

## Clinical and Molecular Study of *Corynebacterium diphtheriae* Systemic Infections in France

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**Diphtheria is a disease with a long history that almost completely disappeared from developed countries. In addition, until 1987, systemic infections involving *Corynebacterium diphtheriae* were rare. However, in 1990, an epidemic occurred in Russia. These two circumstances have provided the stimulus to gain insight into the situation in France. In fact, between 1987 and 1993, a total of 59 *C. diphtheriae* strains were isolated. Epidemiological data were collected for patients from whom 40 strains were isolated from normally sterile sites, including 34 from blood cultures, and half of the bacteremic patients developed endocarditis. Osteoarticular involvement was noted in 11 of these 40 patients, including 5 bacteremic patients. The fatality rate following bacteremia was 36%, despite specific antibiotic treatment (beta-lactams and aminoglycosides). The mean age of the participants was 38 years, with half of the patients subsisting under low socioeconomic conditions and suffering from homelessness or alcoholism. Apparently, the skin turned out to be the major route of transmission in this reemerging disease. Eighty-eight percent of the isolates belonged to the *C. diphtheriae* biotype *mitis*. These were found predominantly in the Paris area, and most were of the same ribotype. Those isolates originating from the overseas territories (Guyana and New Caledonia) belonged to *C. diphtheriae* biotype *gravis*. No strains were positive for the *tox* gene by PCR. This study attests to the persistent circulation in France of *C. diphtheriae* in the form of systemic infections. The matter is especially significant since these strains are nontoxigenic and are of a unique ribotype. The strains are, however, sensitive to most antibiotics, although 20% are rifampin resistant.**

Diphtheria was first described by Bretonneau in 1821, with the causative organism, *Corynebacterium diphtheriae*, subsequently being isolated by Loëffler in 1883 (19). The first case of *C. diphtheriae* bacteremia was reported in 1893. Diphtheria has dramatically decreased in developed countries since the Second World War through the widespread use of the toxoid (23). Only 10 cases of diphtheria were reported in France between 1984 and 1986, and 2 cases were reported in 1987 (1). Since 1990, diphtheria has again emerged in Eastern Europe, especially in Russia and Ukraine (5).

Concomitantly, nontoxigenic strains continue to circulate throughout the world, including countries where diphtheria is epidemic. An increase in nontoxigenic *C. diphtheriae* systemic disease has been observed in France, while a few cases have been reported in other countries (Switzerland, Australia, the United Kingdom, and Singapore) (2, 5, 26, 27).

Only 58 cases of bacteremic infections due to toxigenic or nontoxigenic *C. diphtheriae* strains were described between 1893 and 1995 (2, 5, 8, 16, 17, 24, 26, 27). The current report examines the cases diagnosed in France since 1987 and presents the results from collaborative work on the microbiological and molecular characteristics of these isolates.

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### MATERIALS AND METHODS

**Isolates.** The *C. diphtheriae* strains were collected over a 7-year period (1987 to 1993) by the Laboratory of Diagnostic Bacteriology, Institut Pasteur, Paris, France.

**Clinical Data.** The clinical and epidemiological information was gathered through questionnaires submitted to the microbiologists and physicians who had dealt with the problems of *C. diphtheriae* systemic infections. The accumulated data included information on predisposing factors, clinical history, physical examinations, microbiological characteristics, therapy, and disease progression.

**Microbiological data.** The strains of *C. diphtheriae* were identified at the Institut Pasteur. The microbiological studies were performed at the Laboratory of Diagnostic Bacteriology, Institut Pasteur, or by the microbiology laboratories at various hospitals in France.

**(i) Biotyping.** Strains were characterized by colonial morphology and color on Columbia agar, Gram staining, motility, and the API Coryne system profile (bioMérieux, La Balme les Grottes, France). Three biotypes were identified: *C. diphtheriae* biotype *mitis*, *C. diphtheriae* biotype *gravis*, and *C. diphtheriae* biotype *belfanti*, a biochemical variant of *C. diphtheriae* biotype *mitis*. *C. diphtheriae* biotype *belfanti* differs from *C. diphtheriae* biotype *mitis* only by its nitrate reductase-negative reaction.

