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Comparative Study of Five Methods for Intratypic Differentiation of Polioviruses

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A coded panel of 90 poliovirus isolates, 30 of each of the three known serotypes, was used to evaluate five methods for the intratypic differentiation of polioviruses: (i) an enzyme-linked immunosorbent assay with polyclonal cross-absorbed antisera (PAb-E), (ii) a neutralization assay with type-specific monoclonal antibodies (MAb-N), (iii) a restriction fragment length polymorphism (RFLP) assay, (iv) a Sabin vaccine strain-specific PCR assay, and (v) a Sabin vaccine strain-specific cRNA probe hybridization (ProHyb) assay. Sequence analysis was used for the definitive characterization of the strains. The panel was distributed to five laboratories; each laboratory analyzed the strains by at least two methods. Each method was used by three or four laboratories. The total performance scores (percentage correct results per number of tests) of the five methods were 96.7% for PAb-E, 93.9% for MAb-N, 91.9% for RFLP assay, 93.3% for Sabin vaccine strain-specific PCR, and 97.4% for Sabin vaccine strain-specific ProHyb. Consistent results were obtained by each laboratory for 88 of 90 isolates (97.8%) examined by PAb-E, 81 of 90 isolates (90.0%) examined by MAb-N, 78 of 90 isolates (86.7%) examined by RFLP assay, 81 of 90 isolates (90.0%) examined by PCR, and 89 of 90 isolates (98.9%) examined by ProHyb assay. Six strains were classified differently by different methods. It is recommended that at least two methods be used for the intratypic differentiation of poliovirus isolates, and each method should be based on a different principle (i.e., antigenic properties and nucleotide sequence composition). If two assays yield discrepant results, further characterization, preferably by partial sequence determination, will be required for correct identification.

The goal of global eradication of poliomyelitis by the year 2000 was approved by the World Health Assembly in 1988 and was adopted by the World Health Organization (WHO). The program has two main objectives: no more cases of poliomyelitis caused by wild polioviruses and the absence of wild poliovirus circulation, as evidenced by examining specimens from humans and the environment (25). Extensive use of the two available vaccines, the live attenuated oral poliovirus vaccine (OPV), or the Sabin vaccine, and the inactivated poliovirus vaccine, or the Salk vaccine, has dramatically reduced the numbers of poliomyelitis cases caused by wild poliovirus infection (22).

More than 90% of poliovirus infections are asymptomatic. Polioviruses are shed by infected persons and OPV vaccinees in their stools and can survive in the environment for several months (15). The WHO-recommended strategy of mass immunization with OPV to interrupt the transmission of wild poliovirus results in the extensive circulation of vaccine virus in the population and isolation of the vaccine virus in cell cultures. An essential step in the documentation of the absence of wild poliovirus circulation is therefore intratypic differentiation of isolates from healthy persons and from the environment. In addition, vaccination with OPV also sporadically induces paralytic disease, that is, vaccine-associated poliomyelitis (12). It is therefore important to characterize all polioviruses isolated from patients with clinically suspected cases of poliomyelitis as wild or vaccine-related viruses.

In 1981 a WHO-initiated collaborative study on various markers for the intratypic differentiation of polioviruses was conducted (23). The serum neutralization test with cross-absorbed antisera was shown to be superior to all other tests in the study. Since then, new developments in microbiological diagnostics and molecular virology have added new possibilities for the rapid and reliable intratypic differentiation of poliovirus isolates. Cross-absorbed intratype-specific polyclonal rabbit antisera (PAbs) are currently used in an enzyme-linked immunosorbent assay (ELISA) format (7, 13). Panels of monoclonal antibodies (MAbs) that react with almost all wild isolates or all Sabin vaccine isolates have been developed (2, 6) and have been used for intratypic differentiation (5, 9). Differences in the antigenic structure between vaccine-related and wild-type poliovirus strains reflect differences in the viral RNA. Accordingly, PCR tests and hybridization assays have been developed to enable specific characterization and sensitive detection of Sabin vaccine-related strains (4, 17, 26). However, because of the genetic variability of the wild poliovirus, only genotype-specific or strain-specific primers and probes have

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been described for the positive identification of polioviruses from countries where the virus is endemic (3, 18, 27) or that cause epidemics (19). Another principle that has been applied for the genetic differentiation of wild and Sabin vaccine strain-derived polioviruses is that of restriction fragment length polymorphism (RFLP) (1, 16).

