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## Evidence of interspecies transmission and reassortment among avian group A rotaviruses

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### ABSTRACT

Avian rotaviruses are broadly distributed among birds, but only scarcely characterized on the molecular level. The VP4-, VP6-, VP7- and NSP5-encoding sequences of eight group A rotaviruses from chickens and turkeys determined here indicate a low degree of sequence similarity with mammalian rotaviruses. An NSP6-encoding region was missing in all chicken isolates except for isolate Ch2. Four novel genotypes (P[30], P[31], G22 and H8) were assigned by the Rotavirus Classification Working Group. Generally, chicken and turkey isolates clustered into separate branches of phylogenetic trees. However, chicken isolate Ch2 consistently clustered together with turkey isolates. Chicken isolate 06V0661G1 has a VP4-encoding sequence of unknown origin, but possesses VP6, VP7 and NSP5 genotypes typical for chicken isolates. These results might indicate interspecies transmission and reassortment among avian group A rotaviruses under field conditions. PCR protocols enabling amplification of avian and mammalian group A rotaviruses were developed for use in further epidemiological studies.

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### Introduction

Rotaviruses are the leading cause of viral gastroenteritis in young children worldwide.

In developing countries, rotavirus infections cause estimated 460,000 deaths per year (Parashar et al., 2003), mainly due to dehydration. Currently, the wide use of novel rotavirus vaccines containing live attenuated rotavirus strains has been promoted (Ruiz-Palacios et al., 2006; Vesikari et al., 2006; Vesikari et al., 2007), however, their efficacy against a broad variety of field strains in developing countries is yet to be determined.

Rotaviruses are non-enveloped icosahedral particles containing a genome of 11 segments of double-stranded (ds) RNA (Estes and Kapikian, 2007; Ramig et al., 2005). Each segment of RNA encodes one viral protein, however, segment 11 codes for the non-structural protein (NSP) 5 and NSP6 using different open reading frames. The viral particle is formed by three layers of viral structural proteins (VP), with VP2 forming the core, VP6 the inner layer with group and subgroup-specific epitopes, and VP7 and VP4 forming the outer shell which contains the serotype-specific epitopes. VP1 and VP3 are minor structural components of the core. VP4 defines the P (protease-sensitive) types and VP7 the G (glycoprotein) types (Estes and Kapikian, 2007; Gorziglia et al., 1990). Recently, a novel classification system based on the nucleotide sequences of all rotavirus genome

segments has been suggested (Matthijnsens et al., 2008a) enabling a comprehensive characterization of rotavirus strains, which also takes into account possible reassortment events.

Rotaviruses are widespread in several animal species too. Group A rotaviruses are important etiological agents of diarrhoea in calves and piglets (Aich et al., 2007; van der Heide et al., 2005). There is increasing evidence that transmission of rotaviruses from animals to humans occurs and that it significantly contributes to genetic variability of human rotaviruses (Cook et al., 2004; Müller and Johne, 2007; Palombo, 2002). Using complete genome sequence analysis, direct transmission of a lapine rotavirus to humans was demonstrated (Matthijnsens et al., 2006). A common origin of human Wa-like strains with porcine rotaviruses and human DS1-like strains with bovine rotaviruses was proposed based on phylogenetic relationships between rotavirus strains isolated from these three different species (Matthijnsens et al., 2008a). In addition, several analyses show that some human rotaviruses contain genome segments of animal rotaviruses, which have been acquired by reassortment (Ghosh et al., 2007; Khamrin et al., 2006; Mascarenhas et al., 2007; Nguyen et al., 2007; Rahman et al., 2007; Matthijnsens et al., 2008c).

Despite a great variety of rotaviruses detected in avian species (McNulty et al., 1980; Otto et al., 2006; Otto et al., 2007), data on genome sequences and interspecies transmission of these viruses are scarce. Rotaviruses of groups A, D, F and G have been detected in different avian species, but their causative role for diarrhoea and a chronic disease designated as runting and stunting syndrome in chickens or turkeys is not completely understood (Day et al., 2007;

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McNulty, 2003; Otto et al., 2006). Infections with rotaviruses from different groups might explain the highly variable clinical signs.

For a long time, the full-length genome sequence of the pigeon group A isolate PO-13 (genotype G7P[17]) was the only one available for an avian rotavirus (Ito et al., 2001). Recently, the complete genome sequence of chicken rotavirus 02V0002G3 (Ch-2G3, genotype G19P[30]) has been additionally determined (Trojnar et al., 2009). Some additional partial sequences are available, e.g. for the chicken strains Ch1 and Ch2 (genotypes G19 and G7, respectively) and the turkey strains Ty1 and Ty3 (genotypes G17 and G7, respectively), which had been originally isolated in Northern Ireland in 1979 (McNulty et al., 1979). Although a close relationship to other isolates from chicken or pigeon is evident, these sequences are too fragmentary for assignment of genotypes for most of the other genome segments. In general, the available sequences indicate that the avian group A rotaviruses are only distantly related to mammalian rotaviruses. Also, the electrophoretic migration pattern of the genome segments of avian group A rotaviruses (5:1:3:2) is different from that of the mammalian viruses (4:2:3:2) although reactivity of monoclonal antibodies with VP6 indicate a common classification into group A (Minamoto et al., 1993). Interestingly, transmission of PO-13 to mice has been successfully demonstrated, which resulted in clinical disease under experimental conditions (Mori et al., 2001). Also, a group A rotavirus with a high degree of sequence similarity to avian rotaviruses has been isolated from a calf with diarrhoea indicating that rotavirus transmission between avian and mammalian hosts occurs under field conditions (Brüssow et al., 1992; Rohwedder et al., 1995).

To gain insight into the relationships between avian group A rotaviruses and to assess their ability for interspecies transmission, a more detailed genome analysis of eight avian isolates was undertaken here. The genes encoding the capsid proteins VP4, VP6 and VP7 were selected for analysis due to their importance as major antigenic determinants. The NSP5 gene has been included in full-length sequence analyses due to its marked low sequence similarity between PO-13 and the mammalian rotaviruses. Based on these sequences, degenerate primers for the detection of the VP4-, VP6-, VP7- and NSP5-encoding gene segments of mammalian and avian group A rotaviruses were developed, which may be useful in further epidemiological studies.