**(ii) Sensitivity to antibiotics.** The sensitivities to 17 antibiotics were determined by the disk diffusion test. This test was done as prescribed by the National Committee for Clinical Laboratory Standards (NCCLS) on blood agar (Mueller-Hinton medium containing 5% horse blood) into which benzylpenicillin (6 µg), amoxicillin (10 µg), cefixime, cefpodoxime proxetil, aztreonam, cefotaxime, ceftriaxone, pristinamycin, erythromycin, lincomycin, sulfamethoxazole, pefloxacin, vancomycin, fusidic acid, rifampin, minocycline, or imipenem (Pasteur Diagnostics, Marne la Coquette, France) was incorporated (20).

The agar microdilution plates were read at 24 and 48 h according to current NCCLS parameters. Penicillin sensitivity was assessed by using the criteria for *Streptococcus* and *Listeria* as suggested by NCCLS.

**(iii) Toxigenicity.** For strains collected up to 1992, toxin production was detected by direct immunodiffusion (Elek test) (4). The strains were cultured on Bacto KL Virulence Agar supplemented with Bacto KL Virulence Enrichment. Toxigenicity (virulence) was tested by using Bacto KL Antitoxin Strips or Pasteur Merieux Antitoxin Strips. For all strains collected between 1987 and 1993, toxin gene detection was performed by PCR as described by Hauser et al. (12). Known

TABLE 1. *C. diphtheriae* strains isolated in France from 1987 to 1993 by year and site of location<sup>a</sup>

Site of isolation	No. of isolates isolated in the following yr:							Total
	1987	1988	1989	1990	1991	1992	1993	
Blood cultures	2	0	0	5	8	5	14	34
Osteoarticular fluid	0	0	0	0	4	2	3	9
Perineal abscess	0	0	0	0	0	1	1	2
Nasopharyngeal swabs	0	1	0	0	2	4	1	8
Respiratory tract	0	1	0	0	2	4	0	7
Skin <sup>b</sup>	0	0	0	0	2	1	0	3
Other sites	0	0	0	0	1	0	2 <sup>c</sup>	3

<sup>a</sup> The same strains were isolated from more than one site in several instances. The strains were collected by the Institut Pasteur, Paris, France.

<sup>b</sup> *C. diphtheriae* was isolated from nonsuppurative lesions.

<sup>c</sup> Including one isolate from peritoneal fluid.

toxigenic (Park and Williams no. 8, CIP 6928, and A 102) and nontoxigenic (CIP 100-721) strains were used as reference strains in all tests.

Both Elek-positive and -negative strains were additionally tested in vivo for comparative virulence by using the guinea pig subcutaneous test. Cultures grown on agar were suspended in 2 ml of normal serum. Pairs of female guinea pigs (weight, 250 g) were inoculated subcutaneously with each test strain (0.2 ml of inoculum), with one of the pair simultaneously receiving 100 IU (0.5 ml) of antitoxin intraperitoneally (Diphtheria Antitoxin; Pasteur Mérieux S.V.), according to the World Health Organization prescription for the laboratory diagnosis of diphtheria (4).

(iv) **Ribotyping.** The chromosomal DNAs of the strains were extracted, purified, and cleaved with restriction endonuclease *Bst*EII (Boehringer) in accordance with the manufacturer's instructions. The restriction fragments were subjected to agarose gel electrophoresis and were blotted onto a nylon membrane, and then hybridized with 5' <sup>32</sup>P-labelled *Escherichia coli* 16S and 23S rRNA for 16 h at 60°C, as elaborated by Grimont and Grimont (6).

## RESULTS

**Clinical data.** Over a study period of 7 years (1987 to 1993), a set of 59 strains of *C. diphtheriae* was collected in France (Table 1). Forty of these strains were isolated from normally sterile sites and were responsible for systemic infections. The same strain could be found in more than one site in a patient. The mean age of the 40 patients suffering from these systemic infections was 38 years (range, 4 to 87 years). The male-to-female distribution gave a sex ratio of 3.1. Two distribution peaks were observed, the first one corresponding to pediatric infections (7 cases) and the second one corresponding to the 40- to 49-year-old age group (10 cases). These strains were examined in this study and were compared with respect to their clinical and microbiological data.

