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Molecular epidemiology of multidrug-resistant *Shigella dysenteriae* type 1 causing dysentery outbreaks in Central African Republic, 2003—2004

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Summary *Shigella dysenteriae* type 1 (Sd1) represents a particular threat in developing countries because of the severity of the infection and its epidemic potential. Antimicrobial susceptibility testing and molecular subtyping by pulsed-field gel electrophoresis (PFGE) and plasmid profiling (PP) of Sd1 isolates collected during two dysentery outbreaks (2013 and 445 cases of bloody diarrhoea) in Central African Republic (CAR) during the period 2003—2004 were reported. Eleven Sd1 comparison strains (CS) acquired by travellers or residents of Africa ($n = 10$) or Asia ($n = 1$) between 1993 and 2003 were also analysed. The 19 Sd1 isolates recovered from CAR outbreaks were multidrug resistant, although susceptible to quinolones and fluoroquinolones. Molecular subtyping by PFGE was more discriminatory than PP. The PFGE using *Xba*I and *Not*I restriction enzymes indicated that the two outbreaks were due to two different clones and also revealed a genetic diversity among the CS recovered from outbreak or sporadic cases between 1993 and 2003. This study was the result of a fruitful collaboration between field physicians and microbiologists. The data collected will serve as the basis for establishing long-term monitoring of Sd1 in CAR.

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1. Introduction

Among *Shigella* species, *Shigella dysenteriae* type 1 (Sd1) is of particular importance, because: (1) Sd1 produces the Shiga toxin and causes severe infections; (2) Sd1 is associated with large dysentery epidemics in developing countries; and (3) Sd1 strains isolated worldwide are often multidrug resistant (MDR), with plasmid-mediated resistance to commonly used antimicrobials such as ampicillin, tetracycline, chloramphenicol and sulfamethoxazole-trimethoprim, whereas additional resistance to quinolones, due to chromosomal mutation(s) is mainly observed in South Asia (Dutta et al., 2003b; Pazhani et al., 2004; Sarkar et al., 2003; Talukder et al., 2004; WHO, 2005).

In 1979, a major epidemic caused by MDR Sd1 started in North East Zaire (now Democratic Republic of Congo) and spread rapidly through Zaire, Rwanda, Burundi and Tanzania by 1981—1982 (Bogaerts et al., 1997; Ebright et al., 1984; Frost et al., 1981, 1985; Mhalu et al., 1984; Petat et al., 1987). Afterwards, other MDR Sd1 outbreaks were reported in Central, East, South and West African countries between 1983 and 2004 (Aragon et al., 1995; Cunin et al., 1999; Diallo et al., 2001; Engels et al., 1995; Gebre-Yohannes and Drasar, 1990; Goma Epidemiology Group, 1995; Guerin et al., 2003; Kariuki et al., 1996; Mudzamiri et al., 1996; Pillay et al., 1997; Ries et al., 1994; Tuttle et al., 1995; WHO, 2003, 2004, 2005).

In 2003—2004, two large outbreaks of dysentery occurred in two Préfectures (administrative subdivisions) of Central African Republic (CAR) (WHO, 2003) (Médecins Sans Frontières, unpublished results). CAR is a 623 000 km^2 Central African country with an estimated population of 3 285 000 inhabitants. Outbreak A occurred between July 2003 and February 2004 in the Préfecture of Ouaham-Pende in northwest CAR, about 450 km from the capital, Bangui (Figure 1) (WHO, 2003) (Médecins Sans Frontières, unpublished results). In total, 2013 cases of bloody diarrhoea were reported in the towns of Bozoum and Paoua and in villages along the 140-km-long road between these towns. Forty-one deaths attributed to dysentery were recorded during the outbreak period, giving an overall case fatality rate (CFR) of 2.04%. Outbreak B occurred between August and December 2004 in the Kaga-Bandoro area in the Préfecture of Nana-Grebizi in northern CAR, about 350 km from Bangui (Figure 1) (Médecins Sans Frontières, unpublished results). Outbreak B involved 445 cases of bloody diarrhoea. A total of 34 deaths (overall CFR 7.6%) attributed to dysentery were reported.

