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**Porcine rotavirus infections in Belgian piglets and assessment of
their evolutionary relationship with human rotaviruses**

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Dissertation submitted in fulfillment of the requirements for the
degree of Doctor in Veterinary Sciences (PhD), 2015

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Vertaling van de titel naar het Nederlands:

Porciene rotavirus infecties in Belgische biggen en de bepaling van hun evolutionaire verwantschap met humane rotavirussen

Theuns S. (2015). Porcine rotavirus infections in Belgian piglets and assessment of their evolutionary relationship with human rotaviruses. Dissertation, Ghent University, Belgium

ISBN 978-90-58-64-427-5

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This work was supported by grants from the Faculty Commission for Scientific Research of the Faculty of Veterinary Medicine, and the Special Research Fund (BOF) of Ghent University.

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List of abbreviations

5-HT	5-hydroxytryptamine
A	Interferon antagonist
aa	amino acid
ATP	adenosine triphosphate
C	C ore protein
CaCl ₂	calcium chloride
Caco-2	carcinous colon cell line 2
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
DLP	double layered particle
dsRNA	double stranded ribonucleic acid
E	e nterotoxin
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EIA	enzyme immunoassay
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	Eukaryotic translation initiation factor 4G
EM	electron microscopy
ETEC	Enterotoxigenic <i>Escherichia coli</i>
EU	European Union
EuroRotaNet	European Rotavirus Surveillance Network
G	G lycoprotein
H	p hosphoprotein
HBGA	Histo-blood group antigen
I	I nnner capsid protein
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
IFNAR	interferon- α/β receptor
IL	interleukin
IPS	interferon- β promoter stimulator 1
IRF	interferon regulatory factor
I κ B	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
L-Ara4N	4-amino-4-deoxy-L-Arabinose
LPS	lipopolysaccharide
LT	thermo-labile enterotoxin
M	M ethyltransferase
MDCK	Madin-Darby Canine Kidney
MgCl ₂	magnesium chloride

MgSO ₄	magnesium sulphate
MyD88	Myeloid differentiation primary response gene (88)
NaCl	sodium chloride
N	NTPase
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSP	non structural protein
nt	nucleotide
OAS	pligoadenylate synthetase
OSU	Ohio State University
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDB	Protein Data Bank
pDC	plasmacytoid dendritic cell
ORF	open reading frame
P	Protease sensitive protein
PEDV	porcine epidemic diarrhea virus
PGE2	prostaglandin E2
pIgR	polymorphic immunoglobulin receptor
PKR	protein kinase RNA-activated
PRR	pattern recognition receptor
qPCR	real-time or quantitative polymerase chain reaction
R	RNA -dependent RNA polymerase
RCWG	Rotavirus Classification Working Group
RLR	retinoic acid-inducible gene 1-like receptor
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RT-qPCR	reverse transcriptase real-time polymerase chain reaction
RVA	group A rotavirus
RVB	group B rotavirus
RVC	group C rotavirus
RVD	group D rotavirus
RVE	group E rotavirus
RVF	group F rotavirus
RVG	group G rotavirus
RVH	group H rotavirus
RVI	group I rotavirus
SIgA	secretory immunoglobulin A antibody
ST	thermo-stabile enterotoxin
STAT	signal transducer and activator of transcription
stx2e	Shiga toxine 2e
T	translation enhancer
TGEV	transmissible gastroenteritis virus
TGF-β	transforming growth factor-β

TLP	triple layered particle
TLR	Toll-like receptor
TRIF	TIR-domain-containing adapter-inducing interferon- β
UDP	Uridine-diphospho-glucose
USA	United States of America
VHH	variable heavy chain
VIP	vasoactive intestinal peptide
VP	viral protein
β -TrCP	beta-transducin repeat containing protein

Chapter I

Introduction

1.1. Diarrhea: an important cause of production losses in pig industry

In 2012, the global production of pig meat was 109 million tonnes, of which 21.9 million tonnes were produced in the former EU-27, nowadays EU-28 (FAO Statistical Year Book 2013). In Europe, the main pig producing regions are located in the axis from Flanders (Belgium) to The Netherlands, North West Germany and Denmark, and also in Brittany (France), Catalunya (Spain) and West Poland. Belgium represents a small area within Europe, but is an important contributor of pork production (1.1 million tonnes in 2012). Ninety five percent of the Belgian pig farms are located in the region of Flanders, and the economical value of their production has been estimated to reach €1.8 billion each year. Thus, pig production is a major agricultural and economical activity in Flanders.

It takes 5 to 6 months before pork can be sold to the consumer. Sows are artificially inseminated, and piglets are born approximately 114 days later. Suckling pigs are housed together with the sow in the farrowing units for 3 to 4 weeks, during which they are suckling milk from the sow (Figure 1). Then, pigs are weaned and brought to the weaning barn, where they are housed in groups of approximately 25 pigs (Figure 1). At the same time, the sow is prepared again for insemination and production of a new litter of pigs. When weaned piglets reach a weight of approximately 20kg, they are transferred to larger units for further fattening stages. They are finally slaughtered when their weight reaches approximately 115kg. During their lives, pigs are transported often from one stable to another, which will bring them in contact with different environments and pathogens, often leading to health

problems. Diarrhea is the major health problem that young pigs are confronted with. It occurs most frequently during two critical periods: during their stay in the farrowing house and shortly after weaning. It is mainly a result of infections with enteric pathogens such as viruses, bacteria and parasites. After birth, suckling piglets are protected against enteric infections by maternal secretory immunoglobulin A (SIgA) antibodies present in the sow's milk [1]. When SIgA against certain pathogens are absent or present at very low levels, enteric pathogens will be able to replicate in the intestine, which may lead to diarrhea. In general, concentrations of maternal antibodies in milk of primiparous sows (gilts, farrowing for the first time) are lower than that of multiparous sows. Most diarrheic problems are thus observed in litters from gilts [1]. Weaning at an age of 3 to 4 weeks causes an abrupt disappearance of the lactogenic immunity, making the pigs fully susceptible for infections with enteric pathogens. Diarrheic problems lead to impaired growth, increased administration of antimicrobial drugs, mortality, and cause serious economic losses.

Rotavirus is a major cause of diarrhea in young piglets and in children, and is considered to be a zoonotic agent. This virus will be the main area of focus of the present doctoral thesis. Therefore, a thorough description of pig and human rotaviruses and the evolutionary link between rotaviruses from both host species will be delineated hereafter. At the end of this chapter, some important differentials of diarrhea in the young piglet and the impact of co-infections with rotaviruses will be briefly introduced.



Fig. 1. Young pigs in the farrowing unit (left) and weaning barn (right).

1.2. Rotavirus

1.2.1. History

Rotaviruses were first discovered in feces of diarrheic mice (1963), monkeys (1963) and calves (1969) [2-4]. Bishop and colleagues recognized rotavirus as a cause of diarrhea in humans in 1973, by investigating duodenal biopsies of diarrheic children in Australia [5]. Not long hereafter, rotavirus was also detected in feces of pigs (1975) [6]. The virus was detected for the first time in Belgium by Debouck and Pensaert in 1977 and associated with diarrhea and vomiting in pigs [7]. In Latin 'rota' means 'wheel', which resembles the shape of the virus as demonstrated by transmission electron microscope analysis (Figure 2). Consequently, the name 'rotavirus' was given by Flewett and colleagues in 1974 [8].

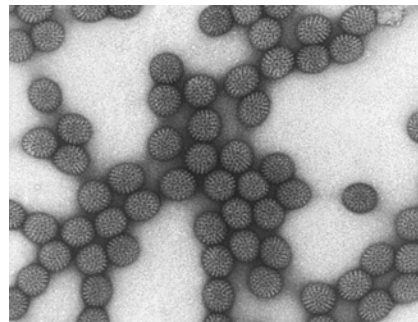


Fig. 2. Transmission electron microscope image of rotavirus particles (from: www.phil.cdc.org).

1.2.2. Structural biology

1.2.2.1. Genome

Eleven gene segments of double stranded RNA reside in the rotavirus particle. The total genome size is 18.5 kb and encodes 6 structural (VP1-VP4, VP6 and VP7) and 6 non-structural proteins (NSP1-NSP6). Each gene segment encodes one viral protein, except for gene segment eleven which encodes NSP5 and NSP6 [9]. The protein encoding sequence of each gene is flanked by untranslated sequences of variable lengths at the 5' and 3' ends. Furthermore, a methylated cap is present at the 5' end of each gene segment. A polyadenylation signal is absent at the 3' end, but a conserved UGUG sequence is present instead [10-12]. An overview of the rotavirus genome organization is presented in Figure 3.

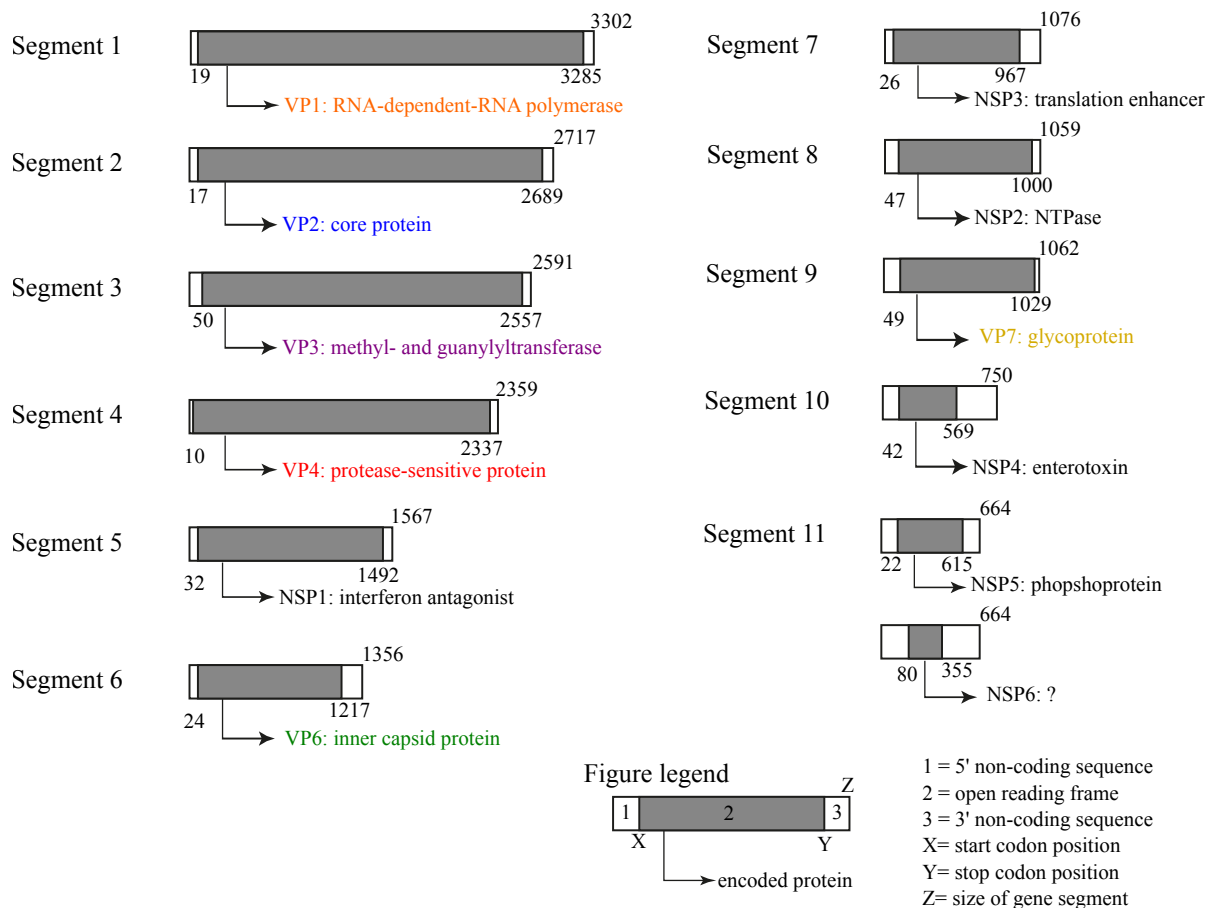


Fig. 3. Rotavirus genome organization based on porcine reference strain Gottfried. The color-code will be used further throughout the introduction to address the different structural viral proteins.

1.2.2.2. Rotavirus particle

Rotavirus particles consist of genomic segments enclosed by an icosahedral capsid, composed of 3 layers. As non-enveloped particles, they are relatively large with a size of approximately 70 nm [13, 14]. The structure of a rotavirus particle with the different capsid layers is shown in Figure 4. From inside to outside, the following layers can be distinguished: core layer (VP2), inner capsid layer (VP6) and outer capsid layer (VP7 and VP4).

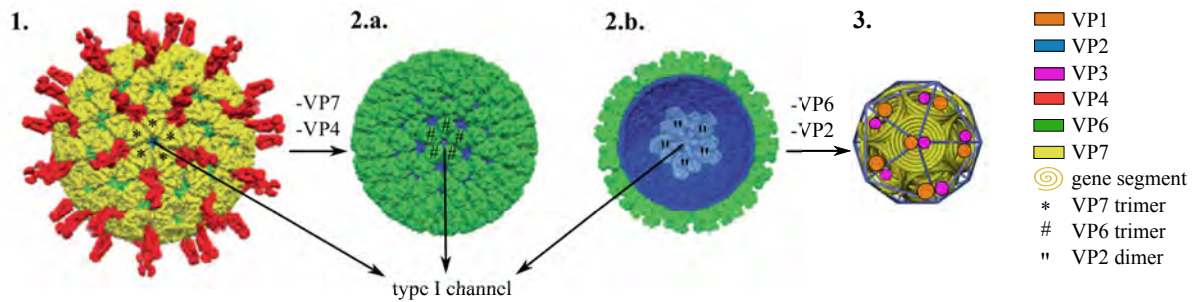


Fig. 4. Structure of a rotavirus particle: 1. mature triple layered virus particle; 2.a. double layered virus particle; 2.b. cross-section through double layered particle showing the core (blue) built of VP2 decamers (light blue); 3. icosahedral organization of gene segments and VP1 and VP3 proteins (Adapted from [13, 14]).

Layer 1: core

The core is built by 120 copies of the VP2 protein and has an icosahedral symmetry possessing 12 five-fold axes, each axis surrounded by five VP2 dimers [15]. A small pore (type I channel) is present in the center of each decamer. Within the capsid core, each of the eleven gene segments is twisted like a spiral and projects towards one of the twelve five-fold axes of the icosahedral core particle. At these vertices, gene segments are tightly associated with two viral enzymes: the RNA-dependent RNA polymerase (VP1) and the methyl- and guanylyltransferase (VP3) [16, 17]. VP1, the largest rotavirus protein, is the RNA-dependent RNA polymerase and contains 4 entries, each leading to the catalytic site in the center of the protein [18, 19]. These openings function as an entry port for nucleoside triphosphates, an entry port for single stranded RNA, an exit for positive stranded mRNA, and an exit for negative stranded RNA or dsRNA, respectively [18]. Within the core particle, the polymerase is tightly associated with VP3, the capping enzyme. Its guanylyltransferase, (guanine-N7)-methyltransferase and (nucleoside-2'-O)-methyltransferase enzymatic activities result in the formation of a 7-methyl-GpppG cap at the 5' end of transcribed positive stranded rotavirus RNA [20-22].

Layer 2: inner capsid

The core is surrounded by the **inner capsid** layer, which is composed of 780 VP6 proteins organized in 260 trimers. Particles containing the two inner shells are also called double-layered particles (DLPs). The inner capsid layer also has an icosahedral symmetry (T=13) and functions as a scaffold for the outer capsid layer. The VP6-side interacting with the capsid core (VP2) is composed of α -helices, whereas the opposing side consists of β -sheets interacting with VP7 and VP4 of the outer capsid layer (Figure 5) [15, 23].

Layer 3: outer capsid

Two hundred and sixty trimers of VP7 proteins, stabilized by calcium ions, make up the outer shell of rotavirus particles [24]. VP7 contains N-linked glycosylation sites and is also called the ‘glycosylated protein’ or ‘glycoprotein’. Sixty trimers of VP4 proteins are plugged in this outer shell of VP7 proteins and protrude like spikes from the viral surface [14, 25]. VP4 can be subdivided into two domains, VP5* and VP8*. With its base, VP5* is connected with VP6 through a pore in the VP7 layer and further extends outside the viral surface (Figures 4 and 5) [26, 27]. On top of the VP5* subdomain sits the globular VP8* domain, which is highly variable and known to interact with carbohydrate moieties (Figure 5) [28, 29]. A trypsin cleavage site is present between both domains, and for this reason VP4 is also called the ‘protease-sensitive protein’. After proteolytic cleavage both domains remain associated by hydrophobic interactions [30, 31].

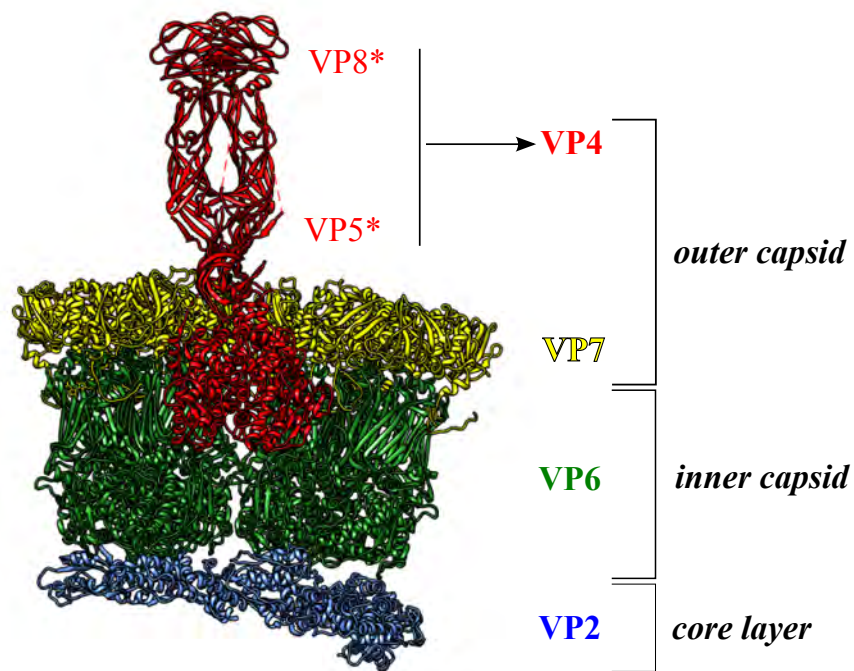


Fig. 5. Cross-section through the different layers of a rotavirus particle, demonstrating the interaction sites between the structural viral proteins (image constructed using PDB 4V7Q).

1.2.3. Classification

1.2.3.1. Taxonomy of the Reoviridae

Rotaviruses belong to genus *rotavirus* and are members of the subfamily *Sedoreovirinae* within the *Reoviridae* family. Members of the *Sedoreovirinae* subfamily have a rather smooth surface, whereas those of the *Spinareovirinae* subfamily possess a turreted capsid. Within the *Reoviridae* family, some other important veterinary pathogens can be found, namely blue tongue virus and African horse sickness virus, both members of the genus *orbivirus* [32, 33].

1.2.3.2. Rotavirus groups or species

Within the *rotavirus* genus, a further subdivision in groups or species has been established. This classification was first based on the reactivity of antibodies directed against the VP6 group antigen [34]. Rotaviruses from different species can also be recognized by a typical migration pattern of their dsRNA segments using polyacrylamide gelelectrophoresis followed by silver staining. Currently, the subdivision into species is being performed using sequences of the VP6 encoding genes. An amino acid identity cut-off value of 53% has been proposed to demarcate RV species A to H, and they have now been officially recognized by the International Committee on Taxonomy of Viruses (ICTV) [35]. Recently, a putative new RV species I was detected in dogs from Hungary [36]. Rotavirus species A (RVA) is clinically and epidemiologically the most important species and can be found in humans, pigs, ruminants, horses, dogs, cats, monkeys, bats, poultry and a variety of other species [37, 38]. Furthermore, RVB and RVC have also been detected in humans, pigs, cattle and other species [37, 38]. Pigs are so far the only identified host for RVE, whereas RVH has been isolated from pigs and humans [37, 39-43]. RVD, RVF and RVG have so far only been detected in feces of poultry [44]. Figure 6 shows the evolutionary relationship between different rotavirus species A to I. Each branch of the phylogenetic tree represents a VP6 gene segment of a rotavirus strain. A node connecting two different branches represents a common evolutionary ancestor of both strains. However, the longer the length of the branches, the more nucleotide differences are present between two strains, and the more evolutionary divergent they are. Using these principles of phylogeny, it can be concluded from Figure 6 that RVA is evolutionary more related to RVC, RVD and RVF, than to RVB, RVG, RVH and RVI.

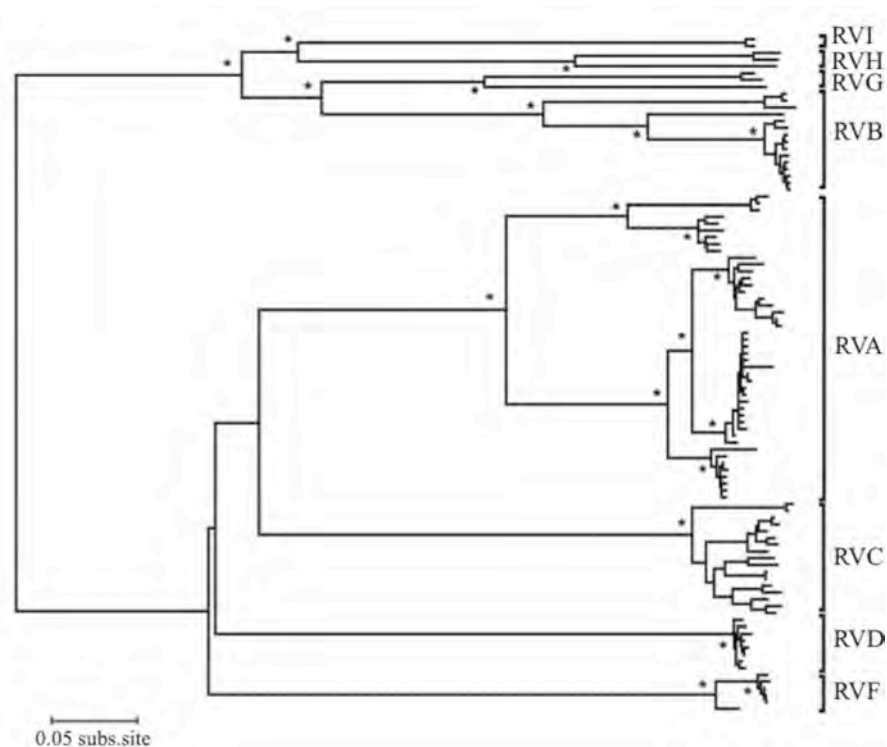


Fig. 6. Phylogenetic relationship of different rotavirus species, based on VP6 gene sequences (adapted from [36]).

1.2.3.3. Classification of group A rotaviruses

RVA strains can be further assigned to different serotypes or genotypes based on cross-reactivities of antibodies directed against the outer capsid proteins VP7 and VP4, or based on sequences identities of their coding genes, respectively.

Serotyping of VP7 and VP4

The outer capsid proteins VP7 (glycoprotein) and VP4 (protease-sensitive protein) independently induce neutralizing antibodies. This ability led to the development of a dual *serotype* classification system for VP7 and VP4 of group A rotaviruses using cross-neutralization assays in MA104 cells. This classification is not perfect, because serotype-specific antibodies are not always available and serotype classification should be performed on susceptible enterocyte cultures, which are not available yet [45-47].

Genotyping of VP7 and VP4

To overcome the difficulties associated with RVA serotyping, a dual VP7 and VP4 *genotyping* system was established [48]. A nucleotide sequence identity cut-off value of 80% was proposed to assign VP7 and VP4 genes to certain genotypes [48, 49]. For VP7, the genotyping classification system matches the serotype classification. However, for VP4, more genotypes than serotypes have been described. As such, the antigenic and genetic

properties of VP4 are being described as follows: P1A[8], in which 1A is the serotype and [8] is the genotype [48]. Until now, 27 G-genotypes for VP7 (Glycoprotein; G1-G27) and 37 P-genotypes for VP4 (Protease-sensitive protein; P[1]-P[37]) have been detected in different species [50, 51].

