Molecular Evolution of Metallo- β -Lactamase-Producing *Pseudomonas aeruginosa* in a Nosocomial Setting of High-Level Endemicity

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An outbreak of multidrug-resistant *Pseudomonas aeruginosa* strains producing VIM-type metallo- β -lactamases (MBLs) has occurred in an Italian hospital since 2000 (C. Lagatolla, E. A. Tonin, C. Monti-Bragadin, L. Dolzani, F. Gombac, C. Bearzi, E. Edalucci, F. Gionechetti, and G. M. Rossolini, *Emerg. Infect. Dis.* 10:535–538, 2004). In this work, using molecular methods, we characterized 128 carbapenem-resistant isolates (including 98 VIM-positive isolates) collected from that hospital from 2000 to 2002 to investigate the dynamics of the dissemination of MBL producers in the clinical setting. Genotyping by random amplification of polymorphic DNA and pulsed-field gel electrophoresis showed that most VIM-positive isolates belonged to two different clonal lineages, producing either a VIM-1- or a VIM-2-like MBL, whose ancestors were detected for the first time in the hospital in 1999, suggesting that clonal expansion played a predominant role in the dissemination of these isolates. The 86 clonally related isolates carrying a *bla*_{VIM-1}-like gene on an In70-like integron were clearly related to a VIM-1-positive *P. aeruginosa* clone circulating in various Italian hospitals since the late 1990s. VIM-negative *P. aeruginosa* strains related to the VIM-1-positive clone were detected during the same period, suggesting that the latter strain was derived from a clonal lineage already circulating in the hospital. In the VIM-2-like positive clone, the MBL gene was carried by an unusual class 1 integron, named In71, lacking the 3' conserved sequence region typical of *sulI*-associated integrons. A different class 1 integron with an original structure carrying a *bla*_{VIM-2} determinant, named In74, was detected in a sporadic isolate. A retrospective investigation did not reveal the presence of strains related to any of the VIM-producing isolates earlier than 1997.

Pseudomonas aeruginosa remains one of the most important pathogens in the nosocomial setting, where it is a common causative agent of bacteremia, pneumonia, and urinary tract infections (10). Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antibiotic class (9, 24, 31). β -Lactams are among the first-choice agents for chemotherapy of *P. aeruginosa* infections, and acquired resistance to the antipseudomonal β -lactams is a matter of major concern. Several mechanisms can contribute to acquired β -lactam resistance in *P. aeruginosa*, including β -lactamase production, outer membrane impermeability, and active efflux mediated by RND-type efflux systems.

Concerning β -lactamases, during the last decade, the acquired metallo- β -lactamases (MBLs) have emerged as a new threatening mechanism of broad-spectrum β -lactam resistance in *P. aeruginosa* (2, 14, 21). In fact, these enzymes can efficiently degrade virtually all antipseudomonal β -lactams (except aztreonam), while they are not susceptible to therapeutic β -lactamase inhibitors (such as clavulanate or sulfones) (20, 25, 27). The most common and widespread acquired MBLs are those of the IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are known

(<http://www.lahey.org/Studies/>). Their genetic determinants are carried on mobile gene cassettes inserted into chromosome- or plasmid-borne integrons and can rapidly disseminate in the clinical setting via the integron system and the associated mobile DNA elements (12, 21).

P. aeruginosa strains producing acquired MBLs have mostly been reported as sporadic isolates or as causing small nosocomial outbreaks (4, 34). However, a number of major outbreaks have now been reported (15, 33, 35), including in our hospital, where recently, *P. aeruginosa* strains producing VIM-type enzymes have rapidly achieved remarkable diffusion (11).

In this work, we report the molecular characterization of the MBL-producing *P. aeruginosa* strains involved in the major outbreak observed in this hospital. Results provided original insights into the dynamics of dissemination of MBL-producing *P. aeruginosa* strains.

MATERIALS AND METHODS

Bacterial isolates. A total of 174 clinical isolates of *P. aeruginosa* collected at the Laboratory of Clinical Microbiology of the Trieste University Hospital (northern Italy) were investigated in this study. Each isolate was obtained from a different patient. The collection included 128 imipenem-resistant isolates randomly selected among those isolated during the first semesters of 2000, 2001, and 2002 and 46 isolates obtained from October 1996 to March 1997. The first two isolates producing VIM-type enzymes detected in the hospital in February 1999 (isolates TS-832035 and TS-832347) (32) were also included in the present study.