In this study, we report laboratory data of these two Sd1 dysentery outbreaks, which are to our knowledge the first outbreaks due to MDR Sd1 reported in CAR. Antimicrobial susceptibility testing and molecular subtyping by plasmid profiling (PP) and pulsed-field gel electrophoresis (PFGE) using enzymes *Xba*I and *Not*I were performed on 19 Sd1 strains isolated during these outbreaks. Eleven Sd1 comparison strains (CS) acquired by travellers or residents of Africa ($n = 10$) or Asia ($n = 1$) between 1993 and 2003 were also analysed.

2. Materials and methods

2.1. Sampling

The investigation of two large outbreaks of dysentery in CAR during the period 2003—2004 was conducted by the Médecins Sans Frontières (MSF) team based in Bangui, on behalf of the CAR Public Health Ministry (CPMH). The mobile teams, composed of personnel from MSF and CPMH, provided adapted hygiene advice, soaps and treatment, assessed water sources, and collected field data and stool samples. The stool samples were placed in Cary-Blair transport medium (Oxoid, Basingstoke, UK) and stored at 4 °C.

2.2. Bacterial strains

The stool samples were processed in the Clinical Microbiology Laboratory of the Institut Pasteur de Bangui on the following media: Hektoen (bioMérieux, Marcy l'Etoile, France), Bromcresol Purple agar plate (bioMérieux), Mac Conkey-Sorbitol (Becton-Dickinson, Sparks, MD, USA), Karmali (Oxoid) and Selenite medium (Bio-Rad, Marnes la Coquette, France) for enrichment. Sd1 strains were identified using API 20E strips (bioMérieux), and confirmed by

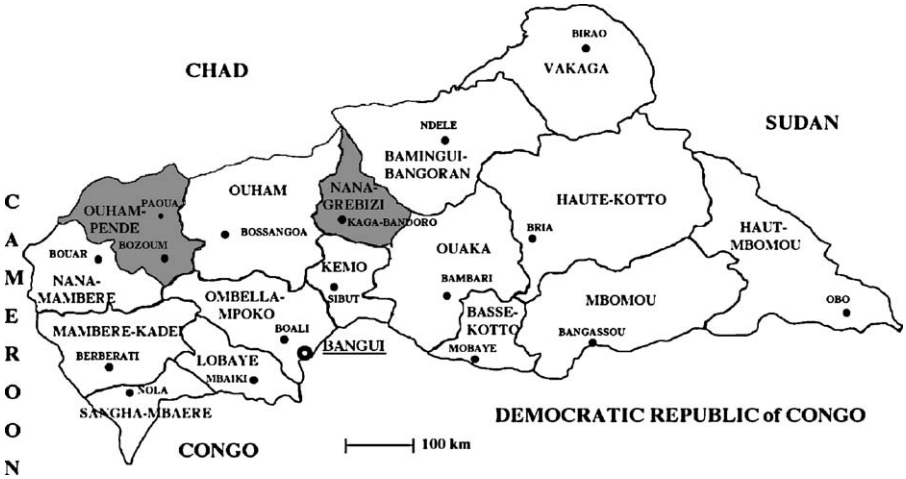

Figure 1 Areas affected by epidemic dysentery, Central African Republic, 2003—2004.

Table 1 Phenotypic and molecular characteristics of the *Shigella dysenteriae* type 1 (Sd1) isolates under study

