HETEROGENEITY AMONG THREE FOOT-AND-MOUTH DISEASE SUBTYPE A24 CRUZEIRO VIRUS STRAINS USED FOR THE PRODUCTION OF VACCINES

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Abstract. **Three foot-and-mouth disease (FMD) subtype A_{24} Cruzeiro virus strains used for the production of vaccines were compared with each other by T_1 mapping (fingerprinting) and by polyacrylamide gel electrophoresis of the capsid proteins. Although indistinguishable by complement fixation tests, genomic variations were found among them by T_1 mapping, reaching values of up to 4.0%. These differences were reflected in the electrophoretic mobility of the main immunogenic protein VP_1. In one of the strains, migration patterns of structural proteins VP_2 and VP_3 were also affected. The results illustrate the potential genomic diversity of strains used for production of FMD vaccines.**

Control of foot-and-mouth disease (FMD) in endemic regions is partly based on systematic immunization with chemically inactivated vaccines. Although currently effective, their production and use are affected by the extensive antigenic diversity of the virus. There are seven distinct serologic types, distributed unevenly throughout the world (*21*). In most areas, vaccines to two or more serotypes are used routinely in prophylaxis.

Each serotype comprises an ever-increasing number of subtypes, which arise more frequently in the A group (*20, 23*). Not all variants within a subtype provide cross-protection. Conversely, vaccines derived from some strains can protect against viruses from more than one subtype (*11, 19*). This illustrates the need to select vaccine strains which are capable of neutralizing effectively as broad a range of field samples as possible. In fact, it is generally accepted that new antigenic variants emerging in the field may be brought under control by using vaccines which induce broadly reactive antibodies.

Once an adequate vaccine variant strain has been selected, a major concern is to ensure its stability during vaccine production. This process usually involves multiple growth cycles in cell culture. However, during the passage of the virus in cell lines, the potential for FMDV diversity has been demonstrated. Thus, various authors (*5, 10, 15*) observed plaque variants associated with antigenic shifts following passages in baby hamster kidney (BHK) cells, while Sobrino et als. (*25*), described the emergence of genomic variants.

Since diversity is in itself the result of genetic variation (reviewed by Domingo et al. *8*), the application of molecular methods constitutes a valuable tool for variability studies, as they can identify genomic mutations which may lead to antigenic changes.

To obtain preliminary information on the impact that FMDV diversity has on individual

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vaccine strains, the T_1 maps of genomic RNA and the electrophoretic mobility on polyacrylamide gels (PAGE) of the structural proteins of three subtype A_{24} Cruzeiro vaccine strains were analyzed. As shown by the results, it was possible to establish substantial genetic differences between two of the strains studied.

MATERIALS AND METHODS

Cells and viruses

Three viral strains belonging to subtype A_{24} Cruzeiro and designated A_{24} Cruzeiro Brasil 1/55 (A_{24} 1/55), A_{24} Cruzeiro Brasil 2/55 (A_{24} 2/55), and A_{24} Cruzeiro Brasil 3/55 (A_{24} 3/55) were used in this study. The former two have been used for vaccine production at the Pan American Foot-and-Mouth Disease Center, of which strain A_{24} 1/55 is currently in use. Strain A_{24} 3/55 was obtained from the Animal Virus Research Institute, Pirbright, United Kingdom. Viruses were passaged in BHK-21, clone 13 cell monolayer cultures to the minimal extent needed for the studies.

RNA isolation and T_1 fingerprinting

^{32}P labeling of FMDV RNA and extraction of cytoplasmic RNA were carried out as described previously (*1*). The method of separation of T_1 oligonucleotides used was a modification of earlier techniques, and was performed as described (*2*).

Polyacrylamide gel electrophoresis of viral structural proteins

Structural proteins were prepared for each strain by infection of BHK-21 cell monolayers at a multiplicity of infection of 10 PFU/cell. After complete cytopathic effect was recorded, the chilled, clarified media was shaken with 10% trichlorotrifluorethane and centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant adjusted to a final concentration of NET buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA).

Viruses were concentrated by centrifugation through a cushion of 20% sucrose in NET buffer, at 95,000 x g, 3h at 4°C. The pellet was resuspended in the same buffer, and further purified by sucrose gradient sedimentation according to Denoya et al. (*6*).