Full genome-based genotype classification

RVAs possess a segmented genome, which implicates that genetic/antigenic variation located on other genes/proteins of the virus, may be easily exchanged by reassortment events. In 2008, a genotype classification system was established for the other 9 gene segments of RVAs, using nucleotide sequence identity cut off values presented in Table 1 [49]. As such, the genotype constellation of each RVA strain can be represented by a code of 11 letters for the protein that is encoded by each gene segment, and 11 numbers designating the genotype. The genotype constellation of an RVA strain is thus represented as follows: G_x-P_[x]-I_x-R_x-C_x-M_x-A_x-N_x-T_x-E_x-H_x for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5, respectively [49].

Table 1. Genotypes of eleven gene segments of RVA strains [12, 49].

protein	function	abbreviation	nt identity (%)	number of genotypes
VP7	Glycoprotein	G	80	27
VP4	Protease sensitive protein	P	80	37
VP6	Inner capsid protein	I	85	18
VP1	RNA-dependent RNA polymerase	R	83	9
VP2	Core scaffold protein	C	84	9
VP3	Methyl- and guanylyl transferase	M	81	8
NSP1	Interferon Antagonist	A	79	19
NSP2	NTPase	N	85	10
NSP3	Translation enhancer	T	85	12
NSP4	Enterotoxin	E	85	15
NSP5	Phosphoprotein	H	91	11

1.2.3.4. Classification of group C rotaviruses

Rotavirus like particles were detected in diarrheic intestinal contents of a 27 day old conventional nursing pig in Ohio, United States, by Saif and colleagues (1980). This virus was morphological similar, but antigenically distinct from pig, cow and human RVAs, suggesting that this virus belonged to another group of rotaviruses [52]. This virus was called 'Pararotavirus'. It caused villus atrophy, diarrhea and mortality after inoculation of

gnotobiotic pigs [53]. At least two distinct serotypes were detected within these group C rotaviruses (RVCs). One serotype contained the bovine RVC strain Shintoku, whereas the other serotype contained porcine reference strain Cowden, serotypically similar to the human RVC strain Ehime 86-542 from Japan. A possible third serotype contained pig RVC strain HF [54]. The difficulty to isolate RVC in cell culture has led to the development of a classification system for RVC based on nucleotide sequence information [55, 56]. An amino acid identity cut-off value of 89% was proposed for VP7, resulting in the classification of RVC strains into 6 types [56]. More recent, this classification was extended to the nucleotide sequences of genes encoding VP7 proteins of RVC. A nucleotide identity cut-off value of 85% was used, resulting in 9 VP7 genotypes (G1 to G9) [55]. A classification system for RVC VP4 genes or serotypes does not exist. When RVC susceptible cells become available in the future, it will be investigated how good this genotype classification corresponds to the antigenic classification (serotypes).

1.2.3.5. Rotavirus strain nomenclature

A uniform nomenclature for rotaviruses was proposed by the Rotavirus Classification Working Group (RCWG). In this nomenclature system, each strain is abbreviated as follows: rotavirus group/species of origin/country of identification/common name/year of identification/G- and P-genotype. As an example human RVA reference strain Wa is named as follows: RVA/Human-tc/USA/Wa/1974/G1P[8] [50].

1.2.4. Epidemiology

1.2.4.1. Stability and infectivity

Rotaviruses are very infectious and stable in the environment. The virus is transmitted by the feco-oral route. Calf rotavirus is stable for 1 year in closed recipient, whereas exposure to air may reduce the infectivity after a couple of days [57]. Inactivation studies have been performed using the simian strain RVA/Simian-tc/ZAF/SA11-H96/1958/G3P[2]. Rotavirus is resistant to ether and chloroform, but is sensitive to treatment with 5M EDTA, 5M EGTA, acidic pH and alkalic pH. Heating rotavirus at 50°C for 30 minutes abolished infectivity with 99%, whereas adding 2M MgCl₂, 2M CaCl₂ or 2M NaCl already reduced the infectivity with 98% after 15 min at 50°C. In contrast, addition of MgSO₄ had a stabilizing effect on rotavirus infectivity [58]. The minimal infectious dose of pig rotavirus strain OSU is 1 plaque forming unit, demonstrating its high infectivity [59]. Ethanol 95% is an effective inactivator of SA11 rotavirus [60]. However, rotavirus show high resistance

against sodium hydrochlorite, iodophors and quaternary ammonium disinfectants [61]. Viral inactivation is obtained upon exposure to 10% formol for 2 hours [57].

1.2.4.2. Epizootiology of rotavirus infections in pigs

Prevalence of RVA infections in pigs

Several prevalence studies on the occurrence of RVA infections in both diarrheic as well as non-diarrheic piglets have been performed in the last decade. As shown in Table 2, prevalences of RVA infections clearly varied between different age groups, between diarrheic and non-diarrheic piglets and between different countries studied.

Table 2. Prevalence of RVA infections in diarrheic and non-diarrheic pigs.

Country	Year	Diagnostic test	Age (days)	Symptoms	n=	% RVA positive	Reference
USA, Canada, Mexico	2009-2011	RT-qPCR	1-3	D	954	30%	[62]
			4-21	D	2144	46%	
			22-55	D	2538	84%	
			>55	D	1207	61%	
Argentina	1999	PAGE + antigen EIA	<45	ND	901	3.3%	[63]
Canada	2005-2007	RT-PCR	Slaughter	ND	96	8.3%	[64]
			>24	ND	50*	16.0%	
Denmark	2006-2007	EIA	1-28	D	308	10%	[65]
Germany	nd	EM	1-21	D	102	2.0%	[66]
Italy	2004-2006	RT-PCR	28-84	D	102	71.5	[67]
Ireland	2005-2007	RT-PCR	28-63	ND	292	6.5%	[68]
Slovenia	2004-2005	RT-PCR	1-21	D	6	50%	[69]
				ND	121	11.6%	
			22-70	D	14	35.7%	
				ND	133	25.6%	
			>70	D	13	46.2%	
Japan	2000-2002	PAGE	suckling weaning	D	36	18 outbreaks	[70]
				ND	119		
South Korea	2006-2007	nested RT-PCR	3-70	D	475	38.3%	[71]
Thailand	2000-2001	antigen EIA	7-49	D	175	22.3%	[72]
Vietnam	2012	RT-qPCR	all ages	D	76	19.7%	[73]
				ND	654	24.9%	

Legend: D diarrheic; ND non-diarrheic; EIA enzyme immunoassay; EM electron microscopy; PAGE polyacrylamide gel electrophoresis; * mixed samples from multiple animals

It needs to be emphasized that in most of these studies different diagnostic approaches with varying sensitivities have been applied, which likely has influenced the observed detection rates [62-73]. Recently, it was demonstrated in Japan by repeated sampling at fixed time intervals and RT-PCR analysis of fecal samples collected from 10 pigs, that pigs may become infected with different RVA strains during different production stages from birth to slaughter [74]. It has also been described that a single G/P combination can be predominant

on a farm during one season, whereas it may be replaced by another strain during the next season [75].

Antigen ELISA and immunochromatographic strips are most frequently being used as tools to detect group A rotaviruses in fecal samples of piglets with diarrhea at Belgian diagnostic laboratories. These rapid detection methods are especially designed for the detection of human rotaviruses in clinical samples from children and make use of antibodies elicited against the VP6 protein of a bovine rotavirus strain. In a study performed in Belgium in 2012 on euthanized pigs with weaning diarrhoea, 16 % of the pigs had rotaviral antigens in their small intestinal content when tested using an antigen ELISA. However, also 8% of the control piglets were rotavirus positive (Coddens et al., unpublished results). In the 1980s, Debouck and Pensaert followed the excretion of rotavirus with one week intervals in suckling and weaned piglets and sows, on 3 Belgian pig farms. Almost all piglets shed the virus before the age of 5-6 weeks and most of them demonstrated recurrent sheddings. However, no association could be made between rotavirus infection and diarrheic symptoms at that moment [76].

RVA strain distribution in pigs: VP7 and VP4 genotypes

So far, 12 G-genotypes (G1 to G6, G8 to G12 and G26) and 13 P-genotypes (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], P[34]) have been detected in diarrheic and non-diarrheic pigs [37, 77]. In a recent systematic review from Papp and colleagues, 55 papers about the characterization of the genes encoding outer capsid proteins VP7 and VP4 were analyzed. G5P[7] was the dominant G/P combination detected in pigs around the world. Nonetheless, differences in strain predominance between continents and countries were present [77]. In the United Kingdom, G4P[6] strains, followed by G5P[7] strains were most frequently detected in fecal samples collected from pigs with confirmed RVA infections [78]. In Japan, the G9 genotype was predominantly detected in diarrheic outbreaks in young pigs, mainly in association with genotype P[13], P[23] or P[6] [70]. The G9P[13] genotype was also most frequently detected in a recent study in Ohio, United States [79]. In South Korea, the G5P[7] combination was most detected in diarrheic feces, but only strains successfully isolated in cell culture were characterized, which could have biased the results [71].

Prevalence of RVC infections in pigs

Recently, more and more efforts are undertaken to study the importance of RVC infections in pigs. Marthaler and colleagues demonstrated that RVC infections in the United States,

Canada and Mexico were in general most frequently detected in very young pigs (<3 days old), whereas RVA and RVB infections were generally more frequently detected in the 21-55 days, and >55 days age groups, respectively [55, 62]. RVC was also detected in diarrheic fecal samples collected from 1 to 3 month old piglets in Italy during 2 outbreaks. However, RVC positive samples were mostly coinfecting with RVA or enteric caliciviruses as well [67]. Pig RVCs were also detected in feces of asymptotically infected 4 to 5 week old Irish pigs, but at a low rate (4.4% of 292 samples) [80].

1.2.4.3. Epidemiology of RVA in humans

RVA disease in children

In humans, RVA is the leading cause of diarrhea in children under 5 years, followed by *Cryptosporidium*, *Shigella* and enterotoxigenic *Escherichia coli* [81]. Approximately 453.000 children die each year as a result of a rotavirus infection, and most of these deaths occur in India, with an estimated number of 78.000 annual fatal cases [82, 83]. Overall, the highest mortality is seen in African and Asian developing countries [82].

Two globally licensed oral attenuated vaccines are available to induce active immunity in babies: Rotarix[®] (GlaxoSmithKline) and RotaTeq[®] (Merck). Rotarix[®] is a monovalent vaccine based on a human G1P[8] strain and attenuated by serial passaging in African Green Monkey kidney epithelial cells and Vero cells. RotaTeq[®] is pentavalent vaccine containing 5 human-bovine reassortant vaccine strains. A modified-Jennerian approach was the rationale for the development of this vaccine, as it was observed that animal rotavirus strains are naturally attenuated in heterologous hosts. Each of these 5 strains in RotaTeq[®] contains one human gene segment (VP7 or VP4), whereas the other 10 gene segments are derived from the bovine G6P[5] WC3 strain [84]. The efficacy of both vaccines is high in well-developed countries (Europe, USA, Australia...), whereas it is generally lower in developing countries from Africa and Asia [85-88]. Recently, a new vaccine was developed by Bahrat Biotech in India: Rotavac[®]. This vaccine contains a natural reassortant G9P[11] strain (116E), whereof 10 gene segments are of human origin and 1 gene segment (VP4) is of bovine origin. In a clinical phase III trial conducted in India, efficacy against severe rotavirus gastroenteritis was 53.6% [89]. Since their release in 2006, rotavirus vaccines have been implemented in national immunization programs of many countries, including Belgium and other well-developed countries. In Asia, where the rotavirus burden is most strikingly, vaccines have only been introduced in the Philippines. It was announced at the Eleventh International Rotavirus Symposium in 2014 (New Delhi, India), that rotavirus immunization would be

introduced in the national immunization program of India to reduce the high burden of gastrointestinal diseases in children.

RVA strain diversity in humans: VP7 and VP4 genotypes

Introduction of rotavirus vaccination may lead to the emergence of strains that are uncovered or less covered by the vaccines. Therefore, rotavirus surveillance programs have been established to characterize circulating strains and to rapidly recognize emerging strains in the human population. An overview of the predominant strains in important regions around the world is presented in Figure 7.

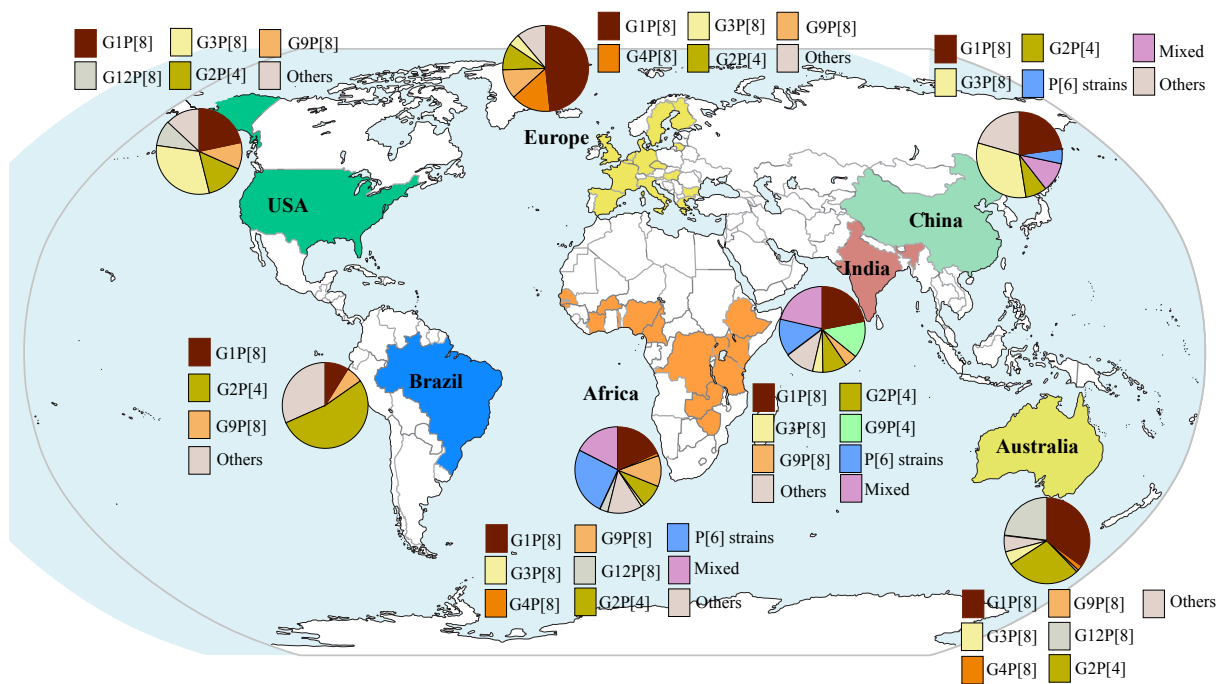


Fig. 7. Human RVA strain predominance in globally important regions. Strain prevalences are based on references [90-95].

Five genotype combinations contribute to 80-90% of all RVA infections in humans across the world: G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]. Of these, G1P[8] is the most prevalent G/P combination worldwide [91, 96]. Results from the European rotavirus surveillance network EuroRotaNet, demonstrated the predominance of G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] strains in 16 European countries in the period 2006-2009, with G1P[8] strains being the leading cause of rotavirus gastroenteritis [90]. In the US and China, similar findings have been described, but more recently G3P[8] strains were shown to be most prevalent [91, 94]. Remarkable is the high burden of infections with P[6]-strains in combination with G1, G2, G3, G4, G8 and G9 in Africa [91, 92]. A higher proportion of

infections with G2P[4] strains was seen in some countries, like Belgium and Brazil, after introduction of the monovalent Rotarix[®] vaccine in their national immunization program in 2006, which might be the result of lower cross-protection induced by the G1P[8] strain in the vaccine [97, 98]. However, natural fluctuations in the prevalence of G2P[4] strains have also been described in non-vaccinating countries. Therefore it is difficult to conclude that variations seen in vaccinating countries are only due to the introduction of the monovalent vaccine [98].



1.2.4.4. The evolutionary relationship between pig and human rotaviruses

As described above, each gene segment of an RVA strain can be given a genotype. Whereas the number of pig RVA strains of which the VP7 and VP4 genotypes have been characterized is increasing, only little information is available about the genotypes found for the other 9 gene segments of pig RVA strains. Complete genome analyses of old cell-culture grown pig RVA strains (RVA/Pig-tc/USA/OSU/1977/G5P[7], RVA/Pig-tc/USA/Gottfried/1983/G4P[6], RVA/Pig-tc/MEX/YM/1983/G11P[7], RVA/Pig-tc/IND/RU172/2002/G12P[7]) were performed earlier [99, 100]. In the past years, some additional pig strains with different VP7/VP4 genotype combinations from Canada, Thailand, South Korea and Italy were completely characterized. Most of the strains characterized so far possessed an I5-C1-R1-M1-A8-N1-T1-E1-H1 genotype constellation for the 9 remaining genes [64, 101-103]. However, the T7 genotype for NSP3 has frequently been detected in these studies as well [64, 102]. A rarity was the detection of the dual I5+I14 genotype for VP6 in a G2P[34] strain from Canada, which also possessed the rarely detected E9 genotype for NSP4 [64]. Still, the number of completely characterized pig RVA genomes is rather limited. Furthermore, the majority of characterized strains possessed the G9P[23] genotype combination for VP7 and VP4, which is not fully representative for the wide diversity of G/P combinations that can be found in pigs.

In contrast, many more human RVA strains from human origin have been fully genetically characterized, with around 500 complete human RVA genomes deposited into Genbank in 2014. As demonstrated above, only a few G/P genotype combinations are predominant in humans worldwide. When the genotypes of the other 9 gene segments of human RVA strains are analyzed, two major and one minor genotype constellation can most frequently be found: Gx-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 and G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, and G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3, respectively. These genotype constellations are also called Wa-like or genogroup 1, DS-1-like or genogroup 2, and AU-1-like or genogroup 3, respectively [104]. As depicted in Table 3, it is clear that genotype 1

genes (in green) are shared between the human Wa-like and pig strains. Based on these findings, it was suggested that human and pig RVA strains are evolutionary related to each other, and that they likely possess a common evolutionary ancestor. A similar evolutionary relatedness was believed to exist between human DS-1-like strains and bovine RVA strains, as they share several genotype 2 genes. Finally, it has been thought that human AU-1-like strains originate from cats, based on the shared genotype 3 genes (orange) [49]. While an evolutionary relationship, which is based on the large number of shared genotypes, seems to exist between certain human and animal RVA strains, several other genes from human and animal strains possess different genotypes. In the present thesis, it will be further investigated to which extent human Wa-like and pig RVA strains are evolutionary related and if there are any genetic factors existing, which may hamper or facilitate the spread in a new host species. Interestingly, for RVC strains such a complete genotype classification does not yet exist, and there is less evidence available that there might be an evolutionary relationship between RVC strains from different host species. Still, viruses are constantly evolving by different mechanisms which will be described in the section hereafter.

Table 3. Genotype constellations of RVA strains from different host species.

Species	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
 <i>Wa-like</i>	G1	P[8]	T1	R1	C1	M1	A1	N1	T1	E1	H1
<i>DS-1-like</i>	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
<i>AU-1-like</i>	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
 pig	G5	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
cow	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
cat	G3	P[9]	I3	R3	C2	M3	A3	N1	T3	E3	H3

1.2.5. Mechanisms driving rotavirus evolution

1.2.5.1. Point mutations

Rotaviruses use an RNA-dependent RNA polymerase for transcription and copying of their gene segments. However, this enzyme lacks proofreading capacity which leads easily to the introduction of nucleotide mutations in the viral genome. Blackhall and colleagues estimated that one nucleotide change is introduced in the rotavirus genome during each replication cycle [105].

The outer capsid proteins VP7 and VP4 are directly exposed to the environment and form a major target for the induction of neutralizing antibodies. Thus, it is logically that these proteins are under strong evolutionary pressure. Nucleotide changes in genes encoding VP7

and VP4 may result in amino acid mutations leading to mutant viruses able to escape immunity. The evolutionary rates for several VP7 encoding genes of different genotypes have been calculated. As an example, a substitution rate of 1.87×10^{-3} nucleotide substitutions/site/year was calculated for G9 strains, whereas a similar evolutionary rate (1.66×10^{-3}) was found for G12 strains [106]. Similar evolutionary rates were calculated for G1 (0.93×10^{-3}), G2 (1.45×10^{-3}), and G9 (1.07×10^{-3}) RVA strains from Bangladesh collected over a time period of 22 years [107]. The evolutionary rate of the outer capsid protein VP4 was calculated to be slightly lower than that of VP7, at a rate of 0.58×10^{-3} substitutions/site/year [108]. A similar evolutionary rate was determined for the N1 genotype of NSP2, although this is a protein that is generally considered to be rather conserved, which suggested that most nucleotide substitutions were synonymous [109].

1.2.5.2. Reassortment of gene segments between human RVA strains

The shuffling of gene segments from two RVA strains coinfecting the same cell may rapidly result in the formation of genetic or antigenic variant RVA strains possessing a mix of gene segments from both parental strains (genetic or antigenic shift). The occurrence of gene reassortment has been thoroughly studied using a unique collection of RVA strains (G1P[8], G3P[8], G4P[8]) in fecal samples from children with RVA gastroenteritis collected between 1974 and 1991 at the Children's Hospital National Medical Center in Washington. The genomes of all strains characterized in these studies possessed a genogroup 1 genotype constellation (Gx-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1). Multiple subgenotypic alleles existed within the genotype 1 of each gene segment, and multiple allele genotype constellations were circulating within a single rotavirus season. However, some of these allele constellations persisted in the community over a long period of time. It was suggested that proteins encoded by such predominant genotype allele constellations operated better together. Consequently, exchange of gene segments between strains occurs less frequently than previously thought [110-113]. Zhang and coworkers identified strong intermolecular connections between VP4 and VP2 proteins of the aforementioned USA RVA strains, indicating that mutations in one protein drive mutations in the other [113]. G2P[4] strains are also epidemiologically important in humans and most of these strains possess a genogroup 2 genotype constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2). Reassortment events between genogroup 1 and genogroup 2 strains have been described, but not frequently, which indicates that mixing genotype 1 and genotype 2 constellations results in a reduced viral fitness of these mosaic viruses [114-118]. Heiman and colleagues also identified genotype specific mutations between proteins of different human genotypes, which may result in

better interactions between RVA proteins, or between RVA proteins and cellular proteins [119].

1.2.5.3. Interspecies transmission and reassortment of animal RVA strains

Rotaviruses from animal origin are not seldomly detected in fecal samples of diarrheic children in rotavirus surveillance programs. Human RVA strains with atypical G/P genotypes that are normally found in animal RVA strains are suspected to be the result of interspecies transmission events from animals to humans. Approximately 0.3% of the strains characterized by EuroRotaNet were probably interspecies transmission events or zoonoses from animals to humans [90]. However, most interspecies transmission events from animals to humans are dead-end infections. Nonetheless, interspecies jumps of animal rotaviruses to humans are considered as a risk for the creation of animal-human reassortant viruses, which may lead to the introduction of animal gene segments in the human population and *vice versa*. Around 1.7% of RVA strains surveilled by EuroRotaNet were categorized as animal-human reassortant viruses [90].

It is not unlogical that interspecies transmission occurs more frequently in developing countries from Africa or Asia, where people live in close proximity with animals. Nonetheless, species crossings have also been detected in well-developed countries such as Belgium [92, 120]. As an example, a rare G9P[6] strain, RVA/Human-wt/BEL/BE2001/2009/G9P[6] was detected in a one month old child in Belgium during a surveillance study conducted in 2009. The father of the kid had been working on a pig farm in the week that the child became ill. Analysis of the complete genome revealed that the gene sequences of this RVA strain were much more related to those of pig RVA strains rather than to those of human RVA strains circulating in Belgium. As a consequence, this strain was most probably a pig-to-human interspecies transmission event [120]. In Cameroon, a G5P[7] strain with characteristics of pig and human RVA strains caused diarrhea in a child [121]. Moreover, it was suggested earlier that the spread of human P[6] strains in the human population was the result of multiple independent interspecies transmission events from pigs to humans [122, 123]. In many countries, direct interspecies transmission events of pig P[6] strains to humans or infections with pig-human reassortant P[6] strains in children have been described [120, 124-130]. Also infections with atypical G9P[19] strains in Asian children were thought to be of pig origin [131-134]. Most of these interspecies crossings result in dead-end infections, but some reassortant viruses gain the capacity to spread successfully in the human population. In Brazil, an epidemic of G5P[8] strains occurred during the 1980s. The VP7 gene (G5) of these strains was of pig origin and

the other 10 gene segments were of human origin. Later, G5 strains were also demonstrated in other countries of the South American continent, demonstrating the capacity of reassortant strains to cause local epidemics in humans [119, 135-138]. The recent emergence of G9 strains in the human population worldwide is also thought to be the result of an interspecies transmission event from pig G9 strains to humans, followed by reassortment with human Wa-like RVA strains. These G9 strains were able to spread around the world in only a couple of decades, and became the fifth most important genotype worldwide [106].