Isolate	Date	Sex/age group[a]	Geographic origin (CAR Préfecture)[b]	Antimicrobial resistance pattern[c]	*Xba*I-PFGE type	*Not*I-PFGE type	Plasmid profile[d]
CAR Sd1 isolates							
1	Oct 03	M/IV	Bozoum (OP)	ASCSulTmpSxtTe	X1	N1	P5
2	Oct 03	M/II	Bozoum (OP)	ASCSulTmpSxtTe	X1	N1	P5
3	Oct 03	M/IV	Paoua (OP)	ASCSulTmpSxtTe	X4	N2	P5
4	Oct 03	F/IV	Paoua (OP)	ASCSulTmpSxtTe	X1	N1	P5
5	Feb 04	M/IV	Balaou (OP)	ACTmpTe	X2	N3	P4
6	Feb 04	M/IV	Taley (OP)	ASCSulTmpSxtTe	X3	N4	P5
7	Oct 04	F/IV	Dogo-Yalo (NG)	ASSpCTe	X5	N5	P4
8	Oct 04	M/III	Grevai (NG)	ASSpCTe	X5	N5	P4
9	Oct 04	M/IV	Kaga-Bandoro (NG)	ASSpCTe	X7	N7	P4
10	Oct 04	F/III	Kaga-Bandoro (NG)	ASSpCSulTmpSxtTe	X6	N6	P6
11	Nov 04	M/IV	Bangui	ASSpCTe	X7	N7	P4
12	Nov 04	F/III	Bangui	ASSpCSulTmpSxtTe	X6	N6	P6
13	Nov 04	F/III	Grevai (NG)	ASSpCTe	X5	N5	P4
14	Nov 04	F/IV	Yangoubanda (NG)	ASSpCTe	X5	N5	P4
15	Nov 04	F/IV	Yangoubanda (NG)	ASSpCTe	X5	N5	P4
16	Nov 04	F/III	Grevai (NG)	ASSpCTe	X5	N5	P4
17	Nov 04	M/II	Grevai (NG)	ASSpCTe	X5	N5	P4
18	Nov 04	M/IV	Grevai (NG)	ASSpCTe	X5	N5	P4
19	Dec 04	F/IV	Yangoubanda (NG)	ASSpCTe	X5	N5	P4
Sd1 comparison strains							
C1	Jan 93	F/II	Madagascar	ASSpCTmpTe	X8	N8	P9
C2	Aug 97	F/IV	Pakistan	ASSpCSulTmpSxtTe	X18	N17	P4
C3	Jul 99	M/IV	Mali	ACTmpTe	X9	N9	P8
C4	Apr 99	M/IV	Egypt	ASSpCTmpTe	X10	N10	P2
C5	Nov 99	M/IV	Senegal	ASCSulTmpSxtTe	X11	N11	UT
C6	Dec 99	F/IV	Mauritania	ACTmpTe	X12	N12	P7
C7	Nov 99	M/IV	Egypt	ASSpCTe	X13	N13	P3
C8	Aug 00	F/IV	Ivory Coast	ACTmpTe	X14	N14	P1
C9	Oct 00	M/IV	Senegal	ACTmpTe	X15	UT	UT
C10	Mar 01	F/IV	Burkina-Faso	ACTmpTe	X16	N15	P1
C11	May 03	F/IV	Niger	ACTmpTe	X17	N16	P4

UT: untypeable (lysis during PFGE or no plasmid detected for PP).
[a] Age group: I, <1 year; II, 1—5 years; III, 6—14 years; IV, 15—64 years; V, >65 years.
[b] Central African Republic (CAR) Préfectures: OP, Ouham-Pende; NG, Nana-Grebizi.
[c] Resistance patterns: A, amoxicillin; S, streptomycin; Sp, spectinomycin; C, chloramphenicol; Te, tetracycline; Sul, sulfonamides; Tmp, trimethroprim; Sxt, sulfamethoxazole-trimethoprim.
[d] Plasmid profiles are shown in Figure 3.

agglutination test using monospecific Sd1 serum (Bio-Rad). Isolates were sent to the French National Reference Center for *Escherichia coli* and *Shigella* (NRCECS), located at Institut Pasteur, Paris, France, for molecular typing studies. Eleven Sd1 strains used as CS were from the NRCECS collection (Table 1). Two CS, C1 and C5, were recovered during outbreaks in Madagascar in 1993 and in Senegal in 1999, respectively. The remaining CS were not known to come from outbreaks of dysentery.

2.3. Antimicrobial susceptibility testing

Antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton (MH) agar according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (Soussy et al., 2000). The following antimicrobials (Bio-Rad) were tested: amoxicillin (A), amoxicillin-clavulanic acid, ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cephalothin, cefamandole, cefoperazone, cefoxitin, ceftriaxone, ceftazidime, cefepime, aztreonam, moxalactam, imipenem, streptomycin (S), spectinomycin (Sp), kanamycin, tobramycin, netilmicin, gentamicin, amikacin, isepamicin, nalidixic acid (Nal), ofloxacin, ciprofloxacin (Cip), sulphonamides (Sul), trimethoprim (Tmp), sulfamethoxazole-trimethoprim (Sxt), chloramphenicol (C) and tetracycline (Te). *Escherichia coli* ATCC 25922 was used as a control.

2.4. Plasmid profiling

Plasmid DNA was purified from all the Sd1 isolates under study by an alkaline lysis procedure (Takahashi and Nagano,

1984) and subjected to 0.8% agarose gel electrophoresis. Molecular sizes of plasmids were estimated using Taxotron software (Institut Pasteur, Paris, France) by reference to the following plasmids of known sizes (RP4, 54 kb; pIP173, 126 kb; TP116, 215 kb) and to a supercoiled DNA ladder (Invitrogen, Groningen, The Netherlands).

