccharide is a virulence factor with a key role in the bacteremic phase. Other bacterial attributes contribute to *E. coli* survival in serum and may thus be involved in the pathogenesis of ECNM, such as the iron uptake systems encoded by the loci *iro* and *chu*, the siderophores aerobactin (*aer*) and yersiniabactin (*fuyA*), and also hemolysin (6). Our *E. coli* meningitis strain did not show the K1 capsular polysaccharide; it showed only three virulence factors (*fuyA*, *aer*, and *iroN*) of the eight tested for. Several authors have reported mainly group B2 and, to a lesser extent, group D as the groups including numerous highly pathogenic isolates that were frequently involved in extraintestinal infection (6, 30). In contrast, *E. coli* strains of groups A (our strain) and B1 are usually isolated as commensals and are devoid of virulence (30). However, based on MLST data, our meningitis strain belonged to phylogenetic group C (clonal complex 66), which was identified as group A by the triplex PCR phylogrouping technique (2, 12) but was closely related to phylogenetic group B1. Few data are available on these strains, but they can be found as commensals in nonhuman primates (ECOR 70), as well as in human septicemia (21). This work shows that strains belonging to phylogenetic group C/clonal complex 66 can have a high potential for gut colonization, transmission, and as proven by the mouse septicemia assay in our study, real virulence. Five days before developing meningitis, the baby tested positive for the epidemic strain by rectal swab but we did not explore the level of intestinal carriage of the strain. A high level could make translocation of the strain into the blood and its penetration of the blood-brain barrier easier, leading to meningitis. On the other hand, negative urine culture allowed us to rule out the role of pyelonephritis with bacteremia as a cause of meningitis.

Unfortunately, the strain's virulence, as shown by the mouse septicemia assay, was combined with ESC resistance. In our hospital, the first-line treatment of neonatal infections usually combined cefotaxime and gentamicin, since ESBL-producing isolates remained rare within our neonate population. Fortunately, the neonate with meningitis was already known to be colonized by ESBL-producing *E. coli* and thus was immediately treated with the appropriate antimicrobial drug combination of imipenem, gentamicin, and ciprofloxacin. Ciprofloxacin was added to imipenem because of its excellent brain tissue penetration to prevent the abscess complications frequently observed in neonatal meningitis due to Gram-negative bacilli (24). While *K. pneumoniae* was the ESBL-producing species most frequently involved in nosocomial outbreaks (1, 23, 35), this has changed since the 1990s. In France, from 1998 to 2005, the prevalence of ESBL-producing *K. pneumoniae* isolates decreased from 9.4% to 3.7%, whereas that of ESBL-producing *E. coli* isolates increased from 0.2% to 1.99% (17). Some cases of serious neonatal ESBL-producing *E. coli* infections were reported, mainly involving CTX-M-15-producing strains (7) and never TEM-52-producing strains, to our knowledge. The TEM-52 ESBL was first described in 1998 in *K. pneumoniae* (31) and then in *Salmonella* and also in *E. coli* (8, 9) and has now been found in several European countries (13). However, TEM-52 was infrequently reported in French *E. coli* isolates compared to CTX-M-15 (25). TEM-52 was mainly associated with a broad-host-range IncI1 plasmid replicon, in contrast to other TEM ESBL, which were carried preferentially on IncA/C replicons, or CTX-M-15 ESBL carried by IncF replicons (27).

This outbreak in a neonatal ward, with a case of meningitis, was out of control for more than 1 month. It was caused by an

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E. coli strain belonging to an infrequent phylogenetic group (group C or clonal complex 66) and exhibiting intrinsic virulence in a mouse septicemia assay. The strain also showed an unusual ESBL, TEM-52, carried by the IncI1 plasmid replicon.

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