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Klebsiella pneumoniae resistant to third-generation cephalosporins in five African and two Vietnamese major towns: multiclonal population structure with two major international clonal groups, CG15 and CG258

S. Breurec[1], N. Guessennd [2], M. Timinouni[3], T. A. H. Le[4], V. Cao[5], A. Ngandjio[6], F. Randrianirina[7], J. M. Thiberge[8], A. Kinana[1], A. Dufougeray[1], J. D. Perrier-Gros-Claude[3], P. Boisier[6], B. Garin[1,7] and S. Brisse[8]

1) Institut Pasteur, Dakar, Senegal, 2) Institut Pasteur, Abidjan, Côte d'Ivoire, 3) Institut Pasteur, Casablanca, Morocco, 4) Institut National d'Hygiène et d'Épidémiologie, Hanoi, Vietnam, 5) Institut Pasteur, Ho Chi Minh City, Vietnam, 6) Centre Pasteur, Yaounde, Cameroon, 7) Institut Pasteur, Antananarivo, Madagascar and 8) Institut Pasteur, Genotyping of Pathogens and Public Health, Paris, France

Abstract

The molecular epidemiology of third-generation cephalosporin-resistant (3GC-R) *Klebsiella pneumoniae* in developing countries is poorly documented. From February 2007 to March 2008, we collected 135 3GC-R *K. pneumoniae* isolates from seven major towns in Maghreb (Morocco), West Africa (Senegal, Côte d'Ivoire), Central Africa (Cameroon), East Africa (Madagascar) and Southeast Asia (Vietnam). Their genetic diversity, assessed by multilocus sequence typing, was high (60 sequence types), reflecting multiclonality. However, two major clonal groups, CG15 (n = 23, 17% of isolates) and CG258 (n = 18, 13%), were detected in almost all participating centres. The two major clonal groups have previously been described in other parts of the world, indicating their global spread. The high diversity of enterobacterial repetitive intergenic consensus sequence-PCR banding patterns at the local level indicates that most isolates were epidemiologically unrelated. The isolates were characterized by the presence of multiple resistance determinants, most notably the concomitant presence of the *aac(6′)-Ib-cr*, *qnr* and $bla_{\text{CTX-M-15}}$ genes in 61 isolates (45%) belonging to 31 sequence types. These isolates were detected across a large geographical area including Cameroon (n = 1), Vietnam (n = 4), Madagascar (n = 10), Côte d'Ivoire (n = 12), Morocco (n = 13) and Senegal (n = 21). These results have major implications for patient management and highlight a potential reservoir for resistance determinants.

Keywords: Africa, antimicrobial resistance, CG15, CG258, CTX-M-15, epidemiology, extended-spectrum β-lactamase, *Klebsiella pneumoniae*, multilocus sequence typing, Vietnam
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Corresponding author: S. Breurec, Unité de Biologie Médicale et Environnementale, Institut Pasteur, 36 avenue Pasteur, BP 220, Dakar, Senegal
E-mail: sbreurec@pasteur.sn

Introduction

Third-generation cephalosporin-resistant (3GC-R) *Enterobacteriaceae* have spread throughout the world, initially in the hospital setting and more recently in the community. This resistance is mainly mediated by acquired extended-spectrum β-lactamase (ESBL) genes located on mobile genetic elements such as plasmids or transposons [1]. This situation is of great concern, as ESBL enzymes can hydrolyse almost all β-lactams (except for carbapenems and cephamycins), and are frequently associated with resistance genes against antimicrobial agents of multiple families.

The ESBL-producing *Klebsiella pneumoniae* constitute one of the most common multidrug-resistant groups of gram-negative bacteria worldwide. CTX-M has emerged as the dominant ESBL family in *K. pneumoniae* strains, although TEM and SHV enzymes as well as members of the other three Ambler classes of β-lactamases (B, C and D) are also common. Molecular studies of ESBL isolates using multilocus sequence typing have shown that the spread of ESBL-producing *K. pneumoniae* is

largely multiclonal, contrary to KPC-producing isolates of this species, which mainly belong to sequence type (ST) 258 or related STs [2]. Few data on the population diversity of *K. pneumoniae* strains and the antibiotic resistance genes they harbour are available for Africa [3] and Southeast Asia [4]. This information is important for understanding the global spread of multidrug-resistant *K. pneumoniae*.