Bacterial identification and susceptibility tests. Identification and routine antibiograms of the isolates were carried out using the Vitek system (bioMérieux, Marcy l'Étoile, France). MICs of imipenem were also determined by the agar

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dilution method according to CLSI guidelines (3). *P. aeruginosa* ATCC 27853 was used as a reference strain for quality control in susceptibility testing.

Identification of isolates carrying *bla*_{IMP} and *bla*_{VIM} determinants. Genomic DNAs of the isolates were extracted as previously described (12), spotted (0.5 µg per spot) onto positively charged nylon membranes (ZetaProbe; Bio-Rad, Hercules, Calif.), and probed by dot blot hybridization using digoxigenin-labeled DNA probes obtained by amplification of an internal fragment of the *bla*_{IMP-2} and *bla*_{VIM-1} genes with primers IMP-DIA (22) and VIM-DIA (29), respectively, in the presence of 70 µM dig-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany). Amplification conditions were previously described (19). Hybridization, carried out at 64°C on the basis of preliminary experiments, and hybrid detection were performed as recommended by the manufacturer (1). In the *bla*_{VIM}-positive isolates, the nature of the allelic variant was identified as *bla*_{VIM-1} or *bla*_{VIM-2}-like by analysis of the restriction fragment length polymorphism (RFLP) of the amplicons obtained with the VIM-DIA primers after digestion with *Rsa*I (New England Biolabs, Beverly, Mass.) as described previously (16).

Characterization of the variable regions of the *bla*_{VIM}-containing integrons. The *bla*_{VIM-2} determinant of isolate TS-832347 was mapped, by Southern blot analysis using the *bla*_{VIM} probe (see above), into a 3.5-kb *Bam*HI fragment of the genomic DNA. This fragment was cloned into the plasmid vector pBC-SK (Stratagene Inc., La Jolla, Calif.) and subjected to sequence analysis on both strands as described previously (28). The structure of the variable regions of the *bla*_{VIM}-containing integrons was investigated by a PCR mapping approach using the VIM-DIA/f and 3' conserved sequence 3'-CS (13) primers or the 5'-CS (13) and INV1 (5'-TCGGTTCAGCCGCATAAA) primers, designed on the conserved integron sequences flanking the cassette array. Amplification reactions were carried out in a 25-µl volume using 1 U of AmpliTaq Gold enzyme (Applied Biosystems, Foster City, Calif.), 20 ng template DNA, and 0.5 µM of each primer in the reaction buffer recommended by the enzyme manufacturer. Cycling parameters were as follows: an initial denaturation step for 4 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 57°C (for VIM-DIA/f and 3'-CS) or 55°C (for 5'-CS and INV1), and 5 min at 72°C (with 5 s added to the extension time at each cycle). The amplification products obtained with primers VIM-DIA/f and 3'-CS were subjected to RFLP analysis with *Sty*I or *Mse*I. The amplification products obtained with primers 5'-CS and INV1 were subjected to RFLP analysis with *Dde*I or *Sal*I. The variable region of the integron from the sporadic isolate TS-284 was amplified with primers 5'-CS and 3'-CS, cloned into the plasmid vector pCR 2.1 using the TA cloning kit (Invitrogen, Carlsbad, Calif.), and subjected to sequence analysis on both strands. The authenticity of the sequence was confirmed by direct sequencing of the PCR product obtained in an independent amplification reaction.

Investigation of the genetic environment and location of the *bla*_{VIM}-containing integrons. Approximately 1.5 µg of genomic DNA, either undigested or restricted with 50 U of enzymes, which did not cut inside the integrons (*Pst*I [New England Biolabs] for *bla*_{VIM-1}-positive isolates and either *Mse*I or *Eco*RI [New England Biolabs] for *bla*_{VIM-2}-positive isolates), was subjected to Southern blot analysis. DNA fragments were separated on a 0.8% agarose gel at 1.3 V/cm for 20 h at 4°C, capillary blotted onto nylon membranes (Hybond-N⁺; Amersham International), and hybridized with the digoxigenin-labeled *bla*_{VIM}-specific probe as described above.

