Human, porcine and bovine rotaviruses in Slovenia: evidence of interspecies transmission and genome reassortment

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A surveillance of human, porcine and bovine rotaviruses was carried out in Slovenia in 2004 and 2005. Stool samples were collected from a total of 406 pigs (373 from asymptomatic animals), 132 cattle (126 from asymptomatic animals) and 241 humans (all with diarrhoea), tested for group A rotaviruses using RT-PCR and analysed by sequencing. The aims of the study were to determine the incidence of asymptomatic rotavirus infection in animals, to look for evidence of zoonotic transmission and to detect reassortment among rotaviruses. The rates of asymptomatic shedding of rotaviruses in pigs and cattle were 18.0 % (67/373) and 4.0 % (5/126), respectively. Evidence for zoonotic transmission was detected in one human rotavirus strain, SI-MB6, with the G3P[6] genotype combination, as the nucleotide and predicted amino acid sequences of the VP6, VP7, VP8* and NSP4 genes of strain SI-MB6 and of porcine strains showed high nucleotide and amino acid sequence identity. Two porcine rotavirus strains carried VP7 of probable human origin, suggesting an interspecies reassortment event in the past.

INTRODUCTION

Rotaviruses are an important cause of acute childhood diarrhoea worldwide and are an important pathogen in various animal species (Estes, 2001). It has been estimated that up to 600 000 deaths a year are caused by rotavirus disease worldwide (Parashar et al., 2003). Rotaviruses belong to the family Reoviridae and are non-enveloped viruses, 75 nm in diameter. Their genome consists of 11 segments of dsRNA and encodes six structural (VP1–VP4, VP6, VP7) and six non-structural (NSP1–NSP6) proteins (Estes, 2001). Rotaviruses can be distinguished as seven serogroups (A–G), based on the major capsid protein VP6. The most prevalent rotavirus group in humans and animals is group A (Parashar et al., 1998). According to the antigenic characteristics of the glycoprotein VP7 and the protease-sensitive protein VP4 of the outer layer, group A rotaviruses are further classified into different G and P types, respectively (Estes, 2001). In addition, rotaviruses are classified into different G and P genotypes based on differences in the nucleotide sequences of the genes encoding these proteins. As both epitopes are important for host immunity, eliciting neutralizing antibodies, a dual classification system for rotaviruses is used. To date at least 15 G and 27 P genotypes have been characterized using molecular techniques (Estes, 2001; Hoshino et al., 2002; Khamrin et al., 2007; Liprandi et al., 2003; Martella et al., 2003, 2006a, 2006b; McNeal et al., 2005; Rahman et al., 2005; Rao et al., 2000; Steyer et al., 2007a). Some of these genotypes have been found only in certain animal species or in humans and appear to be host-restricted. Moreover, specific genotypes have been found in humans and in some animal species. Some of these show high levels of nucleotide and amino acid homology, suggesting interspecies transmission of rotaviruses (Gentsch et al., 2005).

As the rotavirus genome is segmented, it is possible and not uncommon for rotaviruses to undergo reassortment events. This has been demonstrated in previous studies (Iturriza-Gómara et al., 2001; Midthun et al., 1987). Reassortment was detected within rotaviruses infecting one host species and also in rotaviruses detected in different hosts. Reassortment events result in new rotavirus strains with variable antigenic properties (Gentsch et al., 2005; Matthijnssens et al., 2006). The introduction of a new human–animal reassortant rotavirus strain into the human population could have a huge impact on the spread of these viruses.
of rotavirus disease and also on prevention measures. The efficiency of rotavirus vaccines on the market has to be followed with particular attention to new, emerging rotavirus genotypes and strains with unusual genotype combinations.

The aim of this study was to determine the incidence of asymptomatic shedding of rotaviruses in healthy pigs and cattle. In addition, genetic relatedness between human, porcine and bovine rotaviruses was examined based on nucleotide and predicted amino acid sequences of four major genes in rotaviruses: VP4 (VP8*), VP6, VP7 and NSP4. Human, porcine and bovine rotavirus strains were collected in parallel in Slovenia for the first time and molecular analysis was carried out. To date, only one study in Europe has been conducted comparing human and animal rotavirus strains collected simultaneously in one defined area (Van der Heide et al., 2005), but in that study no evidence of interspecies transmission was detected.