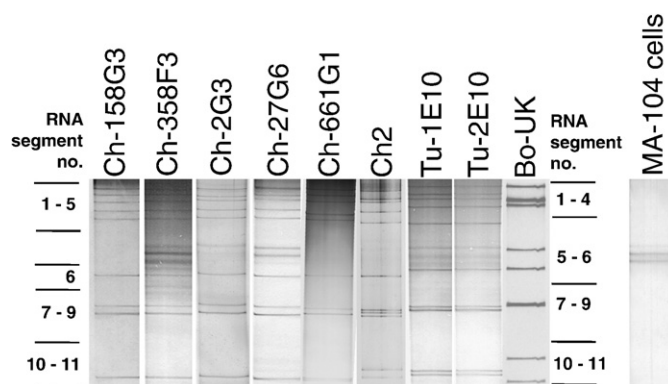
## Results

### Virus isolation and determination of electropherotypes

A cytopathic effect in MA-104 cells appeared between passages 3 and 5, characterized by granular cells and cells detached from the monolayer. The bands obtained by PAGE of the dsRNA prepared from cell culture supernatant are relatively weak with some background staining which most probably reflect the lower titres of the avian isolates (Fig. 1). However, all isolates showed a migration pattern of 5:1:3:2 which is typical for avian group A rotaviruses and which is in contrast to most mammalian group A rotaviruses showing a 4:2:3:2 pattern. Two electropherotypes of the avian isolates are evident, with one electropherotype (isolates Tu-1E10, Tu-2E10 and Ch2) showing separated segments 10 and 11 and the other electropherotype showing a close migration of both segments (all other avian isolates).

### Sequence analysis

Coding sequences for VP4, VP6, VP7 and NSP5 of the six chicken isolates and two turkey isolates, which were not available at the GenBank database (Supplemental Data 2), were amplified by RT-PCR, cloned and sequenced. Despite the use of a range of degenerate primer pairs, only the first 1596 nucleotides of the VP4 gene of isolates Ch2, Tu-1E10 and Tu-2E10 could be determined, thus each sequence lacked approximately 300 nucleotides from the 3'-end of this gene. In all



**Fig. 1.** Genome profiles of group A rotavirus isolates from chickens (Ch-158G3, Ch-358F3, Ch-2G3, Ch-27G6, Ch-661G1, Ch2), turkeys (Tu-1E10, Tu-2E10) and cattle (Bo-UK) after polyacrylamide gel electrophoresis (PAGE) of their genomic RNA. The banding pattern of the RNA is indicated beside the picture. A preparation of non-infected MA-104 cells is shown at the right.

other cases the entire coding regions were determined. The sequences were aligned to full-length (and some partial) avian rotavirus sequences available in the GenBank database as well as to selected sequences of mammalian group A rotaviruses. Human group C rotavirus was included in the analysis as an outgroup sequence. Alignments were performed based on nucleotides (nt) or on amino acid (aa) sequences, however, the latter generally resulted in a more robust grouping of the sequences as determined by bootstrap analysis and by the use of different alignment algorithms.

Generally, the phylogenetic trees established on the basis of the alignments show that for all genes the avian isolates (together with the avian-like bovine isolate 993-83) and mammalian isolates cluster in separate branches (Fig. 2). However, both sequence groups are more closely related to each other than to the group C rotavirus confirming the assignment of the analyzed avian rotaviruses to group A. Within the avian branch, two phylogenetic clusters can be distinguished for most of the analyzed genes; however, a high degree of heterogeneity is evident for the VP7-encoding sequences. Generally, the isolates from chickens are grouped into one phylogenetic cluster and those from turkeys or pigeons (or from cattle in the case of the avian-like isolate 993-83) are grouped into the other. However, all of the available sequences from the chicken isolate Ch2 are most closely related to those of turkeys, which may indicate an interspecies transmission of the virus from turkeys to chickens. The sequences encoding VP6, VP7 and NSP5 of the chicken isolate Ch-661G1 cluster together with sequences derived from chicken isolates. However, its VP4-encoding sequence did not cluster together with the sequences of other chicken, turkey or pigeon isolates, thus suggesting a reassortment event between a chicken virus and another avian virus of unknown source. Genotyping of the investigated avian strains is described in the next paragraphs in more detail; an overview on their genotype constellations is presented in Table 1.

### VP4

The VP4 aa sequences of avian rotaviruses reveal aa identities of 56.6% to 62.7% with the VP4 of mammalian group A rotaviruses. Based on the guidelines for rotavirus classification (Matthijssens et al., 2008b), which uses an 80% nt sequence identity cut-off to distinguish between different P-genotypes, the chicken isolates Ch-7G6, Ch-2G3, Ch-158G3, Ch-358F3, which only show identities up to 77.1% compared to other strains, should belong to a new P-genotype. The sequences were sent to the RCWG and P[30] was assigned. Isolate Ch-661G1 has up to 76.2% nt sequence identity to the other avian strains and has therefore been classified into a novel genotype P[31]. Comparing these findings to the traditional classification scheme

with an 89% aa sequence identity cut-off (Estes and Kapikian, 2007; Gorziglia et al., 1990), the same genotype grouping is evident as the isolate Ch-661G1 and the above mentioned chicken strains show only up to 82.9% and 85.1% aa sequence identity, respectively, to other strains. The isolates Tu-1E10, Tu-2E10 and Ch2 are most closely related to PO-13 (genotype P[17]). However, a final classification was not possible for these strains because only partial sequences were available and the calculated identities (up to 78.5%) were too low for assignment to a known genotype according to the guidelines for classification of partial sequences as described by Matthijnssens et al. (2008b). Because of the short lengths of the available VP4-encoding sequences of Ty1, Ty3 and Ch1, P-genotypes could not be assigned for these isolates.

All avian rotavirus VP4 full-length sequences consist of 770 amino acids; this is slightly shorter than the mammalian counterparts (772 to 776 amino acids) included in the analysis. A detailed comparison of avian and mammalian rotavirus sequences indicated conserved aa residues at most positions (Arg 231, Arg/Lys 247, Lys 259, Arg 467, Arg/Lys 582, aa numbering according to SA-11) known to be important for trypsin-induced activation of VP4 (Crawford et al., 2001; Gilbert and Greenberg, 1998). However, arginine at position 241 was only found in mammalian rotaviruses, but not in the avian isolates. The  $\alpha 2\beta 1$  integrin binding sequence Asp-Gly-Glu (aa positions 308 to 310 in SA-11) (Graham et al., 2006) was found to be strictly conserved in all strains. Two VP4 domains involved in membrane association and permeation by the rhesus rotavirus (Golantsova et al., 2004) are largely conserved in the avian rotavirus isolates, whereas most of the aa positions identified in sialic acid-binding of rhesus rotavirus (Dormitzer et al., 2002) are mutated in the avian VP4 sequences (aa 101, aa 146, aa 155, aa 187, aa 190).