A previous comparative study describing an analysis of 57 poliovirus type 1 (PV1) strains by four methods for intratypic differentiation of polioviruses (PAb neutralization, PAb ELISA, MAb neutralization, PCR) indicated discrepancies in wild or vaccine-related classification for as many as 20 strains (20). However, only a few laboratories have experience with more than one method of intratypic differentiation. Because classification of poliovirus isolates from humans and the environment has important implications for control measures within the eradication program, a more extensive comparative study was needed. At the request of WHO, a new collaborative study was initiated to evaluate five methods for the intratypic differentiation of polioviruses; each method was based on a different principle: (i) ELISA with polyclonal cross-absorbed antisera (PAb-E), (ii) the neutralization test that uses MAbs (MAb-N), and genotyping (iii) by PCR, (iv) RFLP, and (v) probe hybridization (ProHyb). The results of this collaborative study will form the basis of recommendations for the use of these methods in the laboratory network supporting the WHO poliomyelitis eradication program (22).

MATERIALS AND METHODS

Design of the collaborative study. A panel of 90 poliovirus isolates, 30 of each serotype, was prepared at the Laboratory of Virology of the National Institute of Public Health and Environmental Protection (Rijksinstituut voor Volkgezondheid en Milienhygiëne [RIVM]), Bilthoven, The Netherlands. After coding at WHO Headquarters, Geneva, Switzerland, the panels were shipped to the National Public Health Institute in Helsinki, Finland; the Centers for Disease Control and Prevention in Atlanta, Ga.; the Institut Pasteur in Paris, France; the National Institute for Biological Standards and Control (NIBSC) in Potters Bar, England; and RIVM. All five participating laboratories are designated by WHO as Specialized Laboratories for Reference and Research on Poliomyelitis. Each laboratory analyzed the samples by at least two methods. Each method was used by at least three laboratories. The results were reported to WHO Headquarters. RNA sequence information was used as the standard in the evaluation.

Viruses. Ninety isolates were selected from the collection at RIVM. Strains were selected on the basis of the country of origin (broad geographical spread), year of isolation (between 1977 and 1992), and their characteristics in various tests for intratypic differentiation ($\pm$20 wild or non-Sabin-like strains and $\pm$10 vaccine-related or Sabin-like [SL] strains of each serotype). A few problem strains with known aberrant antigenic or genetic properties and two mixtures of a wild virus and a Sabin virus of the same serotype were also included in the panel. The absence of heterologous polioviruses and other viruses was checked by neutralization assays. Virus stocks containing approximately 10^6 50% cell culture infective doses ($CCID_{50}$s) per ml were stabilized with 1 M $MgCl_2$ before shipment. Viruses were cultivated in each laboratory in HEp-2 Cincinnati cells (WHO cell bank at RIVM) prior to application of the various methods for intratypic differentiation.

PAb-E. The PAb-E was performed in four laboratories as described earlier (13, 21). In the protocol, rather strict rules were given for the interpretation of the ELISA results to avoid mistakes such as the failure to detect the second component, in case the sample contained a mixture of a Sabin and a wild-type virus of the same serotype. Two laboratories (laboratories 1 and 3) deviated from the original protocol by using alkaline phosphatase-labeled conjugate with the appropriate substrate instead of horseradish peroxidase-labeled conjugate. One laboratory (laboratory 3) used 2% milk as the blocking reagent instead of 5% fetal calf serum, as described in the original protocol.

MAb-N. MAb-N was performed in four laboratories with a panel of Sabin-specific and serotype-specific MAbs: two for PV1; NIBSC numbers 955 and 234), two for PV2 NIBSC numbers 1233 and 697), and two for PV3 NIBSC numbers 889 and 485) (9). This is a simplification of a previously described procedure which used six MAbs per serotype (24). The residual challenge virus infectivity of 100 $CCID_{50}$s was measured after incubation with various dilutions of the appropriate MAbs (9). Results were read after fixation and staining of the infected cell layers on days 5 to 7 after infection.