Here, we report the molecular characterization of 3GC-R *K. pneumoniae* isolates collected in five African and two Vietnamese major towns by means of multilocus sequence typing and antimicrobial-resistance gene profiling.

Materials and Methods

Study population

Between February 2007 and March 2008, 135 non-duplicate 3GC-R *K. pneumoniae* were collected in 14 major health institutions (including ten hospitals) located in five major African towns, namely Abidjan (Côte d'Ivoire), Antananarivo (Madagascar), Casablanca (Morocco), Dakar (Senegal) and Yaounde (Cameroon), and in two major towns in Vietnam (Southeast Asia), namely Ho Chi Minh City and Hanoi. Written informed consent was obtained from all the patients and the study protocol was approved by local national ethics committees.

Microbiological analysis and DNA extraction

The isolates were identified with the API-20E system (bioMérieux, Marcy L'Etoile, France) and confirmed by sequence analysis of the *rpoB* gene coding for the RNA polymerase β-subunit, as previously described [5]. Genomic DNA was extracted with the QIAmp™ kit (Qiagen, Courtaboeuf, France).

Antimicrobial susceptibility

Susceptibility to amoxicillin, ticarcillin, cefalotin, cefoxitin, cefotaxim, ceftazidim, imipenem, gentamicin, tobramycin, kanamycin, amikacin, trimethoprim–sulphamethoxazol, ciprofloxacin and fosfomycin was determined by the disc diffusion method on Mueller–Hinton agar (Bio-Rad, Marnes La Coquette, France), according to the guidelines of the French Society of Microbiology. Production of ESBL was detected by the double-disc synergy test with an amoxicillin–clavulanic acid disc surrounded at a radius of 20 mm by cefotaxim, ceftazidim and aztreonam discs. External quality control was ensured by the French National Antibiotic Reference Centre (Institut Pasteur, Paris, France).

Detection of β-lactamase and plasmid-mediated quinolone resistance genes

Previously described PCR methods were used to screen for genes encoding carbapenem-hydrolysing β-lactamases (bla_{VIM}, bla_{IMP} and bla_{KPC}), as well as plasmid-encoded bla_{CTX-M}, bla_{AmpC}, bla_{SHV}, bla_{TEM}, bla_{GES}, bla_{VEB}, β-lactamase genes, the *aac(6′)-Ib* gene, and the quinolone resistance *qnr*A/B/S/C/D and *qepA* genes [6–9]. Both bla_{CTX-M} and bla_{SHV} were then characterized by direct DNA sequencing of the PCR products, as was bla_{TEM} for bla_{CTX-M} and ESBL bla_{SHV} gene negative strains. All *aac(6′)-Ib*-positive strains were further analysed by digestion of the PCR product with BtsCI (New England Biolabs, Ipswich, MA, USA) to identify *aac(6′)-Ib-cr*, which lacks the BtsCI restriction site present in the wild-type gene [10]. Isolates resistant to cefoxitin were tested for the presence of six families of plasmid-borne *ampC* genes (FOX, ACC, EBC, MOX, CIT, DHA) [11] and bla_{CMY-2}.

Multilocus sequence typing and enterobacterial repetitive intergenic consensus sequence-PCR

Multilocus sequence typing was performed as described elsewhere [5,12]. In order to assess the degree of clonal relationships between isolates at local level, enterobacterial repetitive intergenic consensus sequence (ERIC) -PCR was performed on strains assigned to the same ST isolated in the same town. Strains with an identical ERIC-PCR banding pattern were defined as related.