Virions (2 µg) were disrupted by treatment at 90°C for 2 min in a buffer containing 80 mM Tris-HCl, pH 6.8, 10% sodium dodecyl sulphate (SDS), 8M urea, 1.2 M 2-mercaptoethanol, 20% glycerol and 0.02% bromophenol blue. Proteins were resolved by electrophoresis on SDS-12.5% PAGE (*14*), containing 8M urea (SDS-urea-PAGE).

RESULTS

T_1 fingerprinting of RNAs from A_{24} vaccine strains

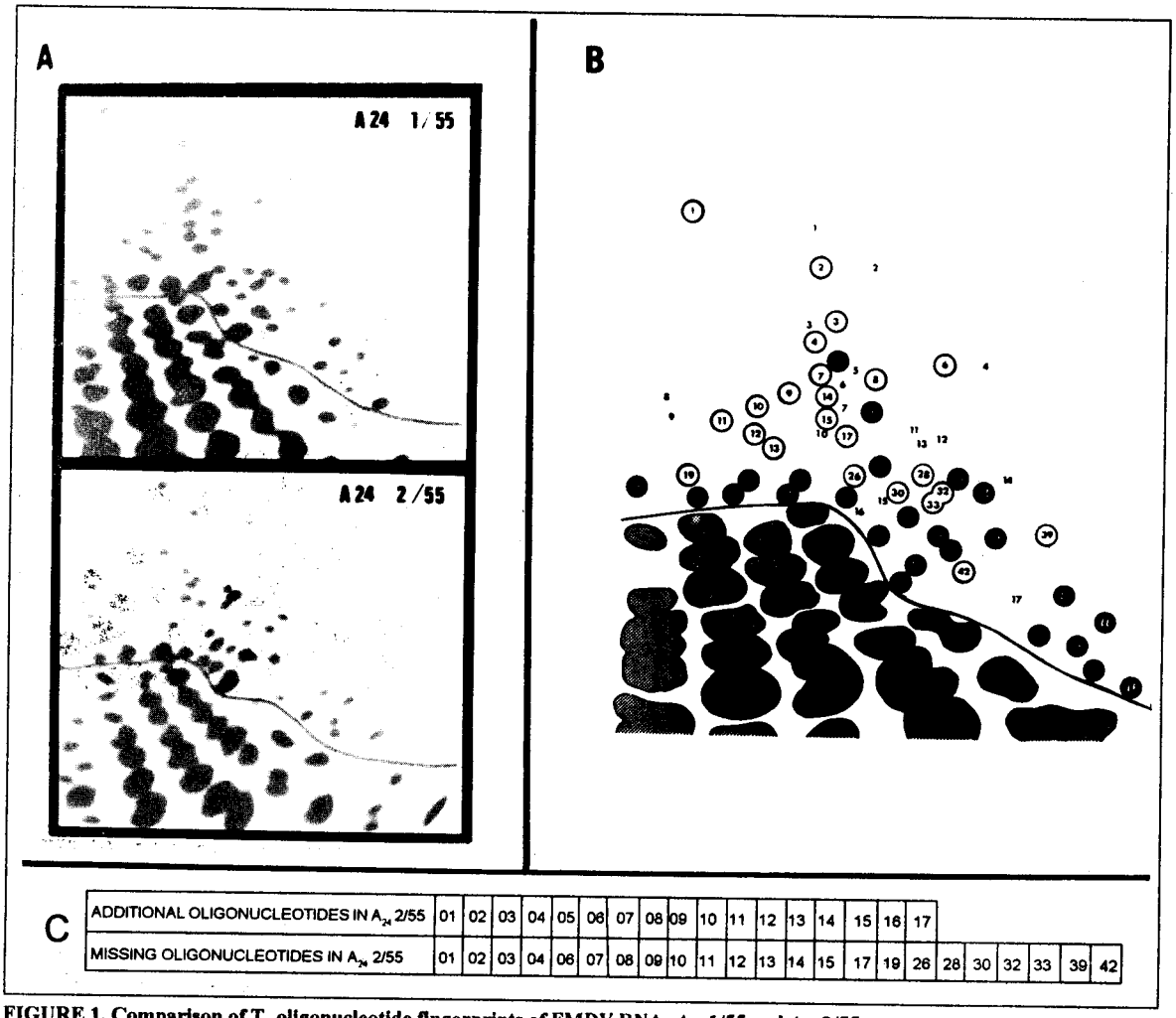
Figure 1A shows the T_1 maps from the RNAs of vaccine strains A_{24} 1/55 and A_{24} 2/55. A schematic representation of the differences between the two strains is summarized in Fig. 1B. Missing and additional spots were catalogued and the results are presented in part C of the figure. Similarly, comparisons between strains A_{24} 1/55 and A_{24} 3/55, as well as between strains A_{24} 2/55 and A_{24} 3/55 are presented in Figs. 2 and 3, respectively.

