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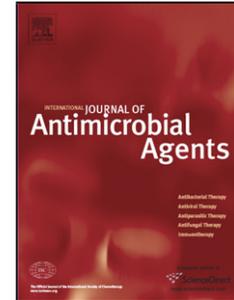
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Outbreak of a novel *Enterobacter* sp. carrying *bla*_{CTX-M-15} in a neonatal unit of a tertiary hospital in Tanzania

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ABSTRACT

Enterobacter hormaechei and *Cronobacter sakazakii* are among the most important causes of outbreaks of neonatal sepsis associated with powdered milk. In this study, we report for the first time an outbreak of a novel *Enterobacter* sp. harbouring *bla*_{CTX-M-15} in a neonatal unit in Tanzania. Seventeen Gram-negative enteric isolates from neonatal blood cultures were studied. Antibiotic susceptibility was assessed by disk diffusion testing, and the presence of the *bla*_{CTX-M-15} gene was established by polymerase chain reaction (PCR) and sequencing. Isolates were typed by pulsed-field gel electrophoresis (PFGE). Identification by biochemical profiling was followed by nucleotide sequencing of 16S ribosomal DNA (rDNA), *rpoB* and *hsp60* alleles. Environmental sampling was done and control measures were established. Isolates were initially misidentified based on their fermentation characteristics and agglutination as *Salmonella enterica* serotype Paratyphi. All isolates were resistant to multiple antibiotics, except for ciprofloxacin and carbapenems, and were found to harbour *bla*_{CTX-M-15} on a 291-kb narrow-range plasmid. PFGE analysis indicated the clonal outbreak of a single strain, infecting 17 neonates with a case fatality rate of 35%. The same strain was isolated from a milk bucket. Phylogenetic analysis using 16S rDNA, *rpoB* and *hsp60* sequences permitted no definitive identification, clustering the strains in the *Enterobacter cloacae* complex with similarities of 92–98.8%. The data describe an outbreak of a novel *bla*_{CTX-M-15}-positive, multiresistant *Enterobacter* strain in an African neonatal unit that can easily be misidentified taxonomically. These data highlight the need for constant surveillance of bacteria harbouring extended-spectrum β -lactamases as well as improvements in hygiene measures in developing countries.

1. Introduction

Enterobacter spp., especially *Cronobacter* (*Enterobacter*) *sakazakii*, have been associated with neonatal sepsis through ingestion of contaminated powdered milk [1]. A number of outbreaks of *C. sakazakii* have been reported in developed countries [2,3], whereas information from developing countries is limited. Bacteria of the genus are ubiquitous in nature and can cause severe infection, especially in immunocompromised neonates [4]. Members of the *Enterobacter cloacae* complex are most frequently isolated from clinical samples. In this complex, *Enterobacter hormaechei* isolates appear to be prone to carrying extended spectrum β -lactamase (ESBL) genes [5] and can be phenotypically misidentified as *C. sakazakii* when using commercially available biochemical kits [2]. Identification of *Enterobacter* spp. is routinely done by phenotypic methods. Most clinical laboratories in developed countries employ commercially available kits or semiautomated systems, whilst in developing countries simple in-house biochemical panels are commonly applied [6]. For further identification and discrimination of the species in this genus, 16S ribosomal RNA (rRNA), *rpoB* and *hsp60* gene sequencing have been used. Here we report the outbreak of a novel species of *Enterobacter* carrying *bla*_{CTX-M-15}, which can easily be misidentified using routinely applied biochemical kits [2,4,6].

2. Methods

2.1. Bacterial isolates

Bacterial strains were isolated from blood cultures of neonates from the Bugando Medical Centre (Mwanza, Tanzania) between December 2009 and February 2010. Environmental sampling was performed, with samples obtained of solutions, milk, suction tubes, sinks and mattresses.

2.2. Procedures

Bacteria were identified using in-house biochemical and fermentation assays (Triple sugar iron agar, citrate utilisation, indole production, urease test, hydrogen sulphide production, motility testing). A *Salmonella* polyvalent latex agglutination test (Oxoid Ltd., Basingstoke, UK) was performed for all isolates. Further identification was done using API 20E (bioMérieux, Marcy l'Étoile, France), VITEK 2 GN ID Card (bioMérieux) and Phoenix-NMIC/ID-64 (Becton Dickinson, Heidelberg, Germany). Fatty acid analysis was performed by the German Resource Centre for Biological Materials (DSMZ). In addition, strains were examined for their ability to form biofilms on plastic surfaces as described by O'Toole et al. [7].