Results and Discussion

Bacterial strains and antimicrobial susceptibility

A total of 135 3GC-R *K. pneumoniae* isolates were collected during the study period. Twelve isolates (9%) were recovered in Antananarivo (Madagascar), 13 (10%) in Yaounde (Cameroon), 17 (13%) in Casablanca (Morocco), 22 (16%) in Abidjan (Côte d'Ivoire), 31 (23%) in Vietnam (15 in Ho Chi Minh City and 16 in Hanoi) and 40 (30%) in Dakar (Senegal; Fig. 1). Forty-six isolates (34%) were associated with urinary/genital tract infections, 44 (32%) with wound infections, 31 (23%) with bacteraemia/septicaemia, 13 (10%) with pulmonary infection and one with diarrhoea. No significant association was observed between the genetic background of the isolates (see next section) and the type of infection (data not shown). All *K. pneumoniae* isolates were resistant to all the β-lactams tested, except for cefoxitin (7% resistance, n = 10), and all were susceptible to imipenem. In addition, they were characterized by high rates of resistance to tobramycin (99%), gentamicin (84%), ciprofloxacin (87%) and cotrimoxazole (90%), and high rates of susceptibility to amikacin (64%), kanamycin (71%) and fosfomycin (99%). The double-disc synergy test was positive only for strains susceptible to cefoxitin.

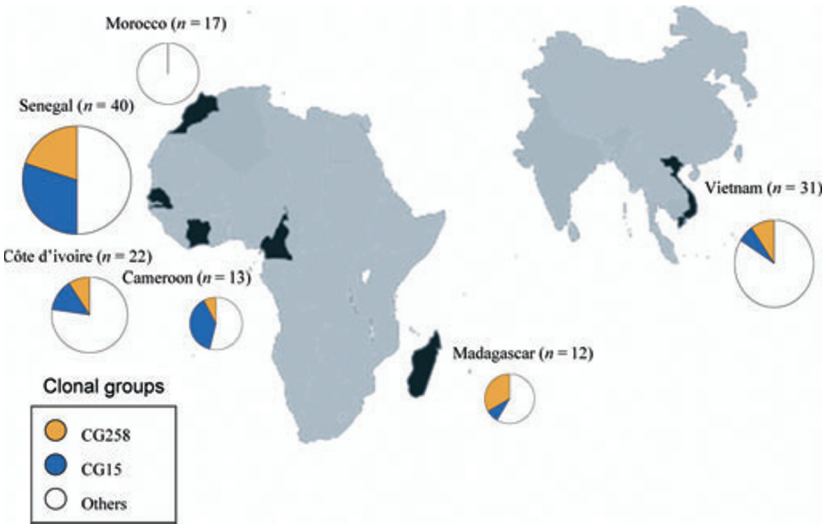

FIG. 1. Distribution of third-generation cephalosporin-resistant *Klebsiella pneumoniae* in five African and two Vietnamese major towns. The proportions of clonal groups at each sampling location are displayed as pie charts, with size indicating the number of isolates.

Genetic background of *K. pneumoniae* isolates and identification of two widespread clonal groups

The 135 *K. pneumoniae* isolates belonged to 60 STs (see Supplementary material, Table S1), in keeping with the high genotypic diversity of this species [5,12]. To define groups of related STs, we first defined clonal complexes (CC) using the most stringent criterion, i.e. as groups of STs differing by a single allelic mismatch relative to at least one other member of the group. Singletons were defined as STs that were not grouped into CCs, i.e. differing by at least two allelic mismatches from any other ST. As the composition and distinctness of CCs is highly dependent on the sample, we attempted to link the CCs identified in the present study with those found in the global multilocus sequence typing database (http://www.pasteur.fr/mlst), that contained 650 STs (as of 12 September 2011). This large dataset resulted in the identification of 62 CCs and 302 singletons. However, the largest CC contained 139 STs, in a heterogeneous and straggly CC, probably resulting from the merging of unrelated STs because of the high rate of homologous recombination [12,13], rather than from diversification from a single common ancestor. Therefore, to define epidemiologically more meaningful groups of STs, we defined clonal groups (CGs) as subsets of this large CC that included (i) one central genotype; (ii) its single-locus variants (first circle SLVs); and (iii) the SLVs of the first-circle SLVs. The CGs were named according to the central ST that was chosen for their definition (e.g. CG258 includes ST258 as its central genotype, plus the SLVs of ST258, plus their SLVs).

Using this definition, the 60 identified STs were assigned to 25 CGs and 20 singletons (see Supplementary material, Table S1), in keeping with the previously described multiclonal spread of ESBL isolates. Only two CGs included more than six strains: CG15 (n = 23, 17%) and CG258 (n = 18, 13%; see Supplementary material,Table S1; Fig. 1). Other frequent groups of isolates were CG394 (n = 5), CG395 (n = 5), CG17 (n = 6), CG42 (n = 6) and one singleton, ST70 (n = 6).