2.5. Pulsed-field gel electrophoresis

PFGE using *Xba*I and *Not*I (Roche, Mannheim, Germany) was carried out with a CHEF-DR III system (Bio-Rad) as described previously (Weill et al., 2004). The running conditions were 6 V/cm at 14 °C for 20 h (22 h with *Not*I) with pulse times ramped from 2.2 to 63.8 s. *Salmonella enterica* serotype Braenderup H9812 was used as molecular size marker. Image normalization and construction of similarity matrices were carried out using BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Bands were assigned manually, and clustering was performed using the unweighted pair-group method with arithmetic averages (UPGMA) based on the Dice similarity index, utilizing an optimization parameter of 0% and a 0.5% band position tolerance. Each pro-

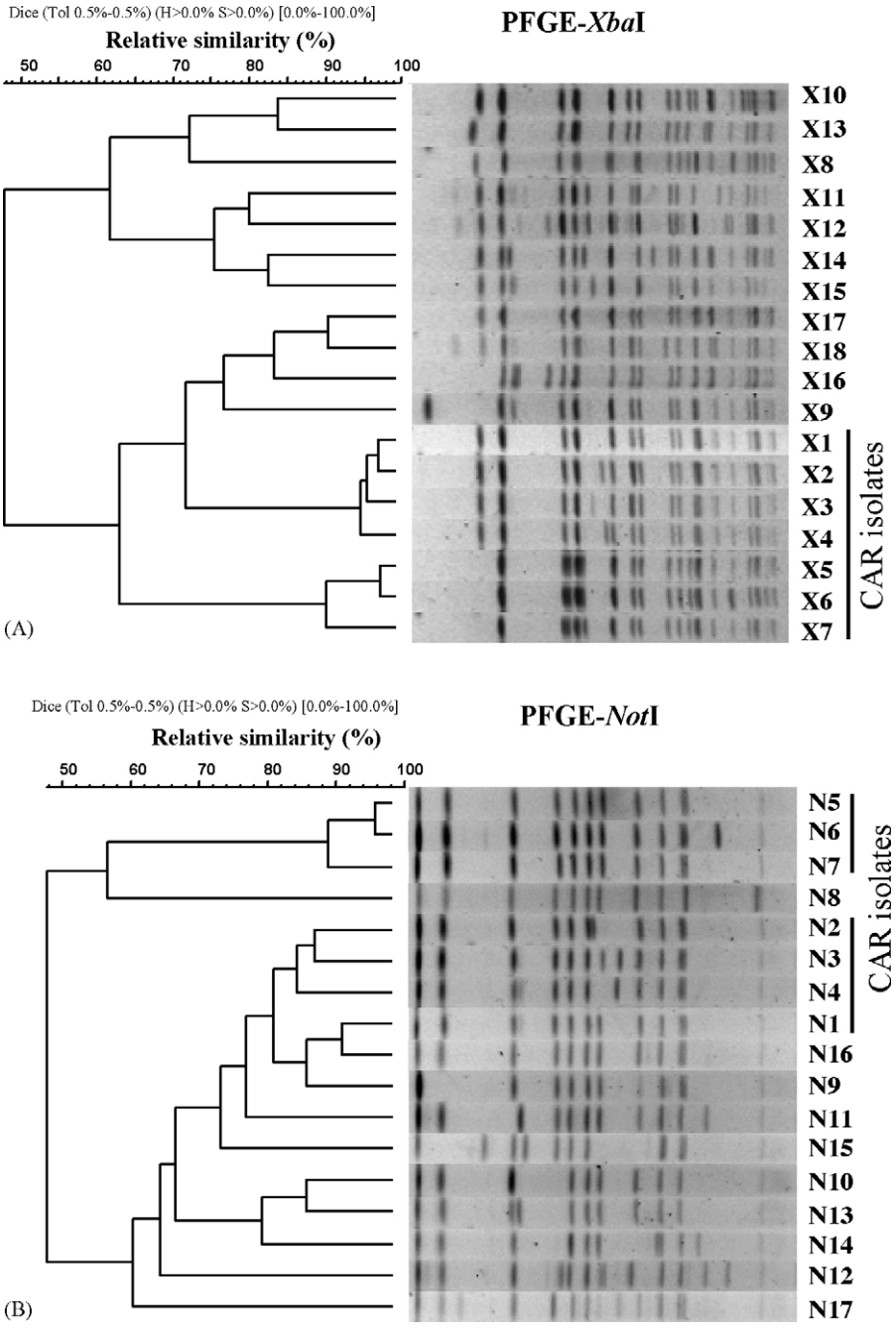

Figure 2 Dendrograms generated by BioNumerics, showing the results of cluster analysis of the 18 *Xba*I-PFGE profiles (A) and the 17 *Not*I-PFGE profiles (B) observed among the 30 *Shigella dysenteriae* type 1 isolates under study. Similarity analysis was performed using the Dice coefficient, and clustering was by unweighted pair-group method with arithmetic averages. CAR: Central African Republic.

file that differed by one or more bands was assigned a type.

3. Results

3.1. Sd1 isolates

Six Sd1 strains were isolated at Institut Pasteur de Bangui during outbreak A (among 47 specimens collected) and 11 during outbreak B (among 48 specimens collected). Two Sd1 isolates recovered from sporadic dysentery cases in Bangui during the time of outbreak B were also included in the study. Table 1 shows details of the 19 Sd1 isolates and the 11 CS included in this study.

3.2. Antimicrobial susceptibility testing of Sd1 isolates

All 30 Sd1 strains included in the study were resistant to A, Te and C by disk diffusion. All strains were susceptible to extended-spectrum cephalosporins, aminoglycosides, quinolones (Nal) and fluoroquinolones (ofloxacin and Cip). Six different antibiotic resistance phenotypes (R-types) were observed (Table 1). ASCSulTmpSxtTe R-type was found in five of the six CAR Sd1 isolates collected from October 2003 to February 2004. ASSpCTe R-type was found in 11 of the 13 CAR Sd1 isolates collected from October to December 2004. ACTeTmp R-type was predominant among the CS (6/11, 55%).

3.3. Pulsed-field gel electrophoresis