Susceptibility patterns to ampicillin, amoxicillin/clavulanic acid (AMC), sulfamethoxazole/trimethoprim (SXT), tetracycline, gentamicin, ciprofloxacin, chloramphenicol, fosfomicin, ceftriaxone, cefotaxime, cefepime, ceftazidime and meropenem were determined by disk diffusion following Clinical and Laboratory

Standards Institute (CLSI) guidelines [8]. *Escherichia coli* ATCC 25922 was used as the quality control strain. ESBL production was confirmed using disk approximation methods [8].

Pulsed-field gel electrophoresis (PFGE) analysis was performed as previously described (<http://www.cdc.gov/pulsenet/protocols.htm>). Briefly, genomic DNA was prepared in agarose blocks and was digested with the restriction enzyme *Xba*I (Fermentas, St Leon-Rot, Germany). DNA products were separated by PFGE on a CHEF-DRIVE III apparatus (Bio-Rad, La Jolla, CA) in 0.5× Tris–borate–ethylene diamine tetra-acetic acid (TBE) buffer for 20 h at 6 V/cm at 13 °C, 5–50 s for 20 h. A dendrogram illustrating the genetic similarity of 18 strains was generated from banding patterns using GelCompar II® (Applied Maths, Sint-Martens-Latem, Belgium). Strains showing ≥80% similarity were classified as genetically related and were assigned to the same clusters.

The presence of ESBLs was established by polymerase chain reaction (PCR) with specific primers targeting SHV, TEM and CTX-M genes [9]. Sequencing of the PCR products was done to establish the corresponding ESBL allele (LGC Genomics GmbH, Berlin, Germany). To determine the location of ESBL genes, conjugation experiments were carried out as described previously [10] with two isolates as donors and *E. coli* CC118 (Rif^R, Str^R, Lac⁻, plasmid-free) as a recipient strain. Transconjugants were selected on lysogeny broth (LB) plates supplemented with 30 µg/mL cefotaxime and 300 µg/mL rifampicin and were confirmed for the presence of ESBL genes using phenotypic and molecular methods. In situ digestion of agarose blocks with S1 nuclease was used

to determine plasmid size [10,11] as described previously, followed by Southern blotting and hybridisation using digoxigenin (DIG)-labelled *bla*_{CTX-M-15} amplicon probes, prepared according to the manufacturers instructions (DIG High Prime DNA Labelling and Detection Starter Kit II; Roche, Mannheim, Germany).

A 1444-bp 16S rDNA template was amplified and sequenced using the primers 49F (TWAYACATGCAAGTCGRRCG), 1504R (CTTGTTACGACTTCACCCCAG), 355R (GCTGCCTCCCGTAGCAGTCTGG) and 1092F (AAGTCCCGCAACGAGCGCAAC), followed by *rpoB* and *hsp60* phylogeny studies [12].

3. Results and discussion

During December 2009 to February 2010, a Gram-negative bacterium with identical antibiotic profile was isolated from blood samples of 17 neonates (Table 1). A similar strain was isolated from the milk bucket. The first case came from the neonatal Intensive Care Unit (NICU) and was followed by more cases in the NICU as well as the general neonatal unit. Twelve cases (71%) were admitted in January 2010 (Table 1). Using in-house phenotypic biochemical profiling, the isolates were classified as non-lactose-fermenters, motile, indole-negative, citrate-positive, urease-negative with no production of hydrogen sulphide. All strains gave a positive agglutination test using a polyvalent *Salmonella* kit; thus, the identification of *Salmonella enterica* serotype Paratyphi was made.

By disk diffusion test, all strains were found to be resistant to ampicillin [30 µg; mean ± standard deviation (S.D.) zone diameter 6.06 ± 0.2356 mm], AMC (20/10 µg; mean ± S.D. zone diameter 11.78 ± 0.428 mm), gentamicin (10 µg; mean ± S.D. zone diameter 6.22 ± 0.428 mm), SXT (25/23.75 µg; mean ± S.D. zone diameter 6.56 ± 0.856 mm), tetracycline (30 µg; mean ± S.D. zone diameter 6 ± 0.000 mm), fosfomycin (200 µg; mean ± S.D. zone diameter 8.67 ± 0.485 mm), chloramphenicol (30 µg; mean ± S.D. zone diameter 10.78 ± 0.428 mm), cefotaxime (30 µg; mean ± S.D. zone diameter 11.72 ± 0.461 mm), ceftriaxone (30 µg; mean ± S.D. zone diameter 12.44 ± 0.511 mm), ceftazidime (30 µg; mean ± S.D. zone diameter 13.78 ± 0.428 mm) and cefepime (30 µg; mean ± S.D. zone diameter 11.89 ± 0.676 mm), whilst being sensitive to ciprofloxacin (5 µg; mean ± S.D. zone diameter 23.44 ± 0.511 mm) and meropenem (10 µg; mean ± S.D. zone diameter 18.33 ± 18.33 mm) (Fig. 1). All strains were found to produce ESBL by the double disk synergy test as described previously [8]. A sample swab from a bucket used to prepare milk was positive for a similar isolate as found in the blood cultures of the infected neonates. All strains were able to form biofilms on plastic using the method described by O'Toole et al. [7].