Genotyping of isolates. Random amplification of polymorphic DNA (RAPD) typing was carried out using 80 ng of genomic DNA and 40 pmol of the 10-mer primer 208 (5'-ACGGCCGACC) as described previously (17). Analysis of the macrorestriction profiles of chromosomal DNA by pulsed-field gel electrophoresis (PFGE) was carried out using a Chef-DR III apparatus (Bio-Rad) as described previously (7). Pictures of RAPD and PFGE gels were scanned, stored in tagged image file format, and processed with GelCompareII 4.0 software (Applied Maths, Kortrijk, Belgium). For RAPD patterns, only bands in the size range of 400 to 2,200 bp were considered for comparisons. For PFGE, the complete banding pattern was considered for comparisons. Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed by the unweighted-pair group method with average linkages.

OprD expression. Expression of OprD was investigated by Western blot analysis, as described previously by Epp et al. (6), using *P. aeruginosa* PAO1 and PASE1 as positive and negative controls, respectively.

Nucleotide sequence accession numbers. The nucleotide sequences of the variable regions of the *bla*_{VIM-2}-containing integrons from isolates TS-832347 and TS-284 were submitted to the GenBank/EMBL database and assigned accession numbers AM180753 and DQ353808, respectively.

FIG. 1. PFGE profiles of some of the isolates after digestion with *Spe*I. Lanes: S, λ ladder; A to E, *bla*_{VIM-2}-positive isolates (lane D, TS-832347); F to O, *bla*_{VIM-1}-positive isolates (lane F, TS-832035).

RESULTS

Characterization of the first VIM-positive *P. aeruginosa* isolates detected in the hospital. At the Trieste University Hospital, the first VIM-positive *P. aeruginosa* isolates (TS-832035 and TS-832347) were detected in February 1999 and were epidemiologically unrelated to each other (11). TS-832035 has already been subjected to molecular characterization: it was found to produce VIM-1 and to be clonally related (although not identical) to other VIM-1-producing isolates from different hospitals in northern Italy, including the index strain VR-143/97 (29), all of which contain conserved *bla*_{VIM-1}-containing integron structures related to that of In70 (30).

Comparison of TS-832437 with TS-832035 by RAPD typing and by PFGE analysis of genomic DNA digested with *Spe*I revealed that the two isolates were not clonally related to each other (Fig. 1). The variable region of the *bla*_{VIM}-containing integron of TS-832347 could not be amplified using primers VIM-DIA/f and 3'-CS. The *bla*_{VIM} gene from this isolate was mapped, by Southern blot, into a 3.5-kb *Bam*HI genomic DNA fragment (data not shown), which was cloned and sequenced. Sequence analysis revealed the presence of a *bla*_{VIM-2} allele carried on a gene cassette with a complete *attC* recombination site, identical to those of In56 from *P. aeruginosa* COL (25) and several other *bla*_{VIM-2}-containing integrons. The cassette belonged to a class 1 integron and was followed by an *aacA4* gene cassette. Interestingly, the cassette array was not followed by a 3'-CS typical of *sulI*-associated integrons (8) but was followed by a *tniC* gene typical of the transposition module of Tn402 (26) (Fig. 2). This unusual class 1 integron was named In71. Southern blot analysis with undigested genomic DNA using a *bla*_{VIM} probe revealed, in both cases, a single hybridization signal corresponding to the band of chromosomal DNA (data not shown), suggesting a chromosomal location of the *bla*_{VIM}-containing integrons in both strains.

Characterization of VIM-positive *P. aeruginosa* isolates that have spread in the hospital. Previous surveillance work showed that in 2001, VIM-producing *P. aeruginosa* isolates underwent a remarkable dissemination in the hospital, rep-

FIG. 2. Structure of the variable region of the *bla*_{VIM-2}-containing integrons from *P. aeruginosa* TS-832347 (In71) and TS-284 (In74). Open reading frames are indicated by arrows; the *attC* recombination sites of gene cassettes are indicated by ovals.

representing approximately two-thirds of all the carbapenem-resistant isolates (11).