PCR. The Sabin genotype-specific PCR test was performed in three laboratories by the protocol described by Yang et al. (27). Since the isolates were typed previously, all laboratories used a single primer set for each of the serotypes. All primers recognize sequences from the VP1 region of the viral genomes.

RFLP assay. The RFLP assay was performed in three laboratories as described by Balanant et al. (1).

ProHyb assay. The ProHyb assay was performed by a protocol developed at the Centers for Disease Control and Prevention (4).

RNA sequence analysis. Partial RNA sequences from several strains have been published earlier (10, 14). Sequencing of viral RNAs from yet unsequenced strains with contradicting results in the various tests for intratypic differentiation was carried out as described by Mulders et al. (11).

RESULTS

The results obtained by the five laboratories by the various methods for the intratypic differentiation of poliovirus are summarized schematically in Fig. 1A for the 30 PV1 isolates, in Fig. 1B for the 30 PV2 isolates, and in Fig. 1C for the 30 PV3 isolates. The five laboratories tested the 90 isolates by a total of 17 individual assays, for a total of 1,498 assay results. RNA sequencing was used to resolve the discrepancies obtained in all cases. Nonreactive results were considered failures to obtain a positive test result and therefore were not scored as discrepancies.

PAb-E. Results of the analyses of the coded panel by PAb-E showed remarkable consistency between laboratories: results were identical for 88 of 90 strains in four laboratories. The few differences that were observed did not correlate with the laboratories that used slight modifications of the standard method.

MAb-N. Four laboratories examined the panel of poliovirus strains by MAb-N. Identical results were obtained for 81 strains. Three laboratories reported for some strains (three PV1, four PV2, and one PV3 strain) the appearance of a cytopathic effect in the reaction with the Sabin-specific MAbs only after 4 to 6 days, just overlapping the time span of 5 to 7 days after infection that was indicated in the protocol for fixation of the cell monolayers. In all cases, this late breakthrough of virus was interpreted as the result of a suboptimal ratio between virus and antibody concentration and was therefore scored as a neutralization-positive result.

RFLP assay. Three laboratories used the RFLP assay for the intratypic differentiation of the panel of polioviruses. Identical results were obtained for all but three of the strains (one PV1 and two PV2 strains) that could be analyzed. However, failure to produce the 480-bp amplicon was reported in a total of 12 cases (concerning nine strains two PV1, two PV2, and five PV3 strains) by two laboratories.

PCR. Identical results were obtained for 81 strains by the three laboratories that carried out the PCR test. One laboratory (laboratory 4) reported its inability to produce a Sabin type 2-specific signal even with the reference strain.

ProHyb test. The results of the analyses of the 90 strains by the ProHyb test were very consistent among the three laboratories that performed the test. Only PV1 strain 29 was characterized as vaccine related by one laboratory, while the other two laboratories reported a result of a wild-type strain.

Comparison of the results from the five tests. Identical and correct results were obtained by all tests in all laboratories for 18 PV1, 21 PV2, and 20 PV3 strains. Sequence information was available for 17 of these strains and confirmed the results obtained by the five methods of intratypic differentiation. RNA sequencing was used to resolve the discrepancies obtained in all other cases. Among the 12 PV1 strains with discrepant results, 5 (strains 9, 10, 12, 19, and 24) occurred in only one laboratory and by only one assay. For three strains discrepant results were observed by a single method in two laboratories (strain 13 by MAb-N; strains 8 and 20 by PCR). PV1 strain 29 was characterized in laboratory 4 as SL by PCR and the Pro-