A majority of the cases were found within the Paris area ( $n = 25$ ). Five patients came from the north or northwest of France, four came from the west of France, and two came from the south or southeast of France. Two strains were isolated in New Caledonia, one strain was isolated in Guyana, and one strain was isolated in Papeete (Tahiti).

The main predisposing factors were low socioeconomic level and poor hygienic conditions (31% of the patients were homeless) and the extent of alcoholism or hepatic cirrhosis (half of all the patients and 63.6% of the patients with osteoarticular involvement). One homeless man with cirrhosis and bacteremia with cutaneous lesions was infected with the human immunodeficiency virus. Two other patients were immunocompromised: an elderly woman with pemphigoid being treated with corticosteroids and a 22-year-old woman with a thoracic tumor.

The skin appeared to be the main route of transmission for these patients (open fracture, bullous pemphigoid, scabies, or other cutaneous lesions). The upper respiratory tract was probably the portal of entry for the organism in four of the patients.

Thirty-four patients demonstrated bacteremia, including 15

patients (44%) exhibiting endocarditis. Three patients had prosthetic valves, and three suffered from rheumatic heart disease. Endocarditis was associated with meningitis in one instance and with cerebral abscess or mycotic aneurysm in two instances. One patient also had myocarditis, while four had developed arthritis.

Osteoarthritis was reported in 11 patients (27.5%), but *C. diphtheriae* was found in joint fluid or a bone specimen in only nine patients. Only one case occurred in a child. The child had osteitis of the femur after an open fracture. Cutaneous lesions were noticed in 10 patients. The knees were the most frequently affected joint in patients with osteoarthritis. The other joints involved were the wrist, ankle, and elbow. In four patients more than one articulation was affected, whereas two patients showed a perineal cutaneous abscess.

Beta-lactams were the agents most commonly prescribed for therapy, including benzylpenicillin ( $n = 4$ ), amoxicillin ( $n = 19$ ), amoxicillin-clavulanic acid ( $n = 7$ ), and broad-spectrum cephalosporins ( $n = 3$ ). The other antibiotics used were macrolides, glycopeptides, fluoroquinolones, and rifampin, which was used twice. These were usually used together with aminoglycosides. The duration of the treatment extended from 4 to 150 days, with an average of 45 days. Surgery was performed on two patients with endocarditis and one patient with osteoarthritis. The mortality rate from *C. diphtheriae* bacteremia was 36%.

**Microbiological data. (i) Biotypes of systemic strains.** *C. diphtheriae* biotype *mitis* was observed to be the predominant biotype (88%) of strains. Two strains were biotyped as *C. diphtheriae* biotype *belfanti*. Three strains of *C. diphtheriae* biotype *gravis* came from New Caledonia and Guyana and were isolated from cultures of blood from patients with endocarditis.

(ii) **Antibiotic susceptibility.** *C. diphtheriae* was sensitive to most beta-lactams; only aztreonam was found to be ineffective. No resistance to benzylpenicillin was noted. Among the broad-spectrum cephalosporins, only cefotaxime and ceftriaxone were always dependable. All of the strains were resistant to cefixime, and only 36% were sensitive to cefpodoxime proxetil.

Nearly all strains were sensitive to erythromycin and lincomycin (97.5 and 94.5%, respectively). Pristinamycin, fluoroquinolones, glycopeptides, chloramphenicol, and carbapenem were always effective. The following susceptibilities were also recorded: 94.5% of the strains were susceptible to tetracyclines, 94.5% of the strains were susceptible to fusidic acid, and 81% of the strains were susceptible to rifampin. The *C. diphtheriae* strains were consistently resistant to fosfomycin.

(iii) **Toxicogenicity.** The Elek test was performed with the first 41 of 59 strains isolated between 1987 and 1993. Twenty-seven