1.2.6. Replication cycle

Rotaviruses have a tropism for enterocytes of the small intestine of many species. The replication cycle consists of several chronological steps, which are demonstrated in Figure 8 and discussed hereafter.

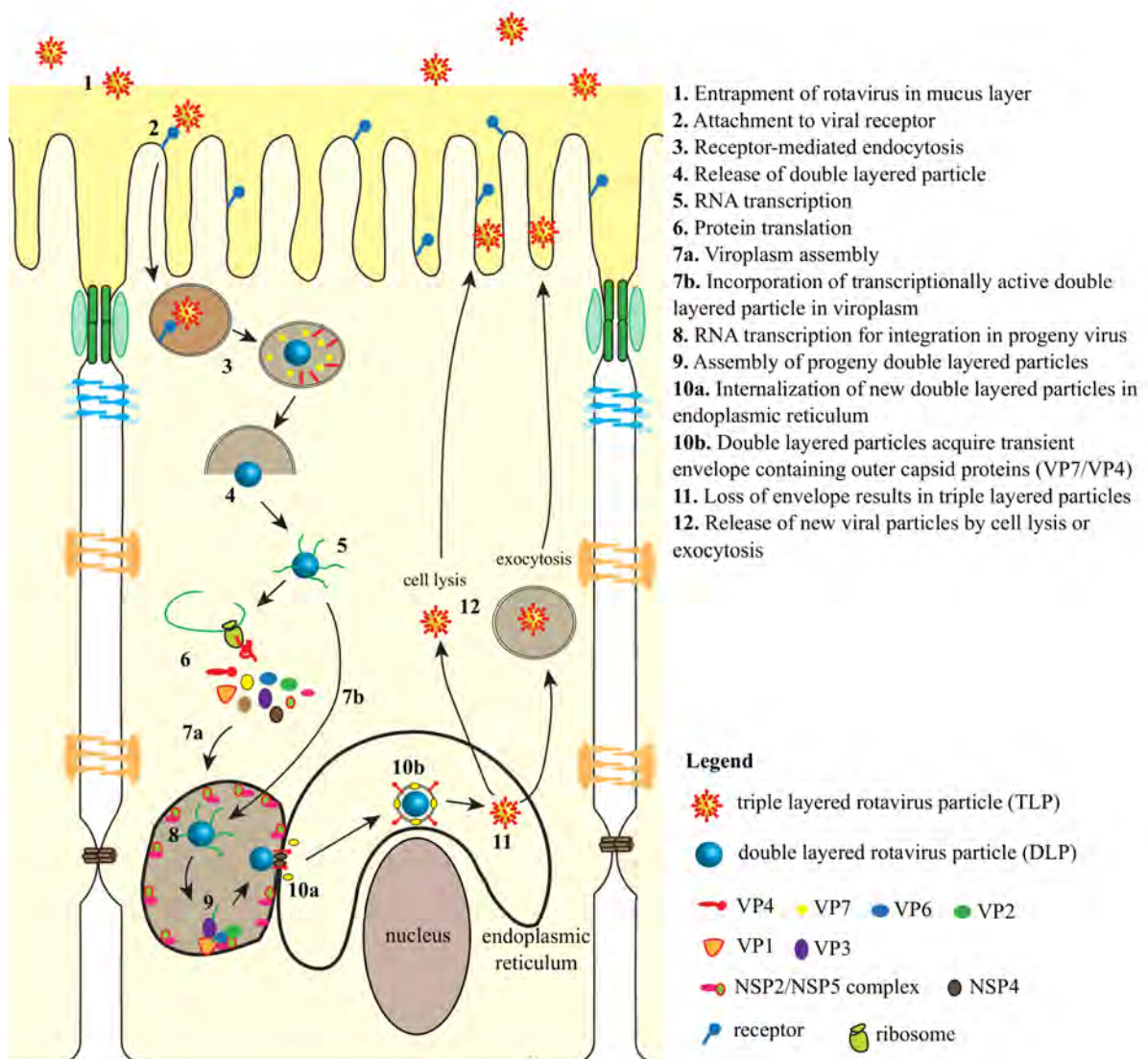


Figure 8. Rotavirus replication cycle

1.2.6.1. Attachment and internalization

VP8 - carbohydrate interactions*

Rotavirus particles contain many VP4 proteins extruding from their surface. In the intestinal lumen, trypsin cleaves rotavirus VP4 proteins between their VP8* and VP5* domains. Nonetheless, both subunits remain together by hydrophobic interactions [30, 31]. It has been demonstrated that VP8* functions as a viral lectin and interacts with carbohydrate moieties on the cellular surface [28, 29]. This interaction leads to entrapment of viral particles into mucus or at the cellular apical membrane to avoid removal by the highly motile intestinal lumen. Some animal rotaviruses have been shown to be dependent on sialic acids for the interaction with the cell, such as those with a P[1], P[2], P[3] and P[7] genotype for VP4, found in pigs, cows and monkeys [139-141]. For some pig rotavirus strains, the sialylated GM3 ganglioside was proposed as cellular receptor [142]. However, other animal and human rotaviruses are independent of sialic acids for their entry into cells. Treatment of cells with *Arthrobacter ureafaciens* neuraminidase has rendered these cells more susceptible for infection with human sialic acid-independent strains. Attachment of these strains to the cell surface was thought to be dependent on subterminal sialic acid residues, which were not removed by *A. ureafaciens* neuraminidase treatment [143, 144]. Furthermore, a role for GM1 gangliosides in rotavirus entry was proposed [145].

Recent studies have indicated that the major human genotypes P[4], P[6] and P[8] are independent of terminal sialic acids for attachment. Indeed, they recognize internal sialic acids on histo-blood group antigens (HBGA). These HBGAs are built by a concerted action of several cellular enzymes. Type 1 chain HBGA precursors are expressed in the intestine and can be fucosylated at the terminal galactose by action of the enzyme encoded by the secretor gene (FUT2), generating H-type 1 antigens. Another enzyme, encoded by the FUT3 gene, results in fucosylation of type 1 precursors or H-type 1 antigens, generating Lewis a or Lewis b antigens, respectively. Furthermore, a terminal N-acetylgalactosamine residue or a galactose can be added to the H-type 1 antigen at its terminal galactose, resulting in A type 1 antigens or B type 1 antigens, respectively. These A type 1 and B type 1 antigens may also be fucosylated by the FUT3 encoded enzyme, resulting in the ALe^b and BLe^b phenotypes [146]. A recent study by Huang and colleagues has indicated preferential binding of P[4] and P[8] VP8* domains to Lewis b antigens or H-type 1 antigens [147]. Moreover, children with FUT2 polymorphisms, also called non-secretors, have been shown to be resistant to infections with P[8] strains [148]. Furthermore, it was demonstrated in a study of Nordgren and colleagues in Burkina Faso that not only the FUT2 secretor phenotype, but also the

Lewis phenotype is necessary for susceptibility to P[8] strains. In contrast, P[6] strains mostly infect Lewis negative children, independent of their secretor (FUT2) phenotype [146]. Moreover, in the study of Huang and colleagues, no interactions between P[6] VP8* proteins and Le^b antigens were observed, although they interacted with H type 1 antigens [147]. Remarkably, only 4-6% of the European and North American population is Lewis negative, whereas this is the case for approximately 30% of the African and Latin American population [146]. In the aforementioned Caucasian population, P[4] and P[8] are dominant VP4 genotypes, whereas P[6] strains are more frequently found in Africans. The latter can be explained by the higher proportion of people carrying the Lewis negative phenotype in this ethnic population. These findings may also explain the reduced vaccine efficacy in Africa, since the P[8] strain enclosed in Rotarix[®] may have a reduced ability to infect Lewis negative children [146].

It was also demonstrated that human P[14] strains depend on interactions with type A antigens for attachment to the cell surface [149, 150]. The same was observed for genotypes P[9] and P[25] [149]. Strains possessing the latter three genotypes infect both humans and animals, whereof human P[14] strains have likely emerged from *Artiodactylae* [149, 151]. On the other hand, P[9] strains are an important genotype in cats. Occurrence of type A antigens both in humans and animals may facilitate interspecies transmission of these strains. Furthermore, naturally occurring human-bovine reassortant neonatal rotavirus strains with bovine P[11] genotypes for VP4 have been shown to bind type 2 antigens, the precursor for H type 2 HBGAs [152].

Post-attachment receptors

Several cellular proteins are used as post-attachment receptors for rotavirus infection in cell cultures. As an example, integrins ($\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha \chi\beta 2$) and heat shock cognate protein 70 have been proposed as co-receptor [153-157]. However, all these studies for determination of rotavirus co-receptors have been performed in the continuous kidney epithelial cell line MA104 derived from an African Green Monkey. Thus, the relevance of these findings should be interpreted carefully, as the small intestinal enterocyte is the real target cell for rotavirus infection.

Endocytosis

Endocytosis has been shown as an internalization pathway of different rotavirus strains from different species. As an example, human strain Wa, porcine strain TFR-41 and bovine strain UK enter MA104 cells by clathrin-mediated endocytosis dependent on cholesterol and

dynamamin. In contrast, monkey strain RRV enters these cells by a clathrin- and caveolin-independent pathway, which is dependent on cholesterol and dynamamin [158]. However, contrasting results have been described for RRV strains by other research groups, as internalization of this strain would not be dependent on cholesterol and dynamamin in MA104 and MDCK cells [159]. Consequently, due to lowering of the Ca^{2+} -concentration in endosomes, the integrity of the VP7 layer is abolished, resulting in the escape of double-layered particles in the cytoplasm. It is thought that the hydrophobic domain located apically on VP5* is involved in permeabilization of endosomes [160, 161].

1.2.6.2. RNA transcription

Once the double-layered particle is released in the cytosol, it becomes transcriptionally active. Conformational changes in VP6 and VP2 resulting from the release of the VP7 layer, cause the widening of type I channels, thereby rendering the cores of the viral particles accessible for nucleotides and ions [162, 163]. Transcription is performed by the enzymatic complex VP1 and VP3 [20]. These ssRNA strands are released from the double-layered particles through type I channels at the five-fold vertices. As such an mRNA transcript is created from each gene segment. RNA transcription can already be observed within one hour after infection [164].

1.2.6.3. Translation

Viral mRNA is used as a template for viral protein synthesis. Protein synthesis is enhanced by the action of NSP3, which recognizes a GACC translation enhancer-motif at the 3' end of the mRNA strand. NSP3 also interacts with eIF4G, which in turn binds eIF4E [165-168]. The circle is closed by recognition of the 5' end cap by eIF4E. As such, transcriptionally highly active polysomes are formed and recycle ribosomes. Furthermore, host protein translation is downregulated by binding of NSP3 to eIF4G [169, 170].

1.2.6.4. Viroplasm synthesis and formation of double-layered particles

After viral translation of viral proteins, NSP2 and NSP5 induce the formation of cytoplasmic inclusion bodies called 'viroplasms' [171-173]. Cytoplasmic NSP2s induce depolymerization of microtubuli and recruit tubulin in the viroplasm structure [174-176].

NSP4 interacts with the autophagosomal marker LC3 and recruits autophagosomes to viroplasms. Fusion of viroplasms and autophagosomes results in enlargement of these viroplasms and enhancement of viral replication [177]. Energy is supplied to these viroplasms by lipid droplets. Replication complexes of VP1, VP2 and ssRNA are enclosed within these viroplasms [176]. Interaction of VP2 proteins leads to the formation of core

capsid particles or single layered particles. Next, the VP2 layer is surrounded by the VP6 inner capsid proteins. As a result, double-layered particles are formed [12, 13].

1.2.6.5. Budding through endoplasmic reticulum (ER) and release of viral particles

To obtain an outer capsid shell, DLPs bud through the ER. NSP4 is present in the membrane of the ER and functions as a receptor for internalization of DLPs in the ER [178]. During the passage through the ER-lumen, DLPs obtain a transient bilipid envelope layer. This layer is lost due to incorporation of the outer capsid layer composed of VP7 and VP4 [12, 13]. Next, two mechanisms of viral egress have been described: rotavirus is released by cell lysis in MA104 cells, but is released by budding in Caco-2 cells [179, 180]. This also emphasizes that care should be taken for using MA104 cells or carcinogenic cell lines for the study of rotavirus replication.

1.2.7. Pathogenesis, pathology and clinical symptoms

Rotaviruses were detected for the first time in the seventies in humans and pigs, and several pathogenesis studies were undertaken shortly hereafter in colostrum-deprived pigs. In a study of Pearson and McNulty, diarrhea was observed within 16-24 hours after inoculation of one day old piglets with rotavirus containing fecal filtrates. Infected epithelial cells were observed at the villus tips in the duodenum and ileum, and along the villus axis in the jejunum [181]. Lecce and King observed that 3 weeks old pigs developed rotavirus diarrhea 3 to 5 days after weaning. Weight gain was also dramatically reduced in the first two weeks after weaning. The disease was reproduced by feeding bacteria-free intestinal contents to new pigs, resulting in villus blunting and shortening [182]. In 1977, pig rotavirus was detected for the first time in Belgium. Three conventional pigs were kept in isolation for experimental purposes at an age of 2 days old. Four days after isolation, watery diarrhea was observed, and the piglets died due to dehydration. A pooled bacteria-free fecal sample was used for inoculation of a colostrum-deprived piglet, which induced vomiting and diarrhea 24 hours after inoculation. This isolate was named RV277 [7]. More recently, the pathogenesis of 2 Korean G9 pig RVA strains was studied in colostrum-deprived piglets. One day after inoculation, piglets showed diarrhea and virus shedding that lasted until 8 and 10 days after inoculation, respectively. Villus atrophy and crypt hyperplasia were observed, and large amounts of viral RNA were detected in mesenteric lymph nodes. Viral RNA was also detected in serum at day 3 and 5 post inoculation, and viral antigens could even be detected in the liver, lungs and choroid plexus, indicating a limited systemic spread of RVA in pigs

[183]. In pigs, villus shortening and blunting due to rotavirus infection is in general less pronounced than those induced by coronaviruses [184].

Different pathogenesis mechanisms have been described for rotavirus-induced diarrhea. First, viral replication results in shedding of infected enterocytes in the lumen, which may impair the absorptive capacity of the intestine. Accumulation of unabsorbed nutrients in the lumen attracts water by osmotic forces, leading to osmotic or malabsorption diarrhea. Furthermore, the enteric nervous system (ENS) is activated upon rotavirus infection and induces hypersecretion of electrolytes and water. Triggering of the ENS may be the result of local inflammation, induced by rotavirus infection, or a direct NSP4-mediated effect [185]. NSP4 is released from infected enterocytes and induces an increase of the intracellular Ca^{2+} -concentration in enterochromaffine (EC) cells, a subset of enteroendocrine cells functioning as sensors in the intestinal lumen. Granules with 5-hydroxytryptamine (5-HT or serotonin) are released upon the intracellular Ca^{2+} concentration increase, causing a stimulation of 5-HT₃ receptors on intrinsic and extrinsic afferent nerves. Intrinsic activation triggers the release of vasoactive intestinal peptide (VIP) by efferent nerve ends of the ENS, leading to hypersecretion. Activation of extrinsic afferent vagal nerve ends can induce vomiting, as these nerve ends are connected with the vomiting center of the brain (nucleus tractus solitarii and area postrema) [186].

1.2.8. Immunology

1.2.8.1. Innate immunity

When cells are infected by viruses, certain viral components (RNA, viral proteins) or replication products will be recognized by pattern recognition receptors (PRRs) present in the host cell. These PRRs can be Toll-like receptors (TLRs), nucleotide-oligomerization domain-like receptors (NLRs) or retinoic acid-inducible gene 1-like receptors (RLRs). These sensors recognize pathogen associated molecular patterns (PAMPs) and trigger a complex cascade of protein-protein reactions in the cell, resulting in transcription and translation of interferon, which in its turn induces an antiviral state in the host cell [169, 187-189].

As an example, pattern recognition receptors RIG-1 and MDA-5 have been shown to be involved in the induction of IFN- β via a pathway dependent on the IFN- β promotor stimulator 1 (IPS-1) upon rotavirus infection. Though, it is not known which viral components are activating these PRRs [190, 191].

On the other hand, rotavirus infection also stimulates TLR3, TLR5 and TLR7. Rotaviral dsRNA is recognized by TLR5 and TLR7, and these receptors seem to play a major role in

induction of the interferon response in human plasmacytoid dendritic cells [192, 193]. The role of signaling proteins MyD88 and TRIF after activation of TLRs by rotavirus infection is unclear [169].

Following stimulation of these PRRs, two major pathways dependent on NF- κ B or interferon-regulatory factors are inducing the transcription of IFN genes. In uninfected cells, NF- κ B is held in an inactive form together with I κ B. Viral infection induces the phosphorylation and ubiquitination of I κ B, which is executed by a complex of an E3 ubiquitin ligase and β -TrCP. In this way, I κ B is degraded by the proteasome, whereas NF- κ B can translocate to the nucleus, activating the transcription of IFN genes. On the other hand, phosphorylation of IRF3, IRF5 and IRF7 upon viral infection results in activation of these regulatory factors and also induces transcription of IFN genes. Finally, activation of these interferon genes results in the production and release of IFN by the cells, and consequently results in inhibition of viral replication.

The most important subclass of interferons involved in antiviral immunity is type I IFN, which recognizes the IFN-1 receptor, composed of 2 subunits: IFNAR1 and IFNAR2. This binding results in the phosphorylation of the IFN-1 receptor by tyrosine kinase 2 and JAK1. Next, signal transducers and activators of transcription (STAT) proteins are recruited and phosphorylated. Phosphorylated STAT1 and STAT2 form IFN-stimulated gene factor 3 (ISGF3) together with IRF9. This complex is translocated to the nucleus where it stimulates the expression of IFN-induced genes that suppress viral replication by four main pathways: ISG15 pathway, Mx protein pathway, 2',5'oligoadenylate synthetase (OAS) and RNaseL pathway and a protein kinase R pathway [194].

OAS proteins function as cytoplasmic sensors for dsRNA, and recognition leads to their activation, generating adenosine oligomers by polymerization of ATP molecules. These oligomers activate in their turn RNaseL enzymes which are able to cleave dsRNA in small pieces. In turn, these dsRNA fragments activate RIG-1 and MDA-5 proteins, as described above, thereby amplifying the interferon response. On the other hand, viral RNA also results in the activation of protein kinase R (PKR), of which its cytoplasmic expression is also upregulated by IFN. Activation of PKR results in phosphorylation of eIF2(α) and shut-down of cellular and viral protein production [169, 194].

Rotaviruses have developed several strategies to overcome the host IFN response in favor of viral replication. The most important viral protein suppressing type I IFN responses is NSP1, also called the interferon antagonist. NSP1 is the most variable protein among rotaviruses. Nonetheless, a highly conserved domain (aa 42-79) is present at the N-terminal region of this

protein. This region contains a putative zinc finger or RING domain, which contains conserved cysteine and histidine residues [195]. Similar domains are present in the cellular E3 ligase, which cooperates with an E1 activating enzyme and an E2 conjugating enzyme for ubiquitination of proteins and subsequent targeting to the proteasome. Thus, NSP1 can function as an E3 ligase [196]. Interferon regulatory factor 3 is recognized at its dimerization domain by the C-terminal half of the NSP1 protein and ubiquitination of this factor by NSP1's E3 ligase activity results in proteasomal degradation of this regulatory factor, leading to suppression of the host's innate immune response [196-200]. Furthermore, NSP1 also has a degradative effect on IRF5 and IRF7 [201]. Animal rotaviruses are able to inactivate IRF3, IRF5 and IRF7, whereas human rotaviruses only seem to target the latter two [169, 201]. Another target of NSP1 in the interferon pathway is β -TrCP. An ancient pig strain OSU, which possesses an A1 genotype for NSP1, is able to induce the proteasomal degradation of β -TrCP, resulting in down regulation of IFN- β production [169, 202]. As a summary, these activities of NSP1 control the expression of IFN genes. However, NSP1 and other viral proteins can also act downstream of the IFN expression pathway, by directly suppressing some of the above mentioned IFN-induced pathways. As an example, NSP1 is able to inhibit the phosphorylation of STAT1, preventing the formation of a STAT1/STAT2 heterodimer and induction of IFN responses in the cell [203-206]. Recent studies have also identified a C-terminal 2H-phosphodiesterase domain in the rotavirus VP3 protein that is capable of cleaving the adenosine-oligomers that are induced by OAS activation. Breakdown of these oligomers prevents the activation of RNaseL and cleavage of viral RNA [21, 207]. Furthermore, cellular translation, but not viral translation is inhibited in rotavirus infected cells by phosphorylation of eIF2a in a PKR dependent manner [208, 209].

1.2.8.2. Acquired immunity

Besides the activation of antiviral interferon responses, the body also reacts with humoral and cellular immune responses against rotavirus infections. Humoral immunity is based on the production of neutralizing antibodies targeting infectious pathogens. Infections with enteric pathogens result in the presentation of antigens to T helper cells. Next, these T_H cells are activated and stimulate B cells in germinal centers of gut-associated lymphoid tissues such as the Peyer's patches and lymphoid follicles, and mesenteric lymph nodes. Production of TGF- β and CD40 signaling results in hypermutational mutation and class-switch recombination in these B cells with the help of IL-10, IL-6, IL-4 and IL-2, resulting in the development of high affinity IgA antibodies in germinal centers of lymphoid organs [210, 211]. B cells expressing the homing factor $\alpha 4\beta 7$ are important for protection against

rotavirus infection [210, 212]. Finally these B cells differentiate into plasma cells that are in fact IgA antibody producing factories. IgA antibodies form dimers, stabilized by the joining (J)-chain. At the basolateral side of the enterocytes, a polymeric Ig receptor (pIgR) is present, which is involved in internalization and transcytosis of these dimeric IgA complexes through the epithelium [211, 213]. During this passage, they acquire a secretory component (SIgA) that protects IgA antibodies against harsh intestinal conditions, thus preventing their rapid degradation. Intestinal SIgA antibodies have been identified as key-components in the protection against rotavirus infections, because they are able to block viral attachment to the cellular surface, preventing infection [214].

Remarkably, in T cell knockout mice, 60% of rotavirus-specific IgA is still being induced, indicating that T cell independent activation of the humoral response is also very important in combating rotavirus infection [215]. Indeed, it has been shown that B cells located in the Peyer's patches are rapidly (<24-48h) activated after rotavirus infection by type I IFN- α and IFN- β , produced by plasmacytoid dendritic cells (pDC) in humans and mice [192, 216]. Moreover, these pDCs form a link between the adaptive immune response and innate response.

Important targets for induction of neutralizing antibodies are outer capsid proteins VP7 and VP4. Two major antigenic sites have been described for VP7. Epitope 7-1 encloses epitopes 7-1a and 7-1b, lying on adjacent VP7 molecules (Figure 9). Antibodies against this epitope are thought to stabilize the outer protein shell. Every VP7 protein also holds a smaller epitope, designated 7-2. Furthermore, 4 antigenic regions (8-1 to 8-4) are recognized on the globular VP8* domain of VP4 (Figure 9). Antibodies targeting these regions are thought to hamper viral attachment to the cell surface, whereas antibodies against antigenic regions on VP5* (5-1 to 5-5) are believed to block membrane permeabilization during internalization (Figure 9) [24, 29, 111, 217]. The outer capsid proteins, and their encoding genes, are antigenically and genetically diverse in humans and animals. At least 27 G-genotypes for VP7 and 37 P-genotypes for VP4 have been detected to date, and this genetic and antigenic diversity may explain the decrease of cross-protection between different sero- and genotypes.

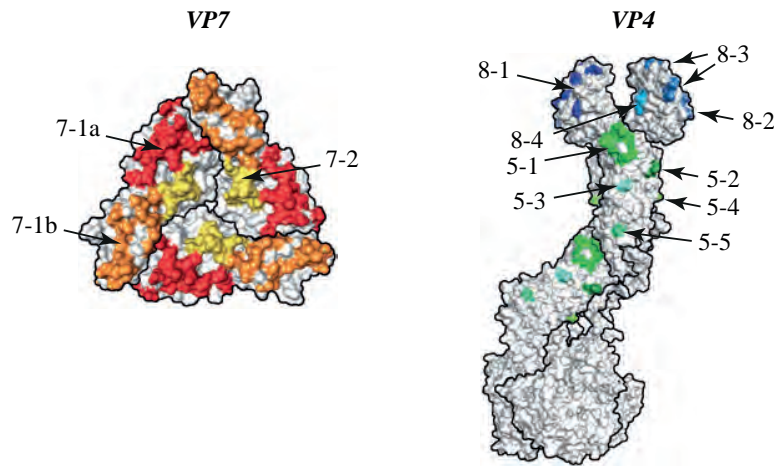


Fig 9. Antigenic regions on VP7 and VP4 (adapted from [13]).