Most of the neonates (12/17; 71%) were premature infants on artificial feeding (Table 1). All neonates were initially treated with ampicillin, gentamicin and cefotaxime without clinical improvement. Owing to the unavailability of carbapenems, the decision was made to use ciprofloxacin. Despite initiating this treatment, overall mortality was 35% (6/17). The outbreak was controlled by extensive hygiene measures, including contact

precautions, education of health workers, surveillance, thorough disinfection and washing of all instruments in the unit.

Genetic relatedness of the isolated strains using *Xba*I-restricted chromosomal DNA PFGE indicated that all strains, including the one from the milk bucket, were clonal (Fig. 1), indicating the spread of a single strain either from a common source or from one patient to another. The strains could not be accurately identified using commercial biochemical kits, with the closest result indicating *E. cloacae* when using API 20E, VITEK 2 GN ID Card and Phoenix-NMIC/ID-64, yielding 56%, 95% and 98% identity, respectively. The isolates could be distinguished from *E. cloacae* as being non-lactose-fermenters, esculin hydrolysis-positive and all strains were urease-positive after 48 h of incubation (Table 2). Fatty acid analysis classified the strain in the Enterobacteriaceae family with a similarity index of 0.471, 0.438, 0.419, 0.409 and 0.366 to *Proteus vulgaris*, *Enterobacter gergoviae*, *Kluyvera ascorbata*, *Serratia plymuthica* and *E. coli*, respectively.

A 16S rDNA phylogeny analysis was performed following sequencing of a 1444-bp fragment of the 16S rDNA (GenBank accession no. HQ122932) and was compared with 16S rDNA sequences for other *Enterobacter* spp. in the National Center for Biotechnology Information (NCBI) GenBank database. A neighbour-joining tree was created using MegAlign software (DNASTAR, Madison, WI). The sequence was 97–98.8% similar to other *Enterobacter* spp. (Supplementary Fig. 1); the closest similarity (Table 2) was seen with *Enterobacter asburiae* (98.86%), *Enterobacter cancerogenus* (98.82%), *Enterobacter kobei* (98.57%), *Enterobacter ludwigii* (98.55%) and *E. cloacae*

(98.52%). A similarity of 99% was observed with the sequence of an uncultured bacterium (GenBank accession no. EF179826) obtained from the gut of an anopheles mosquito. Generally, 16S rDNA analysis grouped the strain in the cluster of the *E. cloacae* complex. For *Enterobacter* spp., the general 16S rDNA intraspecific similarity is ca. 98.7–99% across the entire length of the 16S rRNA gene (ca. 1300–1500 bp) [12,13]. As 16S rRNA gene sequencing provides genus identification in most cases (>90%), but less so with regard to species [12,14,15], the *rpoB* gene, which has been found to have a high resolution in identifying species in the genus, was used as an additional marker [16]. A 982-bp fragment (GenBank accession no. HQ148298) was obtained and was compared with other *Enterobacter* spp. (Table 2). The closest relation was *E. hormaechei*, with a divergence of 1.2%, whilst the 16S rDNA similarity towards this species was 97.9%, thus making it very unlikely to be the same species, particularly also because of its different biochemical properties (Table 2) [15,16]. Finally, a 360-bp fragment of the *hsp60* gene was obtained (GenBank accession no. HQ148299) using previously described primers [6]. The results (Table 2) were 80–97% similar to the known *Enterobacter hsp60* gene sequences [17].

None of the methods could definitively classify the strains among the known species in the genus *Enterobacter*, suggesting that this may be a novel *Enterobacter* sp. that can cause similar diseases and outbreaks as other *Enterobacter* spp.