#### VP6

The VP6 gene (Fig. 2B) is the most conserved gene among those analyzed in this study, with 69.8% to 74.9% aa sequence identity between avian and mammalian sequences. All of the isolates analyzed in this study cluster together with known avian rotavirus sequences: the isolates Ch-2G3 (Elschner et al., 2005), Ch-27G6, Ch-158G3, Ch-358F3, and Ch-661G1 belong to genotype I11 (Matthijnssens et al., 2008a) together with Ch1, and the isolates Tu-1E10, Tu-2E10 and Ch2 belong to genotype I4 (Matthijnssens et al., 2008a) together with PO-13.

VP6 has a length of 397 amino acids in all avian and mammalian rotavirus isolates analyzed. Amino acid differences between avian and mammalian rotavirus VP6 sequences are scattered over the whole sequence without obvious accumulation at specific regions. The histidine residue at position 153 involved in zinc binding (Erk et al., 2003; Mathieu et al., 2001) is strictly conserved in all isolates. The regions identified to be responsible for binding of VP2 (Charpilienne et al., 2002) are strictly conserved (aa 63 to 76) or contain only conservative aa substitutions (aa 31 to 38) between avian and mammalian isolates. Among the immunodominant T-cell epitopes of human rotavirus VP6 identified in mice, one epitope (aa 289 to 302; Choi et al., 2000) seems to be largely conserved with 11 identical amino acids out of 14, whereas the other epitope (aa 242 to 259; McNeal et al., 2007) has only 10 identical amino acids out of 18.

#### VP7

Avian and mammalian rotavirus VP7 genes show aa sequence identities of 56.4% to 62.6% while a high degree of sequence heterogeneity (66.1% to 99.4% aa sequence identity) is evident within the avian branch. Matthijnssens et al. calculated a cut-off of 80% nt sequence identity for the definition of genotypes and classified Ch2 (Nishikawa et al., 1991) and Ty3 as genotype G7, Ty1 as genotype G17, PO-13 as genotype G18 and Ch1 as genotype G19 (Matthijnssens et al., 2008a). As the chicken isolates Ch-27G6, Ch-158G3, Ch-358F3, Ch-661G1 and Ch-2G3 show high nt sequence identities of 94.8% to 95.1%

compared to Ch1, they should be classified as G19 according to the genotyping guidelines (Matthijnssens et al., 2008b). Since the VP7 genes of the turkey isolates Tu-1E10 and Tu-2E10 have only up to 79.7% nt sequence identities compared to other rotavirus sequences (just below the 80% cut-off value for VP7), they might belong to a new genotype, which was verified by RCWG, and a new genotype G22 was assigned. This classification is also supported by using the cut-off of 89% aa sequence identity of the traditional classification scheme (Estes and Kapikian, 2007; Gorziglia et al., 1990), as Tu-1E10 and Tu-2E10 have only up to 84.2% aa sequence identity to other avian sequences.

The highest degree of heterogeneity between the VP7 sequences of avian and mammalian rotaviruses is found in the amino terminal 50 amino acids. All avian isolates have an insertion of three amino acids in this region leading to a total length of 329 aa compared to 326 aa in most mammalian VP7 sequences. The  $\alpha V\beta 3$  integrin binding site at positions 161 to 169 (Zarate et al., 2004) (aa numbering according to SA-11) is largely conserved, however, Asn161 is exchanged for Tyr in all avian isolates and Pro167 is exchanged for Glu in the majority of the avian sequences. The  $\alpha X\beta 2$  integrin binding site Gly-Pro-Arg, present in most mammalian isolates (Graham et al., 2003) is not found within the avian VP7 sequences.

#### NSP5/NSP6

The avian NSP5 sequences show only low aa sequence identities (48.7%–53.3%) with those of the mammalian rotaviruses. Also, the two phylogenetic clusters within the avian branch are well separated from each other (67.9%–70.3% aa sequence identity). Using a cut-off of 91% nt sequence identity for definition of genotypes (Matthijnssens et al., 2008a), the isolates Tu-1E10, Tu-2E10 and Ch2 have to be grouped together with PO-13 into genotype H4. The NSP5 sequences of the isolates Ch-27G6, Ch-158G3, Ch-358F3, Ch-661G1 and Ch-2G3 are placed into a novel genotype H8, as was verified by the RCWG, due to nt sequence identities of only 71.1% to 73.2% to the other avian sequences.

A detailed analysis of the sequences shows significant differences in the lengths of the NSP5 sequences between the different phylogenetic clusters due to deletions or insertions of different size within the 3'-end of this gene (Fig. 3B). This leads to a length of 208 aa, 218 aa and 197–200 aa for the NSP5 derived from chickens, turkeys (additionally including PO-13 and Ch2) and mammals, respectively (Fig. 3A). Out of four serine residues previously shown to be phosphorylated in the mammalian rotavirus NSP5 by casein kinase II (Eichwald et al., 2002), only the serine at a position corresponding to serine 153 in SA-11 is also conserved in avian sequences. Highly conserved sequences are found at aa positions 57 to 69 including serine 67, which is involved in hyperphosphorylation (Eichwald et al., 2004), and at the carboxy-terminus, which is essential for dimerization of NSP5 (Torres-Vega et al., 2000).

For the chicken isolates Ch-27G6, Ch-158G3, Ch-358F3, Ch-661G1 and Ch-2G3, no open reading frame (ORF) for NSP6 is evident (Fig. 3A). Although the region carrying the initiation codon for this protein is also highly conserved in these isolates, six stop codons are present within this reading frame resulting in the first interruption of translation after aa position 21. Also, no other ORF with a coding capacity for more than 28 aa and starting with the codon AUG is present in these sequences. The NSP6 protein of the other avian isolates has only 39.6% to 45.8% aa sequence identity to mammalian rotavirus NSP6. Conserved aa sequences are mainly found in the amino-terminal half of the sequences, and between aa positions 60 to 65, whereas the carboxy-terminal sequences are highly divergent between these two groups.