In this work, a total of 128 imipenem-resistant nonreplicate isolates randomly selected among those isolated at the Clinical Microbiology Laboratory of the hospital during the first semesters of the years 2000, 2001, and 2002 were investigated for the presence of *bla*_{IMP} and *bla*_{VIM} alleles. Dot blot hybridization using specific probes revealed that 98 (76.6%) of these isolates carried a *bla*_{VIM} determinant, while none of them carried a *bla*_{IMP} determinant. The percentage of *bla*_{VIM}-positive isolates was 55% in 2000, 80% in 2001, and 82% in 2002 (Table 1).

PCR-RFLP of *bla*_{VIM} alleles showed that 86 (87.7%) of the *bla*_{VIM}-positive isolates carried a *bla*_{VIM-1}-like allele, while 12 (12.3%) isolates carried a *bla*_{VIM-2}-like allele. The prevalence of *bla*_{VIM-1}-like alleles among *bla*_{VIM}-positive isolates ranged from 83 to 95% during various years (Table 1).

The degree of genomic relatedness of the 128 isolates from the 2000 to 2002 was preliminarily investigated by RAPD analysis. Isolates sharing a Dice similarity coefficient higher than 0.85 were assigned to the same cluster. RAPD results showed that all 86 *bla*_{VIM-1}-like allele-positive isolates and 10 of the *bla*_{VIM}-negative ones belonged to the same cluster, named cluster A, which also included TS-832035. On the other hand, 11 of the *bla*_{VIM-2}-like allele-positive isolates belonged to another cluster, named cluster B, which also included TS-832347. The remaining 21 isolates, including 1 *bla*_{VIM-2}-positive isolate (TS-284) and 20 *bla*_{VIM}-negative isolates, appeared to belong to additional clusters unrelated to each other and to the two major clusters including *bla*_{VIM}-positive isolates (Table 1). PFGE analysis was used to confirm the genomic relatedness of

FIG. 3. Clustering of isolates based on PFGE profiles. Thirty-eight isolates collected during different periods of the survey are compared. The dendrogram is based on cluster analysis by the unweighted-pair group method with average linkages. Dots and stars indicate the clusters identified by RAPD analysis (●, cluster A; ★, cluster B). The first two VIM-producing isolates are indicated in boxes. Dates are shown as day/month/year.

isolates belonging to the same RAPD clusters and the unrelatedness of TS-284. A close relationship among all members of cluster A (Dice similarity coefficient of ≥ 0.87 , corresponding to a difference of a maximum of six bands) was confirmed by PFGE analysis, except for two *bla*_{VIM}-negative isolates (Fig. 3).

TABLE 1. Prevalence of isolates carrying *bla*_{VIM-1}-like or *bla*_{VIM-2}-like genes during the study period and their genetic relatedness revealed by RAPD analysis^a

RAPD type	No. of isolates									Total no. of isolates
	<i>bla</i> _{VIM} negative			<i>bla</i> _{VIM-1}			<i>bla</i> _{VIM-2}			
	2000	2001	2002	2000	2001	2002	2000	2001	2002	
A	1	5	4	10	41	35				96
B							2	8	1	11
C	6	3								9
Sporadic	3	4	4						1	12
Total no. of isolates	10	12	8	10	41	35	2	8	2	128

^a The VIM-type MBL producers TS-832035 and TS-832347 were also analyzed and appeared strictly related with cluster A and cluster B, respectively.

PFGE analysis also confirmed the genomic relatedness among members of cluster B, although in this case, the degree of genomic diversity appeared to be slightly higher than that within cluster A (Dice similarity coefficient of ≥ 0.82 , corresponding to a difference of a maximum of eight bands) (Fig. 3). Finally, PFGE analysis confirmed the apparent lack of relatedness of TS-284 with members of either cluster (Fig. 3).

The two major VIM-producing clones (cluster A and cluster B) were detected in 22 different wards, including 15 medical wards, 5 surgical wards, and 2 intensive care units.

Antimicrobial susceptibility of the *bla*_{VIM}-positive isolates.