Although the three viruses were indistinguishable by complement fixation tests, each RNA had unique oligonucleotides, and the number of total spot changes estimated as described by Nakajima et al. (*16*) ranged from 2.5 (between A_{24} 1/55 and A_{24} 3/55) to 32.5 (between A_{24} 2/55 and A_{24} 3/55). These differences represent variations in 0.3-4.0% of the genomic nucleotides (Table 1).

Analysis of structural polypeptides on SDS-urea-PAGE

To establish whether the genomic changes were reflected in the structural proteins, viral purified polypeptides were resolved on SDS-urea-PAGE.

As can be seen in Fig. 4, differences in the electrophoretic mobility of capsid polypeptide VP_1 (1D) were detected in all three strains.



C

ADDITIONAL OLIGONUCLEOTIDES IN A_{24} 2/55	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17

MISSING OLIGONUCLEOTIDES IN A_{24} 2/55	01	02	03	04	06	07	08	09	10	11	12	13	14	15	17	19	26	28	30	32	33	39	42

FIGURE 1. Comparison of T_1 oligonucleotide fingerprints of FMDV RNAs A_{24} 1/55 and A_{24} 2/55
A. Autoradiographs of the ribonuclease T_1 ^{32}P-labeled oligonucleotide fingerprints of the indicated RNAs.
B. Comparative fingerprinting analyses of viruses indicated in the figure. When required, the identity of spots in the different samples was confirmed by co-electrophoresis of mixtures containing equivalent amounts of RNase T_1 digests. The black line marks an arbitrary limit between the small and large oligonucleotides, the latter (48 in the strain A_{24} 1/55) being used for comparisons. Keys for comparative analysis: Numbered solid circles correspond to spots present in both of the strains compared; circled or uncircled numbers represent oligonucleotides present only in sample A_{24} 1/55 or A_{24} 2/55, respectively.
C. Catalog of missing and additional oligonucleotides of strain A_{24} 2/55 with respect to A_{24} 1/55. Only spots in which differences were seen are listed.