The clonal relatedness of the 19 Sd1 isolates from CAR and the 11 Sd1 CS acquired by travellers or residents of Africa ($n = 10$) or Asia ($n = 1$), was assessed by PFGE using *Xba*I and *Not*I (Table 1 and Figure 2).

Seven distinct *Xba*I profiles were observed among the 19 Sd1 isolates from CAR and 11 profiles among the CS. *Xba*I-PFGE profiles from CAR isolates recovered during outbreak A (X1 to X4) belonged to one cluster, with a genetic similarity of about 95%, while those from isolates recovered during outbreak B (X5 to X7) were grouped in a second cluster, with a genetic similarity of about 90%. All CS displayed distinct *Xba*I-PFGE profiles. However, profiles X9 and X16—X18 observed in four CS were grouped close to the cluster of profiles obtained from CAR isolates recovered during outbreak A (similarity percentage of about 77%).

By using a second restriction enzyme, *Not*I, seven distinct profiles were observed among the 19 CAR isolates and 10 among the 10 typeable CS. *Not*I-PFGE profiles from CAR Sd1 isolates were also grouped into two separate clusters. PFGE profiles (N1—N4) from isolates recovered during outbreak A belonged to a cluster with a genetic similarity of about 81%, while those (N5—N7) from isolates recovered during outbreak B were grouped in a cluster with a genetic similarity of about 89%. *Not*I-PFGE profiles from CS were unique. However, profiles N16 and N9 observed in two CS acquired in Mali (1999) and Niger (2003) belonged to the same cluster of profiles obtained from CAR isolates recovered during outbreak A. Results from *Xba*I- and *Not*I-PFGE were concordant and clearly indicated that the isolates collected during outbreak A were unrelated to those collected during outbreak B. During outbreak A, combined profile X1/N1 was found in three of the six isolates collected, while other isolates displayed closely related combined profiles: X4/N2, X2/N3 and X3/N4. Among the isolates collected during outbreak B, combined profile X5/N5 was found in 81.8% of the isolates (9/11), while the remaining isolates displayed closely related combined profiles: X7/N7 and X6/N6, also seen in two isolates recovered from sporadic cases in Bangui in November 2004. We do not know if the two patients had travelled to the outbreak area or if they were in contact with a person coming from the Kaga-Bandoro area.

