Genomic Characterization of Porcine Rotaviruses in Italy

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Received 25 July 2000/Returned for modification 12 September 2000/Accepted 11 October 2000

A total of 23 rotavirus strains isolated from pigs were analyzed. Twenty strains had been isolated from diarrheic piglets from an outbreak that occurred in northern Italy in 1983. Three strains had been isolated in 1984 from swine herds located in distinct areas of northern Italy. All 23 strains were characterized as type G6P[5] by PCR. The isolation from piglets of rotaviruses displaying typical bovine G- and P-type specificities points out the high frequency of rotavirus transmission between cattle and pigs.

Group A rotaviruses are a major cause of acute gastroenteritis in a variety of mammalian and avian species. They belong to the Reoviridae family and possess a genome consisting of 11 segments of double-stranded RNA (dsRNA) enclosed in a triple-layered capsid. The outer layer is composed of two proteins, VP7 and VP4, that elicit neutralizing antibody responses and that form the basis of the current dual classification system in G (VP7) and P (VP4) types (5, 6).

There are at least 14 different G types, distinguishable on the basis of both serological and genomic techniques, with a substantial correlation between G serotypes and genotypes. Since the VP4 protein carries the minor neutralizing antigen, the serological distinction of the P types is much more difficult than the classification based on genomic analysis. To date, at least 10 P serotypes and 20 different P genotypes have been described. Nevertheless, there is not a complete correlation between P serotypes and genotypes, so that a different designation has been adopted (open numbers for P serotypes, numbers in brackets for P genotypes) (5).

The main G types previously identified in pigs are G3 (CRW-8 type), G4 (Gottfried type), G5 (OSU type), and G11 (YM type) (5), although human types G1, G2, and G9 (1, 4, 22, 24) and bovine types G6, G8, and G10 have also been described (14, 19). The most common P types of pigs are P2B[6] and P9[7], which are Gottfried- and OSU-like types, respectively. However, other porcine P genotypes, P[14] (MDR13 type) and P[19] (4F type), have been recognized (2, 5, 14, 26, 27). Furthermore, typical human P genotypes P[8] and P[6] (M37-like type) (22, 24) and bovine P genotypes P[1], P[5], and P[11] have also been detected (13, 19). Nevertheless, data are fragmentary because of the limited number of viruses examined, the noncontemporaneous characterization of both viral outer proteins, and, finally, the typing methods used.

In this study, the characterization of several rotavirus strains isolated in northern Italy from piglets with diarrhea is reported.

MATERIALS AND METHODS

Viruses. The outbreak occurred in 1983 in a herd of 250 sows with 20 litters. Signs of severe enteritis were observed in 12- to 30-day-old suckling pigs from six litters, with mortality rates ranging from 5 to 9%. A total of 20 rotavirus strains were isolated on MA-104 (monkey kidney) cells from fecal samples of diarrheic animals (8). In addition to the 20 strains isolated in the outbreak described above, three strains isolated from swine herds from different geographic areas of northern Italy (strains 84/52F, 84/100F, and 84/158F) (8) were also analyzed. All the viruses were cultured on MA-104 cells with the addition of 5 µg of trypsin per ml to the maintenance medium, and viral growth was monitored by observation of a cytopathic effect and by indirect immunofluorescence with a rabbit antiserum to rotavirus. The following viruses were used as reference strains: porcine rotaviruses Gottfried, G4P[2B][6], and OSU, G5P[7], and human strain YO, which is type G3P[1A][8]. Furthermore, bovine rotaviruses RV157/99-8224, RV13/95, and RV157/99-716, previously characterized as G6P[4], G8P[7], and G10P[9][1], respectively, were used as controls.

RNA extraction. For RNA extraction, cell culture-adapted viruses were used at the third passage. The genomic dsRNA of each isolate was extracted from the infected MA-104 cells showing a 50% cytopathic effect with the Rneaky test (Qiagen GmbH, Hilden, Germany). The RNA extracted was resuspended in RNase-free water and was stored at −80°C.