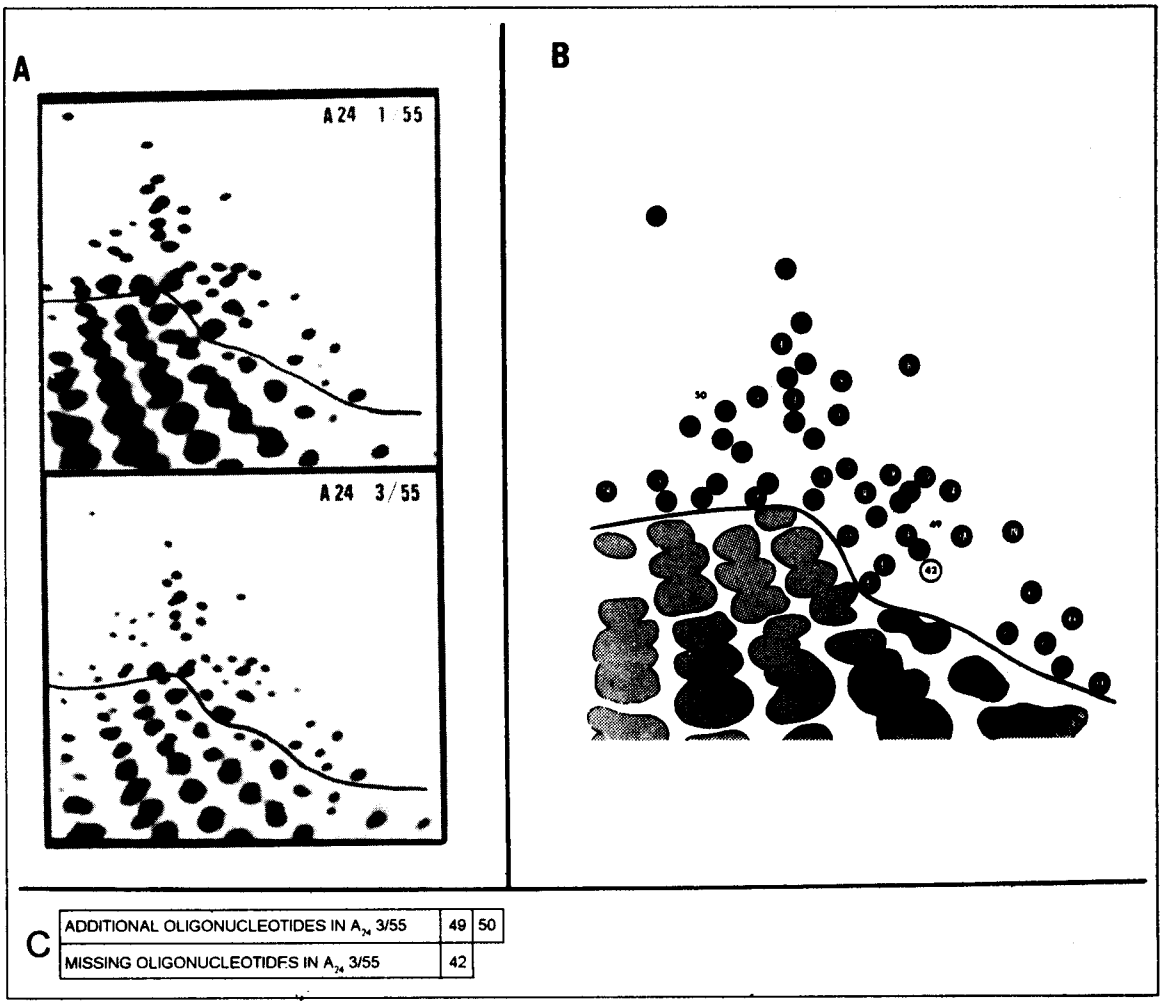


FIGURE 2. Comparison of T_1 oligonucleotide fingerprints of FMDV RNAs A_{24} 1/55 and A_{24} 3/55. Legends are as in Fig. 1.
A. Autoradiographs of the ribonuclease T_1 ^{32}P-labeled oligonucleotide fingerprints of the indicated RNAs.
B. Circled numbers indicate spots present only in sample A_{24} 1/55; uncircled numbers denote spots belonging only to strain A_{24} 3/55.
C. Catalog of missing and additional spots of strain A_{24} 3/55 with respect to strain A_{24} 1/55.

Structural polypeptides VP2 (1B) and VP_3 (1C) showed indistinguishable migration patterns in viruses A_{24} 1/55 and A_{24} 3/55. However, an increased electrophoretic mobility of these proteins was observed when the mentioned strains were compared with strain A_{24} 2/55.

DISCUSSION

The results presented herein provide evidence of heterogeneity among FMD subtype A_{24} virus strains, used for the production of vaccines. This genetic variation affected the immunogenic protein VP_1, as shown by the results of SDS-urea-PAGE (Fig. 4) and nucleotide sequencing (manuscript in preparation).

Although detailed information on the cell culture passages undergone by these vaccine strains is not available, changes observed may be attributed to their different growth histories.

In fact, the emergence of genetic variants with altered antigenic and/or immunogenic properties during replication of FMDV in cell culture has been demonstrated (*12, 13, 24, 25*), even after extensive plaque purification (*27*), or in the absence of specific antibodies against the virus (*7*).