It was suggested that primary rotavirus infections mainly induce antibody responses that provides good protection against the same strain (homotypic protection). Subsequent infections broaden the immune response, thereby inducing protection against a wider variety of strains (heterotypic protection) [218-220]. When Rotarix[®] (GlaxoSmithKline), a monovalent G1P[8] vaccine is administered to children, homotypic protection is induced. This protection does not completely protect against re-infection, but protects children against severe rotavirus diarrhea. However, mild re-infections broaden the immune response, resulting in heterotypic protection. In contrast, RotaTeq[®] (Merck) contains 5 bovine-human reassortant rotavirus strains whereof it is expected that each strain induces homotypic protection against each of the 5 human rotavirus components present in the vaccine [218, 219, 221].

However, high level of serum IgA antibodies against structural proteins VP6 and VP2 are also elicited after rotavirus infection. Indeed, the detected antibody levels against VP6 were even higher than those induced against VP7 and VP4, indicating that antibodies against the inner capsid protein may play an important role in protection against infection [222]. Recently, it has been demonstrated that anti-VP6 specific antibodies block rotavirus replication by recognition of a quaternary epitope on VP6 trimers. VP6-specific SIgA antibodies are internalized into enterocytes by recognition of the pIgR and inhibit virus replication intracellularly. The epitope, which is targeted by VP6-specific antibodies, consists of 2 highly conserved regions A (aa 231-260) and B (265-292). Binding of antibodies to these epitopes results in a sterical blockade of type I channels, hampering the release of transcribed mRNA from transcriptionally active DLPs [223, 224]. It has been suggested that antibodies direct against NSP2 and NSP4 may use a similar strategy to control rotavirus infection [218].

1.2.8.3. Passive protection of offspring

To protect the offspring against enteric pathogens, neutralizing antibodies are secreted in milk of animals and humans. As described above, an enteric infection leads to a local stimulation of the immunity, but in monogastric animals (including pigs and humans), there exists an immunological link between the gut-associated lymphoid tissues and the mammary gland. As such, stimulated B cells are also able to home to the mammary gland, followed by differentiation into plasma cells and production of IgA antibodies. These IgA antibodies are then secreted in the milk as SIgA by passage through the mammary epithelium. To sustain good lactogenic protection, the sow should regularly become reinfected with these pathogens [1, 225]. In contrast to pigs and humans, the IgA concentration in colostrum and milk of cows (3.9 mg/ml and 0.14 mg/ml) is much lower, whereas IgG1 concentrations are much higher (47.6 mg/ml and 0.59 mg/ml) [226]. Therefore, vaccination is used to boost IgG1 concentrations in colostrum, and good colostrum management is essential to prevent infectious diseases in young calves [225, 227].

1.2.9. Prevention of rotavirus infections in pigs

When preventive measures against rotavirus problems need to be installed on pig farms, a distinction should be made between piglets in the farrowing house and piglets shortly after weaning. During the suckling period, young piglets are predominantly fed by the milk of the sow. At this stage, only limited amounts of creep feed are actively taken up, which makes the use of feed and drinking water medication useless at these ages. Strict hygiene measures and vaccination of gilts and sows are preferred measures to prevent diarrhea in suckling piglets. After weaning, piglets will consume solid feed and drinking water wherein components can be added to prevent the emergence of diarrhea and other diseases after weaning. Regrettably, effective measures to control rotavirus infections are relatively scarce on the European veterinary pharmaceutical market.

1.2.9.1. Hygiene measures

One way to reduce the incidence of rotavirus infections is ameliorating the sanitary status of a farm. Hygiene measures should be one of the top priorities to control infectious diseases on modern pig farms, as especially piglets in the farrowing house and weaning barns are extremely vulnerable to them. As such, hygiene measures are not solely installed to reduce rotavirus infections, but are generally needed to ameliorate the general health status of a farm. At the end of each production round, the stable should be emptied, cleaned and disinfected. The use of an all-in all-out system is encouraged. Visible manure should be

removed first, as viruses, bacteria and eggs of parasites may survive for a long period of time in this organic material [228]. Next, all surfaces should be cleaned using a detergent, since pig feces is relatively proteinaceous and fatty, and therefore difficult to remove using water-based methods alone. Including a soaking period with a detergent did reduce the total number of aerobic bacteria on concrete or metal surfaces, but did not reduce the number of *Enterobacteriaceae* [229]. Afterwards, the barns should be high pressure washed with water, followed by disinfection. However, most disinfectants are inactivated by the presence of organic material, which emphasizes the need for thorough cleaning [229, 230]. Drying of the surfaces is essential, as most viruses and bacteria have reduced capacity for survival when they are exposed to drying [229]. Fogging can also be applied after disinfection, which leads to an overall better air quality by reducing ammonia concentrations, fungal spores and dust particles, rather than having a direct bactericidal effect [231]. Unfortunately the effectiveness of cleaning and disinfection procedures to control rotavirus infections has not been studied under field circumstances.

1.2.9.2. Vaccination

Vaccination might be used to improve passive lactogenic immunity or to induce active immunity. Several efforts were already undertaken in the past to develop vaccines for the prevention of rotavirus induced diarrhea in pigs. An attenuated G5P[7] RVA strain was administered to pregnant and lactating sows, and offered partial protection against RVA infection under field conditions [1]. Furthermore, inactivated vaccines failed to induce a protective local immunity in young pigs [232]. Better results were obtained after administration of the aforementioned attenuated G5P[7] strain, which induced protection against challenge under experimental conditions, but failed to protect piglets under field conditions [1, 232-234]. Still, a number of pig rotavirus vaccines are commercially available worldwide (Table 4), but not in the EU-28.

Table 4. Pig RVA vaccines registered in non-EU countries.

Name	Type	Producer	Country	Against	Target age
Prosystem [®] Rota	Attenuated	MSD AH	VS	RVA	Suckling pigs
Prosystem [®] TGE/Rota	Attenuated	MSD AH	VS	RVA TGEV	Gilts and sows
Vaccine against TGEV and Rotavirus (adsorbed)	Attenuated	Narvac	Russia	RVA TGEV	Young pigs, gilts and sows
Vaccine against TGEV and rotavirus (oil-emulsion)	Inactivated	Narvac	Russia	RVA TGEV	Gilts and sows
Vaccine against TGEV and rotavirus	Inactivated	Rue Institute*	Belarus	Rotavirus TGEV	Gilts and sows
TGEV/Rotavirus vaccine	Inactivated	Green Cross Veterinary Products	South Korea	Rotavirus TGEV	Gilts and sows

*Rue Institute of Experimental Veterinary Medicine of S.N. Vyshel'sky

1.2.9.3. Spray dried plasma

Another additive that is frequently used in feed for pigs after weaning is spray dried plasma. At slaughter, pig blood is collected and citric acid is added to prevent clotting. Erythrocytes are centrifuged and the remaining plasma is spray dried, resulting in three fractions containing proteins of different molecular weights: a low, medium (mainly albumin) and high (mainly immunoglobulins) molecular weight fraction. The application of plasma has two main reasons: first it is a nutritional component, but secondly it is also used to passively protect weaned piglets against enteric pathogens by the neutralizing effects of antibodies or glycosylated proteins present in these products [235, 236]. Several studies have been performed and positive effects on growth performances after weaning have been attributed to these products. Fifteen studies were included in a meta-analysis of van Dijk and colleagues and it was concluded that adding spray dried plasma to feed at concentrations of up to 6% resulted in an increased average daily weight gain, increased average daily feed intake and a reduced feed conversion rate. The higher feed intake was suggested to be due to a better palatability of feed containing spray dried plasma [235]. In a rotavirus infection model of pigs, no diarrhea was observed in the group that was treated with spray dried plasma. However, shedding of rotavirus could not be prevented, but it was not clear if the virus was shed at lower titers in the spray dried plasma group, because quantification was not performed [237].

1.2.9.4. Alternative antibody based preventive measures

Chicken IgY antibodies derived from egg yolk of hens immunized with human Wa rotavirus were fed to gnotobiotic piglets. These antibodies protected gnotobiotic piglets against diarrhea, and also reduced viral shedding after challenge with a human virulent Wa strain [238]. Similar results were obtained when llama-derived VHH antibodies directed against the VP6 protein of a bovine G6P[1] strain, were fed to gnotobiotic piglets challenged with the human Wa rotavirus strain [239]. The latter strategy is currently being further developed for application in immunodeficient children, and efforts are being performed to express these anti-rotavirus specific VHH on the surface of probiotics such as *Lactobacilli*, or in transgenic rice [240, 241]. At a certain time, these VHH-antibodies, or *Lactobacilli* producing these antibodies, or plants expressing these antibodies may also be incorporated in pig feed and may form an interesting alternative strategy to help control rotavirus infections in young pigs. VHH antibodies directed against F4, and linked to the Fc part of pig IgA antibodies were produced in *Arabidopsis thaliana*. Pigs fed with these seeds shed lower amounts of ETEC after challenge and had a better weight gain than pigs fed VHH-IgG

containing seeds or wild-type seeds [242]. It is clear that such strategies may eventually find a way into the field, forming a durable alternative to prevent enteric infections.

1.3. Other causes of diarrhea in young piglets

As mentioned earlier, not only rotaviruses can cause diarrhea in young piglets. Attention should also be given to other non-infectious as well as infectious factors that might facilitate the development of diarrhea. Hereafter, some important non-infectious factors will be addressed first. In suckling and weaned piglets, the emergence of diarrhea can be a consequence of too low environmental temperature, which weakens piglets because energy is used to maintain body temperature. A reduced or no uptake of milk by suckling piglets may also result in diarrheic problems as piglets will be unprotected by lactogenic antibodies against enteric infections, and because they cannot maintain their body temperature by a reduced uptake of energy [243]. At weaning, piglets are confronted with a change from a liquid milk diet to solid feed. Poorly formulated linker feeds which are not easily digestible may lead to disturbance of the microbiota, which is the intestinal bacterial flora [244]. A high concentration of essential amino acids is needed for growth, but in the meanwhile a high level of crude protein may result in fermentation of these proteins by *Bacteroides* spp., *Propionibacterium* spp., *Streptococcus* and *Clostridium*, generating potentially toxic products such as ammonia, amines and phenols [245]. Furthermore, soybean, a frequently used protein source may cause hypersensitivity in young piglets and can result in loose feces or growth impairment. Heat treatment or fermentation of soybean can aid to overcome this problem [244, 246]. In contrast to high levels of protein, the presence of a high concentration of fermentable carbohydrates stimulates proliferation of beneficial members of the microbiota, namely *Lactobacillus* and *Bifidobacterium*, which may lead to competitive exclusion of pathogenic bacteria. Carbohydrate fermentation also leads to formation of short chain fatty acids (acetic acid, propionic acid and butyrate), which are used as energy sources by intestinal epithelial cells [245]. Disbalance in the microbiota may allow other pathogens to proliferate and cause disease.

A comprehensive list of the most important infectious causes of diarrhea in pigs, including their age distribution, clinical symptoms and associated pathological lesions, is shown in Table 5.

Table 5. Enteric pathogens found in feces of diarrhetic piglets at different ages, and associated pathological findings.

Cause	Age (weeks)							Clinical symptoms	Gross lesions	Histological findings	Type	References
	N	1	2	3	W	4	5					
Viruses rotavirus	■	■	■	■	■	■	■	pasty to watery diarrhea subclinical infections	thin walled intestines fluid intestinal content	moderate villous atrophy enterocyte exfoliation at villus tips	Mal Secr	[7, 181, 182]
PEDV	■	■	■	■	■	■	■	watery diarrhea, (vomiting) rapid spreading of disease mortality (some strains)	thin walled intestines curdled milk in stomach	severe villous atrophy severe enterocyte ex- foliation	Mal	[247-251]
TGEV	■	■	■	■	■	■	■	watery diarrhea, (vomiting) rapid spreading of disease mortality	thin walled intestines curdled milk in stomach	severe villous atrophy severe enterocyte ex- foliation	Mal	[252, 253]
Bacteria enterotoxigenic <i>Escherichia coli</i>	■	■	■	■	■	■	■	yellowish, watery diarrhea mortality edema disease (F18 ⁺ ETEC)	curdled milk/feed in stomach hyperemic intestine & mesenterium	bacteria adhering to villi no/mild villous atrophy no/mild exfoliation	Sec	[228, 254]
<i>Clostridium perfringens</i> type A	■	■	■	■	■	■	■	non-hemorrhagic, mucoid diarrhea	thin walled, gas-filled gut (pseudomembranes)	mild mucosal necrosis	Secr	[255]
<i>Clostridium perfringens</i> type C	■	■	■	■	■	■	■	bloody diarrhea acute death	hemorrhagic intestines emphysema	severe hemorrhagic, (fibrino)neurotic enteritis gram + bacteria	Secr	[255] [256, 257]
<i>Clostridium difficile</i>	■	■	■	■	■	■	■	pasty-to-watery diarrhea	mesocolonic edema	multifocal epithelial necrosis neutrophil accumulation	Infl	[255, 258]
<i>Salmonella</i> spp.	■	■	■	■	■	■	■	yellow, bloody, mucoid diarrhea mostly subclinical	necrotic enteritis, colitis and typhlitis	necrosis of epithelium infiltration of leukocytes	Infl	[259]
<i>Brachyspira</i> spp.	■	■	■	■	■	■	■	mucohemorrhagic diarrhea	hyperemia and edema of large intestine fibrinous exsudate	epithelial necrosis (colitis) neutrophil accumulation spirochetes	Infl	[260-262]
<i>Lawsonia intracellularis</i>	■	■	■	■	■	■	■	loose to watery/black tarry feces subclinical infections	thickened mucosa	proliferation of crypt cells	Mal	[263, 264]
Parasites <i>Cystoisospora suis</i>	■	■	■	■	■	■	■	Yellow, grey, creamy to liquid diarrhea	mucosal edema fibrinonecrotic membranes	villous atrophy mild epithelial necrosis	Mal	[265, 266]

N is neonatal period (first 3 days after birth); W is weaning; Mal is malabsorptive diarrhea, Secr is secretory diarrhea, Infl is inflammatory diarrhea

Hereafter, some interesting aspects concerning the diagnosis of these pathogens are briefly summarized. To start with, the porcine enteric coronaviruses (PEDV and TGEV) are considered as a major cause of diarrhea, but haven't been detected in the Belgian swine population for several decades, until recently. Since January 2015, some sporadic cases of PEDV were diagnosed on different Belgian pig farms, affected by diarrhea outbreaks without mortality. The strains found were genetically almost identical to German and US INDEL strains, which are associated with milder disease symptoms [267, 268]. These INDEL strains are not only genetically different from the highly virulent US strains that emerged in spring 2013 and which are related to PEDV strains from Asia, but are also different from the prototype European PEDV strain CV777 that circulated in Europe in the 1970s-1990s [249, 250, 269-275]. PEDV is most efficiently being diagnosed using RT-qPCR analysis of RNA extracted from diarrheic feces. The circulation of TGEV on Belgian swine farms has not been studied recently, but the herd was considered serologically negative during a study conducted in 1991-1992 [276].

Enterotoxigenic *E. coli* strains (ETECs) express adhesive fimbriae (F) on their surface, which allow their attachment to enterocytes, followed by colonization of the intestinal tract. F5⁺, F6⁺ and F41⁺ strains can be found in diarrheic neonatal pigs. F4⁺ can also be found in newborn pigs, but also in older suckling pigs and weaned pigs. F18⁺ strains are only being found in weaned piglets, and strains that also express the Stx2e toxin are the cause of edema disease [228, 277]. In routine veterinary practice, diagnosis of *Escherichia coli* infection is mostly performed by isolation of the bacteria in selective agars (e.g. MacConkey) or blood agars under aerobic conditions [278]. F4⁺, F6⁺ and F18⁺ ETECs may produce alpha-hemolysin, which induces hemolysis when bacteria are grown on blood agars. However, 12.2% of pathogenic ETECs from diarrheic weaned piglets in Denmark were not hemolytic upon isolation on blood agar plates, which means that absence or presence of hemolysis is not a complete correlate of virulence [279]. It was suggested that the detection of virulence factors (toxins or fimbriae) is a better, but more expensive approach to assess ETEC pathogenicity [279]. *Clostridium perfringens* are spore-forming gram-positive anaerobic bacteria. Five toxinotypes (A to E) exist according to the major toxins they produce (alpha, beta, epsilon and/or iota) [255, 257]. *Clostridium perfringens* type C is not a normal inhabitant of the intestines of healthy pigs [255]. Type C strains produce the alpha and beta toxin, but the latter has been considered as the primary toxin responsible for disease induction [255-257]. In contrast, *Clostridium perfringens* type A strains are normal intestinal inhabitants which produce alpha toxins [255, 280]. Many strains also produce the beta2

toxin, but recently it was suggested that this toxin is not involved in the pathogenesis of diarrhea [281]. *Clostridium perfringens* can be isolated using Columbia agar under anaerobic conditions. As *Clostridia* are part of the normal intestinal flora, only the shedding of high numbers of bacteria (up to 10^9 CFU/g feces) can be suggestive for pathogenicity [255]. Detection of the beta toxin using enzyme immunoassays is confirmatory for type C infections, whereas detection of the alpha toxin does not confirm that *Cl. perfringens* type A or C were the cause of diarrhea [255]. *Clostridium difficile* infections are being diagnosed by detection of bacterial toxins in intestinal contents using enzyme immunoassays [255, 258]. Diagnosis of *Cystoisospora suis* infections in pigs is performed by microscopical investigation of pig feces after concentrating using the sedimentation and flottation method. *Salmonella enterica* serotype *Typhimurium* can cause enterocolitis in pigs of different ages. *Salmonella* enterocolitis is in general more common in fattening pigs and most infections occur asymptotically [259]. Diagnosis can be performed by isolation of the bacteria in aerobic culture. Brilliant Green agar is frequently being used, mostly in combination with an enrichment step in Rappaport-Vassiliadis broth. This method is widely used and approved by the International Organization for Standardisation (ISO 6579:2002) [282].

Swine dysentery is one of the most important enteric diseases affecting older growing and finisher pigs, and is caused by infections with *Brachyspira hyodysenteriae* [260]. However, other species of *Brachyspira* have been found in clinically affected pigs as well [261, 262] [283]. Mahu and colleagues recommended a combination of bacterial isolation and PCR, followed by sequencing, for the diagnosis of *Brachyspira* infections [283]. Finally, *Lawsonia intracellularis* is a bacteria that affects weaning and growing pigs between 6 weeks and 4 months old. Clinically affected animals can be diagnosed using immunocytochemistry, demonstrating infected cells [264]. Collins and Barchia demonstrated that pigs shedding between 10^7 and 10^8 copies of *L. intracellularis* per gram suffered from a significantly reduced daily weight gain. Real-time PCR may thus be a useful tool to detect subclinical cases in addition to serology [284].

1.4. Co-infections between rotavirus and other pathogens

Many studies already demonstrated that co-infections with different pathogens can be found in diarrheic suckling or weaned pigs. Bohl and colleagues demonstrated the capability of rotavirus to cause diarrhea in pigs and investigated ten diarrheic, rotavirus infected herds. In most of them, diarrhea emerged in suckling piglets between 1-4 weeks of age. Interestingly, TGEV was also detected in 1 of these herds and in 5 of 8 herds tested *E. coli* was isolated as well. However, the bacteria was not isolated from all clinically affected piglets, and similar

disease symptoms were reproduced by inoculation of piglets with bacteria-free rotavirus containing filtrates. This led the authors to question the primary pathogenic importance of the *E. coli* strains found. In general, the duration of rotavirus diarrhea was short between 1 to 3 days, and milder under good environmental conditions [285]. The exacerbating effect of co-infections between rotavirus and *E. coli* was shown in a study performed by Tzipori and colleagues. Gnotobiotic piglets fed on milk were inoculated at 4 weeks of age with either rotavirus or F4⁺ *E. coli*, resulting in mild and severe diarrhea, respectively. In general, the symptoms were more severe in *E. coli* than rotavirus infected piglets. However, when both pathogens were inoculated sequentially, this resulted in a more severe disease [286]. Similar results were obtained in a study from Lecce and coworkers. Piglets were weaned at 3 weeks of age under 2 artificial conditions, namely a “sanitary” and “unsanitary” environment, the latter being contaminated with rotavirus and *E. coli* expressing ST and LT enterotoxins. In the first condition, rotavirus diarrhea was only noticed at 16 days after weaning, but no hemolytic *E. coli* was isolated from these pigs. In contrast, diarrheic symptoms commenced already 3 days after weaning under unsanitary conditions. Rotavirus and non-hemolytic *E. coli* was found in the feces of these piglets, but after a couple of days a hemolytic *E. coli* flora became dominant, which shifted back to non-hemolytic *E. coli* after recovery [287]. Benfield and colleagues inoculated 3 day old piglets with either rotavirus, F5⁺ *E. coli* or both. Disease was more severe in *E. coli* than in rotavirus infected animals. Nonetheless, prior inoculation of rotavirus did not facilitate colonization of the intestine by *E. coli* [288]. In suckling pigs, infections with rotavirus and *Isospora* resulted in more severe lesions and symptoms than infections with coccidia alone [289, 290]. Steel and Torres-Medina also observed a direct effect of low environmental temperature on mortality after rotavirus infection in newborn piglets. Those chilled at 4°C for 4 hours, followed by transfer to a room kept at 26°C showed high mortality rates (70-90%) after inoculation with a human rotavirus strain, whereas no mortality was seen in piglets kept at an environmental temperature of 35°C [291]. Pigs fed a diet which contained lower protein-energy content showed a longer duration of diarrhea in comparison to piglets fed a high protein-energy diet. The first group also showed reduced recovery of the intestinal tissue. However, a control group including non-infected malnourished piglets was not included, so this could have been an effect of the diet itself [292].

Katsuda et al, demonstrated that 22.2% of diarrheic samples from suckling pigs contained more than one pathogen. Co-infections between rotavirus and *E. coli* (6.5%), and between rotavirus and coccidiosis (7.2%) were most often detected. In weaned pigs, co-infections

were detected in 47.7% of the diarrheic samples, and those containing both rotavirus and *E. coli* were most dominant (19.0%) [293]. In Cuban pigs, 25% of enteropathogen positive samples from suckling and weaned pigs contained mixed infections [282]. Also in a recent study from Marthaler and colleagues, it was shown that fecal samples from diarrheic piglets can contain multiple rotavirus species (A, B and/or C) [62]. These studies demonstrate that co-infections between different pathogens may play an important role in the pathogenesis of piglet diarrhea. Further research is needed to investigate this.

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Chapter II

Aims

Diarrhea is one of the most important health problems affecting young pigs, and can be caused by non-infectious as well as infectious factor, including infections with viruses, bacteria and parasites. Group A rotaviruses (RVAs) have been recognized as primary enteric pathogens soon after their discovery. The role of non-RVA strains in the pathogenesis of piglet diarrhea is currently being explored. Despite the reported pathogenicity of pig rotaviruses in the past, their importance in the Belgian pig population nowadays is unreported, and is likely being underestimated. In contrast to pigs, RVA infections are recognized as the major cause of diarrhea in children under 5 years of age. Each year approximately 450.000 children die of severe gastroenteritis caused by RVA infections worldwide, the majority in underdeveloped regions. This has led to the development of oral attenuated vaccines, which are used in national immunization programs for children in many countries. Effective strategies to prevent rotavirus enteritis in pigs are not available in Europe, but prophylactic antimicrobial drugs are very often administered in an attempt to control diarrheic problems in young piglets. This facilitates the selection for antibiotic resistance and is disastrous for pig and human health in the long run. Moreover, pig rotaviruses are capable of crossing to different host species, and the existence of an evolutionary relationship between pig and human rotaviruses has been suggested. Therefore, controlling rotavirus infections in the pig population may reduce the risks for interspecies jumps to humans. Unfortunately, the limited knowledge on pig rotaviruses is hampering our understanding of the risk for their efficiency of spreading in the human population, whether or not preceded by gene reassortment.