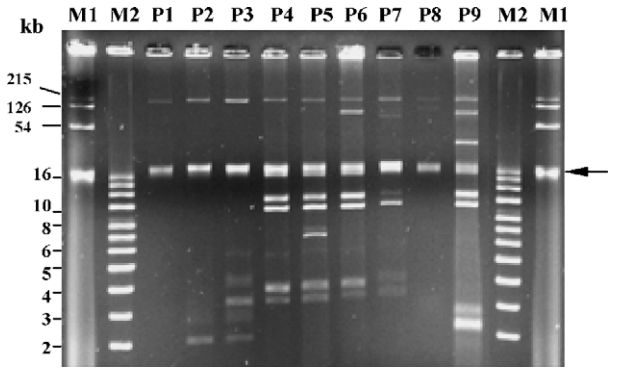

Figure 3 Representative plasmid profiles (P1 to P9) obtained from the 28 typeable *Shigella dysenteriae* type 1 isolates under study. Chromosome position is indicated by an arrowhead. M1 and M2: molecular size markers (band sizes in kilobase pairs); M1: mix of RP4 (54 kb), pIP173 (126 kb) and TP116 (215 kb); M2: supercoiled DNA ladder (Invitrogen).

3.4. Plasmid profiling

Nine plasmid profiles were seen among the 19 CAR Sd1 isolates and the nine typeable CS (Table 1). Plasmid profiles (P1—P9) are shown in Figure 3. Each typeable Sd1 strain had a large plasmid, approximately 200 kb in size. Other plasmids of various sizes (2 to 120 kb) were also detected, depending on the pattern. Profile P5 was seen in five of the six isolates recovered during outbreak A. None of the CS exhibited profile P5. Profile P4 (lacking a 7-kb plasmid compared with P5) was the most frequently encountered profile in the isolates of outbreak B (10/11). It was also observed in one isolate from outbreak A and in two CS.

Profile P6 (with an additional 95-kb plasmid compared with P5) was observed in isolate 10 included in the second outbreak and isolate 12 recovered in Bangui. Both isolates shared PFGE combined profile X6/N6 and ASSpCSuTmpSxtTe R-type. Seven profiles (P1—P4, P7—P9) were encountered among the nine typeable CS.

4. Discussion

These two outbreaks are the first epidemics caused by Sd1 reported in CAR recently. They occurred in a country with extremely poor economic and sanitary conditions, particularly in these remote Préfectures, where water supply and

sanitation facilities were almost non-existent and health care facilities often inoperative because of political instability. Furthermore, a large number of internally displaced persons lived in both regions since the October 2002 to March 2003 rebellion.

The low proportion of Sd1 isolates recovered from stool specimens (12.8 and 22.9% of the samples were positive for Sd1 during outbreaks A and B, respectively) is possibly due to a longer than optimal storage duration. *Shigella*, and especially Sd1, are fragile organisms, and the yield decreases after the first 2 days of storage (WHO, 2005). In our investigation, there was an average time lag of 1 week between collection of the samples in the affected areas and their inoculation at Institut Pasteur de Bangui. The mobile teams were in the field for 10 to 15 d at a time; and at the end of their assignment, they had to drive 10 to 14 h from these remote Préfectures to Bangui, due to the poor quality of the roads (especially during the rainy season between May and October). It would be very interesting to develop a small mobile laboratory as an alternative solution to the problems encountered with sample transport.

Plasmid profiling was the unique molecular subtyping method used for Sd1 isolates in the past (Frost et al., 1981, 1985; Haider et al., 1988; Gebre-Yohannes and Drasar, 1990). There were several limitations to this method due, in particular, to the instability of plasmid DNA, with possible acquisition or loss of one or more plasmids by an isolate. Since 1990, chromosomal DNA fingerprinting techniques have been developed, and it is now well established that PFGE using restriction enzymes *Xba*I and/or *Not*I is the method of choice for molecular subtyping of Sd1 (Dutta et al., 2003a; Kariuki et al., 1996; Pazhani et al., 2004; Soldati and Piffaretti, 1991; Talukder et al., 1999). Furthermore, PulseNet's standardized protocol for PFGE of food-borne bacterial pathogens is now widely used, facilitating international comparisons (Hunter et al., 2005).