Furthermore, genetic variation has been described as a result of adaptation of FMDV to different susceptible cell systems. Rowlands et al (*22*) reported that a number of variants within the known immunogenic region of VP_1 were selected after a single passage of bovine-derived FMDV subtype A_{12} in BHK cells. Variations in this region have also been described among different cDNA clones of the A_5 and A_{24} subtypes (*26*). Bolwell et al (*4*) showed that an antigenic variant of FMDV, A_{22} Iraq 24/64, containing three coding differences in capsid protein VP_2, was selected upon adaptation of a virus sample from monolayer to suspension cell cultures. The monolayer-adapted strain induced antibodies which neutralized effectively a wider range of field variants than the suspension-adapted virus.

TABLE 1. Pairwise comparisons among the three strain.

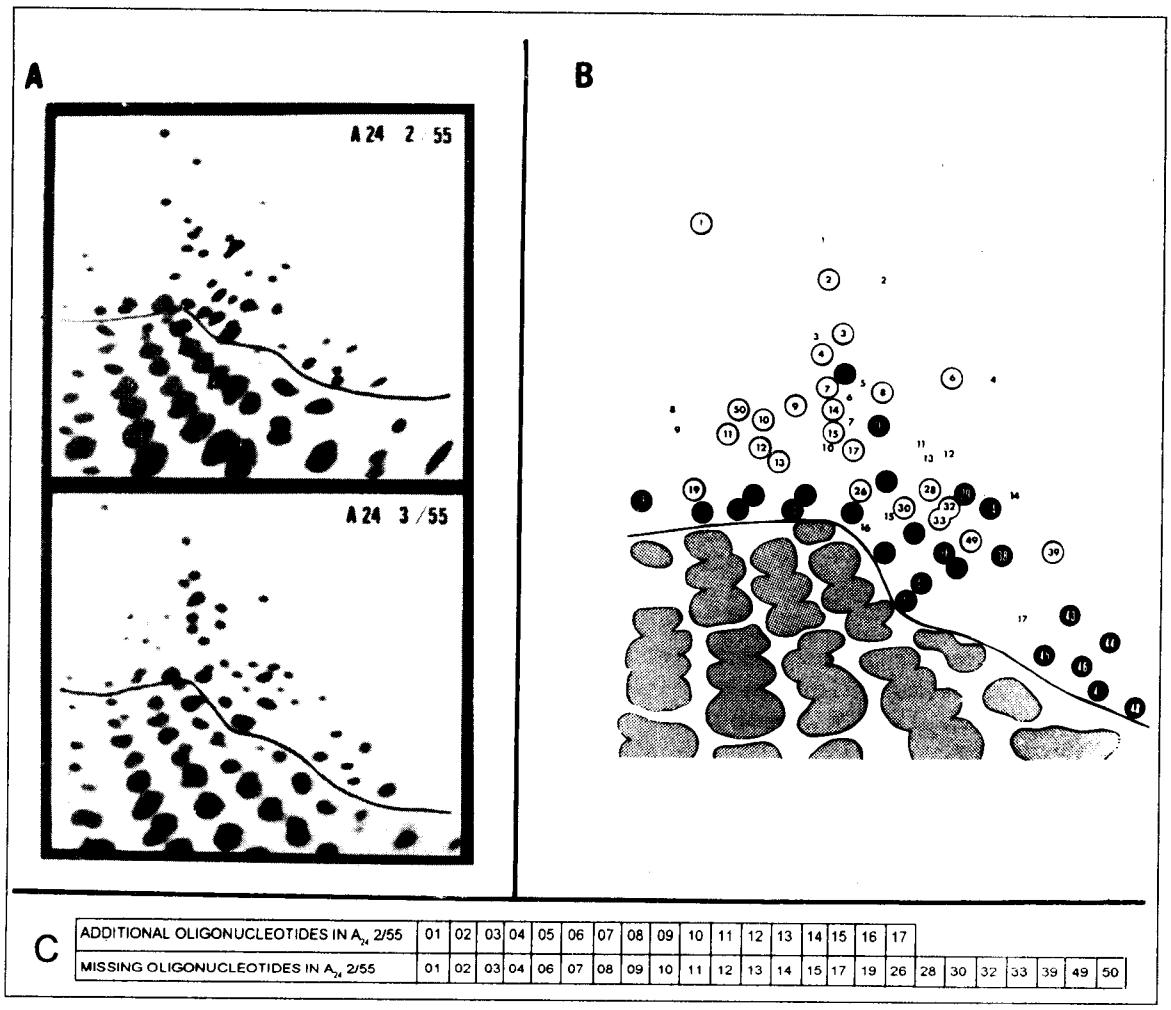
	A_{24} 1/55	A_{24} 2/55	A_{24} 3/55
A_{24} 1/55	-	31,5	2,5
A_{24} 2/55	3,8	-	32,5
A_{24} 3/55	0,3	4,0	-