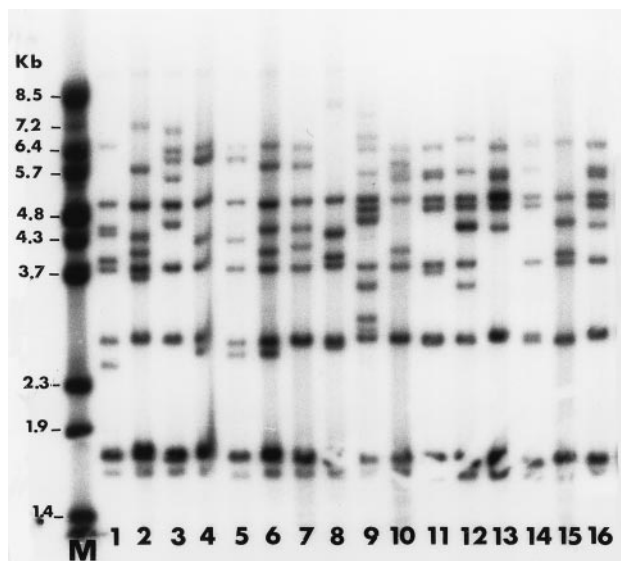


FIG. 1. rRNA gene by restriction endonuclease *Bst*EII analysis of strains corresponding to each ribotype. The lengths of DNA fragments (in kilobases) were obtained by comparison with 5' <sup>32</sup>P-labelled *Bst*EII fragments of bacteriophage lambda DNA (lane M). Lane 1, ribotype 1; lane 2, ribotype 2; lane 3, ribotype 3; lane 4, ribotype 4; lane 5, ribotype 4; lane 6, ribotype 5; lane 7, ribotype 6; lane 8, ribotype 7; lane 9, ribotype 8; lane 10, ribotype 9; lane 11, ribotype 10; lane 12, ribotype 11; lane 13, ribotype 12; lane 14, ribotype 13; lane 15, ribotype 14; lane 16, ribotype 15.

strains were negative, while the other 14 exhibited only late-appearing specific weak bands.

The toxin gene was not detected in any of these strains on the basis of PCR.

Ten strains were tested with the guinea pig model, including toxin-positive (Park and Williams no. 8, CIP 6928, and A 102) and toxin-negative (CIP 100-721) reference strains, three strains with a late-appearing weakly positive Elek test result, and five strains with a negative Elek test result. Toxicogenic manifestations and death at day 4 were observed only with the toxin-positive reference strain.

(iv) **Ribotyping.** Fifteen different ribotypes were obtained following analysis of the collection of 59 strains isolated in France between 1987 and 1993. By using the *Bst*EII system (Fig. 1), eight different ribotypes were obtained with the strains isolated from normally sterile sites (ribotypes 1, 2, 3, 5, 6, 7, 8, and 13). The ribotypes were primarily designated 1 to 15 since a definitive system of nomenclature for *C. diphtheriae* based on ribotyping is not yet available.

Twenty-four strains isolated from blood cultures or osteoarticular fluid showed the same ribotype with *Bst*EII and were all *C. diphtheriae* biotype *mitis* (ribotype 1 in Fig. 1). Nineteen strains were isolated in the Paris area. The three strains (including one from blood cultures) isolated in New Caledonia showed the same ribotype. The other 10 strains had different ribotype patterns. There was no correlation between ribotype and biotype, but the strains belonging to the same ribotype showed a unique biotype.

## DISCUSSION

Diphtheria is still endemic in Eastern Europe and other regions of the world, although it has virtually disappeared in developed countries following mass immunization in the 1940s. *C. diphtheriae* bacteremia is a less well known entity, however, and only a relatively few cases have been documented through-

out the world. Up to 1995, some 58 cases were described in the international literature, including 14 cases reported in France before 1987. The 58 patients alluded to in the international literature by and large had endocarditis. The mean age of the patients described previously was 18 years (age range, 2 to 56 years, but the study subjects included 29 children less than 15 years old) and was much lower relative to the mean age of the patients in the present study. By comparison, only one-quarter of the endocarditis cases observed throughout the world since 1987 have occurred in children. Curiously, the male-to-female sex ratio increased from a previous ratio of 1:3 to one of 3:7.

In the past, the mitral valve was affected in 39 patients, while tricuspid involvement occurred in 4 patients and the aortic valve involvement occurred in 10 patients. However, the diagnosis was not confirmed, especially in the earlier patients because of the absence of autopsy information or echocardiographic data.

The occurrence of joint involvement reported by Tiley et al. (26) (50% of patients) was supported by the present study (27.5%). However, it appears to be a recently described phenomenon. The first reported case was by Guran et al. (8) in France in 1979. Half of the arthritic cases were secondary to bacteremia. Although the scrotum was not described as a site of isolation in the other studies of systemic infection, Harnisch et al. (11) reported two such cases during the outbreak of a *C. diphtheriae* infection in Seattle's Skid Road from 1972 to 1982.