A better understanding of the importance of pig rotaviruses in the etiology of piglet diarrhea will allow us to develop specific strategies to diagnose and control these infections. Therefore, the first specific aim of this thesis was to improve the diagnosis of pig RVA infections by the development of a new RT-qPCR assay. In this study, its performance was validated against other frequently applied diagnostic methods, using a collection of diarrheic and non-diarrheic fecal samples, mainly collected from piglets after weaning. The occurrence of co-infections between RVA and other enteric pathogens was investigated as well. RVA isolates were genotyped in order to obtain better insights in strain diversity among Belgian pigs (**Chapter 3.1**). Having shed more light on RVA infections in Belgian pigs, mainly from the weaning age group, a closer look was taken on the importance of RVA and RVC infections in diarrheic suckling pigs less than 2 week olds. Here, circulating strains of both species were also genotyped, as this will aid future vaccine formulation. It was also investigated if infections with *E. coli*, *Cl. perfringens* and *coccidiosis* were forthcoming, and

it was aimed to formulate advices for diagnostic investigations in routine veterinary practice (**Chapter 3.2**). Next, the shedding of RVAs and *Escherichia coli* was followed in pigs after weaning on a large closed Belgian pig farm, by means of longitudinal pilot study (**Chapter 3.3**).

Furthermore, a better understanding of the evolutionary relationship between pig and human rotaviruses will facilitate the risk assessment for dissemination of pig rotaviruses in the human population, and *vice versa*. Moreover, it will also facilitate the recognition of interspecies transmission events occurring between pigs and humans, in both directions.

Having genotyped the genes encoding the outer capsid proteins (VP7 and VP4) of pig RVAs and RVCs in Chapter 3, the complete genomes of 6 recent and 1 ancient Belgian pig RVA strains were analyzed using Sanger sequencing, and phylogenetically compared to all worldwide available genomes of pig RVAs, and human RVAs (**Chapter 4.1**). Finally, the fecal virome of a non-diarrheic weaned piglet was investigated using *next generation sequencing*, to obtain the complete genomes of an RVA and RVC strain present in this fecal sample. Furthermore, this study predominantly focused on investigating the evolutionary relationship between this pig RVC strain and RVC strains from other host species. Finally the value of fecal virome analysis as a promising veterinary diagnostic tool is discussed (**Chapter 4.2**).

Chapter III

Rotavirus infections in Belgian suckling and weaned piglets

3.1. Porcine group A rotaviruses with heterogeneous VP7/VP4 genotype combinations can be found together with enteric bacteria on Belgian swine farms (2014). Sebastiaan Theuns¹, Lowiese M.B. Desmarets¹, Elisabeth Heylen², Mark Zeller², Annelike Dedeurwaerder¹, Inge D.M. Roukaerts¹, Marc Van Ranst², Jelle Matthijnsens^{2*} and Hans J. Nauwynck^{1*}. *Veterinary Microbiology* **172** (1-2), 23-34

3.2. Presence of pig group A and C rotaviruses in Belgian diarrheic suckling pigs and impact on veterinary diagnostical analyses (2015). Sebastiaan Theuns¹, Philip Vyt³, Lowiese M.B. Desmarets¹, Inge D.M. Roukaerts¹, Elisabeth Heylen², Mark Zeller², Marc Van Ranst², Jelle Matthijnsens² and Hans J. Nauwynck¹. Manuscript in preparation

3.3. Successive subclinical group A rotavirus infections in pigs after weaning on a closed Belgian pig farm (2015). Sebastiaan Theuns¹, Lowiese M.B. Desmarets¹, Inge D.M. Roukaerts¹, Isaura Christiaens¹, Elisabeth Heylen², Mark Zeller², Marc Van Ranst², Jelle Matthijnsens² and Hans J. Nauwynck¹. Manuscript in preparation

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Chapter 3.1.

Porcine group A rotaviruses with heterogenous VP7/VP4 genotype combinations can be found together with enteric bacteria on Belgian swine farms

Summary

Group A rotaviruses (RVA) are an important cause of diarrhea in young piglets, resulting in significant economic losses. However, the role of RVA in the etiology of piglet diarrhea on Belgian swine farms was previously unreported. In the present study, different techniques, including fast antigen detection tests, virus isolation, RT-PCR and RT-qPCR have been applied for detection of RVA in diarrheic (n=28) and asymptomatic (n=6) fecal samples collected on Belgian pig farms. RT-qPCR was shown to be most sensitive. Routine bacteriological analysis of the fecal samples showed that most diarrheic RVA positive samples were also coinfecting with one or more bacterial species, such as *Escherichia coli*, *C. perfringens*, *Salmonella spp.* and/or *Brachyspira spp.* A large intra-genotypic diversity was also apparent for genes encoding outer capsid proteins VP7 and VP4. In conclusion, results of the present study help us better understand the role of RVA in the pathogenesis of piglet diarrhea, and provide better insights into the genetic diversity present among circulating porcine group A rotaviruses.

3.1.1. Introduction

Diarrhea is one of the most important causes of mortality and growth retardation in young piglets and can be evoked by viruses, bacteria and parasites. Among these pathogens, rotavirus, transmissible gastroenteritis virus, porcine epidemic diarrhea virus, *Escherichia coli*, *Salmonella*, *Clostridium perfringens* and *Isospora suis* are considered most important. In general, porcine rotaviruses are an important cause of diarrhea during two critical time points in the pig's life. First, it is often involved in enteritis during the nursery period, mostly in piglets farrowed by gilts with a poor lactogenic immunity. Second, rotavirus also causes diarrhea shortly after weaning due to an abrupt disappearance of lactogenic immunity and the presence of a high infection pressure in the weaner shed [1, 2]. Coinfections between viruses, bacteria and parasites have been detected frequently in diarrheic piglets.

Genus rotavirus is divided in five species or groups (A to E) by the ICTV, and two tentative species (F and G). A potential new species (H) includes "new adult diarrhea rotavirus" isolated from a human in 1997, and a rotavirus isolated from a pig [3]. Although rotavirus species A, B and C can be found in feces from pigs, most research has been conducted on rotaviruses from species A (RVA) [4]. The segmented dsRNA genome of RVA encodes 6 structural (VP1-4, VP6 and VP7) and 6 non-structural proteins (NSP1-NSP5/6). A full genome-based classification system for RVA has been established, giving all 11 gene segments a letter, followed by a number representing the genotype [5]. Genes encoding the outer capsid proteins VP7 (G-genotypes) and VP4 (P-genotypes) are of utmost importance in this classification system, since these proteins induce neutralizing antibodies. To date, 27 G-genotypes and 37 P-genotypes have been identified in many species [6, 7]. So far, 12 G-genotypes (G1 to G6, G8 to G12 and G26) and 13 P-genotypes (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], P[34]) have been disclosed from diarrheic and non-diarrheic pigs [4, 8]. Geographical differences in strain predominance exist [9], with G9P[13] for example being the most prevalent genotype combination detected in Ohio, United States [10]. Moreover, genetically heterogeneous RVA strains can circulate in different units of the same pig farm, with a seasonally changing predominance of certain G/P genotype combinations [11, 12].

Swine production is one of the major agriculture activities in Belgium, with a herd size of approximately 6 million swine, with 1.6 million pigs belonging to the <20 kg weight category. In contrast to other countries, little information is available about the role of porcine RVA infections in the etiology of piglet diarrhea on Belgian pig farms. The use of fast antigen detection tests for diagnosis of RVA infections in samples collected in late phase

of RVA infection, has been thought to hamper a sensitive diagnosis of porcine RVA infections in Belgian veterinary practices, resulting in an underdiagnosis of porcine RVA in the etiology of piglet diarrhea. At present, nothing is known about the genetic constellation of Belgian porcine RVA strains. An exception is the completely characterized genome of the porcine-like human G9P[6] RVA strain, RVA/Human-wt/BEL/BE2001/2009/G9P[6], isolated from a diarrheic child in 2009 in Belgium [13].

Increased surveillance of RVA infections in pigs will help us better understand their etiological role in the pathogenesis of piglet diarrhea. Therefore, different diagnostic approaches were used for detection of RVA in fecal samples, including fast antigen detection tests, virus isolation, RT-PCR and RT-qPCR. RVA strains found in positive samples were further genetically characterized for genes encoding VP7 and VP4. This characterization may help elucidating the evolutionary relationship between porcine and human RVA, and facilitating interpretation of rare interspecies transmission events detected during human RVA surveillance.

3.1.2. Materials and methods

3.1.2.1. Collection and processing of fecal samples

A total of 34 fecal samples were collected on 21 different farms in Belgium. From these, 28 samples were obtained from diarrheic piglets, whereas 6 samples were from non-diarrheic piglets. Twenty samples were obtained from weaning piglets and 5 from suckling piglets. The age of the pigs was not specified for 9 samples. Feces were collected between 2011 and 2012 by private laboratories for diagnosis of enteric pathogens involved in piglet diarrhea. In these laboratories, the presence of RVA antigens was first evaluated using a fast antigen detection test: Prospect[®] Rotavirus Microplate Assay (ThermoScientific, Middletown, USA) or Rota-Strip (Coris Bioconcept, Gembloux, Belgium). Bacteriological examination was also performed in these laboratories according to routine diagnostic procedures. Briefly, *Escherichia coli*, *Clostridium perfringens* and *Salmonella spp.* were detected by isolation, while *Brachyspira spp.* was detected by use of PCR. Presence of *Cryptosporidium parvum* was demonstrated by immunofluorescence. Hereafter, samples were sent to our laboratory, and 20% w/v feces solutions were prepared in phosphate buffered saline (PBS) containing 1000 U/ml penicillin (Continental Pharma, Puurs, Belgium), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/ml gentamicin (Gibco BRL, Merelbeke, Belgium) and 0.01% v/v Fungizone (Bristol-Myers Squibb, Braine l'Alleud, Belgium). Solutions were centrifuged at 793 x g for 3 min, and supernatant was collected for RNA extraction using the

Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. An extraction control with PBS was included to assess contamination during the extraction procedure. Fecal solutions and RNA were stored at -70°C.

3.1.2.2. RT-qPCR assay for porcine RVA gene segment 11

3.1.2.2.1. Primer design and in silico validation

RT-qPCR primers were designed for detection of porcine RVA in fecal samples. The nucleotide sequence of the conserved gene segment 11, coding for NSP5 of strain RVA/Pig-tc/USA/Gottfried/1983/G4P[6] (Genbank GU199491) was used as template during the search for qPCR-suitable primers with the aid of Primerquest (Integrated DNA Technologies, Iowa, USA). Primer-specificity was assessed *in silico*, by use of the Basic Local Alignment Search Tool (BLAST) in public databases, and by aligning these primers to NSP5 encoding genes of contemporary pig strains from Italy, Spain, South Korea, Thailand, India, Russia and Belgium [14]. It was not aimed to target all eleven currently recognized NSP5 genotypes with these primers. In addition, the risk of primer-dimer formation, and the presence of hairpins at the annealing site was analyzed using OligoAnalyzer 3.1 and UNAFold (Integrated DNA Technologies) with a correction for ion concentrations set at 50 mM for Na⁺ and 3 mM for Mg²⁺.

3.1.2.2.2. Preparation of dsRNA standards for absolute quantification

A synthetic fragment of dsRNA with a size of 331 bp was generated *in vitro* to be used as a standard for absolute quantification. Therefore, RNA of the porcine RVA strain RVA/Pig-tc/BEL/RV277/1977/G1P[7] was extracted using the Viral RNA Mini Kit. Strain RV277 was isolated in our laboratory from a pooled sample of watery feces from 3 diarrheic piglets in 1977. Reverse transcription was conducted with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Merelbeke, Belgium), using primerset cDNA_fw/cDNA_rv. Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, USA) was used to incorporate a T7-promotor sequence at the 5' end of both RNA strands using the primerset T7_fw/T7_rv (Table 1), generating a 365 bp fragment. Afterwards, the product was analyzed by electrophoresis on a 1.8% agarose gel and purified with the Nucleospin Gel and PCR-Clean up kit (Macherey-Nagel, Düren, Germany). *In vitro* transcription of dsRNA was performed using the T7 RNA Polymerase-PlusTM Enzyme Mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA), following manufacturer's instructions. Template DNA was removed by treatment of the reaction

mixture with 2U DNase I (Sigma Aldrich, St. Louis, USA). Finally, the *in vitro* generated RNA was purified using the RNeasy Mini Kit (Qiagen). Electrophoresis on 1.8% agarose gel was performed to assess the size of the transcript (331 bp of dsRNA). Synthetic RNA standards were stored in single-use aliquots of 20 µl volume at -70°C. Ten microliters of RNA was used for preparation of a standard curve, and another 6 microliters was used to determine the RNA concentration by means of spectrophotometry at 260 nm with the NanoDrop 2000 system (Thermo Scientific, city, country). The number of dsRNA copies per microliter was then calculated using the ENDMEMO online web tool.

Table 1. Primers used for RT-qPCR and RT-PCR.

Application	Primer name	Gene	Sequence	Reference
RT-qPCR	cDNA_fw	NSP5	5'-TTT AAA AGC GCT ACA GTG ATG-3'	this study
	cDNA_rv	NSP5	5'-CGT TGC TTG AAG GTC GTG ATT-3'	this study
	T7_fw	NSP5	5'- <i>TAA TAC GAC TCA CTA TAG GGT</i> TTA AAA GCG CTA CAG TGA TG-3'	this study
	T7_rv	NSP5	5'- <i>TAA TAC GAC TCA CTA TAG GGC</i> GTT GCT TGA AGG TCG TGA T-3'	this study
	qPCR_fw	NSP5	5'-ATC TAT TGG TAG GAG TGA ACA GTA-3'	this study
	qPCR_rv	NSP5	5'-GTG GGT CGT TTG AAG CAG AA-3'	this study
RT-PCR	Beg9	VP7	5'-GGC TTT AAA AGA GAG AAT TTC CGT CTG G-3'	[15]
	End9	VP7	5'-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3'	[15]
	Gen_VP7F	VP7	5'-ATG TAT GGT ATT GAA TAT ACC AC-3'	[16]
	Gen_VP7R	VP7	5'-AAC TTG CCA CCA TTT TTT CC-3'	[16]
	VP4_1-17F	VP4	5'-GGC TAT AAA ATG GCT TCG C-3'	[17]
	VP4_F	VP4	5'-TAT GCT CCA GTN AAT TGG-3'	[16]
	Con2	VP4	5'-ATT TCG GAC CAT TTA TAA CC-3'	[17]
	VP4R_DEG	VP4	5'-TCY CTR TTR TAT TGC ATY TCY TTC C-3'	this study
	Gen_VP6F	VP6	5'-GGC TTT WAA ACG AAG TCT TC -3	[18]
	Gen_VP6R	VP6	5'-GGT CAC ATC CTC TCA CT -3'	[18]

Legend: T7 promotor sequence in italic

3.1.2.2.3. RT-qPCR reaction conditions and interpretation of results

In order to generate a standard curve, standard RNA was denatured at 95°C for 2 min and immediately chilled on ice. The concentration of RNA was measured as described above, and further serially log₁₀ diluted in nuclease free water. RNA from test samples or H₂O as negative control, was also denatured and chilled on ice. RT-qPCR reaction mixtures (20 µl) consisted of 10 µl Precision OneStep™ qRT-PCR Mastermix with SYBR® Green and ROX (PrimerDesign, Southampton, United Kingdom), 400 nM (0.8 µl/reaction) of qPCR_fw primer, 50 nM (0.1 µl/reaction) of qPCR_rv primer, 6.1 µl nuclease free water (Gibco, Life Technologies) and 3 µl of RNA template or H₂O. Primers were synthesized by Integrated DNA Technologies and purified by standard desalting. Reaction mixtures were loaded in

MicroAmp[®] Optical 96-Well Reaction plates (Applied Biosystems), sealed with MicroAmp[®] Optical Adhesive Films (Applied Biosystems), and experiments were performed in a StepOnePlus apparatus (Applied Biosystems) as follows: 55°C for 10 min, 95°C for 8 min, and 40 cycles at 95°C for 10 sec and 60°C for 60 sec. Fluorescence was measured at the end of each amplification cycle. Results were analyzed using the StepOnePlus Software version 2.2. The baseline was set automatically, and the threshold was placed manually in the exponential phase of the amplification reaction. Melt curve analysis and agarose gel electrophoresis was performed to assess specificity of the reactions. Amplification efficiency was determined by running a standard curve over a linear dynamic range (LDR) from 7 log₁₀ copies/reaction to 1 log₁₀ copies/reaction in log₁₀ dilution steps. Each dilution point of the standard curve was analyzed in triplicate and also 3 nontemplate control reactions (water) were included in each experiment. RNA extracts from fecal samples were analyzed in duplicate reactions. Quantification of the viral titer (copies/g feces) was possible if the C_q values of both reactions fell within the LDR of the RT-qPCR assay. Samples were considered positive, but not quantifiable (+<LOQ), if the C_q value of the reactions fell outside the LDR, but melting curve analysis revealed specific amplification.

3.1.2.3. Infectivity titration of porcine RVA in MA104 cells

Direct titration of porcine RVA in fecal samples was performed in MA104 cells, followed by an immunoperoxidase staining. Therefore, MA104 cells were seeded in 96-well plates and grown in culture medium for 2 days at 37°C and 5% CO₂. Before inoculation, 10-fold dilution series (10⁻¹-10⁻⁸) of the fecal suspensions in serum free culture medium were pretreated with 5 µg/ml trypsin (Sigma Aldrich) for 30 min at 37°C, and cells were washed three times with PBS containing antibiotics. Next, for each dilution, 50 µl of inoculum was brought in 4 separate wells of MA104 cells for 1h at 37°C and 5% CO₂. Hereafter, 100 µl of serum free culture medium was added, and cells were incubated another 5 days at 37°C and 5% CO₂. Since cytopathogenic effect of porcine RVA in MA104 cells is not apparent, RVA infected cells were visualized using immunoperoxidase staining. Plates were brought to room temperature, cells washed with PBS, dried at 37°C, and frozen at -20°C. Next, cells were fixed with paraformaldehyde 4% for 10 min, followed by permeabilization for 5 min with methanol containing H₂O₂. A sheep polyclonal anti-rotavirus antibody (Abcam, Cambridge, United Kingdom) was added for 1 hour at 37°C in a solution containing 10% rabbit serum and 0.1% Tween 80 (Sigma Aldrich). A secondary rabbit anti-sheep IgG

antibody labeled with peroxidase (Dako, Heverlee, Belgium) was subsequently added for an hour at 37°C. Substrate consisted of sodium acetate buffer, amino-ethyl carbazole and H₂O₂, and was brought on the cells and discarded after 10 min at room temperature, followed by the overlay of cells with sodium acetate buffer. Positive wells, containing at least one infected cell, were determined by light microscopy and the tissue culture infective dose (TCID₅₀) was calculated using the formula of Reed and Muench.

3.1.2.4. Serial passaging of porcine RVA in MA104 and IPEC-J2 cells

Serial passaging in MA104 and IPEC-J2 cell lines was another strategy used for isolation of porcine RVA in cell culture. Both cell types were grown in 125 mm roller tubes (Corning) on a roller apparatus. After one week, cells were washed 3 times with PBS containing 100 U/ml penicillin and 0.1 mg/ml streptomycin before inoculation with 200 µl of fecal solutions, pretreated with 5 µg/ml of trypsin during 30 min at 37°C. A positive (first passage of RV277 RVA in MA104 cells) and negative control (FCS-free growth medium), was included, which had been subjected to the same treatment as the field samples during the entire process of virus isolation. After 1 hour of incubation at 37°C on a roller apparatus, fecal solutions were removed from the cells. To reduce cytotoxic effects, cells were immediately washed 2 times and then further incubated in 1 ml FCS-free medium containing 1 µg/ml trypsin for 4-5 days on a roller apparatus. Cells and supernatant were subjected to two freeze-thaw cycles before a second passage was performed in one week old cells. Inoculation was performed the same way as described above, but the inoculum was not removed after one hour of incubation. Instead, 800 µl of serum free medium was added and cells were incubated for 4-5 days. Next, a third passage was performed in cells grown on cell culture inserts in 24-well plates (Nunc). The inoculum of the third passage was prepared in the same manner as for the second passage. After one hour of incubation at 37°C with the cells, inoculum was discarded and cells were washed three times and incubated further for 24 hours in serum free medium containing 1 µg/ml trypsin. After 24 hours, cells of the third passage were fixed with methanol at -20°C. Immunofluorescence staining for RVA infected cells was performed to confirm infection after a third passage. A blocking step with 10% inactivated rabbit serum was performed for 30 min, followed by incubation of the cells with a polyclonal sheep anti-rotavirus antibody for 1 hour. After washing with PBS, a secondary rabbit anti-sheep IgG antibody labeled with FITC (Abcam) was added for another hour at 37°C. Nuclei were stained with Hoechst, and inserts were mounted on microscope slides with glycerin-diazabicyclo-octane (DABCO) (Janssen Chimica, Beerse, Belgium). The

presence of RVA infected cells was analyzed using a fluorescence microscope (Leica Microsystems, Heidelberg, Germany).

3.1.2.5. RT-PCR for porcine RVA genes of VP7 and VP4

RNA was denatured at 95°C for 2 min, followed by RT-PCR using the Qiagen OneStep RT-PCR kit (Qiagen). Reaction mixtures consisted of 5 µl RNA, 5 µl of 5x QIAGEN OneStep RT-PCR Buffer, 1 µl of dNTP Mix, 1.5 µl of forward and reverse primer (8.3 µM), 1 µl of QIAGEN OneStep RT-PCR enzyme mix, and nuclease free water in a total volume of 25 µl per reaction. Reverse transcription was executed at 50°C for 30 min, followed by *Taq* polymerase activation at 95°C for 15 min. Thirty five cycles of amplification were performed at 94°C for 30 sec, 45°C for 30 sec and 72°C during 1.5, 3, or 2 min for VP7, VP4, VP6 respectively. A final extension was performed at 72°C during 10 min. A list of primers used for amplification of fragments of the VP7, VP4 and VP6 genes is provided in Table 1. RT-PCR analysis of the samples was first performed for the genes of VP7 and VP4, using primersets Beg9/End9 and VP4_1-17F/Con2, respectively. Samples negative for both PCR reactions were tested using primer set VP6F/VP6R. Samples testing positive by use of the VP6-specific primerset were then further examined using primersets VP7-F/VP7-R and VP4_1-17F/VP4R-DEG. Next, RT-PCR positive samples were used for sequencing.

Four additional fecal samples (12R053, 13R054, 13R055, 13R068), and one historic Belgian porcine RVA strain (RV277), were also analyzed by RT-PCR and sequenced. They are not included in Table 3, since virus isolation was not performed for these samples.

3.1.2.6. Sequence analysis and genotype determination for VP7 and VP4

Five microliters of RT-PCR product was treated with 1µl of USB ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, California, USA) and sequenced with an ABI Prism BigDye terminator cycle sequencing reaction kit (ABI Prism 3130xl) using forward or reverse primer. Sequence analysis was performed using 4Peaks (Mekentosj, Amsterdam, The Netherlands), and genotypes for VP7 and VP4 were determined using BLAST and the online webtool RotaC^{2.0}. Multiple sequence alignments were performed using the ClustalW plug-in and manually edited in MEGA 5. The software was also used for the construction of neighbour-joining phylogenetic trees, using the Kimura-2 model for calculation of distances at the nucleotide level with bootstrap-analysis set at 1000 replicates.

3.1.2.7. Genbank accession numbers

Genbank accession numbers of the nucleotide sequences of the genes of VP7 and VP4 are provided in Table 2.

Table 2. Genbank accession numbers of VP7 and VP4 gene sequences.