#### Development of a consensus primer RT-PCR

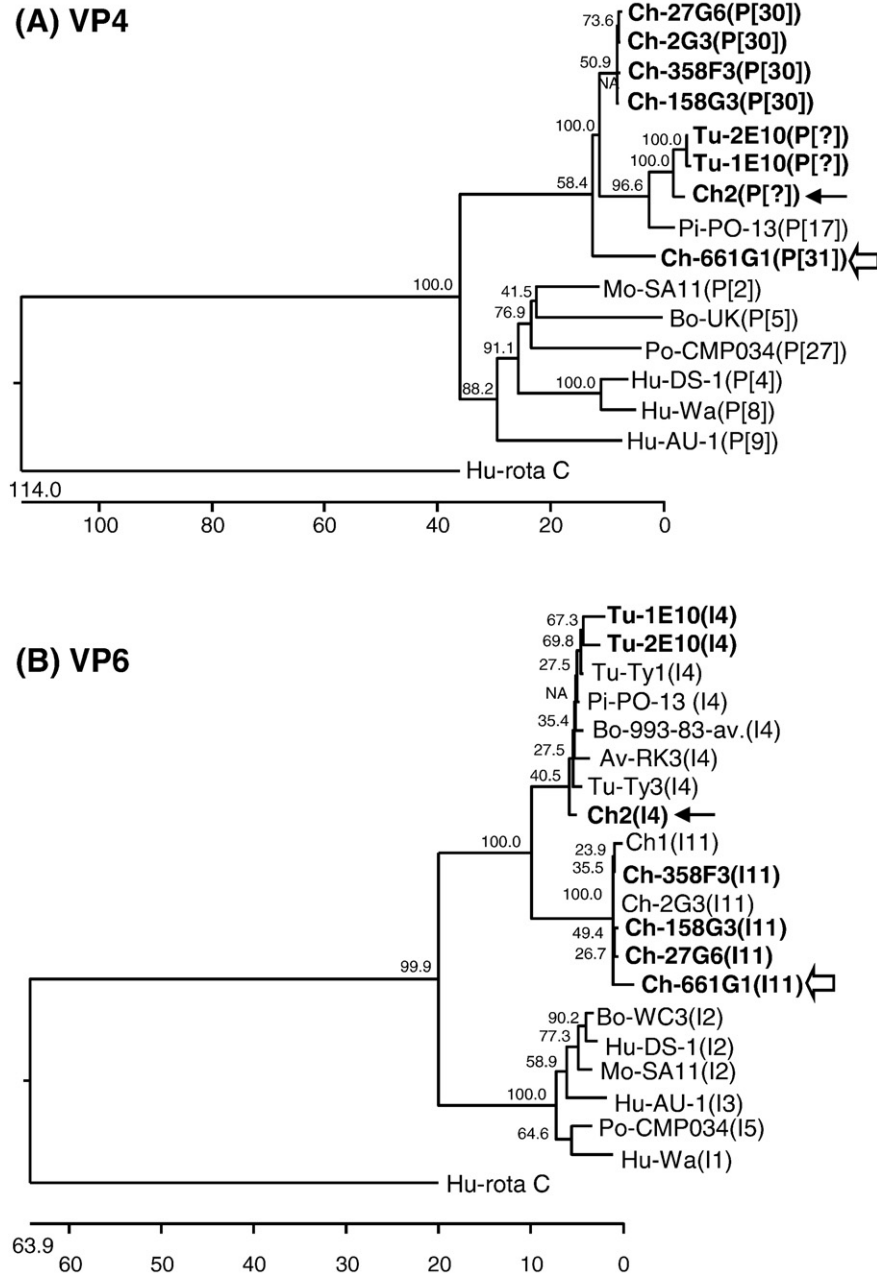
RT-PCR protocols were established based on the avian rotavirus sequences determined here and on selected mammalian rotavirus

sequences (listed in Supplemental Data 2) to enable amplification of parts of genome segments encoding VP4, VP6, VP7 and NSP5 of a broad range of group A rotavirus isolates originating from humans, animals and avian species. The protocols were applied to tissue culture supernatants of the six chicken isolates and the two turkey isolates as well as to the pigeon strain PO-13, the porcine strain OSU, the bovine strain UK, the simian strain SA-11, and the human strain Wa. After electrophoresis, amplicons of the expected length were detected in each case (Fig. 4). Differences in the intensity of the bands were mainly detected for genome segment 4, which probably reflects the high degree of sequence variation within this gene. 16 randomly selected bands derived from the four assays were analyzed by direct

sequencing, which indicated that in each case the PCR products had the expected rotavirus sequence.

**Discussion**

The genetic variability of human rotaviruses is maintained by several mechanisms including (i) point mutations, (ii) genomic reassortment and (iii) genome rearrangements, thus leading to considerable diversity (Estes and Kapikian, 2007; Müller and Johne, 2007). Animal rotaviruses may contribute to this variability by direct transmission to humans (DeGrazia et al., 2007; Matthijnssens et al., 2006), or by reassortment events creating human rotaviruses which



**Fig. 2.** Phylogenetic analysis of the sequences of amino acid positions 1 to 677 of VP4 (A), or the entire amino acid sequences of VP6 (B), VP7 (C) and NSP5 (D) of avian rotavirus isolates and selected mammalian group A rotavirus strains. The human group C rotavirus strain Bristol (Hu-rota C) was included as an outgroup sequence. Clustal W analysis was performed with 1000 bootstrap simulations using the MegAlign module of DNASTAR software package (Lasergene, Madison, USA). The trees are scaled in amino acid substitution units. The animal species from which an individual isolate originated is indicated by the first two letters of its designation: Ch – chicken, Tu – turkey, Pi – pigeon, Bo – bovine, Po – porcine, Mo – monkey, and Hu – human. The genotype is indicated in brackets. The sequences generated in this study are marked in bold, the small arrow indicates the chicken isolate Ch2 and the open arrow indicates the chicken isolate Ch-661G1.

