

# Full-Genome Characterization of a G8P[8] Rotavirus That Emerged among Children with Diarrhea in Croatia in 2006

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**The whole genome of a G8P[8] rotavirus from the 2006 epidemic in Croatia was sequenced and showed a Wa-like genotype constellation. Its VP7 gene clustered with DS-1-like G8 African rotaviruses and a G8P[4] German strain. Remaining genes clustered with contemporary Belgian G1P[8] rotaviruses, suggesting reassortment between human G8 and G1P[8] rotaviruses in Croatia or other European countries.**

Rotavirus A (RVA) causes acute gastroenteritis in humans and animals globally, killing 450,000 children per year, mainly in developing countries (1).

The RVA genome includes 11 double-stranded RNA segments encoding six viral proteins (VP1 to VP4, VP6, VP7) and six non-structural proteins (NSP1 to NSP6) (2). Rotavirus outer layer protein VP7 and spike protein VP4 are neutralization antigens, determining virus genotypes G (glycoprotein; VP7) and P (protease sensitive; VP4). At least 27 G and 37 P genotypes have been identified among human, animal, and avian RVAs (3–5).

Five genotypes (G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]) represent over 90% of global human RVAs (6), which may explain the efficacy of both the monovalent (P[8], G1) Rotarix and the pentavalent (P[8], G1 to G4) RotaTeq vaccines against rotavirus diarrhea worldwide (7).

Serotypes other than G1 to G4 and strains evolving by mutation or genomic reassortment between human and/or animal RVAs represent an emerging threat to children (8–10). Millions of people are exposed to animal rotaviruses worldwide, and several uncommon human genotypes (G6, G8, G12, P3[9], P5A[3]) closely resemble animal RVA strains (11).

Molecular epidemiology and viral typing can help monitor the emergence of novel RVAs, confirming the efficacy of current vaccines against unconventional genotypes.

Reassortment events can involve any double-stranded RNA segments, and G and P typing may be insufficient to investigate RVA origin and interspecies transmission. Genotyping based on sequencing of all 11 genome segments (3, 4) is very helpful, distinguishing 9 VP1 (R), 9 VP2 (C), 8 VP3 (M), 35 VP4 (P), 16 VP6 (I), 27 VP7 (G), 16 NSP1 (A), 9 NSP2 (N), 12 NSP3 (T), 14 NSP4 (E), and 11 NSP5 (H) genotypes.

Bovine-like G8 rotavirus was first reported in an Indonesian child (12), and further G8 RVA human cases occurred worldwide, including in industrialized countries (13–21). Using full-genome sequencing, G8P[8] African RVAs were shown to involve reassortment between at least four human, swine, and bovine strains (22).

This paper reports the whole-genome characterization of a G8P[8] human rotavirus strain that emerged in Croatia in 2006 (23).

Cases showed severe gastroenteritis and included children <5

years old from Croatian hospitals in 2005 and 2006. Stools were screened by use of the Rotalex assay, and RVA was characterized by reverse transcription-PCR (RT-PCR) (23). Agarose gel-purified amplicons were sequenced using RT-PCR primers (8, 18, 24, 25), assembling consensus sequences for all gene segments (18, 22, 26).

Twelve data sets were built and included all NCBI sequences (<http://www.ncbi.nlm.nih.gov/pubmed>) showing >89% similarity with query sequences (between 116 for VP2 and 127 for NSP4).

Sequences were aligned using ClustalX software (27) and edited by Bioedit software. The ModelTest v3.0 (28) with the hierarchical likelihood ratio test was used to select the best-fit models for sequence data analysis.

Bayesian phylogenetic trees were constructed for the VP7, VP4, and NSP4 data sets (29), using the GTR+I+G nucleotide substitution model for VP7 and the HKY+G model for VP4/NSP4.

Markov chain Monte Carlo searches were made ( $50 \times 10^6$  generations, tree sampling every 5,000 generation, and 10% burn-in fraction; clade statistical support followed >0.90 posterior probability).