**METHODS**

**Stool samples and study population.** Stool samples were collected from children up to 5 years of age hospitalized with diarrhea in the two main hospitals of Slovenia: University Medical Centre Ljubljana and University Medical Centre Maribor. Sampling was carried out from January to April and from October to December in 2004 and 2005, respectively. Of 1155 stool samples screened for enteric viruses, a total of 241 rotavirus-positive samples were included in this study.

A total of 406 porcine and 132 bovine stool samples were collected from animals, mostly without diarrhea. The distribution of animals with and without diarrhea is shown in Table 1. Geographically widespread animal farms (not shown) were included in this study to ensure equal distribution of detected rotavirus strains throughout the country. Pigs and cattle were divided into the following age groups: suckling (up to 3 weeks), weanling (3–10 weeks) and fattening (more than 10 weeks). For cattle, only two age groups were formed: calves (up to 150 kg, an approximate age of 4–5 months) and adults (above 150 kg).

A single stool sample was collected from each human and animal subject.

**Case report of a child with rotavirus infection with the porcine-like rotavirus strain SI-MB6.** A 9-month-old child was hospitalized for acute diarrhea, vomiting, fever and mild dehydration. Diarrhea had started 2 days prior to hospitalization. At the time of hospitalization, the patient had mucous and bloody stools. A stool sample was taken for bacterial and virological examinations, which revealed co-infection of *Campylobacter jejuni* and rotavirus of group A. After parenteral rehydration and 5 days of hospitalization, the patient was released. The residence of this child was in a rural part of Slovenia with much agriculture and intensive farming, mostly pig farms. The child had contact with some domestic animals such as dogs, cats, horses, goats and turkeys.

**RNA isolation and RT-PCR.** For both human and animal samples, a 10% suspension of a stool sample was prepared in PBS (pH 7.4). After a short spin for 5 min at 1600 g, supernatant suspensions were used for RNA isolation.

The isolation of total RNA was carried out using TRIzol reagent, following the manufacturer’s instructions (Invitrogen). RNA was resuspended in 30 μl nuclease-free water (Promega) and 2 μl was
used for RT-PCR. For the detection of group A rotaviruses, the VP7 gene was amplified using the primer pair Beg9/End9 as described previously (Gouvea et al., 1990). For amplification of rotavirus genome segments, a one tube/two enzyme RT-PCR system was used following the manufacturer’s instructions (Access RT-PCR; Promega). As the basis for G and P genotyping, the entire VP7 gene (1062 bp) and the VP8* fragment (876 bp) of the VP4 gene were amplified. For amplification of the VP8* fragment, the primer pair con2/con3 was used (Gentsch et al., 1992). For VP6 and NSP4 gene amplification, the primer pairs VP6F/VP6R and NSP4F/NSP4R were used, respectively (Canliife et al., 1997; Iturriza-Gómar et al., 2002). For animal rotavirus strains, previously published VP6 primers (Iturriza-Gómar et al., 2002) were modified slightly as shown in Table 2.

**G and P typing using multiplex nested PCR.** The first-round RT-PCR products of VP7 and VP8* were used as templates for determining G and P types. In a multiplex nested PCR for G genotypes, G1–G6 and G8–G11 type-specific primers were included (Gouvea et al., 1990, 1994). For the determination of P types, primers specific for P[4], P[6], P[8], P[9], P[10] and P[14] were selected (Arista et al., 1999; Gentsch et al., 1992). Multiplex nested PCR was carried out following the instructions of the original publications describing these primers. The products of genotyping were separated on gels and the genotypes were selected for sequence analysis of the VP7, VP6 and NSP4 genes.

**Sequencing and phylogenetic analysis.** Human and animal rotavirus strains that were untypable using multiplex nested PCR were selected for sequence analysis of the VP7, VP6 and NSP4 genes and the VP8* fragment of the VP4 gene. RT-PCR products were purified using an SV Gel PCR Clean-up System (Promega) and direct sequencing was performed. The PCR amplicons obtained were purified using a Centrisep purification system (Princeton Separations) and sequence data were collected using an automated nucleic acid analyser (ABI PRISM 310 Genetic Analyzer; Applied Biosystems).