G-type determination. The G typing consisted of three steps, as described by Gouvea et al. (12, 14), with some modifications. The reverse transcription (RT) and the first PCR amplification were performed with the GeneAmp RNA PCR Core kit (Perkin-Elmer Europe B. V., Monza). Briefly, 2 µl of viral dsRNA was denatured with 1.4 µl of dimethyl sulfoxide (97°C for 5 min) and was immediately cooled on ice. The denatured RNA (2 µl) was added to 18 µl of an RT mixture containing 1× PCR buffer II (KCl, 50 mM; Tris-HCl, 10 mM [pH 8.3]), MgCl2 (2.5 mM), deoxyribonucleoside triphosphates (700 µM), 1 U of RNase inhibitor, 2.5 U of murine leukemia virus reverse transcriptase, and each of the primers (Beg9 and End9) at a concentration of 50 nM. After synthesis of cDNA (42°C for 45 min, 99°C for 5 min), the mixture was brought up to a volume of 100 µl by addition of PCR reagents and distilled H2O to obtain the following mixture: 1× PCR buffer II (KCl, 50 mM; Tris-HCl, 10 mM [pH 8.3]), MgCl2 (1.5 mM), (150 µM), both of the primers (primers Beg9 and End9) at a concentration of 50 nM each, and 2.5 U of DNA polymerase. The PCR mixture was subjected to 25 cycles at 94°C for 1 min, 42°C for 2 min, and 72°C for 1 min. Two microliters of the product of the first PCR, diluted 1:100 in distilled H2O, was used as the template for the second PCR amplification in a 100-µl mixture containing 1× PCR buffer II (KCl, 50 mM; Tris-HCl, 10 mM [pH 8.3]), MgCl2 (1.5 mM), dNTPs (200 µM), 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Europe B. V., Monza), and each of the primers at a concentration of 50 nM. For the G-type characterization, a second PCR amplification was performed separately with different pools of type-specific primers: primer sEnd9 (antisense) with a pool of primers specific for the G1, G2, G3, G4, and G9 (sense) serotypes and primer sBeg9 (sense) with a pool of primers specific for the G5, G6, G8, G10, and G11 (antisense) serotypes. The mixtures were subjected to 10 min of incubation at 94°C for activation of the DNA polymerase and 25 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min. The PCR products were analyzed on a 1.5% TBE (Tris-borate-EDTA [pH 8]) agarose gel and were stained with ethidium bromide, and the G serotype was determined on the basis of the size of the amplicons, as described previously by Gouvea et al. (12, 14). In order to confirm the results, the
strains were also characterized by a PCR strategy analogous to the one described by Isegawa et al. (17) with primer pair Bov9Com5-Bov9Com3 for RT (48°C for 1 min, 50°C for 2 min, and 72°C for 3 min for 25 cycles). The amplicons were partially sequenced, and the sequences obtained were analyzed by the National Center for Biotechnology Information’s and the European Molecular Biology Laboratory’s analysis tools.

### RESULTS

By PCR, the 20 porcine rotaviruses isolated in the same outbreak were all characterized as type G6P[5]. The three strains isolated outside this outbreak (strains 84/52F, 84/106F, and 84/158F) were also characterized as type G6P[5].