Maximum-likelihood (ML) phylogenetic trees of NSP1 to NSP3, NSP5 and NSP6, VP1 to VP3, and VP6 were generated with the PAUP\* v4.0 package (30), using the GTR+I+G model for the VP6, NSP1, and VP1 data sets; the HKY+G model for the NSP6/NSP5 data set; and the TrN+I+G for the NSP2, NSP3, VP2, and VP3 data sets. Statistical robustness and the reliability of the branching order within trees were confirmed by bootstrap analysis (1,000 replicates), with bootstrap values of >70% considered clade support.

Received 9 February 2013 Accepted 14 February 2013

Published ahead of print 20 February 2013

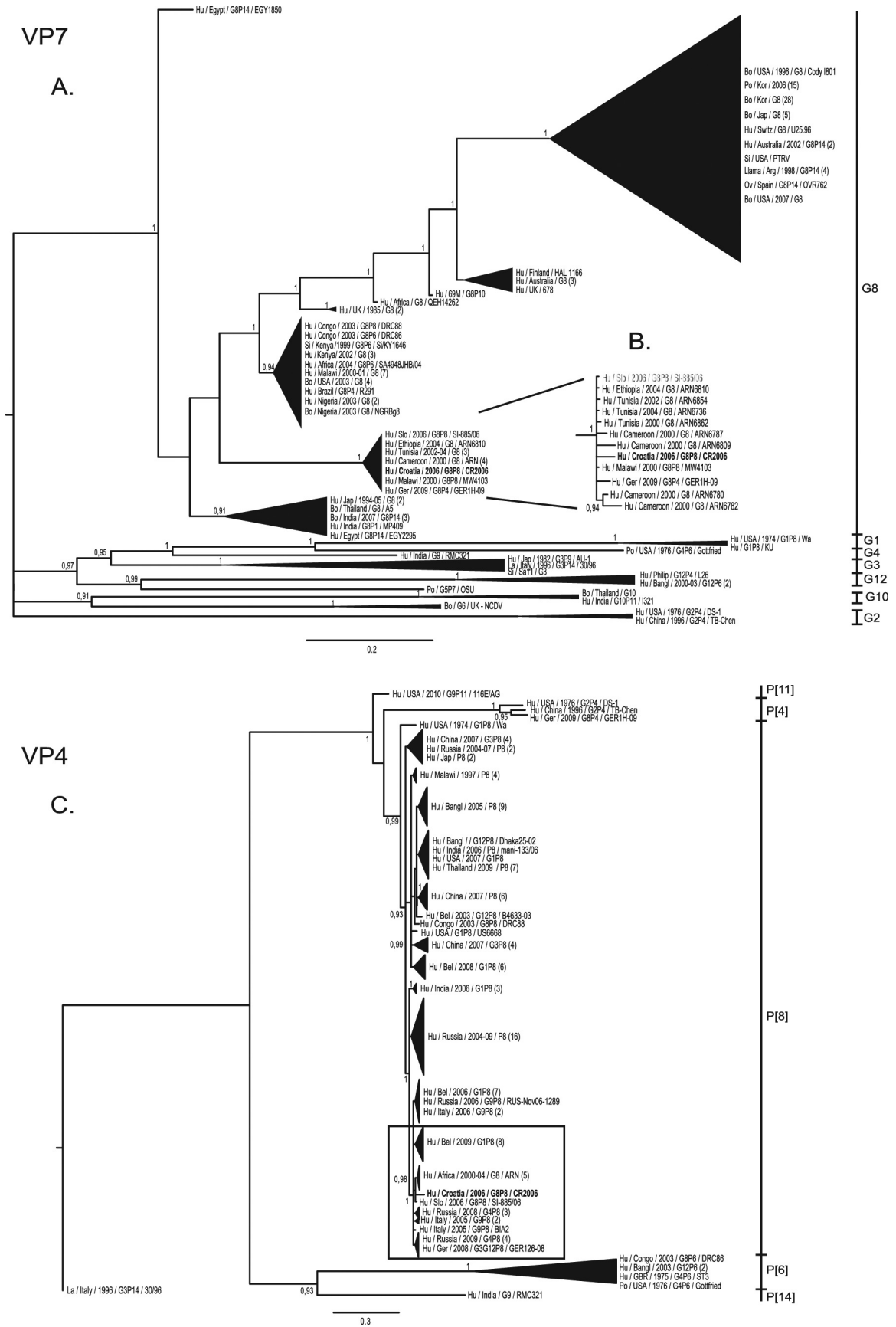
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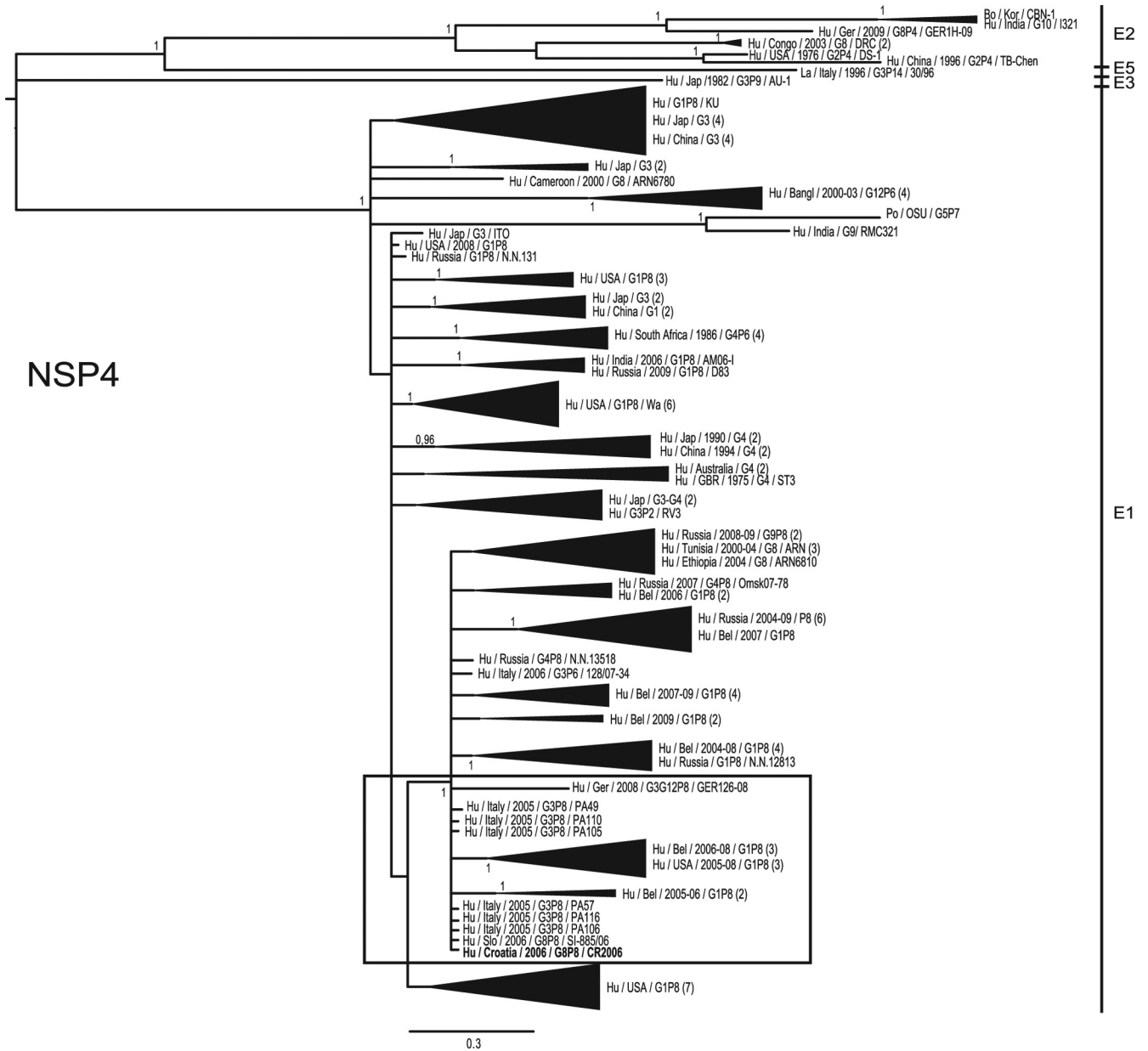
Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.00396-13>.