For nucleotide and predicted amino acid sequence alignment and for phylogenetic analysis, the CLUSTAL_X and MEGA 3.1 software packages were used (Kumar et al., 2004; Thompson et al., 1997). Genetic distances were calculated using the Kimura two-parameter algorithm and phylogenetic trees were constructed based on the neighbour-joining method. Phylogenetic trees were statistically supported by bootstrapping over 1000 replicates. In phylogenetic trees, bootstrap values below 50 are not shown.

**Table 2.** Modification of VP6 forward and reverse primers, published previously by Iturriza-Gómar et al. (2002)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>VP6 annealing site (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP6F</td>
<td>GACGGVGVCRACATCGTGT</td>
<td>747–765</td>
</tr>
<tr>
<td>VP6-Fd*</td>
<td>GAYGGNGCDACATCGTGT</td>
<td>747–765</td>
</tr>
<tr>
<td>VP6-R</td>
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</tr>
<tr>
<td>VP6-Rd*</td>
<td>GTCCAATTCTACGTCGTTG</td>
<td>1107–1126</td>
</tr>
</tbody>
</table>

*Modified VP6 primers.

**RESULTS**

**Incidence of group A rotaviruses in human and animal stool samples and their genotypes**

In human rotavirus-positive stool samples, genotype G1P[8] was the most prevalent, found in 146/241 samples (60.6 %). The second most frequently found rotavirus genotype in humans was G9P[8] (57 samples, 23.7 %), followed by G4P[8] (18 samples, 7.5 %) and G2P[4] (11 samples, 4.6 %). There were also some other rotavirus genotypes at lower prevalence, such as G3, G8 and G12 in combination with P[8] and just one strain with the G3P[6] combination (Table 3).

Of 406 collected porcine samples, 81 (19.9 %) tested positive for group A rotavirus. The highest rate of positive samples was determined in weanling pigs (39/147 samples, 26.5 %). In suckling and fattening pigs, the incidence of group A rotavirus was 13.4 and 18.9 %, respectively. Among the rotavirus-positive pigs, asymptomatic carriage was very frequent. Overall asymptomatic carriage of rotaviruses in porcine stool samples was 82.7 % (67/81). In weanling pigs, the highest percentage of asymptomatic carriage was detected (87.2 %), whereas in suckling and fattening pigs this rate was 82.4 and 76.0 %, respectively (Table 1).

Sixteen different G/P genotype combinations were detected in porcine rotavirus strains (Table 4). The most prevalent rotavirus genotypes in pigs were G3P[6] (15 samples, 18.5 %), G4P[6] (ten samples, 12.3 %) and G5P[7] (11 samples, 13.6 %). The recently recognized rotavirus genotype P[27] (Khamrin et al., 2007; Martella et al., 2007; Steyer et al., 2007a) was found in nine (11.1 %) samples and was combined with three different G types: G1, G2 and G4 (Table 4).

In cattle, only 6/132 samples were positive for group A rotavirus and only one of these was from a diarrhoeic calf, suggesting a high rate of asymptomatic carriage (although based on a small number of animals). The genotypes of bovine rotavirus strains were G6P[1], G6P[5] and G6P[11], with two samples of each.

**Molecular and phylogenetic analysis of human, porcine and bovine rotavirus strains**

Human and animal rotavirus strains of the same G or P genotype were selected for molecular and phylogenetic analysis of the VP7, VP8*, VP6 and NSP4 genes. Only G3, G4 and P[6] were found simultaneously in human and porcine rotavirus strains. In the G3 phylogenetic branch, the human SI-MB6 strain was clustered together with the porcine G3 rotavirus strains (Fig. 1). The VP7 amino acid sequence identity of SI-MB6 and porcine G3 strains was 96.7 %, slightly higher than the identity of SI-MB6 and human G3 strains (96.2 %; data not shown). However, the human SI-MB6 strain clustered unambiguously with the
porcine G3 strains. By contrast, the SI-233/03 strain clustered with the human G3 lineage. The phylogenetic tree separated G4 strains into two branches, one of porcine G4 strains and the other of human G4 strains. It was shown that the two Slovenian G4 porcine strains (SI-P14/3 and SI-O11/4) clustered with human strains in a monophyletic branch, separate from the other described lineages of porcine G4 strains (Fig. 1). The highest amino acid identity between these two Slovenian porcine G4 strains and other human G4 strains was 94.2 % (data not shown).