The two major CGs recovered in our study shared significant features (Table 1): (i) detection in at least five participating centres, indicating their wide geographical distribution; (ii) high diversity of ERIC-PCR banding patterns at the local level, indicating that most of the strains were epidemiologically unrelated; (iii) high prevalence of the CTX-M-15 β-lactamase gene (83% for CG15 and 60% for CG258); and (iv) high rate of resistance to most of the antibiotics tested, apart from fosfomycin for both CGs (4% for CG15 and 5% for CG258) and amikacin (22%) and kanamycin (5%) for CG258.

The first major clonal group, CG15, was composed solely of ST15 isolates, plus a single ST14 isolate. ST15 is a widespread ST previously described in Europe (Denmark and Hungary) [14,15] and Asia (Korea, Malaysia, Singapore and Taiwan) [4]. It was detected in five of the seven towns in the present study, namely Antananarivo (Madagascar, n = 1), Abidjan (n = 3, two unrelated strains) and Dakar (n = 11, six unrelated strains) in West Africa; Yaounde (n = 5, four unrelated strains) in Central Africa; and Hanoi (two unrelated strains) in Southeast Asia (Fig. 1). It was dominant in Dakar (28% of all isolates) and Yaounde (38%; Table 1).

The second major clonal group, CG258, comprised isolates belonging mainly to ST340 (n = 11, 61%) and ST11

TABLE 1. Molecular characteristics of third-generation cephalosporin-resistant *Klebsiella pneumoniae* isolates collected in five African and two Vietnamese major towns. Only clonal groups containing more than four strains are shown.

City (*n*)	CG	ST (*n*)	ERIC-PCR[a]	CTX-M	ESBL-SHV	Non-ESBL SHV	Plasmid-borne AmpC β-lactamases[b]	Non-ESBL Tem[c]	Molecular resistance to C3G (*n*)[d]	PMQR determinants[b]
Abidjan (22)	15	15 (3)	2	CTX-M-15 (18)		SHV-1 (6), -32 (3), -11 (1)	DHA-1 (1)	Tem-1 (1)	18	QnrA (2), QnrB (18), QnrS (3), AAC(6′)-Ibcr (14)
	258	11 (2)	2							
	Others	(17)								
Antananarivo (12)	15	15 (1)	1	CTX-M-15 (10)		SHV-11 (2)			10	QnrB (12), AAC(6′)-Ibcr (12)
	258	340 (4)	3							
	Others	(7)								
Casablanca (17)	Others	(17)		CTX-M-15 (14)	SHV-27 (1)	SHV-1 (5), -11 (4),	DHA-1 (2)	Tem-1 (2)	15	QnrA (1), QnrB (5), QnrS (11) AAC(6′)-Ibcr (16)
Dakar (40)	15	14 (1)	1	CTX-M-15 (37),	SHV-2 (1)	SHV-1 (17), -11 (15),	CMY-2 (3)	Tem-1 (2)	38	QnrA (1), QnrB (29), QnrS (1), AAC(6′)-Ibcr (29)
		15 (11)	6	-3 (1), -8 (1)		-75 (1), -93 (1)				
	258	270 (1)	1							
		340 (6)	5							
		407 (1)	1							
	Others	(20)								
Hanoï (16)	15	15 (2)	2	CTX-M-15 (6),		SHV-1 (3)	DHA-1 (4)		14	QnrA (1) QnrB (3), QnrS (12) AAC(6′)-Ibcr (3)
	258	11 (1)	1	-14 (6), -27 (3)						
	Others	(13)								
Ho Chi Minh City (15)	258	11 (1)	1	CTX-M-15 (5),	SHV-5 (1),	SHV-1 (8),-11 (6)			13	QnrB (2), QnrS (14), QepA (1) AAC(6′)-Ibcr (5)
	Others	(14)		-27 (5), -14 (2)	-2 (1)					
Yaounde (13)	15	15 (5)	4	CTX-M-15 (10)	SHV-5 (2),	SHV-1 (5), -2 (1),		Tem-1 (2)	10	QnrB (4), QnrS (1), AAC(6′)-Ibcr (5)
	258	11 (1)	1		-27 (1)	-11 (5),				
	Others	(7)								
Total (135)				CTX-M-15 (100), -14 (8), -27 (8), -3 (1), -8 (1)	SHV-5 (3), -27 (2), -2 (2)	SHV-1 (44), -11 (33), -32 (3), -75 (1), -93 (1)	DHA-1 (7), CMY-2 (3)		118	QnrA (5), QnrB (73), QnrS (42), AAC(6′)-Ibcr (83), QepA (1)