Before the era of antibiotics or cardiac surgery arrived, most of the patients with *Corynebacterium* bacteremia did not survive. This drastic situation has changed somewhat since 1950, but mortality is still 15 to 50% (26, 27).

From 1990 to 1995, only 14 patients with *C. diphtheriae* systemic infections, apart from those reported in the present study, have been described in the literature; these patients were from Switzerland, Australia, Singapore, and the United Kingdom (2, 5, 26, 27). The only other *C. diphtheriae* systemic infection was described in a homeless patient in Germany; the blood culture isolate was found to be a nontoxicogenic strain of *C. diphtheriae* biotype *mitis* (25a).

Two main predisposing factors appeared in the most recent patients: intravenous drug use and low socioeconomic conditions compounded by alcoholism and homelessness (11). Human immunodeficiency virus infection did not appear to be a usual underlying situation. The skin was the classical colonization site for these bacteria for patients from developing countries (10, 11, 15, 19, 27). This appeared to be the predominant route for bacterial contamination and penetration into the bloodstream (38 to 50%) (9). This transfer was due to various skin lesions that were not unusually found in the past. These included cutaneous ulcers, bullous pemphigoid, scabies, and open fractures.

A study recently carried out in Zurich supports this development regarding the incidence of skin contamination. This finding demonstrated a high rate of isolation of *C. diphtheriae* biotype *mitis* in ulcerated skin specimens obtained from intravenous drug users (7). The characterization of these isolates by ribotyping revealed patterns identical to those of strains isolated from cultures of blood from the same population.

Only 14 of the 58 strains from the earlier studies described in the international literature were toxicogenic. Strictly toxicogenic strains have not been isolated in France since 1987, irrespective of which toxin detection test was used. Some strains collected between 1987 and 1991 and examined by the Elek test were weakly positive after several days of reaction; however, none were positive for the toxin gene by PCR. Guinea pig testing for toxicogenicity correlated with the PCR results, showing the difficulties in obtaining results by the Elek test, which

appeared to be a less specific test than PCR. The validity of detection of the toxin gene by PCR and the ADP ribosylation assay was supported by using reference toxigenic and nontoxigenic strains (12).

The susceptibilities of the strains in the present study were found to be similar to those reported for most antibiotics in other studies (18). Benzylpenicillin or phenoxymethyl penicillin represent the first antibiotics used to treat such systemic infections, usually in combination with an aminoglycoside. Macrolides and streptogramin were also frequently used to treat diphtheria or systemic infections, especially when there was concern about allergies to beta-lactams and the prevention of infection through close contacts (18, 19). In addition, glycopeptides seemed to be a useful alternative to penicillin. Although antibiotic treatment usually appeared to be efficient, fatalities occurred due to exacerbating circumstances of the underlying disease or impoverished living conditions. Penicillin tolerance has recently been hypothesized to be a cause of treatment failure.

Nineteen percent of all strains in the present study were resistant to rifampin. All these strains of *C. diphtheriae* are similar, being *C. diphtheriae* biotype *mitis* and of the same ribotype. By comparison, this sort of result was not observed from a recent Russian study (18). This point is of considerable importance since a more recent Russian study has shown how rapid and efficient this antibiotic is for the prophylaxis of asymptomatic carriers of *C. diphtheriae* (13).

Some similarities were observed between the cases of systemic infections in Switzerland, Germany, Australia, the United Kingdom, and France. All the strains isolated from patients with systemic infections in Europe and Australia were nontoxigenic *C. diphtheriae* biotype *mitis* or biotype *gravis* isolates on the basis of various tests, especially PCR (2, 5, 26, 27). However, Pennie et al. (22) have recently reported on a young boy who presented with bacteremia with endocarditis related to toxigenic *C. diphtheriae* biotype *gravis* infection. Cultures of blood and liver and spleen tissue obtained at autopsy or at postmortem examination revealed toxigenic strains of *C. diphtheriae* during fatal diphtheria (16). The strains emanating from other parts of the world (French Guyana, New Caledonia, and Australia) were *C. diphtheriae* biotype *gravis* but belonged to different ribotypes.