Strain	VP7	VP4
RVA/Pig-wt/BEL/12R001/2012/G9P[7]	KF614038	KF614071
RVA/Pig-wt/BEL/12R002/2012/G5P[7]	KF614039	KF614072
RVA/Pig-wt/BEL/12R003/2012/G9P[23]	KF614040	na
RVA/Pig-wt/BEL/12R004/2012/G9P[7]	KF614041	KF614073
RVA/Pig-wt/BEL/12R005/2012/G4P[7]	KF614042	KF614074
RVA/Pig-wt/BEL/12R006/2012/G3P[6]	KF614043	KF614075
RVA/Pig-wt/BEL/12R008/2012/G5P[6]	KF614044	KF614076
RVA/Pig-wt/BEL/12R010/2012/G4P[13]	KF614045	KF614077
RVA/Pig-wt/BEL/12R013/2012/G5P[13]	na	KF614078
RVA/Pig-wt/BEL/12R014/2012/G5P[x]	KF614046	na
RVA/Pig-wt/BEL/12R016/2012/G4P[x]	KF614047	na
RVA/Pig-wt/BEL/12R017/2012/G5P[7]	KF614048	na
RVA/Pig-wt/BEL/12R018/2012/G5P[7]	KF614049	KF614079
RVA/Pig-wt/BEL/12R020/2012/G4P[x]	KF614050	na
RVA/Pig-wt/BEL/12R021/2012/G11P[27]	KF614051	KF614080
RVA/Pig-wt/BEL/12R022/2012/G2P[27]	KF614052	KF614081
RVA/Pig-wt/BEL/12R032/2012/G9P[13]	KF614053	KF614082
RVA/Pig-wt/BEL/12R041/2012/G9P[13]	KF614054	KF614083
RVA/Pig-wt/BEL/12R042/2012/G4P[6]	KF614055	KF614084
RVA/Pig-wt/BEL/12R043/2012/G9P[13]	KF614056	KF614085
RVA/Pig-wt/BEL/12R045/2012/G4P[6]	KF614057	KF614086
RVA/Pig-wt/BEL/12R046/2012/G9P[23]	KF614058	KF614087
RVA/Pig-wt/BEL/12R047/2012/G9P[23]	KF614059	KF614088
RVA/Pig-wt/BEL/12R048/2012/G5P[7]	KF614060	KF614089
RVA/Pig-wt/BEL/12R049/2012/G5P[7]	KF614061	KF614090
RVA/Pig-wt/BEL/12R050/2012/G5P[7]	KF614062	KF614091
RVA/Pig-wt/BEL/12R052/2012/G4P[6]	KF614065	KF614092
RVA/Pig-wt/BEL/12R053/2012/G4P[6]	KF614064	KF614093
RVA/Pig-wt/BEL/13R054/2013/G5P[7]	na	KF614094
RVA/Pig-wt/BEL/13R055/2013/G3P[7]	KF614066	KF614095
RVA/Pig-wt/BEL/13R068/2013/G9P[7]	KF614067	KF614096
RVA/Pig-tc/BEL/RV277/1977/G1P[7]	KF614068	KF614070

na: no/short sequence was available and not suitable for uploading into Genbank

3.1.3. Results

3.1.3.1. Validation of new RT-qPCR assay for the detection of porcine RVA gene segment eleven

RT-qPCR primers, named qPCR_fw and qPCR_rv (Table 1), were designed against conserved regions of the porcine RVA gene segment 11. *In silico* evaluation of the primers showed a minimal tendency for dimer formation, and the 116 bp target region was free from secondary structures at the primer annealing sites. The amplicon spanned a region from nucleotide positions 117 and 232, based on the sequence from the Gottfried strain. BLAST search for the forward and reverse primers showed that these primers recognized a large variety of contemporary pig RVA strains. The forward primers showed a 100% nucleotide match with NSP5 genes of pig strains from Italy, Spain, Thailand, South Korea, Belgium, India, Russia and the United States. The reverse primer showed a 100% nucleotide match with most of the pig RVA strains. However, 1 nucleotide mismatch (C/T) located at the 5'-end was present in some of the strains analyzed. RT-qPCR efficiencies were derived from the slopes of the standard curves (Figure 1a and 1b) and were calculated to be $98.4 \pm 3.9\%$. Melt curve analysis showed amplification of a specific product with a melting peak at 79.8°C (Figure 1c), which was confirmed by agarose gel electrophoresis analysis. Gel analysis of the non-template control did not show any amplification.

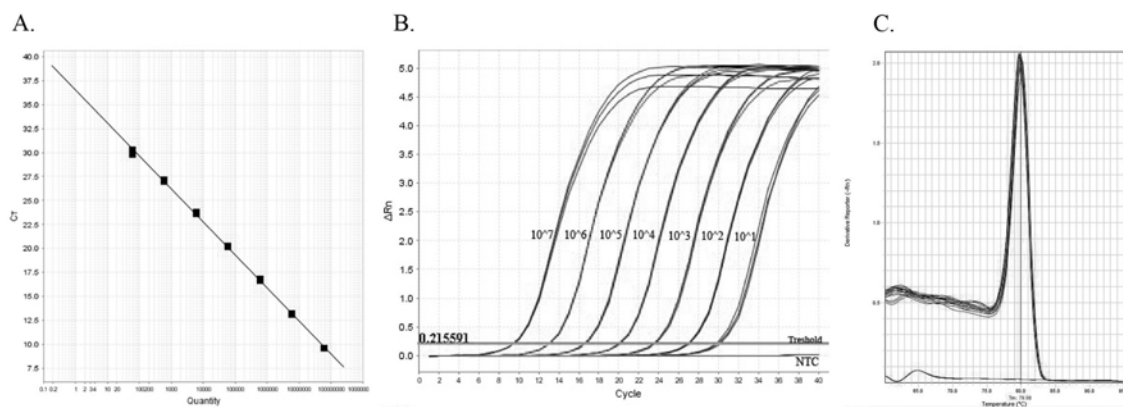


Fig. 1. Standard curve (A), amplification plot (B) and melt curve analysis (C) over a linear dynamic range of 7 log₁₀ to 1 log₁₀ copies/reaction.

3.1.3.2. Detection of porcine RVA in fecal samples using different diagnostic techniques

Different diagnostic techniques were applied to detect RVA in 34 fecal samples collected from diarrheic (n=28, Table 3), and asymptomatic piglets (n=6, Table 4). Using a fast antigen detection test (ELISA or immunochromatographic strip), RVA antigens were detected in 16 diarrheic cases. All these RVA antigen positive samples tested positive by RT-qPCR and RT-PCR, and contained between $10^{7.73}$ and $10^{10.89}$ viral RNA copies/g feces. Virus isolation by direct infectivity titration in MA104 cells was successful for only 13 RVA antigen positive samples. Infective virus titers in these samples ranged from $10^{4.00}$ to $10^{7.67}$ TCID₅₀/g feces. In addition, virus isolation by serial passaging in MA104 and IPEC-J2 cells was performed on RVA antigen positive samples, and using this approach, 13 samples were positive. Nevertheless, some of the samples were successfully passaged, but could not be directly titrated or vice versa.

Further examination of the RVA antigen negative samples from diarrheic and asymptomatic piglets by RT-qPCR and RT-PCR revealed another 13 and 10 positive samples, respectively. Eight cases belonged to the diarrheic group. Five cases were found in the asymptomatic group. Viral load in these RVA antigen negative samples was lower, ranging between $10^{4.81}$ and $10^{7.45}$ copies/g in feces of diarrheic animals, and between $10^{4.79}$ and $10^{5.65}$ copies/g feces from asymptomatic animals. For 2 samples, Cq values were outside the limit of quantification. In contrast to the RVA antigen positive samples, it was not possible to directly titrate porcine strains from RVA antigen negative samples in MA104 cells. Nevertheless, serial passaging in MA104 cells was successful for three RVA antigen negative samples, with 2 of these samples belonging to the non-diarrheic group.

3.1.3.3. Occurrence of bacterial co-infections in RVA positive samples

For 12 of the 16 RVA antigen positive diarrheic samples, the private laboratories provided bacteriological results (Table 3). Non-hemolytic *E. coli* were present in 75% (n=9) of these samples, whereas hemolytic *E. coli* were detected in only 33% (n=4) of the antigen positive samples. In two samples, both non-hemolytic and hemolytic *E. coli* were present together with porcine RVA. Furthermore, *Clostridium perfringens* were found in 50% (n=6) of the RVA antigen positive samples, while *Salmonella* were only present in 17% (n=2) of the RVA antigen positive samples.

In 8 of the RVA antigen negative diarrheic samples in which RVA was detected by RT-qPCR, dual infections were demonstrated with non-hemolytic *E. coli* (50%, n=4), hemolytic

E. coli (75%, n=6), *Salmonella* (38%, n=3), *Brachyspira* (13%, n=1) or *Cryptosporidium* (13%, n=1). In three samples (38%), a triple infection with RVA, hemolytic *E. coli* and *Salmonella* was found.

Table 3. Analysis of fecal samples from diarrheic pigs.

RVA fast diagnostic test	Farm	Sample	RT-PCR VP7/VP4	RT-qPCR titer ¹	Infective titer ²	Serial passage of RVA		E. Coli		Cl.	Sa.	Others
						in MA104	in IPEC-J2	NH	H			
ELISA +	A	12R005	G4P[7]	9.79	7.33	+	+	+	-	+	-	-
STRIP +	B	12R017	G5P[7]	10.40	7.67	+	+	na	na	na	na	na
STRIP +	C	12R018	G5P[7]	9.62	6.33	+	+	na	na	na	na	na
STRIP +	D	12R048	G5P[7]	10.47	7.67	+	+	+	-	+	-	-
STRIP +		12R049	G5P[7]	10.89	7.00	+	+	+	-	+	-	-
STRIP +		12R050	G5P[7]	10.29	7.67	+	+	+	-	+	-	-
ELISA +	E	12R002	G5P[7]	7.84	4.50	+	+	-	+	-	-	-
ELISA +	F	12R001	G9P[7]	10.18	5.99	+	+	+	-	-	-	-
ELISA +		12R004	G9P[7]	9.51	5.50	+	+	+	+	-	-	-
STRIP +	G	12R046	G9P[23]	10.04	4.67	+	+	na	na	na	na	na
STRIP +		12R047	G9P[23]	10.61	5.50	+	+	na	na	na	na	na
ELISA +	H	12R003	G9P[23]	7.95	-	+	+	+	+	+	-	-
ELISA +	I	12R006	G3P[6]	9.74	-	+	+	-	-	-	-	-
ELISA +	J	12R008	G5P[6]	9.77	5.50	-	-	+	-	+	-	-
ELISA +	K	12R042	G4P[6]	7.73	-	-	-	-	+	-	+	-
ELISA -		12R045	G4P[6]	6.91	-	-	-	-	+	-	+	-
ELISA -		12R020	G4P[x]	5.20	-	-	-	-	+	-	-	-
ELISA +	L	12R041	G9P[13]	10.21	4.00	-	-	+	-	-	+	-
ELISA -		12R032	G9P[13]	5.10	-	-	-	+	+	-	+	-
ELISA -	M	12R043	G9P[13]	4.81	-	-	-	+	+	-	-	Bra
ELISA -		12R025	-	-	-	-	-	+	+	-	-	Bra
ELISA -	N	12R013	G5P[13]	4.87	-	+	-	+	-	-	-	-
ELISA -		12R015	-	-	-	-	-	+	-	-	-	-
ELISA -	O	12R014	G5P[x]	7.45	-	-	-	-	+	-	+	-
ELISA -		12R016	G4P[x]	6.89	-	-	-	-	+	-	-	Cryp.
ELISA -	P	12R024	-	-	-	-	-	+	+	-	-	Bra
ELISA -	Q	12R027	-	+(<LOQ)	-	-	-	+	-	-	-	-
ELISA -	R	12R028	-	-	-	-	-	+	-	-	+	-

¹ log₁₀ copy number per gram feces; ² log₁₀ TCID₅₀ per gram feces in MA104 cells; <LOQ: below limit of quantification, NH is non-hemolytic; H is hemolytic; Cl is *Clostridium perfringens*; Sa is *Salmonella* spp.; Bra is *Brachyspira* spp. and Cryp is *Cryptosporidium* are mentioned in the 'Others' column.

Bacteriological examination of 6 fecal samples collected from asymptomatic pigs, showed the presence of non-hemolytic *E. coli* in all samples, while RVA was detected by RT-qPCR in 5 out of 6 samples. One of these samples (12R021) contained RVA, non-hemolytic *E. coli* and *Brachyspira* (Table 4).

Table 4. Analysis of fecal samples from non-diarrheic pigs.

RVA fast diagnostic test	Farm	Sample	RT-PCR VP7/VP4	RT-qPCR titer ¹	Infective titer ²	Serial Passage of RVA		E. Coli		Cl	Sa	Others
						in MA104	in IPEC-J2	NH	H			
ELISA -	S	12R021	G11P[27]	4.79	-	-	-	+	-	-	-	Bra
ELISA -	T	12R022	G2P[27]	5.65	-	-	-	+	-	-	-	-
ELISA -	U	12R009	-	+ (<LOQ)	-	+	-	+	-	-	-	-
ELISA -		12R010	G4P[13]	5.13	-	-	-	+	-	-	-	-
ELISA -		12R011	-	4.94	-	+	-	+	-	-	-	-
ELISA -		12R012	-	-	-	-	-	+	-	-	-	-

¹log₁₀ copy number per gram feces; ² log₁₀ TCID₅₀ per gram feces in MA104 cells; <LOQ: below limit of quantification, NH is non-hemolytic; H is hemolytic; Cl is *Clostridium perfringens*; Sa is *Salmonella* spp.; Bra is *Brachyspira* spp. is mentioned in the 'Others' column.

3.1.3.4. VP7 and VP4 genotype distribution among RVA positive samples

Screening of 34 fecal samples resulted in 26 positive samples by RT-PCR using a combination of different primer sets for VP7, VP4 and VP6. The VP7/VP4 genotype combinations from the strains in the samples are given in Table 3 and 4. A frequency distribution chart, presenting the G/P genotype combinations found in 26 RT-PCR positive fecal samples is shown in Table 5. Six different G-genotypes (G2, G3, G4, G5, G9, and G11), and five different P-genotypes (P[6], P[7], P[13], P[23], and P[27]), were detected in a total of 12 different G/P genotype combinations. G5 (34.6%) was the most abundant genotype, followed by G9 (30.8%) and G4 (23.1%), whereas P[7] (34.6%) was the predominant P-type, followed by P[13] (19.2%) and P[6] (15.4%). Overall, the G5P[7] combination was most frequently detected (23.1%). It was not possible to determine the P-types of strains 12R014, 12R016, and 12R020 using primersets VP4_1-17F/Con2, VP4_1-17F/VP4RDeg, or VP4_F/VP4RDeg. On farms where more than one sample was collected, only one predominant genotype combination was demonstrated, except for farm O, where both a G4P[x] and a G5P[x] strain were found.

Table 5. Frequency distribution of RVA G/P-genotypes detected in porcine feces.

	G2	G3	G4	G5	G9	G11	Total
P[6]		1 (3.9%)	2 (7.7%)	1 (3.9%)			4 (15.4%)
P[7]			1 (3.9%)	6 (23.1%)	2 (7.7%)		9 (34.6%)
P[13]			1 (3.9%)	1 (3.9%)	3 (11.5%)		5 (19.2%)
P[23]					3 (11.5%)		3 (11.5%)
P[27]	1 (3.9%)					1 (3.9%)	2 (7.7%)
P[x]			2 (7.7%)	1 (3.9%)			3 (11.5%)
Total	1 (3.9%)	1 (3.9%)	6 (23.1%)	9 (34.6%)	8 (30.8%)	1 (3.9%)	26 (100%)

3.1.3.5. Phylogenetic analysis for the VP7 gene of porcine RVA

Neighbour-joining trees, based on phylogenetic analysis of the partial VP7 gene sequences, are presented in Figure 2. Genetic similarities at the nucleotide level are mentioned between brackets hereafter. A historic Belgian RVA isolate, defined as strain RV277 was genotyped as G1P[7]. The VP7 gene of RV277 clustered with the unusual human G1P[6] strain AU19 (lineage VI, 93.8%), but was only distantly related to another old G1 porcine strain SW20/21 (lineage X, 89.3%). The genetic distance between RV277 and the Argentinean G1 (lineage VII) RVA strains collected from pigs (C60 and C95) and a cow (T449) was also large (82.7 to 84.6%). Furthermore, the VP7 gene sequence of RV277 was distantly related to those of contemporary human G1 strains (84.1 to 87.2%). In this study, one porcine RVA strain (12R022) was defined as a G2 strain, and was isolated from the feces of an asymptomatic piglet. This strain clustered with other porcine strains from Spain, Thailand, Ireland, Japan and Canada (83.6 to 93.6%). However, 12R022 was clearly divergent from human G2 RVA strains (73.7 to 75.7%), and from the porcine Canadian G2 strain F8-4 (72.7%). Two Belgian G3 strains, 12R006 (G3P[6]) and 12R055 (G3P[7]), showed high genetic relatedness (98.7%). These strains formed a monophyletic cluster with other porcine and some bovine and human strains with porcine characteristics in the G3 genotype (89.5 to 93.6%). However, the old porcine G3 strain A131 from Venezuela was genetically only distantly related to the Belgian G3 strains (84.5 to 85%). Remarkably, the porcine G3 strains from Belgium were genetically more closely related to human G3 strains (82.7 to 86%), than to equine, feline and canine G3 strains (73.8 to 77.9%). A large genetic heterogeneity was detected in the G4 genotype. The genetic distance between the Belgian porcine G4 strains and the prototype porcine G4 strain Gottfried (lineage III) was large (78.1 to 82.8%). Strains 12R010, 12R016 and 12R042 were assigned to lineage V, which also contains porcine (93.8 to 97.1%) and human strains (93.8 to 95.7%) from Asia. Strain 13R053 clustered together with porcine RVA strain JP40-G8 (89.2%) in a separate genetic branch. In addition, strain

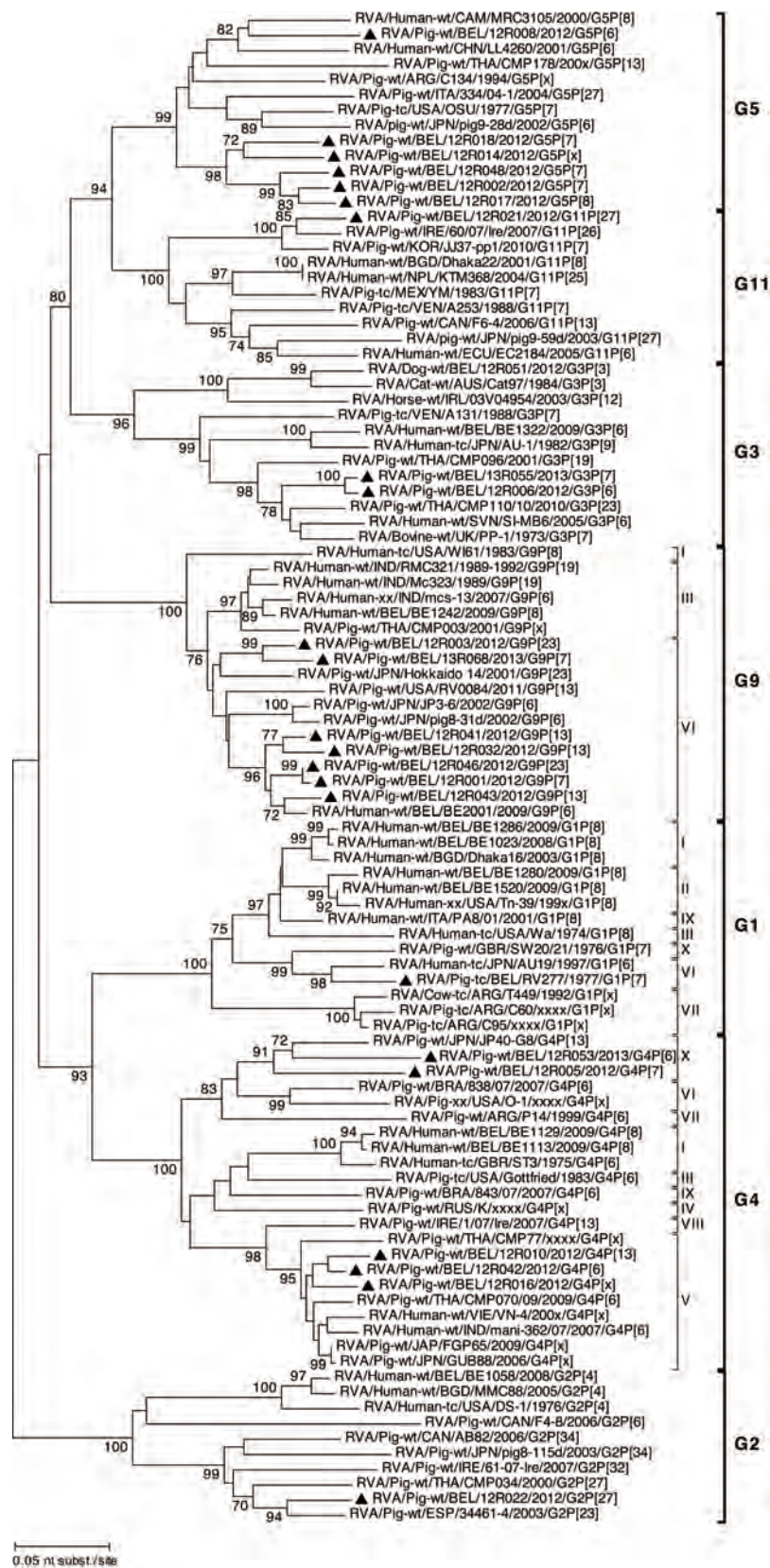


Fig. 2. Phylogenetic tree based on the partial sequences of the VP7 gene. Bootstrap values (1000 replicates) smaller than 70% are not shown. Belgian porcine RVA strains from the present study are marked with a black triangle. Intra-genotypic lineages are indicated by Roman numerals.

12R005 also belonged to a separate branch in the G4 genotype with a large genetic distance from the other Belgian porcine G4 strains (77.8 to 79.1%), except for strain 13R053 (85.9%). Moreover, the Belgian porcine G4 strains were also clearly divergent from contemporary Belgian human G4 strains from lineage I (77.5 to 80.5%). For genotype G5, a major cluster was formed by the Belgian isolates 12R002, 12R014, 12R018, 12R017, and 12R048, but these strains were only distantly related to the porcine reference strain OSU (83 to 84.7%). The nucleotide identity among these 5 Belgian G5 strains was high (87.7 to 96.4%), but one strain (12R008) was clearly genetically divergent from these Belgian isolates (80.2 to 82.8%). This strain, clustered with human G5 strains from China and Cameroon (88 to 88.7%), also rather distantly related to strain OSU (80.4%). Belgian porcine G9 strains were genetically distinct from contemporary human G9 strains belonging to the major lineage III (89.3 to 93.5%). Strains 12R041 and 12R032 were both obtained from pigs on the same farm, but were not completely identical at the nucleotide level (96.6%). A Belgian porcine subcluster was formed in the G9-lineage VI by strains 12R001, 12R032, 12R041, 12R043 and 12R046, which also comprised the Belgian porcine-like human G9P[6] strain BE2001 (94.3 to 96.9%). Remarkably, strains 12R003 and 13R068 were found in a second cluster in this lineage, distinct from the other Belgian porcine cluster (88.2 to 90.3%). Only one G11 strain was detected in the present study, namely 12R021 (G11P[27]), which clustered together with other porcine G11 strains from Ireland and South-Korea (93.8 to 95%). However, a large genetic distance was noticed between this strain and porcine G11 reference strain YM (81.4%). The Belgian porcine G11 strain was only distantly related to human G11 RVA strains detected in Bangladesh, Nepal and Ecuador (81.9 to 82.8%).

3.1.3.6. Phylogenetic analysis for the VP4 gene of porcine RVA

A phylogenetic tree based on partial VP4 sequences is shown in Figure 3. The Belgian porcine P[6] strains were classified in lineage V of the P[6] genotype, and were genetically distinct (74.7 to 77.3%) from porcine reference strain Gottfried (lineage II). Most of the Belgian P[6] strains clustered together in lineage V, showing a high degree of genetic similarity (96.9 to 97.8%) with the porcine-like human G9P[6] strain BE2001. Nevertheless, one Belgian P[6] strain (12R045) formed a new monophyletic branch in the P[6] genotype, and was only distantly related to the other Belgian porcine P[6] strains (83.8 to 85.0%) and the porcine-like human strain BE2001 (85.5%). Contemporary Belgian porcine P[7] RVA strains were only distantly related to the porcine P[7] reference strain OSU (84.4 to 89.3%).