All of the *bla*_{VIM}-positive isolates showed an MDR phenotype, including imipenem (MIC ≥ 64 μ g/ml), meropenem (MIC ≥ 32 μ g/ml), and most other antipseudomonal agents. Seventy of the 98 VIM-producing isolates (71%) were resistant to all of the drugs tested (ceftazidime, piperacillin, aztreonam, amikacin, gentamicin, tobramycin, and ciprofloxacin), while the remaining 28 isolates showed susceptibility to only one (often amikacin) or two of the above-mentioned agents.

On the other hand, the carbapenem MICs for the 30 *bla*_{VIM}-negative isolates were lower overall (imipenem MICs, 16 to 32 μ g/ml; meropenem MICs, 8 to 32 μ g/ml), and the MDR phenotype was considerably less frequent among those isolates (10/30 [33%]), while 13 of them were susceptible to at least three of the tested drugs. In 10 of those isolates (selected to be representative of the different RAPD types), decreased expression of the OprD porin was detected by Western blot analysis (data not shown), but other mechanisms of carbapenem resistance (e.g., upregulation of efflux systems) were not further investigated in this work.

Characterization of the *bla*_{VIM}-containing integrons and their genetic environment. Eleven *bla*_{VIM-1}-positive isolates of cluster A, selected to be representative of different imipenem MICs (64 to 256 μ g/ml), different isolation times (2000, 2001, and 2002), and different wards, and all 12 *bla*_{VIM-2}-positive isolates were subjected to further characterization of the *bla*_{VIM}-containing integrons and their genetic environment by means of a PCR-RFLP approach and Southern blot analysis as described in Materials and Methods. TS-832035 and TS-832347 were also included in this analysis for comparison.

With the 11 *bla*_{VIM-1}-like allele-positive isolates of cluster A, as well as with TS-832035, PCR-RFLP analysis of integron structures yielded identical profiles, suggesting the presence, in all cases, of an In70-like integron carrying a *bla*_{VIM-1} cassette. In Southern blot analysis of genomic DNA digested with PstI, the *bla*_{VIM} probe recognized a fragment of approximately 9.4 kb in all isolates but one (TS-343, detected in the last period of our survey), in which the probe hybridized to a fragment of approximately 15 kb (Fig. 4, lanes A and B, respectively).

With the *bla*_{VIM-2}-like allele-positive isolates of cluster B, PCR-RFLP analysis of integron structures yielded identical profiles with all isolates of cluster B and with TS-832347, suggesting a conserved structure of their *bla*_{VIM-2}-containing integrons. In Southern blot analysis of genomic DNA digested with MseI, the *bla*_{VIM} probe hybridized to a fragment of approximately 4 kb in all isolates (Fig. 4, lane D, and data not shown). However, in three of the isolates (TS-62, TS-90, and TS-122), the probe also recognized an additional fragment of approximately 9.4 kb (Fig. 4, lane C). The presence of two copies of the *bla*_{VIM} determinant in those isolates was con-

FIG. 4. Southern blot analysis of genomic DNAs obtained with the *bla*_{VIM}-specific probe. Lane A, TS-832035; lane B, TS-343; lane C, TS-62; lane D, TS-832347; lane E, TS-832347; lane F, TS-122; lane G, TS-90; lane H, TS-111; lane M, MW Marker II (Roche).

firmed when the experiment was repeated after digestion with EcoRI. In this case, further heterogeneity was observed with some isolates (Fig. 4, lanes E to H).

In the sporadic isolate TS-284, the variable region of the integron containing the *bla*_{VIM} cassette could not be amplified using primer VIM-DIA/f or 5'-CS plus INV-I but was amplified using primers 5'-CS and 3'-CS. Cloning and sequencing of the amplification product obtained with the latter primers revealed a structure different from that of In71, with an *aadB* cassette, identical to that described in a class I integron from *Corynebacterium asperum* (GenBank accession number AJ871915), in the first position followed by a *bla*_{VIM-2} cassette, and its *attC* recombination site, identical to that found in In71. Since this cassette array has never been described before, this integron was named In74 (Fig. 2).