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**TABLE 1. Primers used for G- and P-type characterization**

| Assay and primer | Sequence (5’→3’) | Sense | Position |
|------------------|------------------|-------|----------|
| G typing         |                  |       |          |
| Beg9<sup>e</sup> | GCCTTAAAAGAGAAAGATTTCGTCAGG | +     | 1–28     |
| sBeg9<sup>e</sup> | GCCCTTTAAAAGAGAAAGTTTTC | +     | 1–21     |
| End9<sup>f</sup> | GGTCACATCATACAAATCTAATATTAAG | –     | 1062–1086 |
| sEnd9 (RY9G)<sup>f</sup> |GGTCACATCATAACAAATCTAATAGTC | –     | 1002–1045 |
| G1<sup>a</sup> (aBT1)<sup>e</sup> | CAAAGACTCATAATCAATGATG | +     | 314–335 |
| G2<sup>a</sup> (aCT2)<sup>e</sup> | CAATGATAATTAACACATTTTCGGTG | +     | 411–435 |
| G3<sup>a</sup> (ET3)<sup>e</sup> | CGTTGAAGAAAGTTGCAACAG | +     | 689–709 |
| G4<sup>a</sup> (aDT4)<sup>e</sup> | CGTTTCGTTGAGAAGGTTG | +     | 480–498 |
| G9<sup>a</sup> (aFT9)<sup>e</sup> | CTAGATGAATCTTACATACAT | +     | 757–776 |
| G5<sup>f</sup> (FTS)<sup>e</sup> | CACGTACTGTCGTGATCAGTGC | –     | 779–780 |
| G6<sup>f</sup> (DT6)<sup>e</sup> | CTAGTTCCGTCGTCGATAC | –     | 499–501 |
| G8<sup>a</sup> (HTS)<sup>e</sup> | CGTTCGCGGGATAGACAC | –     | 273–256 |
| G10<sup>a</sup> (E10)<sup>e</sup> | TACCGGGTGTGGCAG TTTCT | –     | 714–697 |
| G11<sup>a</sup> (BT11)<sup>e</sup> | GTCATCAGCAATCTGATGTTGC | –     | 336–316 |
| Bov9Com3<sup>f</sup> | TCACATCATACAAATCTGATCT | +     | 1038–1060 |
| BovCom5<sup>f</sup> | GTAGATGATATTAATTTTGATTTAC | +     | 50–71     |
| G6<sup>e</sup> | GGATCAGCGATTTCTGTTG | –     | 336–315 |
| G10<sup>e</sup> | AACGTTCTGATATTGTTGTGCTT | –     | 692–671 |

| P typing         |                  |       |          |
|------------------|------------------|-------|----------|
| Com2<sup>d</sup> | ATTTCCGGACCATTTAACA | –     | 887–868 |
| Com3<sup>d</sup> | TGCCCTGCTGCTCATTTTAGAC | –     | 11–32    |
| P1<sup>b</sup> (pCDVY)<sup>d</sup> | CGAACCGGGGTTGATGTTG | +     | 269–289 |
| P5<sup>a</sup> (pUKY)<sup>d</sup> | GCCAGTTGTCGATCAG | +     | 336–354 |
| P11<sup>d</sup> (pB223)<sup>f</sup> | GGAACGTATCATTAGGGTGGT | +     | 574–594 |
| P6<sup>a</sup> (pGot)<sup>d</sup> | GCTTCAAGTCTTACATCAG | +     | 465–487 |
| P7<sup>c</sup> (pOSU)<sup>d</sup> | CTTATCCGTTGGAGAATCGTAC | +     | 389–412 |
| Bov4Com5<sup>f</sup> | TTCTATGAGAGGTAGCTACCA | +     | 1067–1088 |
| Bov4Com3<sup>f</sup> | CAAACCACGCTATATGATATC | –     | 1930–1909 |
| P1<sup>d</sup> | TTTATCCTCTCTCTTTTAC | –     | 1526–1505 |
| P5<sup>d</sup> | GGCAGCCTCAGTTAGACAT | –     | 1725–1704 |
| P1<sup>d</sup> | TGACTTCTAATTTGTTGTGCT | –     | 1398–1377 |

### Notes:

a Data from Gouvea et al. (12).
b Data from Gouvea et al. (14).
c Data from Isegawa et al. (17).
d Data from Gouvea et al. (12).
e Data from Gouvea et al. (13).
f Data from Isegawa et al. (17).
g Primers are located as indicated as originally reported in the corresponding reports.