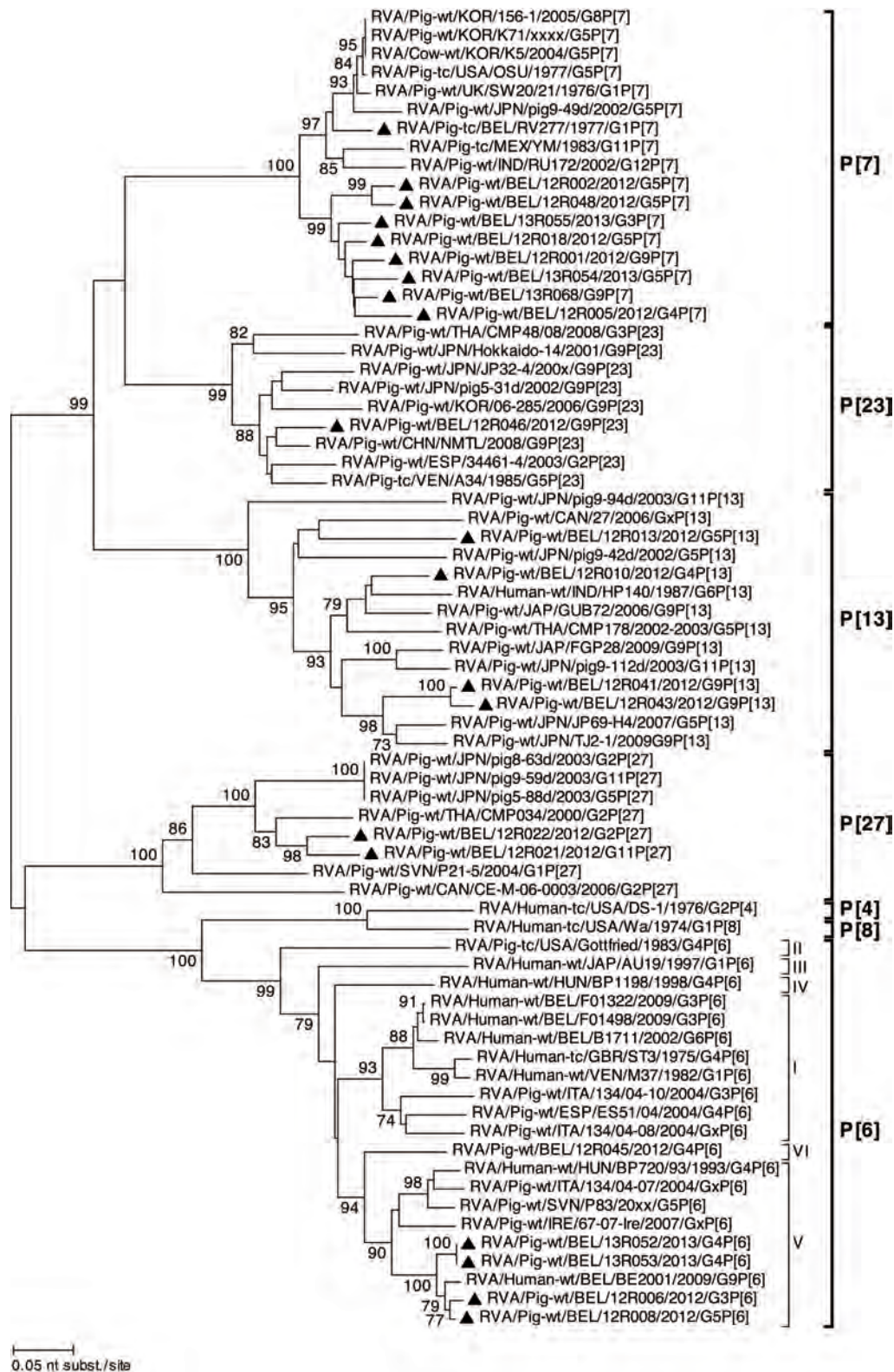


Fig. 3. Phylogenetic tree based on the partial VP8* sequences of the VP4 gene. Bootstrap values (1000 replicates) smaller than 70% are not shown. Belgian porcine RVA strains from the present study are marked with a black triangle. Intra-genotypic lineages are indicated by Roman numerals.

These Belgian strains formed a separate monophyletic cluster in the P[7] genotype, and similarity between these Belgian P[7] strains was between 89.0 and 96.2%. The old Belgian porcine RVA strain RV277 was genetically distantly related to the contemporary Belgian P[7] strains (83.6 to 87.8%), but showed a higher nucleotide similarity (94.1%) with reference strain OSU. High genetic diversity was observed in the genotype P[13]. Little genetic similarity was observed among the Belgian P[13] strains (70.7 to 81.1%), except for strains 12R041 and 12R043 (97.6%). The genetic distance of the Belgian P[13] strains to reference strain HP140 was also large (70.4 to 88.5%). The Belgian P[23] strain was most closely related to porcine G9P[23] RVA strain NMTL from China (93.1%). However, the diversity between this strain and the other P[23] strains was rather high (80.9 to 90.9%). Finally, the similarity between the two Belgian P[27] strains was rather low (92.1%). Furthermore, these strains were only distantly related to other P[27] strains detected in Thailand, Canada and Japan (70.1 to 87.6%).

3.1.4. Discussion

Diarrhea in pigs is an important cause of increased mortality, growth impairment, and economic losses. The role of RVA in the etiology of piglet diarrhea in Belgium was unclear. It was hypothesised that RVA infections were underdiagnosed on Belgian swine farms due to a lower sensitivity of fast diagnostic tests. Therefore, samples from Belgian diarrheic and asymptomatic pigs were collected and analyzed for the presence of RVA using different diagnostic approaches, followed by further genetic characterization of their VP7 and VP4 genes. The NSP5 gene was selected as a target for RT-qPCR, since this gene segment is relatively well conserved among porcine RVA strains. Despite the relatively small sample size of the present study, it was possible to obtain better insights in rotavirus diagnosis and strain diversity.

All samples that were positive after testing with a fast antigen detection test, had been collected from diarrheic pigs, and also tested positive by RT-qPCR and RT-PCR. No positive samples were found upon analysis of asymptomatic samples using fast antigen detection tests. Positive samples contained higher viral RNA copy numbers ($10^{7.73}$ to $10^{10.89}$ RNA copies/g feces), most likely representing an acute stage of RVA infection. Interestingly, the G/P genotype combinations carried by RVA strains in these samples were widely diverse, including G3P[6], G4P[6], G5P[6], G4P[7], G5P[7], G9P[7], G9P[13] and G9P[23]. From these results, broad strain sensitivity can be suggested for the fast antigen detection tests, RT-qPCR and RT-PCR. However, the detection of porcine RVA by RT-

qPCR and RT-PCR in RVA antigen negative samples indicated that molecular techniques such as RT-qPCR and RT-PCR are far more sensitive diagnostic methods in comparison to fast antigen detection tests. Typically, these antigen negative samples contained a lower number of RVA copies, which can be explained by two hypotheses. First, some of the samples have probably been collected from diarrheic animals which were in a later phase of RVA infection, resulting in reduced excretion of virus in feces. Another explanation might be the circulation of avirulent RVA strains, leading to a mild replication and viral shedding. Possible examples of such viruses are the RVA strains detected in asymptomatic samples. In the present study, the rare P[27] genotype was detected in two asymptomatic samples in combination with G2 or G11. Nevertheless, in other studies from Thailand, Italy and Canada, P[27] strains have not only been associated with asymptomatic infections in pigs, but also with diarrhea [12, 19-22]. Furthermore, in our study, a G4P[13] RVA strain (12R010) was also detected in an asymptomatic litter. Samples 12R009 and 12R011 from the same litter were also positive by RT-qPCR analysis and isolation, but genotyping was not successful. Most likely, the same G4P[13] RVA strain was present in these samples, but could not be detected by the less sensitive RT-PCR. However, in the present study, P[13] strains were not only associated with asymptomatic infections, but could also be detected in fecal samples from diarrheic pigs in association with G5 or G9. As an example, naturally attenuated P[6] strains have been described to circulate in neonatal children. Nevertheless, the P[6]-genotype was not necessarily associated with a single G-genotype in these attenuated strains [23]. In contrast, P[6] strains are also frequently associated with diarrhea, being highly prevalent on the African continent, and certain regions of the middle East [24, 25]. As such, there does not seem to be a clear correlation between the G/P genotype combination and outcome of infection. Genotype fluctuations in other genes of the RVA genome will most likely influence RVA strain virulence as well. Therefore it is important that future studies also investigate the complete genotype constellation of porcine RVA strains. Additionally, variations in host factors, such as age, immunity levels and mucus thickness/composition can also determine the outcome of an RVA infection *in vivo*.

Another important factor influencing the clinical outcome of an RVA infection are simultaneous infections with other enteric pathogens. In this study, *E. coli* was most frequently encountered together with RVA. However, the finding of non-hemolytic *E. coli* in fecal samples of pigs is no abnormality, since these can also be encountered in healthy fecal samples. In contrast, the hemolytic form of *E. coli* is stated to be the pathogenic form, and the finding of this variant is thus of more value. Co-infections between RVA and *C.*

perfringens were also common in the present study. *C. perfringens* type A and C have both been associated with diarrhea in piglets, mainly in nursery pigs, but is also commonly detected in the normal pig intestinal flora, albeit at low numbers [26]. In the present study, it was not clear if *C. perfringens* found in the present samples were pathogenic or non-pathogenic, since no further counting or toxin-analysis was performed. Further investigations should be performed on the occurrence of co- or subsequent infections with viruses and bacteria to provide better insights in the pathogenesis of piglet diarrhea. Furthermore, it should also be investigated in the future if group B and C rotaviruses can be detected in feces of Belgian diarrheic pigs, as they have been detected frequently in feces of diarrheic pigs on swine farms in different countries [27-30]. Finally, a better understanding of the role of porcine rotaviruses in the pathogenesis of piglet diarrhea, and their interplay with enteric bacteria will facilitate the development of effective prophylactic measures against rotavirus induced piglet diarrhea, which might also lead to a reduced use of antibiotics in the piglet industry. However, major difficulties in the fight against RVA infections on pig farms will probably be formed by the large genetic heterogeneity among circulating RVA strains. Different sets of primers were necessary for the amplification of partial porcine VP7 and VP4 genes using RT-PCR. This elaborate genetic diversity among the Belgian porcine RVA strains is in large contrast with the relatively restricted number of VP7/VP4 genotype combinations detected in human RVA strains [31]. However, similar findings on the large genetic heterogeneity of group A rotaviruses found in asymptomatic pig in southern Ireland, are reported by Collins and colleagues [32]. The large genetic diversity was also demonstrated by a clear intragenotypic diversity among strains of the present study, which was considerably larger than described for human RVA. A curious finding of the phylogenetic analysis in the present study was the G1 genotype of the G1P[7] strain RV277, which was isolated in Belgium in 1977 from a pool of watery diarrhea of 3 pigs, 4 days after removal from the sow at 2 days of age [33]. This strain was grown for 3 passages in MA104 cells before genotype analysis. Although G1 is the predominant VP7 genotype in humans, it is not frequently encountered in animal feces [4]. Moreover, animal G1 strains, including RV277, are also genetically distinct to contemporary human G1 strains. An exception is the human G1P[6] strain AU19, which clustered with RV277 and possessed a super-short electropherotype as demonstrated by PAGE analysis, indicative for the presence of a rearrangement in the NSP5 gene [34]. Recently, it was reported that the AU19 strain was most possibly an interspecies transmission event from a pig to a child [35]. Furthermore, a striking genetic distance was shown between human G2 RVA strains and

animal G2 RVA strains in the present study. Analysis of the partial VP7 nucleotide sequence of strain 12R022 with RotaC^{2.0} indicated that this strain was a borderline G2 strain. Circulation of borderline G2 strains in the porcine population was already shown by the demonstration of the G2P[23] strain 34461-4 from Spain, and the G2P[32] strain 61-07-Ire in Ireland [36, 37]. Interestingly, human G2 strains are almost exclusively found in association with genotype P[4] or P[6], while the porcine G2 strains have been identified in combination with genotypes P[23], P[27], P[32] and P[34], which also indicates that human and porcine G2 are evolutionary distinct [12, 37]. Surveillance of RVA infections in pigs, and other animal species, will also allow identifying more easily the occurrence of interspecies transmission events in humans. Interestingly, interspecies crossings from pigs to humans have been reported increasingly for P[6] strains in combination with different G genotypes, and much less frequent for other porcine VP4 genotypes [13, 38-40]. Based on the VP4 gene sequences, porcine and human P[6] strains have been shown to fall into multiple phylogenetic clusters relatively closely related to each other [41]. In the present study, most Belgian porcine RVA strains were classified in lineage V, but the existence of a putative new lineage VI formed by strain 12R045 can be proposed by its genetic distance from other P[6] strains [41, 42]. It seems that the threshold for interspecies transmission is lower for P[6] strains. This observation could be explained by a more efficient binding of the P[6] VP4 spikes to human enterocytes as compared to other typical porcine P-genotypes such as P[7] or P[13]. However, this hypothesis needs further investigation.

Regarding the high diversity of porcine RVA strains, it can be concluded that further surveillance of porcine RVA infections is necessary. Molecular diagnostic techniques such as RT-qPCR and RT-PCR have been shown to be better surveillance techniques than fast antigen detection tests and virus isolation. Surveillance will not only be useful for the identification of genotype diversification as a risk for future interspecies transmission events towards humans, but also for a better understanding of the role of RVA in the etiology in piglet diarrhea, which will enhance development of accurate prophylactic measurements against piglet diarrhea.

3.1.5. Acknowledgments

The authors are grateful to Ellen Van Driesche, Charlotte Brosse, Willem Van Praet (DGZ Vlaanderen), Philip Vyt (Dialab), Filip Barbé and Griet Vercauteren (Mediclab) for supplying fecal material of pigs. The authors thank Lieve Sys and Ytse Noppe for their excellent technical assistance. LMD was supported by a doctoral fellowship of The Research

Foundation-Flanders (FWO Vlaanderen). MZ, AD and IR were supported by doctoral fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen).

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Chapter 3.2.

Presence of pig group A and C rotaviruses in Belgian diarrheic suckling pigs and impact on veterinary diagnostical analyses

Summary

The importance of group A and C rotaviruses (RVAs and RVCs) in the pathogenesis of diarrhea in Belgian suckling pigs is poorly investigated. In the present study, viral loads of RVAs and RVCs were quantified in diarrhea samples of suckling piglets less than 2 weeks old, collected on 36 different Belgian farms. On 22 of 36 farms tested (61%), high viral loads of RVA (6.96 to 11.95 log₁₀ copies/g feces) and/or RVC (5.40 to 11.63 log₁₀ copies/g feces) were detected, indicating an important role for both rotavirus species in the pathogenesis of diarrhea in suckling pigs. Seventeen RVA isolates were genotyped for their outer capsid proteins VP7 and VP4. Four different G-genotypes (G3, G4, G5 and G9) for VP7 were found together with 4 different P-genotypes (P[6], P[7], P[13] and P[27]) for VP4, in 8 different G/P combinations. All characterized RVC strains belonged to genotype G6 (VP7), except for one strain possessing the G1 genotype. VP4 genes of Belgian RVC strains were genetically highly heterogeneous. Most rotavirus positive samples were also infected with *Escherichia coli*, whereas *Clostridium perfringens* infections were mainly detected in rotavirus negative samples. These results offer better insights on the importance of RVA and RVC infections in the pathogenesis of diarrhea in Belgian suckling piglets. As a conclusion, routine diagnostic testing for both viral species in cases of diarrhea in suckling pigs is highly recommended. Furthermore, the present findings also offer valuable information on the composition of new rotaviruses vaccines, which are urgently needed if diarrheic problems are to be controlled in a durable manner. Finally, the relatedness between RVC strains from pigs and other host species is described, and its implications for interspecies transmission events are discussed.

3.2.1. Introduction

Diarrhea is one of the most important health problems affecting suckling pigs, and is mainly the result of a combination of poor maternal protection and a high infection pressure of enteric pathogens. Rotaviruses belong to the genus *rotavirus* within the *Reoviridae* family. Eight different species (A to H) have been identified. To date, five species (A to E) have been officially recognized by the International Committee on Taxonomy of Viruses [1]. Rotaviruses A, B, C, E and H have been isolated from piglet feces, whereof A, B, C and H rotaviruses have also been detected in humans [2-4]. Nonetheless, group A rotaviruses (RVA) are being considered as clinically and epidemiologically the most important rotaviruses in pigs and humans. The viral genome, composed of eleven segments of double stranded RNA, encodes 6 structural (VP1 to VP4, VP6 and VP7) and 6 non-structural proteins (NSP1-NSP6). A complete genome classification system has been established for RVAs, in which each gene segment is given a letter, followed by a number representing the genotype [5]. The outer capsid proteins VP7 and VP4 have been considered as most important in this classification system, as they induce neutralizing antibodies. So far, 27 G-genotypes and 37-P genotypes were isolated from different host species [6, 7]. Recently, RVA strains with heterogeneous VP7 (G2, G3, G4, G5, G9 and G11) and VP4 (P[6], P[7], P[13], P[23] and P[27]) genotype combinations were isolated from feces of diarrheic and non-diarrheic Belgian piglets [8]. In contrast to the increasing knowledge becoming available for pig RVA infections, there is less information available on the importance of rotavirus C (RVC) infections in pigs and humans. In the past, RVCs have been isolated from diarrheic piglets, and their ability to cause pathological lesions and diarrhea was reproduced in gnotobiotic piglets. RVCs were shown to be morphologically similar, but antigenically distinct from RVAs [9, 10]. At least two distinct RVC serotypes have been identified: one serotype contained cow strain Shintoku, whereas another serotype contained pig strain Cowden. The latter pig strain was serotypically similar to human RVC strain Ehime 86-542 from Japan. A possible third serotype contained pig RVC strain HF [11]. The difficulty to isolate RVCs in cell culture makes it impossible to further expand this serotyping system, and this has led to the development of a genetic classification system for RVC [12, 13]. Martella et al. (2007) proposed the classification of RVC VP7 genes into 6 types, based on a 89% amino acid identity cut-off value [13]. More recently, Marthaler and colleagues improved this classification system, and a nucleotide identity cut-off value of 85% was established to classify RVC VP7 genes into 9 genotypes (G1 to G9). So far, pig RVCs could be assigned to genotypes G1, G3, G5, G6, G7, G8 and G9, whereas human and cow strains

belonged to G4 and G2, respectively [12-14]. A serotype classification system for VP4 has not been established, but two different studies have recently identified two different nucleotide cut-off values to classify VP4 genes in distinct genotypes [15, 16]. However, there is still no consensus for the classification of VP4. More and more researchers are currently screening for pig RVCs in feces of diarrheic and asymptomatic pigs. Furthermore, the recent availability of a new RT-qPCR assay for RVC will facilitate future screening studies [17]. In the United States, Canada and Mexico, infections with only RVC were frequently detected in young pigs of less than 3 days old [12, 17]. In a regional study conducted in Ohio, United States, RVC was also identified in feces of suckling (23.5%) and weaned pigs (8.5%). In the latter study, all RVC positive cases from suckling pigs were associated with diarrhea, whereas the RVCs isolated from weaned pigs were asymptomatic [18]. Furthermore, RVC was also detected in diarrheic fecal samples collected from 1 to 3 month old Italian piglets during 2 outbreaks. Most RVC positive samples were also co-infected with RVA or enteric caliciviruses [19]. Nonetheless, pig RVCs have also been detected in feces of asymptotically infected 4 to 5 week old Irish pigs, but only at a low rate (4.4% of 292 samples) [20]. Currently, there is no information available on the circulation of RVC infections on Belgian pig farms.

Moreover, co-infections between viruses and other enteric pathogens have been described frequently in suckling and weaned pigs [8, 21-25]. As an example, *Escherichia coli* is one of the most important pathogens involved in the pathogenesis of diarrhea in suckling and weaned pigs. Enterotoxigenic *E. coli* strains from suckling pigs can express F4, F5, F6 or F41 fimbriae (F) on their surface, which allows them to adhere to and colonize the intestinal epithelium of young piglets [26, 27]. The production of thermolabile (LT) or thermostable (STa and STb) enterotoxins results in the development of diarrhea [27].

Another important enteric pathogen affecting suckling pigs is *Clostridium perfringens*. Two major strains can be distinguished, based on their secreted toxins. Type A strains produce alpha toxins, whereas type C strains produce alpha and beta toxins. Type C infections are most severe, and may induce lesions of hemorrhagic and necrotizing enteritis. High bacterial counts can be encountered in affected animals [28]. Diarrhea caused by *Isospora suis*, a parasite belonging to the *Eimeriidae* family, mainly affects 5-15 day old piglets [29, 30].

In the present study, it was aimed to obtain better insights in the importance of RVA and RVC infections in Belgian diarrheic suckling pigs and to provide diagnostical advices for veterinarians working in the field. Furthermore it was aimed to characterize the genes encoding outer capsid proteins VP7 and VP4, which may aid future vaccine formulation.

3.2.2. Materials and methods

3.2.2.1. Collection and processing of diarrheic fecal samples

Fourty five diarrheic fecal samples, collected at 36 different Belgian farms from suckling pigs less than 2 weeks old between January and March 2014, were sent to a private laboratory (Dialab, Belsele, Belgium) for etiological diagnosis. The veterinarians sending the samples specified for which pathogens diagnostic tests should be performed. RVA was detected using a fast antigen detection test (Rota-Strip, Coris BioConcept, Gembloux, Belgium) according to the manufacturer's instructions. *E. coli* was isolated on MacConkey agar no. 3 (Oxoid, Thermo Scientific, Erembodegem, Belgium) under aerobic conditions at 37°C. *Clostridium perfringens* isolation was performed under anaerobic conditions at 37°C on Columbia agar containing naladixic acid, and toxins were demonstrated using a commercially available immunoassay. The X/pect[®] *C. difficile* Toxin A/B test kit (Remel Microbiology Products, Thermo Scientific, Erembodegem, Belgium) was used for detection of *Clostridium difficile* toxins in feces. Coccidia were purified by flottation using a saturated salt- and sugar solution, and oocysts were visualized under a light microscope.

Next, fecal samples were stored at -20°C and sent to the Laboratory of Virology for quantitative virological analysis. Here, 20% fecal suspensions were prepared in phosphate buffered saline (PBS) containing 1000 U/ml penicillin (Continental Pharma, Puurs, Belgium), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/ml gentamicin (Gibco BRL, Merelbeke, Belgium) and 0.01% v/v Fungizone (Bristol-Myers Squibb, Braine l'Alleud, Belgium). Solutions were centrifuged at 793 x g for 5 min and 140 µl of supernatant was collected for RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified RNA and fecal suspensions were stored at -70°C until further use.

3.2.2.2. Quantification of RVA and RVC

Quantification of viral RNA of porcine rotavirus A and C was performed using previously described RT-qPCR assays [8, 17]. Prior to RT-qPCR analysis, RNA was denatured for 2 min at 95°C, and immediately chilled on ice. RT-qPCR was performed in 20 µl reaction volumes containing Precision OneStep qRT-PCR Mastermix with SYBR Green, ROX and inert blue pipetting dye (Primerdesign, Southampton, United Kingdom), and variable amounts of primers and nuclease free water, depending on the virus tested (Table 1). Nucleotide sequences of the primers used for RVA and RVC RT-qPCR are shown in Table

2. Mastermixes and template RNA or nuclease free water were transferred to BrightWhite 96-well Real-time PCR plates (Primerdesign). Plates were sealed with adhesive films (Primerdesign) and loaded in a StepOnePlus apparatus (Applied Biosystems). Reaction conditions were as follows: 55°C for 10 min, 95°C for 8 min, followed by 40 cycles of amplification. Each cycle consisted of a denaturation step for 10 sec at 95°C and an annealing step for 60 sec at 60°C or 56°C for RVA and RVC, respectively. Afterwards, a melt curve analysis was performed to check the specificity of the amplified products. The baseline was set automatically, and the threshold was placed manually in the exponential phase of target amplification. Standard curves, using synthetically generated dsRNA templates for RVA and RVC, were included to determine efficiencies of the assays, and to allow for absolute quantification over a linear dynamic range (LDR) from 6 log₁₀ to 1 log₁₀ copies/reaction [8]. Assays were valid if efficiency was between 90 and 110%. Quantification of the viral load in fecal samples (copies/g feces) was possible if the C_q values of both RT-qPCR reactions fell within the LDR of the assay. Samples were considered unquantifiable, but positive (<LOQ) if C_q values of specific amplicons fell behind the LDR.

Table 1. RT-qPCR reaction mixture composition.

Component (concentration)	RVA	RVC
Precision OneStep qRT-PCR Mastermix	10 µl	10 µl
Forward primer (IDT)	0.8 µl (400 nM)	0.4 µl (200 nM)
Reverse primer (IDT)	0.1 µl (50 nM)	0.1 µl (50 nM)
Nuclease free water	6.1 µl	6.5 µl
RNA template or water	3 µl	3 µl

3.2.2.3. RT-PCR for VP7 and VP4 genes of porcine RVA and RVC

RT-PCR was performed to amplify VP7 and partial VP4 encoding genes of RVA and RVCs. RNA was denatured at 95°C for 2 min, and chilled on ice. RT-PCR was performed using the Qiagen OneStep RT-PCR kit (Qiagen). Reaction mixtures consisted of 5 µl of 5x Qiagen OneStep RT-PCR Buffer, 1µl of dNTP Mix, 3µl of each primer (Table 2), 1 µl of Qiagen OneStep RT-PCR enzyme mix, nuclease free water (10 µl) and 5µl of RNA template in a total volume of 25 µl per reaction. Reverse transcription was executed at 50°C for 30 min, followed by Taq polymerase activation at 95°C for 15 min. Thirty five cycles of amplification were performed at 94°C for 30 s, 45°C for 30 s and 72°C during 1.5 min. A final extension was performed at 72°C during 10 min. Next, 9 µl of PCR product was mixed with 1 µl of loading buffer, loaded on polyacrylamide gels and electrophoresis was executed during 36 min at 200V. Amplicons were visualized using ethidium bromide and UV light.