All four strains involved with diphtheria in Switzerland were nontoxigenic *C. diphtheriae* biotype *mitis* (27). These strains could not be differentiated by ribotyping with *Pvu*II and *Eco*RI restriction enzymes or by evaluation of enzyme activities or antimicrobial resistance patterns. The eight cases of endocarditis observed in Australia between October 1990 and September 1991 were all identified as infections due to nontoxigenic *C. diphtheriae* biotype *gravis* (26). Ribotyping showed that there were three distinct patterns among these isolates; the patterns for six of the cases from New South Wales were identical, but the patterns for the other two strains were different.

The ribotyping results for isolates found in France revealed that among the patients infected with *C. diphtheriae* biotype *mitis*, 27 were infected with isolates belonging to the same ribotype; the patients mainly lived in the Paris area and included 24 patients with systemic infections. At this time, it is not known whether this ribotype refers to the same *C. diphtheriae* biotype *mitis* strain of nontoxigenic *C. diphtheriae* that has circulated in other parts of Western or Eastern Europe or Algeria. The German strain isolated from a patient with a systemic infection was a toxin-negative *C. diphtheriae* biotype *mitis* strain. The ribotype of this strain differed from all 15 ribotypes isolated in France (23a). Elsewhere, *C. diphtheriae* strains isolated in Switzerland differed from the ribotype 1

strain (23a). Ribotyping appears to be a powerful technique for differentiating strains of *C. diphtheriae*. This tool was recently applied during different outbreaks of diphtheria, especially in Sweden and Russia (3). In this instance, pulsed-field gel electrophoresis was also applied, and although the results correlated well with those of ribotyping, it did not provide further information (3).

The majority of the epidemic *C. diphtheriae* strains with regard to the cases in Russia and Ukraine were toxigenic *C. diphtheriae* biotype *gravis*, with a ribotype different from those of French strains.

Many strains of nontoxigenic *C. diphtheriae* are distributed throughout the world. In recent studies, such strains have been isolated from both Eastern and Western Europe. However, little is known about the circulation of strains between different parts of France and between France and other countries.

Over the years, the rate of carriage of toxigenic strains has decreased considerably for an immunized population, whereas the rates of carriage of nontoxigenic strains has remained unchanged (14, 25). Some strains of *C. diphtheriae* are better-known colonizers than other strains. Individuals harboring a certain strain in a particular geographical area tend to be colonized with the same nontoxigenic strain, while different strains will predominate in each area (25). This situation is observed for the main ribotype in the present study and for the Swiss strains and the German strain. The three strains from New Caledonia were found to belong to the same ribotype and to be different from all the other strains tested.

*C. diphtheriae* systemic infections have been considered an emergent disease in France since 1990. The majority of strains are of the same biotype and ribotype. These results are quite unique compared to those obtained by investigators in other countries. However, this is not the only explanation, since the two principal reasons for this situation are the use of newly available diagnostic systems for identifying corynebacteria and the existence of a host population exposed to increasingly harsh socioeconomic conditions. In fact, data from Switzerland and Australia indicate that the situation is the same, with each involving a dominant ribotype.

This study demonstrates the persistence and potential for spread of *C. diphtheriae* infection in France. Theoretically, bacteriophages which carry the *tox* gene could be in contact with and thus infect resident nontoxigenic strains, rendering them toxigenic (5, 21). As a consequence, it might be speculated that diphtheria outbreaks should be anticipated, although these have not been reported.

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#### APPENDIX

The indicated members of the collaborative study were from the following locations: Arles (B. Mery and B. Hautefort), Beaujon, Paris (A. Andreassian, S. Sterker, and B. Bruneau), Bichat, Paris (M. L. Joly), Bondy (J. C. Torlotin and P. Cruaud), Brest (M. Garré and A. Le Lay), Caen (C. Thomassin-Monnier and M. Exmelin), Calais (R. Fernon and A. Leclair), Colombes (P. Cahen and Y. Boussougant), Créteil (J. Duval, M. Habis, J. L. Monin), Grenoble (J. P. Stahl, M. Micoud, P. Le Noc, and G. Manquat), Institut Gustave Roussy (C. Tancrede and I. Charnoy), Institut Pasteur Nouméa, Nouvelle Calé-

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