All strains were found to contain *bla*_{CTX-M-15} (GenBank accession no. HQ175999), and PCR analysis with subsequent sequencing could also demonstrate that CTX-M-15 was associated with an 1800-bp insertion sequence *ISEcp1/tnpA* gene upstream [10]. In

conjugation experiments, gentamicin, tetracycline and fosfomycin resistances were transferrable. S1 nuclease PFGE and subsequent DIG hybridisation with a CTX-M-15-specific gene probe indicated that *bla*_{CTX-M-15} was located on a 291-kb plasmid with a hitherto untypeable replicon in all wild-type isolates. However, in the transconjugants, *bla*_{CTX-M-15} was chromosomally located, thus indicating a translocation of the resistance gene to the recipient chromosome probably following an abortive conjugal transfer (Fig. 1B) and thereby implying a narrow host range for this plasmid.

4. Conclusion

Here we describe a new species of *Enterobacter* that has so far not been categorised using biochemical and genetic markers. The strain was also isolated from milk and is capable of biofilm formation; a property also described for other species of *Enterobacter* and suggesting a possible association with milk powder. The isolate carries the *bla*_{CTX-M-15} gene on a hitherto untypeable plasmid and is resistant to multiple antibiotics, thus limiting treatment options. This is the first report of an outbreak of *Enterobacter* spp. carrying *bla*_{CTX-M-15} in Africa. Surveillance of this strain is needed to determine its epidemiological spread and to identify markers to improve its detection.

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Competing interests

None declared.

Ethical approval

Ethical approval was obtained from Bugando Medical Centre/WBUCHS ethics review board.

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Fig. 1. (A) Pulsed-field gel electrophoresis (PFGE) dendrogram rooted from *Xba*I-digested *Enterobacter cloacae* strain 263 (isolated from blood culture during the outbreak) of 18 strains revealing clonal isolates; isolate MO244 was isolated from a milk bucket. TET, tetracycline; SXT, sulfamethoxazole/trimethoprim; G, gentamicin; CIP, ciprofloxacin; FF, fosfomicin; C, chloramphenicol; MEM, meropenem; R, resistant, S, sensitive. (B) Agarose gel showing S1 nuclease PFGE-based sizing of large plasmids for strain 247BMC (isolate 247, representative of the 18 identical strains). M (λ marker) indicates the molecular weight marker. Plasmid size preparations from isolate 247W (wild-type) and 247T (transconjugant) reveal a plasmid with size of 291 kb in 247W, no plasmid in 247T, and positive chromosomal hybridisation in 247T.

Supplementary Figure 1. (A) Neighbour-joining tree of *Enterobacter* spp. based on 16S rDNA sequences in relation to strain 247BMC (isolate 247, representative of the 18 identical strains). Numbers in parentheses are the GenBank accession no. (B) Neighbour-joining tree of *Enterobacter* spp. based on the *rpoB* gene. A 1444-bp 16S rDNA template was amplified and sequenced with the primers 49F (TWAYACATGCAAGTCGRRCG), 1504R (CTTGTTACGACTTCACCCCAG), 355R (GCTGCCTCCCGTAGCAGTCTGG) and 1092F (AAGTCCCGCAACGAGCGCAAC).

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Table 1Demographics and clinical characteristics of neonates infected with *Enterobacter* sp. nov.

No.	Sex	Age (days)	GA	Date of admission	Diagnosis	Date of sepsis diagnosis	Positive culture	Isolate	Outcome
245	F	5	Premature	23/12/2009	Prematurity	27/12/2009	29/12/2009	<i>Enterobacter</i> sp.	Death
246	F	3	Full term	30/12/2009	Birth asphyxia	1/1/2010	3/1/2010	<i>Enterobacter</i> sp.	Discharge
247	M	7	Premature	1/1/2010	Prematurity	3/1/2010	5/1/2010	<i>Enterobacter</i> sp.	Discharge
248	F	2	Premature	3/1/2010	Prematurity	5/1/2010	7/1/2010	<i>Enterobacter</i> sp.	Death
249	F	14	Full term	1/2/2010	Birth asphyxia	1/1/2010	3/1/2010	<i>Enterobacter</i> sp.	Discharge
250	F	2	Premature	13/1/2010	Prematurity	15/1/2010	17/1/2010	<i>Enterobacter</i> sp.	Death
251	M	6	Full term	14/1/2010	Birth asphyxia	14/1/2010	16/1/2010	<i>Enterobacter</i> sp.	Discharge
252	F	2	Premature	14/1/2010	Prematurity	16/1/2010	18/1/2010	<i>Enterobacter</i> sp.	Death