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doi:10.1128/JCM.00396-13



**FIG 1** Bayesian phylogenetic analysis of human rotavirus VP7 and VP4 nucleotide sequences. The trees were rooted by using the midpoint rooting method. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. For significant statistical support, posterior probabilities of >0.90 are reported. Several clusters are replaced by triangles for simplicity, and the size of each triangle is an indication of the number of sequences that it represents. The number of strains for each place of isolation is indicated in parentheses. The RV strain isolated in Croatia is in boldface type. For each strain, the



**FIG 2** Bayesian phylogenetic analysis of human rotavirus NSP4 nucleotide sequences. The tree was rooted by using the midpoint rooting method. Branch lengths were estimated with the best-fitting nucleotide substitution model according to a hierarchical likelihood ratio test. For significant statistical support, posterior probabilities of >0.90 are reported. Several clusters are replaced by a triangle for simplicity, and the size of the triangle is an indication of the number of sequences that it represents. The number of strains for each place of isolation is indicated in parentheses. The phylogenetic cluster containing CR2006 is boxed, and the CR2006 strain is in boldface type. For each strain, the following data are given: species of origin/place of isolation/year/strain name. The number of substitutions per site is indicated by the scale bar.

Initial sequencing of genes encoding VP4 and NSP4 for 10/31 G8P[8] RVAs showed nearly complete nucleotide sequence identity, suggesting circulation of a single G8P[8] strain in Croatia in 2005 and 2006. The consensus sequence of the VP7 gene of the Croatian strain from 2006 (CR2006) showed

>98% nucleotide sequence identity with the sequences of other human G8 strains whose sequences are in GenBank, particularly sequences from neighboring Slovenia recovered in 2006 and from Africa recovered in 2000 to 2004. CR2006 VP4 and NSP4 recalled human G3P[8] strains reported in Italy in

following data are given: species of origin/place of isolation/year/strain name. The number of substitutions per site is indicated by the scale bar. (A) Phylogenetic tree based on VP7 gene nucleotide sequence; (B) detail of the phylogenetic relationship between RV strains of the G8 genotype; (C) phylogenetic tree based on VP4 gene nucleotide sequence. Kor, South Korea; Jap, Japan; Switz, Switzerland; Slo, Slovenia; Ger, Germany; Philip, The Philippines; Bangl, Bangladesh; Bel, Belgium; GBR, Great Britain; Bo, bovine; Si, simian; Ov, ovine; Po, porcine; La, lapine (rabbit).

2005 (31). To investigate the Croatian G8P[8] origin, full-genome sequencing was performed, using the RotaC tool (<http://rotac.regatools.be>) (32). CR2006 presented a genotype G8P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1.

Bayesian analysis identified CR2006 VP7 in a statistically supported cluster with African strains (Malawi, Tunisia, Cameroon, Ethiopia) and European strains (Slovenia [SI-885/06], Germany [GER1H-09]) (Fig. 1A). In the VP4 and NSP4 trees (Fig. 1C and 2), CR2006 also clustered with European and African RVAs, including human (Hu)/Slovenia/2006/G8P[8]/SI-885/06, Hu/Italy/2005/G3P[8], Hu/Ethiopia/2004/G8P[8]/ARN, and Hu/Tunisia/2000-2004/G8P[8]/ARN.

CR2006 clustered with the Wa-like genotype constellation strains: Hu/Belgium/2005-2009/G1P[8], Hu/Germany/G3G12P[8]/GER126-08, and other global RVAs in NSP1 to NSP6, VP1 to VP3, and VP6 (see Fig. S1 to S9 in the supplemental material).

G8P[8] rotavirus circulated abnormally in Croatia in 2006, reaching 15.8% of children hospitalized with diarrhea (23). Full-genome sequencing of these viruses was performed to investigate links between their emergence and genome characters.

The G8 rotavirus genotype, normally combined with the P[1], P[5], P[11], or P[21] type (11, 33, 34), is typical of bovine strains but has also been sporadically detected in humans since 1978 (35) in association with different P types (13, 16–18, 36–38). Human G8 strains normally exhibit P[4] or P[6] both in developed countries and in Africa, where they are particularly prevalent (15–17, 26). Recently, full-genome sequencing of a human G8P[8] rotavirus in the Democratic Republic of Congo (DRC) in 2003 (18, 22) suggested that this strain originated from reassortment between a bovine G8P[6] ancestor with three or more different rotaviruses, including human Wa- and DS-1-like strains. This strain exhibited a DS-1-like genotype constellation.