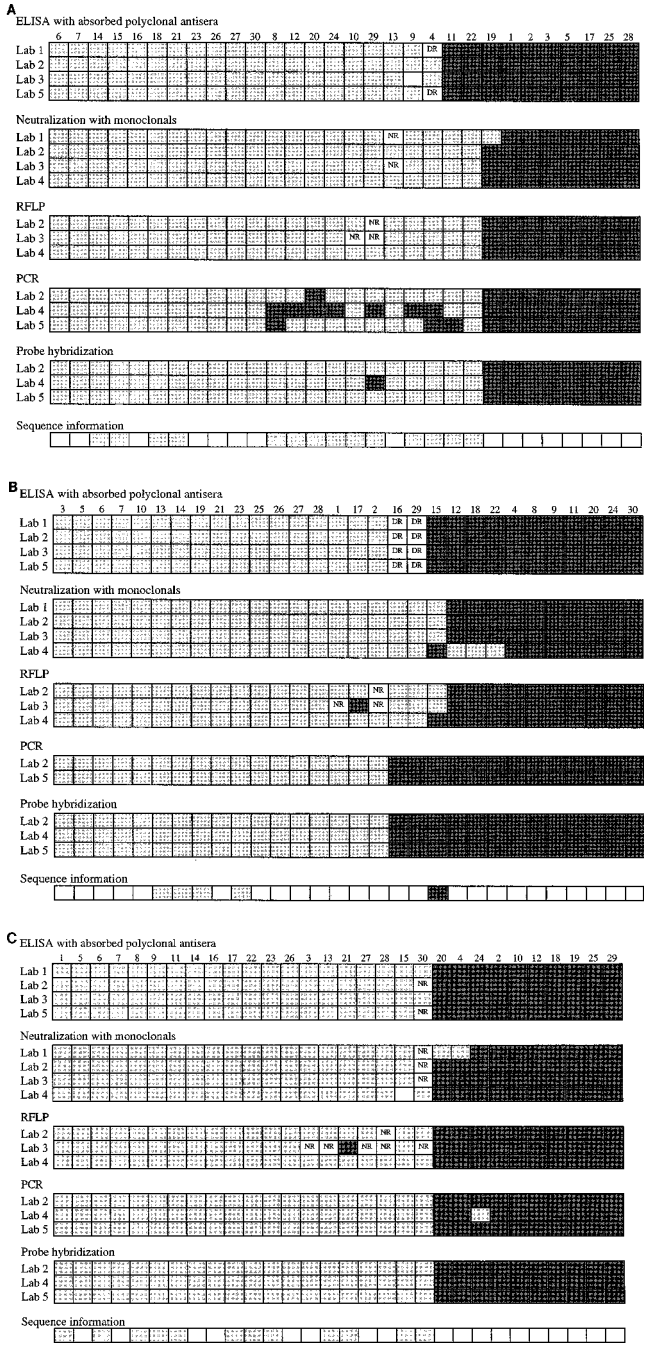

FIG. 1. Schematic representation of the results of five methods for the intratypic differentiation of polioviruses on a panel of 90 strains. The numbers correspond to strain numbers. Each row represents the results of one type of test in one laboratory. All results for a certain strain are shown in the column indicated by the strain number. (A) Results for the 30 PV1 strains. (B) Results for 30 PV2 strains. (C) Results for 30 PV3 strains. A uniform set of symbols was used for all 90 strains in the panel. The following symbols describe the indicated results: □, wild (non-Sabin-like) strain; ■, vaccine-derived (SL) strain; DR, double-reactive strain (only by PAb-E); NR, nonreactive strain; ▢, isolate not analyzed.

Hyb test. The double-reactive result by PAb-E and the SL result by PCR for wild PV1 strain 4 in laboratory 5 are also consistent with each other. The most striking results were obtained for PV1 strains 11 and 22. These stains were labeled consistently, but incorrectly, as vaccine-like by PAb-E, because all other four tests and RNA sequencing characterized them as wild-type strains.

TABLE 1. Performance scores for various tests for intratypic differentiation

Test	Percent		
	Correct result	Incorrect result	No result
PAb-E (n = 359)	96.7	2.8	0.5
MAb-N (n = 359)	93.9	4.7	1.4
RFLP (n = 270)	91.9	3.7	4.4
PCR (n = 240[a])	93.3	6.7	0.0
ProHyb (n = 270)	97.4	2.6	0.0

[a] Laboratory 4 was not able to perform a valid PV2 PCR test.

For PV2 strains 1, 12, 17, 18, and 22, the discrepancies could be attributed to a single experiment in one laboratory with incorrect or nonreactive results. Strain 2 was nonreactive by the RFLP assay in two laboratories. RNA sequencing proved that PV2 strain 15 was vaccine related and, therefore, that its characterization as a wild strain by MAb-N and RFLP assays in several laboratories was incorrect. PV2 strains 16 and 29 were mixtures of a wild strain (major component) and a vaccine strain (minor component) of the same serotype. The results by the various tests were as expected. In the PAb-E a positive reaction was obtained with both the non-Sabin-like-antiserum and the SL-specific antiserum, while the Sabin-specific MAb did not neutralize the wild virus. The RFLP assay recognized only the major wild component, and both PCR assay and the ProHyb test showed a positive reaction with the minor vaccine component, but they were not able to detect the presence of the major wild component.