254	F	3	Premature	15/1/2010	Prematurity	18/1/2010	19/1/2010	<i>Enterobacter</i> sp.	Death
255	M	2	Premature	14/1/2010	Prematurity	16/1/2010	18/1/2010	<i>Enterobacter</i> sp.	Discharge
256	M	2	Full term	15/1/2010	Birth asphyxia	17/1/2010	19/1/2010	<i>Enterobacter</i> sp.	Discharge
258	M	3	Premature	23/1/2010	Prematurity	25/1/2010	27/1/2010	<i>Enterobacter</i> sp.	Discharge
260	F	4	Premature	24/1/2010	Prematurity	28/1/2010	30/1/2010	<i>Enterobacter</i> sp.	Discharge
261	M	3	Full term	4/2/2010	Birth asphyxia	7/2/2010	9/2/2010	<i>Enterobacter</i> sp.	Death
264	M	4	Premature	4/2/2010	Prematurity	8/2/2010	10/2/2010	<i>Enterobacter</i> sp.	Discharge
265	F	3	Premature	5/2/2010	Prematurity	8/2/2010	10/2/2010	<i>Enterobacter</i> sp.	Discharge
267	F	2	Premature	6/2/2010	Prematurity	8/2/2010	10/2/2010	<i>Enterobacter</i> sp.	Discharge

GA, gestational age.

Table 2

Biochemical properties and percentage homology of genetic markers between strain 247BMC (isolate 247, representative of the 18 identical strains) and other closely related *Enterobacter* spp.

	<i>E. aerogenes</i>	<i>E. asburiae</i>	<i>E. cancerogenus</i>	<i>E. cloacae</i>	<i>E. cowanii</i>	<i>E. gergoviae</i>	<i>E. hormaechei</i>	<i>E. kobei</i>	<i>C. sakazakii</i>	<i>E. ludwigii</i>	247BMC
Biochemical properties											
4-NP-	-	-	-	-	-	-	-	-	+	-	N/A
α - Glc											
VP	+	+	+	+	+	+	+	+	+	+	+
ADH	-	V(21)	+	+	+	+	V(78)	+	+		+
ODC	+	+	+	+	-	+	V(91)	+	+		+
SAC	+	+	-	+	+	+	+	V(25)	+	+	+
RAF	+	V(70)	-	+	+	+	-	+	+		+
ARA	+	+	+	+	+	+	+	+	+	+	+
CIT	+	+	+	+	+	+	+	+	+		+
MR	-	+	-	-	-	-	V(57)	-	-	-	-
ADO	+	-	-	V(25)	-	-	-	-	-		-
SOR	+	+	-	+	+	-	-	+	-	+	+
LDC	+	-	-	-	-	+	-	-	-	-	-
LACT	+	V(75)	-	+	+	V(55)	-	+	+	+	-

RHA	+	–	+	+	+	+	+	+	+	+	+	+
MEL	+	–	–	+	+	+	–	+	+	+	+	+
ESC	N/A	N/A	–	V(30)	N/A	N/A	–	N/A	+	– ⁺	+	+
URE	–	V(60)	–	V(65)		+	V(87)	–	–	–	–	+
Yellow colou r	–	–	–	–	V(66)	–	–	–	+	–	–	–
Homology (%)												
16S rDNA a	98.2	98.8	98.8	98.5	97.3	94.8	97.9	98.5	97	98.5	100	
<i>rpoB</i> ^a	95.7	97.4	96.1	98.1	91.5	91.5	98.8	97.8	91	98.0	100	
<i>hsp60</i> a	NA	94.9	92.6	95.2	86.6	80.0	97.3	N/A	89	93.7	100	

4-NP- α -Glc, metabolism of 4-nitrophenyl α -glucoside; VP, Voges–Proskauer test; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; SAC, acid from sucrose; RAF, acid from raffinose; ARA, acid from arabinose; CIT, use of citrate as sole source of carbon; MR, methyl red test; ADO, acid from adonitol; SOR, acid from sorbitol; LDC, lysine decarboxylase; LACT, acid from lactose; RHA, acid from rhamnose; MEL, acid from melibiose; ESC, hydrolysis of esculin; URE, production of urease; +, 90% or more strains are positive; –, 90% or more strains are negative; V, 11–89% of strains are positive; N/A, not available; –⁺; 10–20% are positive.

^a Percentage homology with strain 247BMC.

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