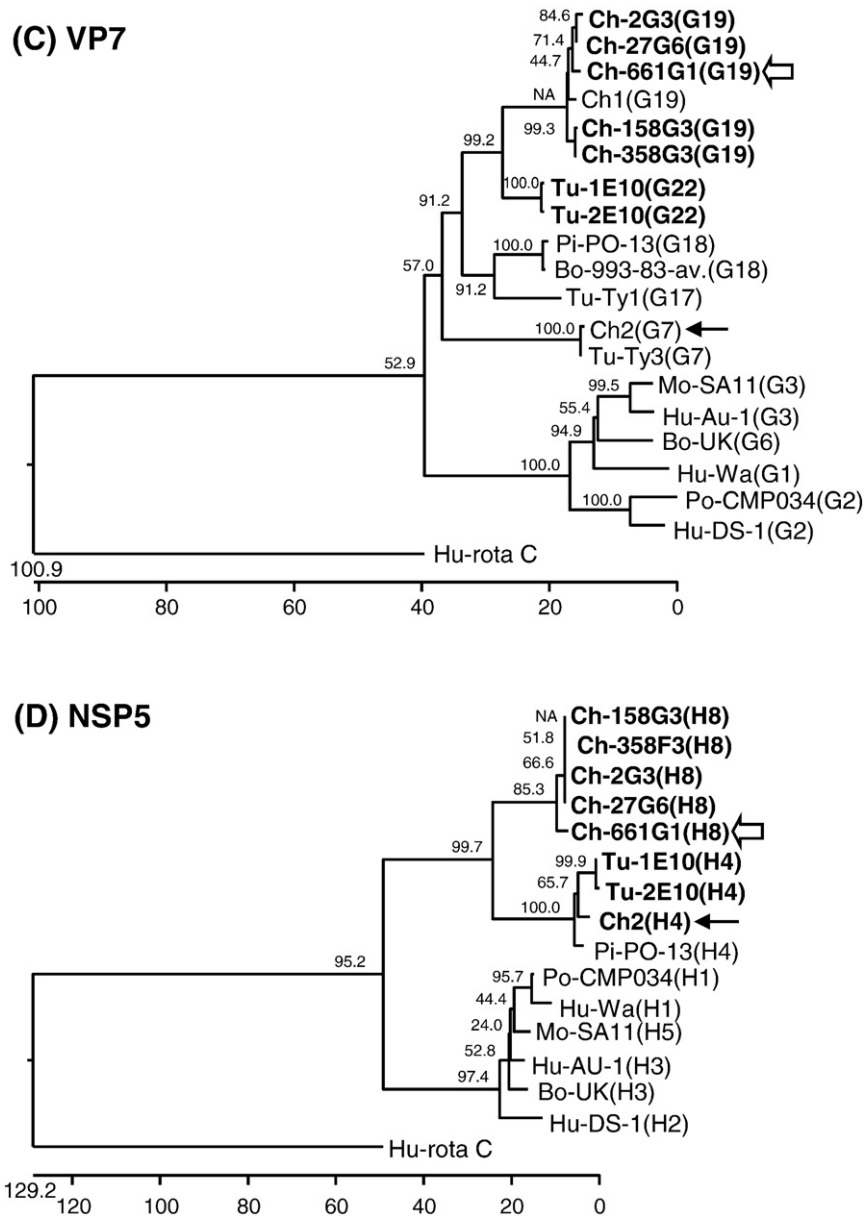


Fig. 2 (continued).

contain only parts of an animal rotavirus genome (Iturriza-Gómara et al., 2002; Khamrin et al., 2006; Mascarenhas et al., 2007; Matthijnssens et al., 2008c). Whereas mammalian rotaviruses are known to be transmitted to humans, the role of avian rotaviruses is still unknown. One of the major drawbacks for assessment of their contribution to human rotavirus variability is the lack of sequence data for avian rotaviruses which would enable tracing of these viruses and detection of their genome fragments in human samples.

In our analysis of the sequences derived from chicken and turkey rotaviruses we identified different types of avian rotaviruses with regard to their hosts and the genotype constellation (Table 1). At least two distinct avian phylogenetic clusters are evident (indicated with green and blue color in Table 1), which preferentially infect different avian hosts. The finding that the sequences of the chicken isolate Ch2 generally cluster with genotypes derived from turkeys may therefore indicate an origin of this virus from turkeys and subsequent transmission to chicken. Against this background, it may also be speculated that the close relationship between pigeon strain PO-13 and turkey strains indicate interspecies transmission between pigeons and turkeys.

Generally, a high degree of sequence heterogeneity was found between the avian isolates, which may significantly contribute to genetic variability of rotaviruses by providing a reservoir of novel genotypes, especially for the antigenic determinants VP4 and VP7. The chicken strain Ch-661G1 has a VP4-allele which has only low similarities to the other avian rotavirus VP4 sequences. As it is most closely related to the pigeon-derived sequence of strain PO-13, it is likely that it originated from an unknown avian host. As all of the other analyzed segment sequences clearly clustered within that of chicken strains, a reassortment event is highly likely from an exchange of the VP4-encoding segment only. Under experimental conditions, reassortants have been generated between pigeon strain PO-13 and turkey strain Ty3 (Mori et al., 2003) as well as between turkey strain Ty-1 and simian RRV rotaviruses (Kool et al., 1992).

Comparison of the avian rotavirus sequences to their mammalian counterparts showed that both groups are only distantly related to each other and that the sequences were consistently well separated in the phylogenetic trees constructed for VP4, VP6, VP7 and NSP5. It has been shown for the chicken isolate Ch-2G3 that this grouping is also