For PV3 strains 3, 4, 13, 15, 20, 21, 24, and 27, all discrepancies could be explained by a single experiment in one laboratory with incorrect or nonreactive results or the specimen was not tested. Strain 28 was nonreactive in the RFLP assay in two laboratories. PV3 strain 30 was not recognized in several laboratories by the wild strain-specific cross-absorbed antiserum in the PAb-E and the type-specific MAb in the MAb-N test. These results confirm the aberrant antigenic properties of this strain that caused the Finnish epidemic in 1984 (8).

The results obtained by each of the five different methods are expressed in Table 1 as a performance score indicating the percentage of correct answers for each test. The PAb-E and the ProHyb assay have the highest scores because of a combination of a low rate of failure at producing a signal and the high degree of specificity of that signal.

DISCUSSION

Intratypic differentiation of all polioviruses, whether isolated from humans or the environment, is of increasing importance as the ultimate goal of wild poliovirus eradication by the year 2000 approaches. In recent years, various new methods for the rapid characterization of a poliovirus isolate as wild or vaccine derived have been developed. The present study of five of these methods, each of which is based on a different principle, shows that none of the evaluated methods reached a score of 100% compared with partial viral RNA sequencing. By each method at least one strain was classified differently by at least one laboratory performing the test. For 18 of the 90 strains (20%), differences in characterization were observed by the same test in various laboratories. Most of the discrepant results were obtained by the laboratories that had implemented the

method only recently and not by the laboratories that had developed the assay. However, the performance scores of the various methods, which varied between 91.9 and 97.4%, indicate much more consistent and correct results than those obtained by classical methods of intratypic differentiation (23).

Remarkably consistent results were obtained by the PAb-E assay, even though two laboratories used a slightly modified protocol. It is noteworthy that the two PV2 mixtures of a wild and a vaccine virus in the panel (PV2 strains 16 and 29) were correctly detected only by PAb-E in all four laboratories. The most striking result in the analysis of the panel by PAb-E was the consistent characterization of PV1 strains 11 and 22 as SL by all four laboratories that performed the test. Analysis by the other four tests and by determination of the 150-nucleotide sequence in the VP1-2A region of the viral RNA indicate the wild character of both strains. The strains are epidemiologically related, because they were isolated in 1990 and 1991 from throat swabs from two children with poliomyelitis from Karachi, Pakistan, 5 and 12 days after the onset of symptoms, respectively. The ELISA results were confirmed by neutralization assays with the absorbed antisera. The SL-specific antiserum, but not the wild-type-specific antiserum, neutralized 100 $CCID_{50}$s of the two viruses up to titers of 64 and 256, respectively. The most likely explanation for the observed phenomenon is that the two strains are representatives of a wild PV1 strain with an immunodominant SL antigenic determinant generated by genetic drift. Further nucleotide sequencing is probably needed to reveal the exact nature of these strains. Because most laboratories have the necessary experience with and equipment for the use of ELISA in the determination of serological parameters for microbiological infections, implementation of the test in other laboratories should present little or no problems, as long as the necessary specific reagents are provided.

Most laboratories serotype poliovirus isolates by neutralization tests, and therefore, MAb-N is a test that makes good use of existing resources, experience, and skills. MAbs also provide improved reagent standardization. In the present investigation, discrepant results were obtained for some vaccine strains that were characterized as wild. In the context of screening for wild poliovirus in a community vaccinated with OPV, this would therefore be a fail-safe result. The drawback of the MAb-N test is the 5- to 7-day incubation period, especially if repeat tests are needed.