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Moreover, the detection of G10 porcine rotavirus genotypes have previously been detected. The G5 (37.3%), P[6] (Gottfried-like type) (23.7%), and P[9] type specificities have been described in Thailand and Brazil (19, 22).

It was possible to isolate 20 viruses sharing the G6P[5] specificity from piglets with diarrhea in northern Italy. With a few exceptions (15, 20), G6P[5] seems to be the most widespread combination among bovine rotaviruses all over the world (3, 9, 25). Partial sequence analysis revealed substantial nucleotide and amino acid sequence identities between strain 83/15F and 83/16F, isolated in the same outbreak of diarrhea in piglets. It is clear that in the outbreak described above, all the viruses represented multiple isolates of the same strain, suggesting a quick and efficient transmission of the virus among the six litters. These pieces of evidence highlight the potential pathogenic role of bovine rotaviruses in piglets as a consequence of interspecies transmission. Nevertheless, the possibility that the G6P[5] porcine isolates are molecular reassortants between porcine and bovine strains may not be excluded. Only sequence analysis of other RNA segments or RNA-RNA hybridization (18) might clarify whether these porcine isolates have resulted from direct interspecies transmission or from interspecies reassortment.

At the moment, the lack of epidemiological data does not to those of strains 83/15F and 83/16F at the nucleotide level and was about 98% similar at the amino acid level. As shown in Tables 2 and 3, the VP7 and VP4 genes of all three porcine isolates showed the highest degree of amino acid sequence similarity to those of bovine strain UK, type G6P[5].

### DISCUSSION

The development of genomic tools for the characterization of rotavirus VP7 and VP4 specificities has quickly led to a better definition of the relative distributions of rotavirus G and P types in humans and bovines. Conversely, epidemiological data relative to porcine rotaviruses are poor. Porcine rotavirus G4, G5, G11, P[6] (Gottfried-like type), and P[7] genotypes have been shown to be common in the United States and Brazil (19, 22).

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At the moment, the lack of epidemiological data does not support the hypothesis of direct transmission from bovine to porcine. The epidemiological similarity of the porcine isolates with bovine isolates suggests that the porcine isolates may be part of the same transmission event. However, further studies are needed to confirm this hypothesis.
allow exclusion of the possibility that typical bovine rotavirus G and P types are more common in pigs than was previously believed. Thus, the isolation in the same years of rotaviruses with G6P[5] specificity from swine herds located in different areas (strains 84/52F, 84/106F, and 84/158F) leads to the hypothesis that interspecies transmission of rotaviruses between cattle and swine occurs frequently. Interestingly, in Italy rotaviruses with G6 specificity have also been detected in children affected by acute gastroenteritis (11). Similarly, in Thailand, where G10 bovine rotaviruses are highly prevalent, G10 rotaviruses have been detected in pigs (19) and humans (26).

In conclusion, this study reports for the first time the repetitive isolation of rotaviruses with typical bovine rotavirus specificities from piglets in different regions of northern Italy during the years of 1983 and 1984. Recent epidemiological studies on the relative distributions of bovine rotavirus G and P types in Italy indicate that the G6P[5] combination is widespread (7, 21).

It is now clear that for the comprehension of rotavirus ecology it is important to define the relative distributions of both human and animal rotavirus G and P types. Despite the relatively small amounts of viruses analyzed and the retrospective nature of this study, the results obtained provide interesting insights into the interspecies circulation of rotaviruses. Further epidemiological studies are required to better define the current relative distributions of porcine rotavirus G and P types in Italy and better understand whether the interspecies circulation that occurs between cows and pigs is occasional or not.

ACKNOWLEDGMENTS

We thank D. Narcisi for excellent technical collaboration and S. Arista (Department of Hygiene and Microbiology, University of Palermo, Palermo, Italy) for supplying human rotavirus strain YO.

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