[a]Unrelated strains using enterobacterial repetitive intergenic consensus sequence (ERIC) -PCR typing.
[b]VEB, GES, KPC, PER, QnrC and QnrD were not found in any of the isolates.
[c]TEM typing was performed on strains both CTX-M-negative and ESBL-SHV-negative.
[d]Strains with CTX-M and/or ESBL-SHV and/or Plasmid-borne AmpC β-lactamases.
CG, clonal group; ST, sequence type; ESBL, extended-spectrum β-lactamase; PMQR, plasmid-mediated quinolone resistance.

(*n* = 5, 28%). It was detected in all the participating centres, apart from Casablanca, and was the dominant clone in Antananarivo (33%). Interestingly, ST258, the central genotype of CG258, which has been reported in the Americas, Europe and Asia, was not found in our study [4,16,17]. In contrast, ST340, found in Antananarivo (four strains, of which three were unrelated), Dakar (six strains, of which five were unrelated) and Hanoi (*n* = 1), has rarely been described elsewhere [4]. ST11 was detected at four sites in our study, namely Abidjan (two unrelated strains), Yaounde (*n* = 1), Hanoi (*n* = 1) and Ho Chi Minh City (*n* = 1). This ST is currently prevalent in China, Korea [18] and Hungary [14] and has also been detected in Brazil [19] and in several countries in Europe [17] and Asia [4].

Clonal group CG258 is a major cause of multidrug-resistant *K. pneumoniae* infections worldwide, but its evolutionary emergence is poorly documented. ST11 and ST340 differ from each other and from ST258 by only a few (two to four) nucleotide changes in their *tonB* alleles. First isolated in 2008 [16], ST258 has almost always exhibited carbapenem resistance, to the best of our knowledge, whereas ST11, isolated as early as 1997, has been associated with ESBL production but rarely with carbapenem resistance. Furthermore, the *tonB* allele of ST258 (*tonB*-79) is only observed in six CG258 STs that are closely related to ST258 (including ST340) and that were identified recently, whereas the *tonB* allele of ST11 (*tonB*-4) is widely distributed in 76 unrelated STs that include strains isolated over several decades (http://www.pasteur.fr/mlst). We infer that ST258 probably arose from ST11 by acquisition of the *tonB*-79 allele, followed by acquisition of carbapenem-resistance genes on mobile elements. More sampling and higher-resolution genotyping are necessary to retrace the emergence of this clonal group.

The remaining CGs and singletons identified herein have previously been detected sporadically in other parts of the world. Interestingly, we identified four strains belonging to ST23 (CC23; one in Vietnam, and two in Madagascar, of which two were unrelated) associated with surgical site infections. This ST belongs to a well-known clone that causes liver abscesses, mainly in Asia but also in Europe and Africa [12,20,21].

Resistance determinants

CTX-M-15 was detected in 100 (74%) of the 135 isolates tested, belonging to 42 of the 60 STs. It was detected at all sites and was the dominant ESBL among the African isolates (Table 1). CTX-M-14 and CTX-M-27 were also detected in strains from Vietnam, in keeping with a previous report [22]. CTX-M-15 ESBL, first detected in 1999 in India, is currently recognized as the most widely distributed CTX-M β-lactamase [1], even on the African continent [3]. Of the 89 SHV-producing *K. pneumoniae* isolates, only seven strains were ESBL-SHV-positive: SHV-5 (*n* = 3), SHV-27 (*n* = 2) and SHV-2 (*n* = 2), with two strains each harbouring SHV-5 and CTX-M-15. TEM typing was only performed on seven strains that were both CTX-M-negative and ESBL-SHV-negative, and all expressed the non-ESBL TEM-1. Among the ten strains that were resistant to cefoxitin, seven were positive for DHA-1 (one in Abidjan, two in Casablanca and four in Hanoi) and three for CMY-2 (Dakar). In all, 17 (13%) strains resistant to third-generation cephalosporins with a positive double-disc synergy test were negative for the tested ESBL, suggesting the presence of ESBL OXA or unknown β-lactamases.