The RFLP assay was reported as a very elegant but technically demanding test. Its ability to produce positive signals (i.e., restriction profiles) with all wild polioviruses, which can be compared with the signals for reference strains, makes the method a very useful tool not only for the intratypic differentiation of polioviruses but also for the rapid characterization of genetically related strains. Several laboratories reported problems in obtaining the 480-bp amplicon for some of the wild strains, possibly because of the presence of PCR inhibitors in conjunction with variability in the viral sequences to which the primers bind. Recultivation of the isolate at a low multiplicity of infection may solve the inhibitor problem. The RFLP method was clearly superior to the PCR method in the present study, in which large quantities of well-typed single isolates were analyzed by the various tests. In cases in which the original isolate is a wild virus with traces of Sabin vaccine virus of the same serotype, the RFLP method will identify the wild virus component, while in the PCR assay a positive Sabin vaccine virus-specific signal, and thus the wrong result, will be found. The incorrect PCR results in the present study are examples of this phenomenon. Another potential drawback of the PCR assay in the format used in the present study is that Sabin vaccine virus preparations in the presence of inhibitors may be labeled incorrectly as wild viruses, because no signal is obtained. A PCR assay with primers that specifically recognize enteroviruses must be used as a positive control (2).

The ProHyb assay appeared to be a very reliable method for the intratypic differentiation of polioviruses and has great advantages in studies in which a large number of isolates must be characterized. In the present study, the RNA-RNA hybrids were detected by chemiluminescence. This technology permits reuse of the filters with the immobilized RNA, after denaturation of the hybrids and removal of the original probe at high salt concentration. The subsequent hybridization assay could, for example, use another probe that is specific for an epidemic wild strain. For laboratories that lack facilities for the development of photographic films, a protocol for the colorimetric detection of the hybrids is also available. In that format, however, the filters can be used only once.

On the basis of the results of the present study, the following recommendations can be given for use of methods for the intratypic differentiation of polioviruses by laboratories in the network supporting the polio eradication initiative.

(i) Methods for intratypic differentiation should only be applied to typed isolates of one serotype. In PAb-E and the RFLP assay a minor heterologous component may not be detected, while it may mask the main component in PCR and the ProHyb assay. In the MAb-N assay such a mixture will lead to a nonconclusive test result. When a mixture of a wild and a vaccine-derived strain of the same serotype is suspected in a sample, positive proof of the presence of both components is needed for definite and final characterization.

(ii) Because none of the methods has a 100% performance record, intratypic differentiation of polioviruses should be performed by at least two methods; preferably, one should be based on antigenic differences and one should be based on genetic differences between wild and vaccine viruses. This recommendation minimizes the risk of incorrect or incomplete results because of the emergence of antigenic and genetic variants of polioviruses. Examples of this phenomenon are the results obtained by PAb-E for the PV1 strains 16 and 29 from Pakistan and also the presence of a wild PV1 genotype in India that is recognized by the Sabin PV1-specific primer pair (9a).

(iii) When discrepant results between the two methods are obtained, further characterization, preferably by partial sequencing of the viral RNA, is necessary for definite characterization of the isolate. Comparison with the already existing and constantly growing database of RNA sequence data from polioviruses can give additional information on the origin of the isolate.

(iv) Because of the enormous sensitivity of the Sabin vaccine strain-specific PCR assay, this method should be used only under conditions that minimize the occurrence of contamination of samples by traces of Sabin vaccine viruses. Furthermore, it is also recommended that a poliovirus or enterovirus-specific PCR assay be performed on the same sample to prove that the sample does not contain substances that interfere with the PCR.

(v) Intratypic differentiation should be performed on poliovirus isolates of epidemiological, strategic, or scientific importance. Because different countries in the world have reached different stages in the eradication program, the need for intratypic differentiation of poliovirus isolates will also differ. As long as there are many cases of poliomyelitis, circulation of wild poliovirus is evident, and intratypic differentiation of only a representative portion of the total amount of isolates will be sufficient to answer all relevant questions. As a country or region reaches the end stage of the program, intratypic differ-

entiation of all isolates will be more important to document the absence of wild poliovirus circulation in humans and the environment.

The abundance of poliovirus isolates from all over the world as a result of the progress made in the eradication program calls for the necessary transfer of methods and reagents for the intratypic differentiation of polioviruses from the laboratories that have developed these methods to laboratories working at the national or regional level in the polio eradication program. This will enable a further important step in the process that is suspected to certify, for the second time in history, after the successful eradication of smallpox in 1979, the elimination of a human epidemic disease, poliomyelitis, and of its cause, the wild polioviruses.

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