In the present study, we used *Xba*I and *Not*I-PFGE (with running conditions and a molecular size marker similar to PulseNet's standardized protocol, thus facilitating international comparisons) and PP. The CAR Sd1 isolates subtyped by PFGE were found to be different from the CS isolated in Africa from 1993 to 2003, while some of the plasmid profiles were similar. This indicates that PFGE was more discriminatory than PP. This study is, to our knowledge, the first study that used comprehensive molecular subtyping methods to assess genetic relatedness of Sd1 isolates from different countries in Africa. PFGE analysis indicated that there is a genetic diversity among African Sd1 isolates. The different reports dealing with Sd1 outbreaks in Africa suggest that the continent has been experiencing an Sd1 pandemic, which began in 1979 in Zaire (Ebright et al., 1984; Frost et al., 1985; Kariuki et al., 1996). However, only plasmid profiling has been done to support this statement. The 11 CS used for the present study originated mostly from West Africa, and it would have been very interesting to perform PFGE on earlier isolates from Central African outbreaks to check if the Sd1 isolates from the 'pandemic' are linked. This genetic diversity has even been observed in subsequent outbreaks in the same country; such examples are the CAR, which experienced two outbreaks in a 1-year period, and Senegal with two isolates (C5 and C9), also collected during a 1-year period. As observed in our study, even during an outbreak some isolates displayed different but closely related PFGE profiles. This suggests that some closely related clones may have arisen from the original clone during the outbreak. A large outbreak affecting several hundreds or thousands of people may facilitate chromosomal events in bacteria that would lead to the emergence of some such closely related clones. The diversity of the PFGE profiles in the CAR outbreak A, which affected 2013 patients (four combined PFGE patterns among six isolates recovered), was higher than those of outbreak B, affecting 445 patients (three combined PFGE patterns among 11 isolates recovered).

The Sd1 CAR isolates were resistant to most common antibiotics, as reported worldwide since 1972, when MDR isolates with additional resistance to ampicillin had been detected in Mexico (Olarte et al., 1976). Both the Sd1 CAR isolates tested, as well as the 11 CS, were susceptible to quinolones (Nal) and fluoroquinolones (ofloxacin and Cip). Nal-resistant Sd1 isolates were reported in Central and Southern Africa (Rwanda since 1985 and Burundi since 1981, Zaire in 1994, South Africa in 1995) soon after the introduction of Nal as treatment for dysentery (Bogaerts et al., 1997; Karas and Pillay, 1996; Ries et al., 1994). Susceptibility to Nal in Sd1 isolates from CAR can be explained by the lack of Sd1 outbreaks in the past and the fact that Nal was neither distributed, nor used, in this country, which is one of the poorest in the world. Resistance to Nal is the first step towards resistance to Cip, which is now recommended by WHO as first-line treatment for all patients, adults and children during Sd1 outbreaks (WHO, 2005). Due to a recent introduction of a generic version of Cip in CAR (2003), it is very important that we continue monitoring antimicrobial susceptibility to quinolones by screening isolates with Nal (either by disk diffusion or by minimal inhibitory concentration [MIC] determination) and, if resistant, by performing MIC to Cip.

Antimicrobial susceptibility testing may also be very helpful for the field bacteriologist as a phenotypic subtyping method when molecular techniques are not available. In this study, the different antimicrobial resistance phenotypes were in accord with PFGE profiles. Use of several antimicrobial disks (including antibiotics used for therapeutic purposes and others used only for phenotypic expression of resistance mechanisms) allowed us to identify different resistance profiles in CAR Sd1 and then to further distinguish the isolates. Thus, aminoglycoside Sp was useful in differentiating the isolates from outbreak A (resistant to Sp) from those from outbreak B (susceptible to Sp). In the same way, concomitant use of Sul, Tmp and Sxt disks allowed us to identify three resistance profiles (susceptibility to all the three antibiotics, resistance to Tmp alone or resistance to all the three antibiotics) instead of the two (susceptibility or resistance to Sxt) that we would have obtained if we had used the Sxt disk alone. However, two isolates (isolate 5 during outbreak A and isolate 10 during outbreak B) exhibited different antibiotic resistance from those collected during the same outbreak. This might be due to the acquisition (isolate 10, plasmid profile 6) or the loss (isolate 5, plasmid profile 4) of a plasmid carrying resistance gene(s). For a better characterization of the mechanisms of resistance to antibiotics (genes involved, plasmidic or chromosomal location etc.) in Sd1 isolates of the present work, a genetic study is ongoing.

In conclusion, our study was the result of a fruitful collaboration between field physicians, epidemiologists and microbiologists. The data collected will serve as the basis for establishing long-term monitoring of Sd1 in CAR, but also, if necessary, in neighbouring countries.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

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