Only one common P genotype, P[6], was found simultaneously in human and porcine rotavirus strains. Genotype G3P[6] was the most prevalent in porcine rotavirus strains and was also found in one rotavirus strain in humans (SI-MB6). Comparing the VP8* fragments of the porcine and the human P[6] rotavirus strains, it was found that they shared high nucleotide and amino acid identities (93.7 and 95.0 %, respectively; data not shown). This was confirmed by phylogenetic analysis where SI-MB6 grouped with porcine rotavirus strains of P[6] genotype, separate from the human strains (Fig. 2).

In the NSP4 gene analysis, it was shown that all of the porcine rotavirus strains were of NSP4-B genotype and represented a separate branch to the human cluster of NSP4-B rotavirus genotype (Fig. 3). The only exception was human rotavirus strain SI-MB6, which was found to be more related to porcine NSP4-B genotype strains (with 96.3–99.3 % amino acid identity) than to human strains (92.7–93.9 % amino acid identity) of NSP4-B genotype (data not shown). Some of the porcine rotavirus strains (SI-P21/5 and SI-P34) were more distantly related to NSP4-B genotype strains. The amino acid identity of these two strains with other strains of NSP4-B genotype was less than 90 % (data not shown). They could represent a new NSP4 genotype or at least a new lineage of the NSP4-B genotype. Human rotavirus strains with the G2P[4] genotype combination were grouped by phylogenetic analysis of the NSP4 gene into genotype NSP4-A, but were distantly related to Slovenian and other bovine rotavirus strains in this genotype (data not shown).

Human and animal strains were also analysed in a region spanning aa 241–367 (nt 747–1126 of the VP6 gene) implicated in subgroup specificity (Tang et al., 1997). As shown in the phylogenetic tree of VP6 (Fig. 4), all analysed porcine rotavirus strains were closely related and grouped separately from the human rotavirus strains. Only the human SI-MB6 rotavirus strain was found to be more closely related to porcine rotavirus strains. Bovine and human rotavirus strains with the G2P[4] genotype combination were detected in the same cluster, but were distantly related in the VP6 gene segment.

Thus, in all four analysed genes, the strain SI-MB6 was more closely related to porcine rotavirus strains than to human ones. The highest identity of amino acid sequences between human SI-MB6 and porcine rotavirus strains was found in the VP6 and NSP4 genes (99.0 and 99.3 %, respectively; data not shown).

### Table 3. Distribution of G and P rotavirus genotypes detected in human stool samples

<table>
<thead>
<tr>
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<th>G3</th>
<th>G4</th>
<th>G8</th>
<th>G9</th>
<th>G12*</th>
<th>Mixed</th>
<th>Total (%)</th>
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<tr>
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<td>241</td>
<td>(100)</td>
</tr>
</tbody>
</table>

*Characterized by sequence analysis.

### Table 4. Distribution of G and P rotavirus genotypes detected in porcine stool samples

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<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G6</th>
<th>G10</th>
<th>G11</th>
<th>Gnt*</th>
<th>Total (%)</th>
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<td>3</td>
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<td>81 (100)</td>
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</table>

*The VP7 genome segment or VP8* fragment of VP4 genome segment could not be amplified.
†Characterized by sequence analysis.
DISCUSSION

It has been discussed previously that bovine and porcine slurry brought onto land could be a possible source of contamination of water sources with animal rotavirus strains via run-off water (Cook et al., 2004). Thus, transmission of animal rotavirus strains to humans is possible not only through direct contact with animals, but also indirectly by contact with contaminated surfaces, food and water. The risk of zoonotic transmission of porcine or bovine rotaviruses is higher in rural areas with farms under intensive or extensive management. Evidence exists to show that zoonotic transmission and reassortment of animal rotaviruses occurs. Zoonotic rotavirus strains are capable of causing not only asymptomatic infection but also mild to severe diarrhoea in humans (Gentsch et al., 2005; Ghosh et al., 2007; Khamrin et al., 2006; Mascarenhas et al., 2007; Matthijnssens et al., 2006b; Palombo, 2002; Varghese et al., 2004).