**Table 1**  
Comparative presentation of genotype constellations for VP7, VP4, VP6 and NSP5 of avian rotaviruses

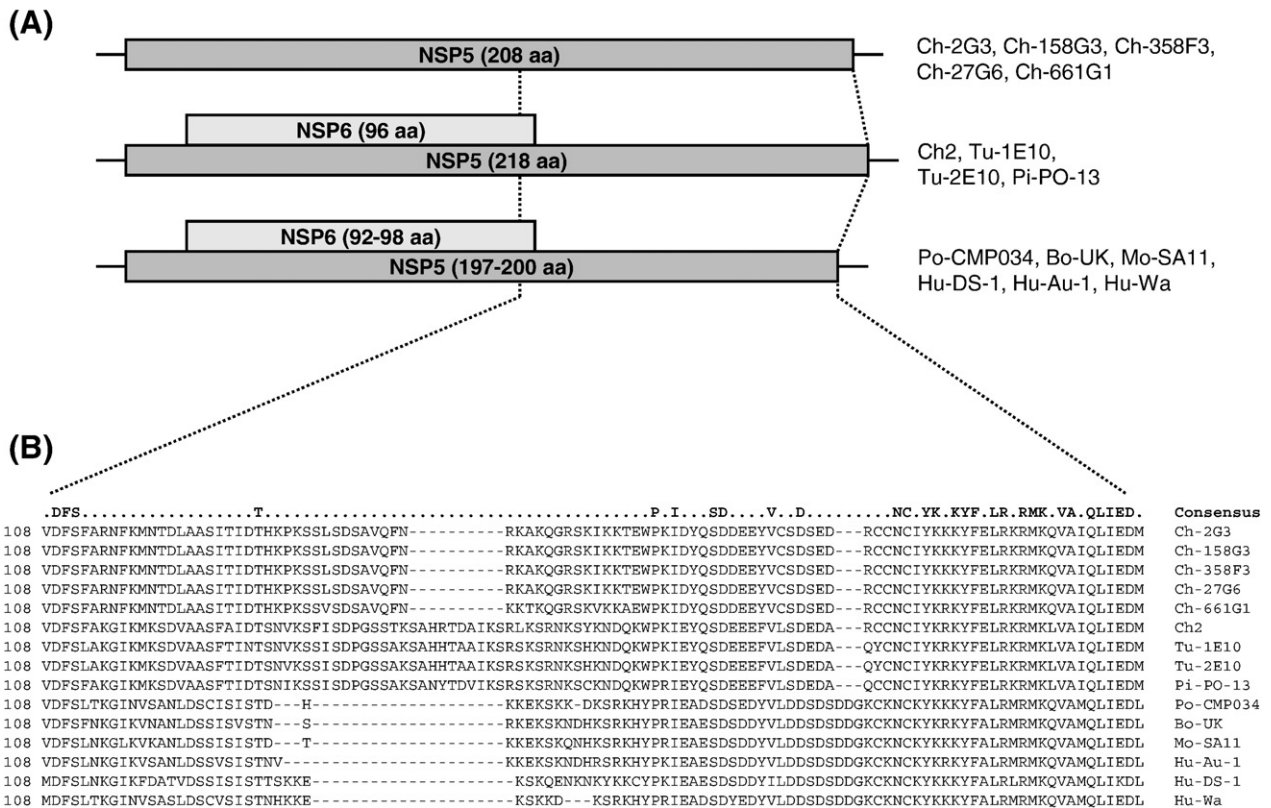
Strain	Abbreviation	Host	VP7	VP4	VP6	NSP5
02V0002G3	Ch-2G3	Chicken	G19	P[30]	I11	H8
03V0158G3	Ch-158G3	Chicken	G19	P[30]	I11	H8
03V0358F3	Ch-358F3	Chicken	G19	P[30]	I11	H8
04V0027G6	Ch-27G6	Chicken	G19	P[30]	I11	H8
06V0661G1	Ch-661G1	Chicken	G19	P[31]	I11	H8
Ch2	Ch2	Chicken	G7	P[?]	I4	H4
03V0001E10	Tu-1E10	Turkey	G22	P[?]	I4	H4
03V0002E10	Tu-2E10	Turkey	G22	P[?]	I4	H4
PO-13	Pi-PO-13	Pigeon	G18	P[17]	I4	H4

Green and dark blue color indicates the genotypes of chicken strain Ch-2G3 and pigeon strain PO-13, respectively. Other genotypes are marked with additional colors.

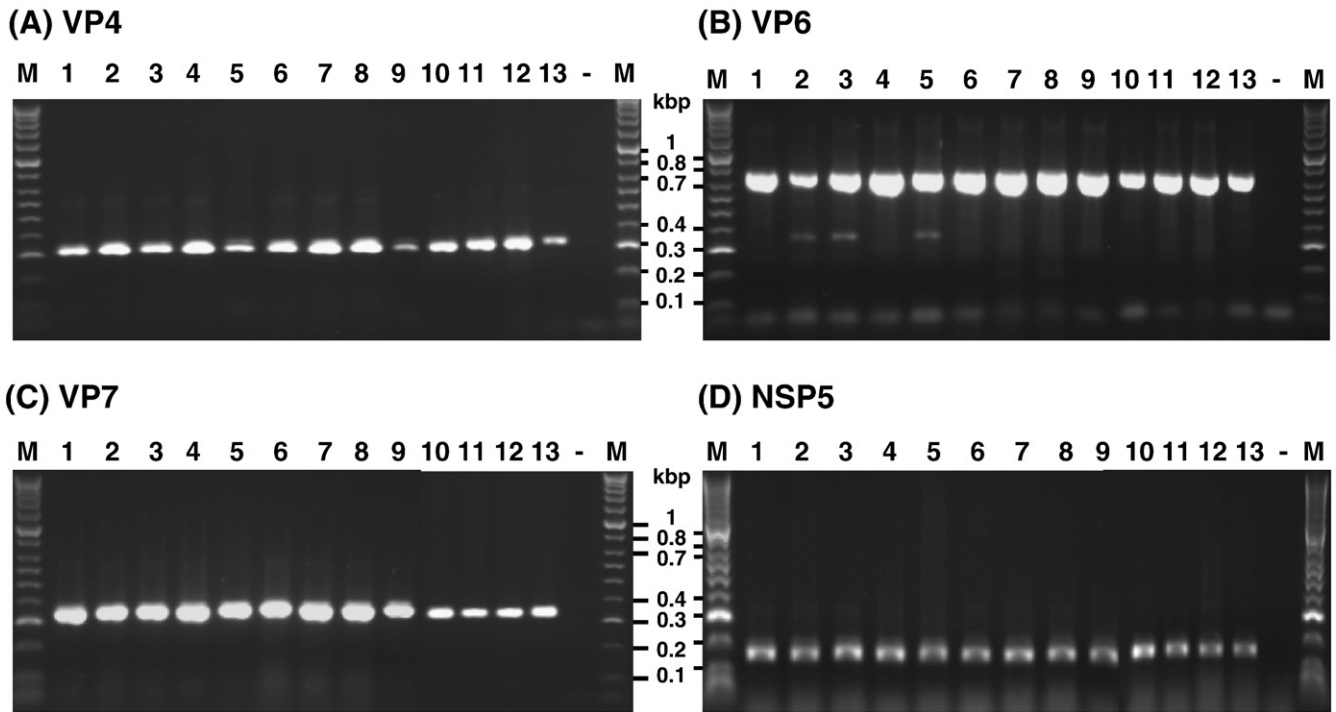
evident for the seven remaining genome segments (Trojnar et al., 2009). All avian rotavirus isolates analyzed here showed an RNA electrophoretic pattern of 5:1:3:2 characteristic of avian group A rotaviruses, which is in contrast to the characteristic migration pattern 4:2:3:2 of mammalian group A rotaviruses. Also, a number of amino acid sequences conserved in mammalian rotavirus proteins have been found to be mutated in the avian rotaviruses. Most of the chicken strains lack an ORF for expression of NSP6, which is present in most of the mammalian rotavirus isolates, however, with some exceptions (Gorziglia et al., 1989; Kojima et al., 1996). NSP6 has been shown to interact with RNA and the viral non-structural protein NSP5 (Rainsford and McCrae, 2007; Torres-Vega et al., 2000). The absence of an ORF encoding NSP6 in the chicken isolates substantiates the assumption that this is not an essential gene of group A rotaviruses. Taking all of these findings together, it may be concluded that transmission of rotaviruses between birds and mammals is unlikely due to the marked genetic and antigenic differences of the viruses of these groups. However, experimental transmission of pigeon rotavirus PO-13 to mice