Croatian CR2006 G8P[8], however, presented the Wa-like constellation (3) G8-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1, with all genes except the VP7 gene, which is related to African and Slovenian human G8 strains, showing a close evolutionary relationship to the prototype G1P[8] human strain Wa. Rotaviruses are usually species specific, and gene reassortment with human strains appears to be necessary for zoonotic adaptation (26, 39–41). Characterization of epidemic CR2006 further supports this view. As VP7 plays a major role in the immune response (42, 43), CR2006 emergence and epidemic spread may have involved reassortment with common rotaviruses preexisting in Croatia.

CR2006 was more frequent in younger children (23) than any other rotavirus genotypes cocirculating in Croatia in 2006. This could be fortuitous or suggest that maternal antibodies protected younger children from CR2006 less than they did from viruses of other serotypes. Maternal immunity fades quickly (44, 45), and it is conceivable that in 2006 older Croatian children were similarly unprotected and hence susceptible to G8 or any other common rotavirus genotypes.

Phylogenetic analyses revealed that, except for the VP7 gene, CR2006 was more closely related to humans G1P[8] strains identified between 2005 and 2009 in Belgium. Therefore, the origin of CR2006 likely involved reassortment of a Wa-like rotavirus circulating in one or more countries of Europe, including Belgium and possibly Croatia, with a cocirculating G8 virus. In 2006, a similar G8P[8] strain was reported and partially sequenced in Slovenia (20), whereas no G8P[8] rotaviruses were detected in other European countries. It is possible that the reassortment event leading to

CR2006 may have occurred in or near Croatia. The low correlation between VP7 of CR2006 and VP7 of G8 DS-1-like rotaviruses characterized in DRC (18) indicates the independent evolution of these G8 reassortants. Conversely, CR2006 shared a similar gene 9 with human G8 strains from northern/central Africa in the early 2000s, suggesting that the G8 gene followed immigration routes from Africa. A closely similar VP7 was found in a G8P[4] human strain identified in Germany 2 years later (21), suggesting that the CR2006 G8 gene participated in additional reassortments with European DS-1-like rotaviruses. The German strain was strictly correlated with the DRC G8 rotaviruses reported earlier, except for VP4 (18), and may represent a further evolution of G8P[8] rotaviruses imported from Africa. The VP7 of all European G8 strains differed markedly from the VP7 of bovine G8 rotaviruses, contrary to a recent human-bovine reassortment event within Europe.

Rotavirus genetic evolution is certainly influenced by immune pressure (46) that may favor the spread of G8 and other uncommon rotavirus G types (19, 20, 47), as occurred for G9 strains that originated from swine (48, 49). International transmission of genotypes is facilitated by globalization and travel, urging worldwide rotavirus strain surveillance to anticipate the emergence of novel strains and animal/human reassortants and monitor the effectiveness of current human rotavirus vaccines.

**Nucleotide sequence accession numbers.** Sequences were deposited in the GenBank database (accession numbers [JQ988894](https://www.ncbi.nlm.nih.gov/nuclot/JQ988894) to [JQ988904](https://www.ncbi.nlm.nih.gov/nuclot/JQ988904)).

## ACKNOWLEDGMENTS

This study was sponsored by the Ministry of Health, Italy (CCM Epidemiologia Molecolare di Rotavirus in Età Pediatrica in Italia; Creazione di una Rete di Sorveglianza per Monitorare la Diffusione e l'Evoluzione di Genotipi Virali [to L.F.] and Strategic Program: Preparedness and Response to Emerging Zoonoses and Exotic Viral Infection through an Integrated Medical and Veterinary Approach [to F.M.R.]), ISS/NIH (Molecular and Antigenic Evolution of Rotavirus Strains of Human and Animal Origin to F.M.R.), and EuroRotaNet (<http://www.eurorota.net>).

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