The *aac(6′)-Ib* gene was detected in 95 (70%) of the 135 isolates, of which 84 (88%) were the *cr* variant. The *qnr* gene was present in 115 isolates (85%) and *qepA* in one isolate from Ho Chi Minh City (ST244). Seventy-one (97%) of the 73 strains carrying both *qnr* and *aac(6′)-Ib-cr* were resistant to ciprofloxacin. This combination was frequent in all sites, which is a major concern because these two mechanisms act additively. Among the 115 *qnr*-positive strains, 73 were *qnrB*, 41 were *qnrS* and five were *qnrA*, with four strains each harbouring two *qnr* genes. The *qnr*B was predominant among strains from Africa (apart from Casablanca) and *qnrS* was predominant among strains from Vietnam. Genes *qnrC* and *qnrD* were not detected.

Sixty-one (45%) isolates, belonging to 31 (52%) of the 60 STs, carried the *aac(6′)-Ib-cr*, *qnr* and $bla_{\text{CTX-M-15}}$ genes. They were detected over a large geographical area, including Ho Chi Minh City (*n* = 4), Yaounde (*n* = 1), Antananarivo (*n* = 10), Casablanca (*n* = 13), Abidjan (*n* = 12) and Dakar (*n* = 21).

Reservoir for ESBL and other resistance determinants

A worrying prevalence of ESBL-producing *Enterobacteriaceae* has been observed in all developing countries for which data are available. For example, ESBL producers accounted for 5.2% of community-acquired urinary tract infections caused by *Escherichia coli* in Senegal [23], 19.3% of *Enterobacteriaceae* in the Central African Republic [24], 32.9% of *Enterobacteriaceae* in Tanzania [25], 26.9% of *Enterobacteriaceae* in India [26] and 36.6% of *E. coli* in Cambodia [27]. In addition, as carbapenems are often the most effective antibiotics used against 3GC-R *Enterobacteriaceae*, the emergence of carbapenem resistance, a phenomenon that has been described in Africa [28], but that was not found in our study, could become a serious challenge for infection control and antibiotic therapy in the future.

The potential role of *K. pneumoniae* and other *Enterobacteriaceae* as a reservoir for ESBL genes and other resistance determinants is a clear cause of concern in countries with

inadequate healthcare systems. Key factors that favour the spread of antimicrobial resistance in these countries include uncontrolled consumption of antimicrobial agents through self-medication, inappropriate antibiotic prescription, the substandard quality of some drugs, and a lack of effective measures to prevent nosocomial infections. The virtual lack of physical barriers between the community and hospital settings in these countries may be an important contributory factor, along with poor living conditions [29].

Conclusions

This study shows that even though the spread of multidrug-resistant *K. pneumoniae* in Africa and Vietnam is mainly multiclonal, two major clonal groups, CG15 and CG258, cause infections in most sites. Even though our findings may not be representative of the overall situation of these countries, it is noteworthy that this study took place in the main health institutions of each country. Clonal groups CG15 and CG258, associated with multidrug resistance, have now been extensively described in other parts of the world. Their widespread geographical distribution is a major concern, both as a source of therapeutic failure and as a potential reservoir of resistance determinants. Human mobility may be the main factor in the spread of multidrug-resistant *K. pneumoniae* clones, as illustrated by the spread of NDM-1-producing strains from India and Pakistan to the UK [30]. Analysis of the allelic diversity within CG258 suggested that the widespread carbapenem-resistant genotype ST258 derived from an ST11 ancestor. High-resolution genotyping studies of isolates collected over larger spatial and temporal scales will be necessary to decipher the dynamics of the emergence of multidrug-resistant *K. pneumoniae* clonal groups.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Distribution of clonal groups and sequence types among 135 third-generation cephalosporin-resistant *Klebsiella pneumoniae* isolates, according to the location.

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