Note: Total spot changes (upper right) estimated as described in Nakajima et al., 1978, and percentage of variation calculated assuming that 10% of the genome is analyzed, and that the large oligonucleotides are representative of the whole genome (lower left).

Consequently, and in agreement with the results presented herein, vaccine strains may display differences between lots, according to their passage history. Heterogeneity among viral preparations used for vaccine production in Spain was also reported (*9*).

Early results by Cowan et al. (*5*) suggested that the fixation of relevant antigenic changes can impair the expected immunogenicity of vaccines. These authors were able to associate the antigenic shifts occurring by culturing FMDV in BHK cells, with a reduced ability to immunize against the parental virus. In contrast, Parry et al. (*17*) found no correlation between antigenic variants and apparent performance in potency tests, of 18 BHK suspension cell-adapted vaccine strains. Studies are in progress to establish whether the modifications observed in the three vaccine strains analyzed here, affected their immunogenicity.

Even when heterogeneity is inherent to FMDV populations, the emergence of new antigenic variants during vaccine production could be minimized by reducing the number of viral growth cycles in a seed lot system. However, in the case of Frenkel's procedure, this is not practicable and variation has been reported after the continuous passage that is inherent in the system (*18*).



C

ADDITIONAL OLIGONUCLEOTIDES IN A_{24} 2/55	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17
MISSING OLIGONUCLEOTIDES IN A_{24} 2/55	01	02	03	04	06	07	08	09	10	11	12	13	14	15	17	19	26	28	30	32	33	39	49	50

FIGURE 3. Comparison of T_1 oligonucleotide fingerprints of FMDV RNAs A_{24} 2/55 and A_{24} 3/55. Legends are as in Fig. 1.
A. Autoradiographs of the ribonuclease T_1 ^{32}P-labeled oligonucleotide fingerprints of the indicated RNAs.
B. Circled numbers indicate spots present only in sample A_{24} 3/55; uncircled numbers denote spots belonging only to strain A_{24} 2/55.
C. Catalog of missing and additional spots of strain A_{24} 2/55 with respect to strain A_{24} 3/55.

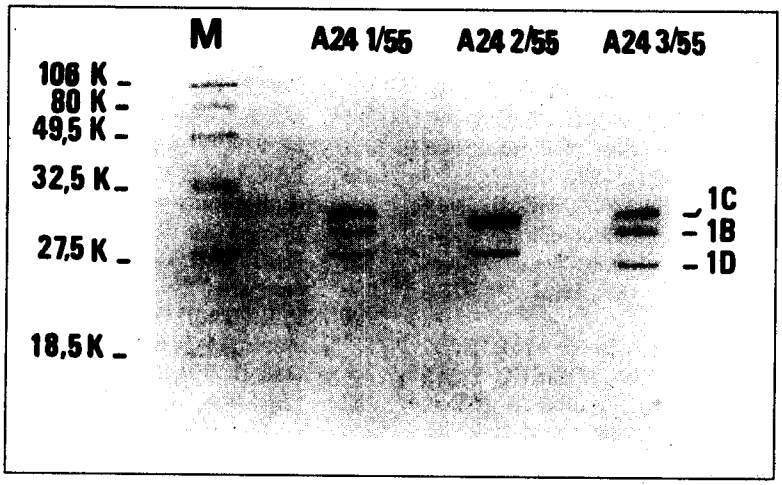


FIGURE 4. Analysis on SDS-urea-PAGE gels of viral structural polypeptides of the studied strains. M: Molecular weight markers.

From a practical standpoint, these studies emphasize the need to test the virus stocks used for production (and challenge) of vaccines. This is particularly important, in view of the modern tendency of selecting broad spectrum vaccine strains (even for highly labile serotypes such as A), for use throughout a large geographical region such as South America.

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