In this study, the incidence of asymptomatic shedding of group A rotaviruses was highest in weanling pigs (34/147, 23.1 %). Lower incidences in suckling and fattening pigs correlate with other studies where weanling pigs were also found to be most often infected with rotaviruses (Gatti et al., 1993; Wieler et al., 2001). There is a limited number of comparable studies throughout the world that have looked for asymptomatic rotavirus infection in piglets. In the recent study of Parra et al. (2007a), only 3.3 % of porcine stool samples (30/901) were positive for rotavirus, and only five of the 30 rotavirus-infected animals had diarrhoea. Regarding this low percentage, it should be noted that, in the study of Parra et al. (2007a), the less sensitive methods of ELISA and PAGE (compared with RT-PCR) were used. However, it is well known that rotavirus infection is endemic in pig herds worldwide (Steele et al., 2004). Reports on antigen detection studies in diarrhoeic pigs have shown that two-thirds of herds are infected with rotavirus and seroprevalence studies in pigs have demonstrated that almost 100 % of animals have been exposed (Bohl et al., 1984; Steele et al., 2004). Thus, it is important to screen for the presence of rotavirus in asymptomatic infections and to characterize rotaviruses in such animals, as they could be an important source of new emerging genotypes; this is also true for humans. In this study, a very
high rate of asymptomatic carriage in piglets was detected (82.7%; Table 1). It should be noted, however, that this carriage was determined by molecular methods only and did not reflect the actual risk of transmissibility.

In our study, at least 16 different G/P rotavirus genotype combinations were detected in pigs, showing the high variability of porcine rotavirus strains. The most prevalent genotypes, G3P[6], G4P[6] and G5P[7], were also the most prevalent in other studies throughout the world (Barreiros et al., 2003; Estes, 2001; Winiarczyk et al., 2002). We detected the recently described new rotavirus genotype P[27] in 11.1% of all positive samples. It is interesting that, in this and other studies, the P[27] genotype was found in four G/P combinations: G1, G2-like, G4 (this study) and G5 (Khamrin et al., 2007; Martella et al., 2007; Steyer et al., 2007a). The rotavirus P[27] genotype strains (CMP034, 344/04-1 and P21-5) independently detected by these three author groups showed high genetic variability, as they shared only 89–90.8% VP4 amino acid sequence identity within this genotype. It has been suggested that these three P[27] strains could already represent three lineages in the P[27] genotype (Parra et al., 2007b). Both characteristics of the P[27] genotype — variability in combination with the G genotype and low amino acid identity within the VP4 genes — suggest that this genotype has probably been circulating in the porcine population for some time.

In cattle, asymptomatic shedding of rotaviruses was detected in 4.0% of healthy calves (5/126), but only one out of six infected calves was symptomatic. In our study, asymptomatic carriage of rotavirus in cattle was higher than described previously by Myers et al. (1984). In comparison with RT-PCR used in the present study, Myers...
and co-workers used the much less sensitive electron microscopy method, which could have resulted in an underestimation of asymptomatic rotavirus shedding in calves. Unfortunately, in our study, only the G6 genotype was detected in bovine rotavirus strains. This finding was not surprising as the G6 genotype has previously been reported to be one of the most prevalent G genotypes in bovine rotavirus strains (Falcone et al., 1999; Snodgrass et al., 1990).

In this study, the most common global human rotavirus genotypes were detected. The most prevalent genotype was G1P[8], followed by G9P[8]. These two genotypes represented more than 80 % of all rotavirus strains analysed in this study. It was interesting that, in our region, G9P[8] has remained the second or third most prevalent genotype from the year of its first detection up to the end of this study (Steyer et al., 2005, 2007b; Tcheremenskaia et al., 2007). This supports the evidence of G9 importance in global rotavirus molecular epidemiology.