has been successfully demonstrated (Mori et al., 2001), and a rotavirus with a high degree of sequence similarity to avian rotaviruses has been isolated from a calf (Brüssow et al., 1992). Also, a mammalian-like rotavirus has been isolated from chickens (Wani et al., 2003).

To our knowledge, avian rotaviruses have not been detected in humans and avian rotavirus sequences have as yet not been found in human rotavirus isolates. This might indicate that infection of humans with avian rotaviruses does not occur, however, it may also reflect an insensitivity of the detection methods used for human rotaviruses with respect to the detection of avian rotaviruses. Only very few protocols are available for specific detection of avian rotaviruses (Day et al., 2007; Pantin-Jackwood et al., 2007). Based on the sequences determined here, we have developed PCR assays capable of detecting a wide range of human, mammalian and avian group A rotaviruses, and which could be useful in further epidemiological studies. Taking into account the widespread use of chickens for food production and the high incidence of rotavirus infections in this animal species (Day et al., 2007; Otto et al., 2007;



**Fig. 3.** Comparison of the structure of genome segment 15 of avian and mammalian rotaviruses. (A) Schematic presentation of the localization of the open reading frames encoding NSP5 and NSP6. (B) Alignment of the carboxyterminal NSP5 amino acid sequences of nine avian and six mammalian rotavirus isolates. The abbreviation of the isolates is the same as in Fig. 2. The consensus shows the amino acid positions which are conserved in all sequences.



**Fig. 4.** Detection of genome segments encoding VP4 (A), VP6 (B), VP7 (C) and NSP5 (D) of avian and mammalian rotavirus isolates by consensus primer RT-PCR. PCR products were separated on ethidium bromide-stained 1.5% agarose gels. M: molecular mass markers, with sizes indicated between the pictures. RNA was isolated from tissue culture supernatant of MA-104 cells infected with isolates Ch-2G3 (lane 1), Ch-158G3 (lane 2), Ch-358F3 (lane 3), Ch-27G6 (lane 4), Ch-661G1 (lane 5), Ch2 (lane 6), Tu-1E10 (lane 7), Tu-2E10 (lane 8), Pi-PO-13 (lane 9), Po-OSU (lane 10), Bo-UK (lane 11), Mo-SA11 (lane 12), Hu-Wa (lane 13), or from uninfected MA-104 cells (–). The abbreviation of the isolates is the same as in Fig. 2.

Pantin-Jackwood et al., 2007), a relatively high exposure of humans with avian rotaviruses would be expected.

## Materials and methods

### Rotavirus strains

The chicken group A rotavirus Ch2 (G7P[?]), isolated in 1979 in Northern Ireland (McNulty et al., 1979), was kindly provided by H.M. Hafez (Institute of Poultry Diseases, Free University Berlin, Germany). The pigeon group A rotavirus PO-13 (G18P[17]) and the mammalian group A rotaviruses from pig (strain OSU, G5P[7]), cattle (strain UK, G6P[5]), monkey (strain SA-11, G3P[2]), and human (strain Wa, G1P[8]) used in the study were obtained from H.-J. Streckert (Dr. Streckert Diagnostika, Witten, Germany).

### Samples

Intestinal contents were obtained from five chickens and two turkeys from flocks in Northern Germany between 2002 and 2006. Details of designation and origin of strains as well as clinical symptoms observed in the host species are presented in Table 2. The samples were suspended at 1:5 (v:v) in phosphate-buffered saline pH 7.4 (PBS), homogenized for 30 s in an ultrasonic water bath at level 4 (UST 20, K.-W. Meinhardt Ultraschalltechnik, Leipzig, Germany) and then clarified at 2700 ×g for 10 min. The supernatants were collected and stored at –20 °C.

### Virus isolation

The samples were selected on the basis of RNA pattern observed in polyacrylamide gel electrophoresis (PAGE). The supernatants were treated with gentamicin solution (Sigma, Taufkirchen, Germany) at a final concentration of 50 µg/ml, and incubated for 60 min at room temperature. Prior to infection of MA-104 cells (Rhesus monkey

kidney cells, No. 142, Friedrich-Loeffler-Institute, Collection of Cell Lines in Veterinary Medicine, Isle of Riems, Germany), the viral inoculum was activated by addition of trypsin (Serva, Heidelberg, Germany) to a final concentration of 0.4 u/ml for 60 min at 37 °C. Confluent monolayers of MA-104 cells were washed with PBS and pretreated with diethylaminoethyl (DEAE)-dextran solution (40 mg/l) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) for 30 min at 37 °C. After removal of the DEAE-dextran solution, the suspensions were inoculated on the cells at 37 °C for 1 h. The inocula were replaced with DMEM containing 0.04 u/ml trypsin and 50 µg/ml gentamicin and incubated at 37 °C in a humidified air atmosphere with 5% CO<sub>2</sub> for 1–5 days. Up to 10 passages were performed, depending on the appearance of a cytopathic effect. All isolates were clone-purified by end-point dilution. Briefly, fourfold log<sub>2</sub>-dilution series of virus suspensions were inoculated into 96-well plates (Nunc, Karlsruhe,

**Table 2**

Origin of avian rotavirus isolates analyzed in the study

Designation	Abbreviation	Species	Clinical signs	Geographical region	Year of isolation
Ch2 <sup>a</sup>	Ch2	Chicken	Diarrhoea	Northern Ireland	1979
02V0002G3	Ch-2G3	Chicken	RSS <sup>b</sup>	Lower Saxony (Germany)	2002
03V0001E10	Tu-1E10	Turkey	Diarrhoea	Saxony-Anhalt (Germany)	2003
03V0002E10	Tu-2E10	Turkey	Diarrhoea	Saxony-Anhalt (Germany)	2003
03V0158G3	Ch-158G3	Chicken	RSS <sup>b</sup>	Lower Saxony (Germany)	2003
03V0358F3	Ch-358F3	Chicken	RSS <sup>b</sup>	Lower Saxony (Germany)	2003
04V0027G6	Ch-27G6	Chicken	RSS <sup>b</sup>	Bavaria (Germany)	2004
06V0661G1	Ch-661G1	Chicken	No clinical signs	Bavaria (Germany)	2006

<sup>a</sup> According to McNulty et al. (1979).