Comparative analysis with *P. aeruginosa* previously circulating in the hospital. A collection of 46 nonreplicate *P. aeruginosa* isolates isolated from clinical specimens at the Clinical Microbiology Laboratory of the Trieste University Hospital from October 1996 to March 1997 (i.e., 2 years earlier than the first detection of VIM producers) was available for investigation. Most of these isolates (34/46 [74%]) were susceptible to carbapenems, while the remaining 12 isolates were imipenem resistant. None of the isolates carried *bla*_{IMP} or *bla*_{VIM} determinants as assayed by dot blot hybridization. RAPD genotyping revealed heterogeneous profiles (data not shown) without any apparent clustering and with no apparent relatedness to any of the VIM-producing clones subsequently detected.

DISCUSSION

P. aeruginosa strains producing acquired VIM-type MBLs were first reported in northern Italy in the late 1990s (12). The University Hospital of Trieste, a large acute-care teaching hospital, was one of the first Italian institutions where VIM-positive isolates were detected, in 1999 (32). By 2001, a rapid and remarkable spread of MDR *P. aeruginosa* isolates producing VIM-type MBLs had occurred in that hospital (11), showing that similar strains indeed have the potential to rapidly and efficiently disseminate in the clinical setting. In this work, we carried out a molecular characterization of VIM-producing isolates collected during that outbreak to investigate the dis-

semination mechanisms and dynamics of VIM-producing strains.

The first two VIM-producing isolates detected in the hospital (approximately at the same time) were representatives of two different clonal lineages unrelated to each other and producing either the VIM-1 or the VIM-2 enzyme. The rapid and massive spread of VIM-producing isolates observed during the following years was largely caused by members of these two clonal lineages, with an overall predominance of that producing VIM-1. By 2002, the two VIM-producing strains were widespread in the hospital, being found in at least 22 different wards and contributing to approximately 80% of the imipenem-resistant *P. aeruginosa* isolates. The essentially clonal nature of the VIM-1- and VIM-2-positive isolates suggests that clonal expansion played a predominant role in the dissemination of these isolates, a view which is also supported by the overall conserved integron structure and genetic environment observed in most isolates. However, some diversity was also observed among members of each major clonal lineage, especially with the *bla*_{VIM-2}-positive isolates, suggesting a remarkable evolutionary potential of the VIM-positive clones. The possibility that VIM-positive isolates of the same clonal lineage showing some differences in their RAPD and PFGE profiles and in the integron genetic environment could be derived from multiple independent events of acquisition of the *bla*_{VIM}-containing integron by different circulating sublineages of the corresponding VIM-negative clone cannot be completely ruled out but seems less likely, at least in most cases. On the other hand, the presence of at least two different clonal lineages carrying the *bla*_{VIM-2} gene in two different integron structures clearly points to a different phylogeny of those isolates and to different events of acquisition of the MBL determinant. This fact, along with the higher genomic heterogeneity observed within the major VIM-2-positive clonal lineage, would support an overall higher mobility of the *bla*_{VIM-2} cassette, as previously suggested by other authors (23, 36). The reason why the VIM-2-positive strains showed an overall lower spreading potential than the VIM-1-positive strain remains to be explained, but it is probably related to the properties of the host strain.

Interestingly, an analysis of VIM-negative carbapenem-resistant *P. aeruginosa* isolates circulating in the hospital during the same period revealed the presence of strains that were genotypically related to the VIM-1-positive strains, suggesting that the latter strains were likely derived from a clonal lineage that was already circulating in the nosocomial setting upon acquisition of the *bla*_{VIM-1} determinant.

A retrospective investigation aimed at looking for the presence of strains of the same clonal lineages as those producing VIM-type enzymes among *P. aeruginosa* isolates from the same hospital collected from 1996 to 1997 did not reveal the presence of similar strains, suggesting that the ancestors of the major VIM-producing clonal lineages arrived in Trieste between 1997 and 1999.

Interestingly, VIM-1-positive isolates belonging to the same clonal lineage as TS-832035 have also already been detected in other Italian hospitals either as sporadic isolates or as causing small outbreaks (29). The reason(s) why such a rapid and massive dissemination of this VIM-1-positive clone has thus far been observed only in Trieste remains to be understood. Sur-

veillance of the dissemination of this highly epidemic clone, however, appears to be an important goal.

In the future, we plan to analyze representatives of different lineages by the recently developed multilocus sequence typing approach (5) to further evaluate their relatedness among each other and with other epidemic lineages circulating in Europe.

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