Comparing human and animal rotavirus genotypes, only the G3P[6] combination was found to be common in human and porcine rotavirus strains. In humans, only one rotavirus strain with the G3P[6] combination was detected, and further molecular analysis confirmed that this was a porcine rotavirus, as it was most closely related to porcine rotavirus strains with G3 and/or P[6] genotypes. Moreover, genes VP6 and NSP4 of this strain (named SI-MB6) also had the characteristics of a porcine rotavirus strain (Figs 1–4). It has previously been reported by other authors that some human and porcine G3 and P[6] genotypes share a high degree of nucleotide and amino acid sequence identity (Gentsch et al., 2005; Iturriza-Gómar et al., 2003a; Martella et al., 2006b). Phylogenetic analysis of human and porcine P[6] strains has shown that human P[6] strains originated from at least three separate porcine–human transmissions as both human and porcine rotavirus P[6] strains were found in P[6] genetic lineages I, III and V (Martella et al., 2006b). This finding was supported by our Slovenian human and porcine P[6] rotavirus strains sharing a high nucleotide and amino acid sequence identity (93.7 and 95.0 %, respectively) in lineage V (Fig. 2). As the human rotavirus strain SI-MB6 was clearly more related to porcine than to human strains in all four genes analysed, we propose that this might be the result of zoonotic transmission of rotavirus from pig to human. The residence of the child infected with the SI-MB6 strain was in a rural area, but it was not reported that the child had close contact with pigs. There was no porcine rotavirus strain in our study with nearly 100 % nucleotide and amino acid sequence identity with the porcine-like human strain SI-MB6. The number of animals included in this study, a wide study area and the high diversity of G3P[6] strains make it almost impossible to identify an actual zoonotic transmission event. For a confirmation of zoonotic transmission, it would be necessary to screen environmental samples as well as porcine stool samples in the surrounding area of the child’s residence. However, indirect infection of humans from contaminated surfaces, food or water could provide additional possibilities for the introduction of animal rotavirus strains into the human population.

The VP7 genome segment of the porcine rotavirus strains SI-P14/3 and SI-O11/4 showed some relatedness between human and porcine rotavirus strains (Fig. 1). The nucleotide and the predicted amino acid sequences of other genome segments (VP8*, VP6 and NSP4) were more closely related to porcine than to human rotavirus strains (Figs 2–4). Due to the low nucleotide and amino acid sequence identities of VP7 of these two strains compared with the human VP7 rotavirus G4 strains, we cannot clearly prove reassortment between human and porcine rotaviruses. However, phylogenetic analysis suggests that this has probably happened in the past.

In phylogenetic analyses of human NSP4 and VP6 genes, a correlation between NSP4 genotype and VP6 subgroup has been described previously (Iturriza-Gómar et al., 2003b). In our VP6 and NSP4 genetic analysis, bovine rotavirus strains and human G2P[4] strains found in a cluster with strains of subgroup I specificity were clustered in the NSP4-A genotype. In contrast, all human rotavirus strains phylogenetically related to rotavirus strains of subgroup II specificity have been grouped together in the NSP4-B genotype. It was demonstrated that even closely related VP6 sequences of porcine rotavirus strains could be of different subgroups (Tang et al., 1997). Thus, we cannot speculate on the subgroup specificity of our porcine rotavirus strains based only on phylogenetic analysis of the VP6 fragment. However, all of them were closely related in VP6 phylogenetic analysis and were grouped in the NSP4-B genotype. In the NSP4 and VP6 analysis, human and animal rotavirus strains were separated in distant phylogenetic branches. The only exception again was the strain SI-MB6, showing porcine specificities in both the VP6 and NSP4 genes. The porcine origin of the SI-MB6 strain was also shown in the NSP4 amino acid species-specific region (aa 135–141), where this strain shared more amino acid homology with porcine strains than with human rotavirus strains (data not shown).

In this study from Slovenia, human, porcine and bovine stool samples were screened simultaneously for the presence of group A rotaviruses, especially in asymptomatic animals. Asymptomatic carriage in humans was not investigated on this occasion. We were able to detect some evidence for zoonotic transmission and genome reassortment within species-specific strains and between human and animal rotavirus strains. Thus, it is likely that interspecies transmission of rotaviruses is not a rare event and in the future more research should be carried out, especially on asymptomatic rotavirus infections in humans and animals. It has to be considered, though, that zoonotic transmission in itself has no direct major public health impact. However, this is the basis for reassortment events with human rotavirus strains, which could result in
efficiently replicating animal–human reassortant rotavirus strains in humans. As human and animal rotaviruses are spread in the environment through fertilization and are very stable in the environment, attention should be given to rotaviruses in environmental samples, water sources and raw food, as they may serve as a source of infection.

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