<sup>b</sup> RSS – Runting and Stunting Syndrome.

**Table 3**

Degenerate primers used for detection of genome segments encoding VP4, VP6, VP7 and NSP5 of avian and mammalian group A rotaviruses

Designation	Gene/segment	Binding position <sup>a</sup>	Sequence <sup>b</sup>	Product size*
Rota-Seg4-s	VP4/Segm.4	766–788	5'-TCTAARACATCATTNTGGAARGA-3'	312 bp
Rota-Seg4-as	VP4/Segm.4	1057–1078	5'-GCTTGTGAATCRTCCCAATC-3'	
Rota-Seg6-s	VP6/Segm.6	317–338	5'-GTAATGGAATWGCDCCNCAATC-3'	777 bp
Rota-Seg6-as	VP6/Segm.6	1072–1094	5'-ATACCTGSWGGAAAWACTGGTCC-3'	
Rota-Seg9-s	VP7/Segm.9	600–621	5'-CTAATAARTGGATWKCNCATGGG-3'	342 bp
Rota-Seg9-as	VP7/Segm.9	920–942	5'-ACTTGCCACCAYTYTTCGAAT-3'	
Rota-Seg11-s	NSP5/Segm.11	108–128	5'-TTCAGGWAAATCTRTTRTAG-3'	170 bp
Rota-Seg11-as	NSP5/Segm.11	257–278	5'-TTTGCRTTGWYTTAACTGCAT-3'	

<sup>a</sup> Corresponding to nucleotide numbering of avian rotavirus PO-13.<sup>b</sup> D = A + G + T, N = A + C + G + T, R = A + G, S = C + G, W = A + T, Y = C + T.

Germany) with monolayer of cells. After incubation for 4 days at 37 °C in a 5% CO<sub>2</sub>–air atmosphere, the virus was harvested by scratching of cells from individual wells of the highest dilution, in which only one rotavirus-positive plaque was observed. The harvested virus suspension was titrated in a second and third cloning procedure.

#### RNA-PAGE

RNA was extracted from virus-containing cell cultures and suspensions of intestinal contents using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The samples were analyzed for their RNA pattern using PAGE and silver staining as described previously (Otto et al., 1999). Gels were dried in a GelAirDryer (Bio-Rad Laboratories, Munich, Germany) and scanned on a GS-700 Imaging Densitometer (Bio-Rad Laboratories).

#### RT-PCR and sequencing

RNA was extracted from 140 µl cell culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen) and a total volume of 60 µl was eluted. After addition of 6 µl dimethylsulfoxide, the double-stranded RNA was denatured by heating at 95 °C for 3 min followed by cooling on ice. RT-PCR was performed with 5 µl of the RNA preparation using the QIAGEN LongRange 2Step RT-PCR Kit (Qiagen) with reaction conditions as recommended by the supplier. Primer sequences for the amplification of the coding regions for VP4, VP6, VP7 and NSP5 were selected by alignment of the 5' termini and 3' termini of the open reading frames of known avian group A rotaviruses. If no specific PCR product could be detected by use of these primers, additional primer sequences with binding sites in conserved regions of avian and mammalian rotavirus sequences were constructed and used for amplification of parts of the genome segments (primers listed in Supplemental Data 1). Reverse transcription was performed in a 20 µl reaction at 42 °C for 90 min. PCR was subsequently performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) using 5 µl of cDNA in 50 µl reactions and 93 °C for 3 min, 35 cycles of 93 °C for 30 s, 56 °C for 30 s and 68 °C for 5 min, and a final incubation at 68 °C for 7 min. The PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Leek, The Netherlands) and subsequently sequenced using M13 Forward and M13 Reverse primers (Invitrogen) in an ABI 3730 DNA Analyzer (Applied Biosystems).

#### Sequence analysis

The entire coding regions were assembled from the sequences using the SeqBuilder module of the DNASTAR software package (Lasergene, Madison, USA) and submitted to the GenBank database with accession numbers EU486956–EU486985. The sequences encoding VP4 (EU486956), VP7 (EU486971) and NSP5 (EU486978) of chicken isolate Ch-2G3 determined here are identical with that determined by Trojnar et al. (2009), however, in the latter case the

non-coding ends of the genome segments are included (accession numbers FJ169856, FJ169861 and FJ16986). Sequence alignments and construction of phylogenetic trees were performed using the MegAlign module of the above mentioned software package. Three human strains of different phylogenetic origin (AU-1, DS-1 and Wa) and strains isolated from monkey, pig, cattle and pigeon, as well as available chicken and turkey strains have been included in phylogenetic analysis (accession numbers shown in the Supplemental Data 2). The CLUSTAL W method was used with the PAM250 residue weight table (Thompson et al., 1994) in alignments, and bootstrap analysis of phylogenetic trees was performed with 1000 trials and 111 random seeds. The assignment of novel genotypes was approved by the Rotavirus Classification Working Group (RCWG) as described by Matthijnssens et al. (2008b).

#### Consensus primer RT-PCR

Primers were constructed with binding sites at highly conserved regions of avian and mammalian group A rotavirus genomes (Table 3). RT-PCR was performed with 5 µl RNA, prepared and denatured as above, using the QIAGEN OneStep RT-PCR Kit (Qiagen) in 25 µl reactions with 50 pmol of each primer and other components as recommended by the supplier. Cycling conditions in a 2720 Thermal Cycler (Applied Biosystems) were as follows: 42 °C for 60 min, 95 °C for 15 min, 40 cycles each with 94 °C for 1 min, 56 °C for 1 min and 74 °C for 1 min, and a final incubation at 74 °C for 7 min. PCR products were visualized by electrophoresis on ethidium bromide-stained 1.5% agarose gels. Selected bands of the expected length (Table 3) were purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the PCR primers as above.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.01.040.

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