Table 2. RT(q)-PCR primers for RVA and RVC.

Virus	Gene	Primer name	Sequence	Ref
RVA	NSP5	NSP5F_qPCR	5'-ATC TAT TGG TAG GAG TGA ACA GTA-3'	[31]
		NSP5R_qPCR	5'-GTG GGT CGT TTG AAG CAG AA-3'	[31]
	VP7	Gen_VP7_F	5'-ATG TAT GGT ATT GAA TAT ACC AC-3'	[32]
		Gen_VP7_R	5'-AAC TTG CCA CCA TTT TTT CC-3'	[32]
		Beg9	5'-GGC TTT AAA AGA GAG AAT TTC CGT CTG G-3'	[33]
	End9		5'-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3'	[33]
		VP4	VP4-1-17F	5'-GGC TAT AAA ATG GCT TCG C-3'
	VP4	VP4F	5'-TAT GCT CCA GTN AAT TGG-3'	[32]
		VP4R_Deg	5'-TCY CTR TTR TAT TGC ATY TCY TTC C-3'	[31]
		RVC	VP6	RVC_Fw_qPCR
RVC	VP6	RVC_Rv_qPCR	5'-ACA TTT CAT CCT CCT GGG GAT C-3'	[17]
		VP7	RVC_VP7-20F	5'-GCT GTC TGA CAA ACT GGT C-3'
VP7	RVC_VP7- 1062R		5'-GCC ACA TGA TCT TGT TTA CGC-3'	[35]
		VP4	RVC_VP4-17F	5'-GAT CRA TGG CGT CYT CAC-3'
VP4	RVC_VP4-1238R		5'-CCT GAT GAA TGT AAT CCW GGA T-3'	[35]

3.2.2.4. Nucleotide sequence analysis

PCR products (5 µl) of RT-PCR positive reactions were treated with 1 µl of USB ExoSAP-IT PCR Product Clean-Up (Affymetrix, Santa Clara, California, USA) before sequencing using the ABI Prism BigDye terminator cycle sequencing reaction kit (ABI Prism 3130xl, Applied Biosystems). Forward primers were used for sequencing. Sequence analysis was performed using 4Peaks (Mekentosj, Amsterdam, The Netherlands). Genotypes for RVA were determined using BLAST or RotaC^{2.0} [36]. Genotypes for the VP7 genes of RVC strains were identified using the 85% nt identity cutoff value defined by Marthaler and colleagues (2013) [12]. Multiple sequence alignments were performed using the ClustalW plug-in in MEGA 5.2.2, followed by manual editing. The substitution model was assessed for each gene separately and the maximum-likelihood method was used to construct phylogenetic trees for partial VP7 and VP4 encoding genes of RVC. For VP7, the Tamura Nei substitution model with gamma distribution and invariant sites was applied, whereas the Tamura 3 model with gamma distribution and invariant sites was used for the partial VP4 genes. Pairwise distance calculation was performed using the *p-distance* model. Statistical bootstrap analysis was set at 500 replicates for construction of the trees and distance calculations.

3.2.3. Results

3.2.3.1. RVA and RVC infections in diarrheic suckling pigs

For 40 fecal samples, diagnostic testing for RVA was performed using a fast antigen detection test, whereas all samples were evaluated using RT-qPCR. The number of RVA positive tests using both methods is shown in Table 3.

Table 3. Results of RVA testing using RT-qPCR and a fast antigen detection strip.

		RVA RT-qPCR		Total
		Positive	Negative	
Strip	Positive	8	1	9
	Negative	12	19	31
	Not tested	2	3	5
	Total	22	23	45

Individual diagnostic results for each sample are shown in Table 4. Eight samples tested positive for RVA using the strip and RT-qPCR, and contained high viral loads between 9.21 and 10.63 log₁₀ copies RNA/g feces. However, 12 samples tested positive with RT-qPCR, but negative with the strip. Five of these samples contained a very low viral load, underneath the limit of quantification (<LOQ), whereas 7 samples contained a high viral load (6.96 to 11.95 log₁₀ copies/g feces). In two of the five samples that were not routinely tested using the strip, an RVA infection was missed. One sample contained a high viral load (8.86 log₁₀ copies RNA/g feces), whereas another had a low viral load (<LOQ). Furthermore, one sample tested positive with the strip but was negative upon RT-qPCR analysis. In summary, 49% (n=22) of the 45 samples were positive for RVA, and 36% percent (n=16) of the 45 samples contained high viral loads (>6.9 log₁₀ copies RNA/g feces).

The presence of RVC was not tested at the private laboratory by the lack of a commercial test. Still, the virus was detected with RT-qPCR in 29% (n=13) of all samples. Viral loads in RVC positive samples ranged between 5.40 and 11.63 log₁₀ copies/g feces, but were low (<LOQ) in some other samples. Eight of the RVC positive samples were negative for RVA, whereas five samples were positive for both rotavirus species.

Table 4. Differential diagnosis of diarrhea in Belgian suckling pigs.

Farm	Sample	Rotavirus A			Rotavirus C		<i>E. coli</i>			<i>Cl. perfr.</i>		<i>Cl. diff.</i>	<i>Cocc.</i>	
		Antigen test	qPCR load ^A	G/P genotype	qPCR load ^A	G genotype	NH	VF	H	VF	Isol.			Toxins
1	14R103	+	9.73	G5P[6]	-		+	-	-	-		+	ND	
2	14R133	+	9.21	G5P[13]	-		-	+	-	-		-	ND	
3	14R163	+	10.63	G9P[23]	-		+	-	-	-		ND	-	
4	14R165	+	9.27	G4P[6]	-		-	-	-	-		ND	ND	
5	14R166	+	10.16	G9P[7]	-		+	-	-	-		ND	ND	
6	14R113	-	<LOQ		-		+	-	-	-		ND	-	
	14R114	+	10.53	G5P[7]	-		+	-	-	-		ND	-	
	14R115	-	-		-		+	-	-	-		ND	-	
7	14R126	+	9.88	G9P[23]	6.31	G6	ND		ND	-		ND	ND	
8	14R160	+	10.63	G5P[7]	<LOQ	Gx	-		-	-		-	ND	
9	14R116	+	-	G9P[7]	-		+		-	-		ND	-	
10	14R124	-	8.67	G4P[23]	-		+		-	-		ND	-	
	14R125	-	9.06	G4P[23]	-		+		-	-		ND	-	
11	14R128	-	11.78	GxP[x]	-		+		-	-		ND	ND	
12	14R155	-	8.55	G3P[7]	-		+		-	-		ND	ND	
13	14R123	-	11.95	GxP[x]	8.51	G6	+	-	-	+	β2	-	ND	
14	14R118	-	-		5.93	G6	+		-	-		-	-	
	14R119	-	6.96	G5P[13]	-		+		-	-		ND	-	
	14R120	-	8.77	G4P[6]	-		+		-	-		-	-	
15	14R108	ND	8.86	G4P[6]	-		ND		ND	ND		ND	-	
16	14R112	-	-		9.94	G6	+		-	+		ND	ND	
17	14R127	-	-		8.54	G6	-		+	-		ND	ND	
18	14R158	-	-		8.67	Gx	+		-	-		ND	I. suis	
19	14R161	-	-		11.63	G6	+		-	+		-	ND	
20	14R137	-	<LOQ		5.40	G6	+		-	-		ND	-	
21	14R145	-	<LOQ		8.72	G1	+		-	-		ND	ND	
22	14R121	ND	-		10.06	G6	+		-	-		ND	ND	
23	14R152	-	<LOQ		-		+		-	ND		ND	ND	
24	14R162	-	<LOQ		-		+		-	+	α + β2	ND	ND	
25	14R107	ND	<LOQ		-		ND		ND	ND		ND	I. suis	
	14R105	ND	-		-		+		-	-		ND	ND	
26	14R104	-	-		<LOQ		+		+	-		-	ND	
27	14R138	-	-		<LOQ	Gx	ND		ND	ND		ND	ND	
	14R139	-	-		-		ND		ND	ND		ND	ND	
28	14R117	-	-		-		+		-	-		-	-	
29	14R136	-	-		-		-		-	-		-	-	
30	14R140	-	-		-		+		-	-		-	ND	
31	14R141	-	-		-		+		-	+	β2	-	-	
	14R142	-	-		-		+		-	+	β2	-	-	
	14R143	-	-		-		+		-	+		-	-	
32	14R154	-	-		-		-		+	*		ND	-	
33	14R156	-	-		-		-		+	+		ND	-	
34	14R157	-	-		-		+		-	+	β + β2	-	-	
35	14R159	-	-		-		+		-	+	α + β2	ND	ND	
36	14R151	ND	-		-		+		-	ND		ND	ND	

<LOQ is positive, but below limit of quantification; A is RT-qPCR titer: log₁₀ copies/g feces; NH is non-hemolytic; H is hemolytic; VF is virulence factors; Isol. is anaerobic isolation; * is virulence factors F18, LT and STb; ND is not tested

3.2.3.2. Presence of *E. coli* in diarrheic samples from suckling pigs

Testing for *E. coli* by aerobic isolation is a routine practice, with 89% of all samples tested. From only three of these samples, *E. coli* could not be isolated. Thirty three (83%) of the tested samples contained non-hemolytic *E. coli*, whereas hemolytic *E. coli* was only detected in a minority of the samples (n=5; 13%). The expression of virulence factors was not frequently assessed under field conditions. Four non-hemolytic samples were investigated, but tested negative. Furthermore, 2 hemolytic *E. coli* strains were further analyzed, whereof one isolate expressed F18 fimbriae, and LT and STb enterotoxins.

3.2.3.3. *Clostridium perfringens* isolation and toxinotyping

Diagnostic isolation of *Clostridium perfringens* was also a routine practice, with 87% of all diarrheic samples analyzed for the presence of this pathogen. Ten (26%) of these 39 samples were positive for *Clostridium perfringens* by isolation under anaerobic conditions. Toxinotyping was not routinely performed. Nonetheless, the beta 2 toxin was most frequently identified (n=6). Only two strains could be identified as type A, and one as type C, based on the detection of alpha and beta toxins.

3.2.3.4. Diagnosis of *Clostridium difficile* and *Coccidiosis*

In contrast to the other enteric pathogens, diagnoses for *Clostridium difficile* and *Coccidiosis* were not routinely requested in this study. Only 33% of the samples were analyzed for *C. difficile*, resulting in one positive test. Flottation analysis was performed for 49% (n=22) of the fecal samples, but *Isospora suis* was only detected in 2 samples.

3.2.3.5. Diagnosis at farm level

Based on diagnostic testing with the RVA fast antigen detection strip, rotavirus (RVA) infections could be identified in only 25% (n=9) of the farms (farms 1-9). In most premises, samples were co-infected with (non)-hemolytic *E. coli*, a single case of *Clostridium difficile* infection was observed on one farm, and no *Clostridium perfringens* or coccidia were detected. Co-infections between RVA and RVC were detected, but only on one farm a significant viral load of RVC was demonstrated in one sample.

RVA infections remained undiagnosed on 16.7% (n=6) of the farms (farms 10-15) when only a fast antigen detection test was used. Co-infections between RVA and non-hemolytic *E. coli* were observed on 4 of these farms. No hemolytic *E. coli*'s were isolated. A quadruple infection with RVA, RVC, non-hemolytic *E. coli* and *Cl. perfringens* was noticed on one farm.

RVC infections were frequently detected on another 19.4% (n=7) of the farms (farms 16-22), with fecal samples containing substantial viral loads (5.40 to 11.63 log₁₀ copies/g feces). All samples, except one, were co-infected with non-hemolytic *E. coli*. The one sample was coinfecting with hemolytic *E. coli*. *Clostridium perfringens* was isolated on two farms, whereas *Isospora suis* was detected on a single farm.

On farms 23 to 27, only very low loads (<LOQ) of RVA and RVC could be detected. If tested, non-hemolytic *E. coli*'s were detected on 4 of these farms. Hemolytic *E. coli*, *Cl. perfringens* type A and *Isospora suis* were isolated on some of these farms as well. Finally, fecal samples of 30.5% (n=11) of the farms tested (farms 28-36) completely negative for both rotavirus species. *Clostridium perfringens* could be isolated on 4 of these rotavirus negative farms.

No pathogens could be identified on farm 29, whereas non-hemolytic *E. coli* was the solely detected potential pathogen on three farms. A case of hemolytic *E. coli*, expressing F18 fimbria and LT and STb enterotoxins, was identified on one farm.

3.2.3.6. Characterization of RVA VP7 and VP4 genes

Seventeen RVA strains were analyzed by RT-PCR for further sequencing of the partial VP7 and VP4 genes (Table 4). The G/P genotype distribution is demonstrated in Table 5. VP7 genotypes G3, G4, G5 and G9, and VP4 genotypes P[6], P[7], P[13] and P[23] were encountered in 8 different G/P combinations. G5 (29.4%) and G4 (29.4%) were the single most detected VP7 genotypes, whereas P[7] (29.4%) was the most frequently isolated VP4 genotype. G4P[6] (17.6%) was the most identified G/P combination, closely followed by G/P combinations G5P[7], G9P[7], G4P[23] and G9P[23] (11.8%). Both the VP7 and VP4 genotypes of 2 strains (14R123 and 14R128) could not be determined. The fecal sample (14R116) that tested positive with the fast antigen test, but negative with RT-qPCR, contained a G9P[7] strain.

Table 5. VP7 and VP4 genotype distribution of RVA strains from diarrheic suckling pigs.

	G3	G4	G5	G9	Gx	Total
P[6]		3 (17.6%)	1 (5.9%)			4 (23.5%)
P[7]	1 (5.9%)		2 (11.8%)	2 (11.8%)		5 (29.4%)
P[13]			2 (11.8%)			2 (11.8%)
P[23]		2 (11.8%)		2 (11.8%)		4 (23.5%)
P[x]					2 (11.8%)	2 (11.8%)
Total	1 (5.9%)	5 (29.4%)	5 (29.4%)	4 (23.5%)	2 (11.8%)	17 (100%)

3.2.3.7. Genetic characterization of partial VP7 encoding genes of pig RVCs

Phylogenetic analysis (Figure 1) demonstrates the existence of three major clades among VP7 encoding genes of RVC strains from pigs, humans and cows. One major clade contained pig genotypes G6 and G8, and cow genotype G2. Most of Belgian pig RVC strains belonged to genotype G6, together with pig strains from the United States, Italy and the Czech Republic. Strains 14R118, 14R121, 14R123, 14R126 and 14R161 were moderately related to each other (92.4 to 95.3% nt similarity) and clustered with Italian strain RVC/Pig-wt/ITA/344-04-7/2007/G6Px (93.5 to 96.0%). Still, within genotype G6, Belgian strains 14R112, 14R127 and 14R137 clustered more distantly from the other Belgian G6 strains (86.9 to 90.8%), demonstrating the overall moderate intragenotypic diversity within the RVC genotype G6.

Another clade contained pig genotypes G1, G4, G5, G7 and G9, and human genotype G4. Human RVC strains of genotype G4 showed a high genetic similarity (95.3 to 99.7%). In contrast with this genotype was the high diversity observed within the G1 genotype, containing pig strains from Italy, the United States, the Czech Republic and Belgium. Belgian strain 14R145 was distantly related to pig reference strain RVC/Pig-tc/USA/Cowden/1980/G1Px (89.6%). Remarkably, the pig genotype G3 clustered distinctly from all other known RVC VP7 genotypes.

3.2.3.8. Genetic characterization of partial VP4 encoding genes of pig RVCs

A phylogenetic tree (Figure 2) was constructed for the partial VP4 sequences of RVC strains as well. A major clade within the tree consisted of human RVC strains, whereas pig and cow RVC strains were assigned to many genetically distinct clusters. Belgian pig RVC VP4 genes clustered distantly together with strain RV0143 from the United States (82.7 to 89.8%) and strain P141 from the Czech Republic (80.2 to 88.3%). Czech strains P8 and P9 formed another pig VP4 subcluster showing a high genetic distance with the Belgian pig strains (62.4 to 66.8%). Other pig strains were assigned to clusters consisting of strains from the Republic of South Korea, the United States, and the Czech Republic, respectively. Furthermore, a major cluster of cow strains could be detected in between the different pig clusters. Most of the cow strains were genetically highly similar, except strain RVC/Cow-wt/JPN/Y/1/04/2004/G2Px.

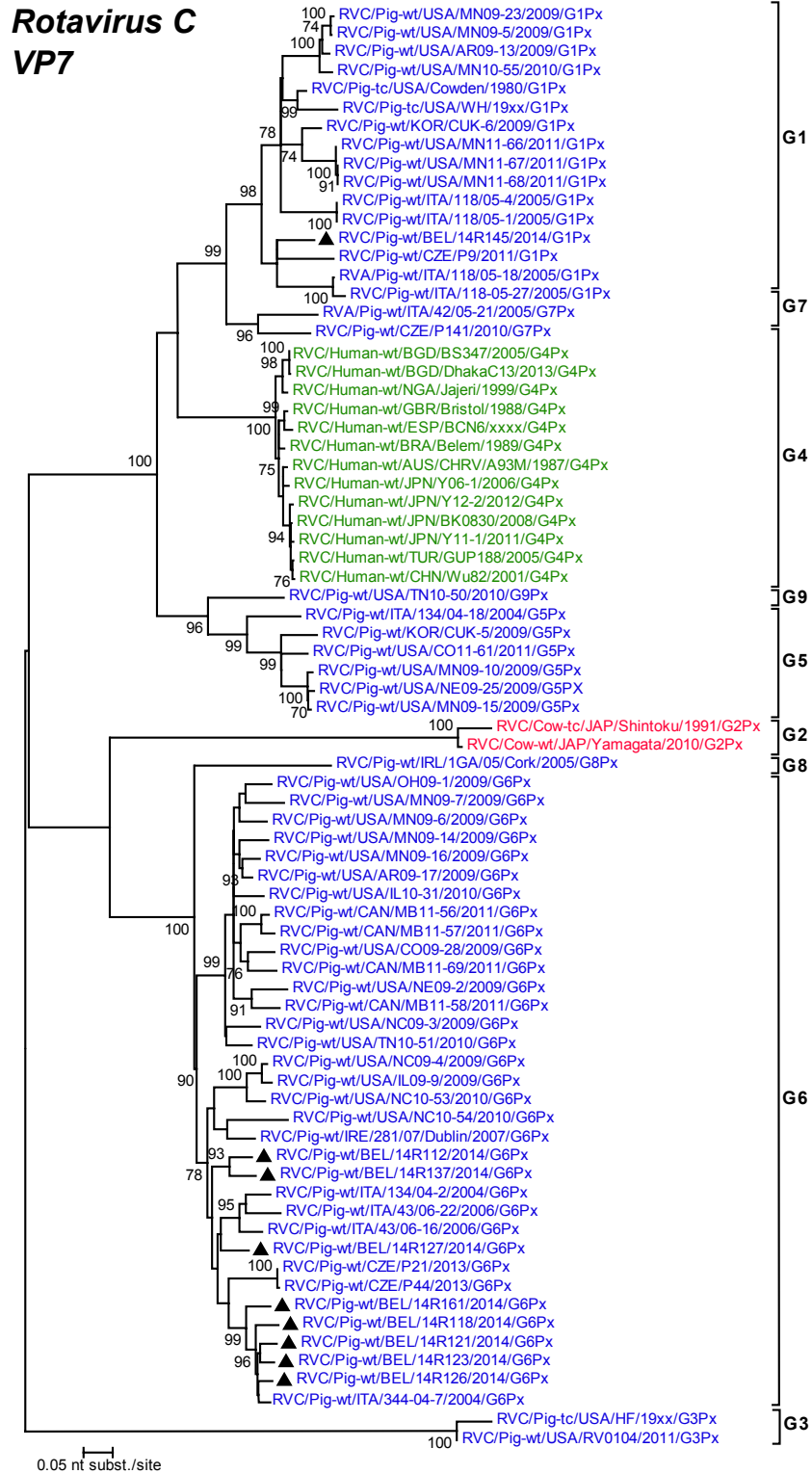


Fig. 1. Maximum-likelihood phylogenetic tree based on partial coding sequences of RVC VP7 genes. Bootstrap values (n=500 replicates) lower than 70% are not shown. Pig, cow and human strains are colored in blue, red and green, respectively. Belgian diarrhetic pig strains are marked with a triangle.

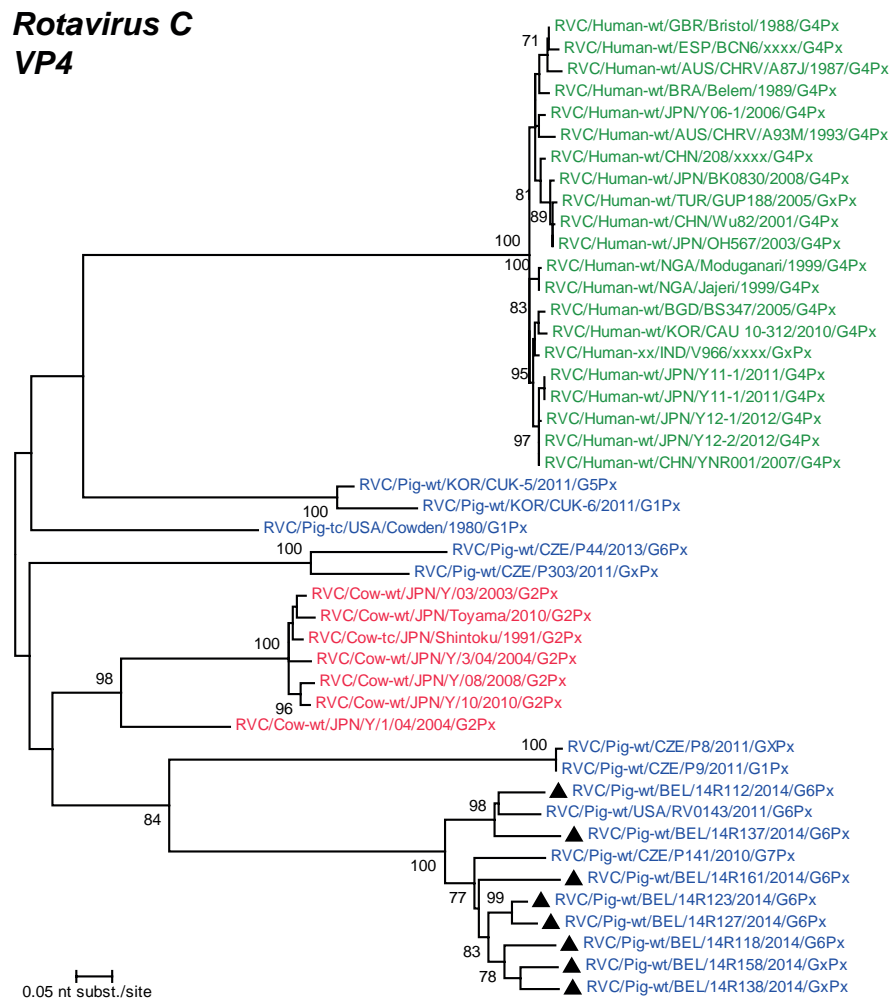


Fig. 2. Maximum-likelihood phylogenetic tree based on partial coding sequences of RVC VP4 genes. Bootstrap values ($n=500$ replicates) lower than 70% are not shown. Pig, cow and human strains are colored in blue, red and green, respectively. Belgian diarrheic pig strains are marked with a triangle.

3.2.4. Discussion

Diarrhea is major health problem during the first weeks of a pig's life and is mostly the result of enteric infections with viruses, bacteria and/or parasites. After birth, pigs are protected by secretory immunoglobulin A (SIgA) antibodies in the milk, which neutralize enteric pathogens in the gut lumen. However, lactogenic immunity provided by gilts is generally less well developed than that of multipare sows. As a consequence, the incidence of rotavirus infections is generally higher in litters farrowed by gilts [37]. Intestinal infections in poorly protected offspring result in diarrhea, growth retardation, increased mortality, high drug use and severe economic losses. For effective treatment and prophylaxis, it is essential to accurately identify the etiological cause(s) of these diarrheic problems, which often remains a challenging task for veterinarians.

Here it was demonstrated that diagnosis of RVA and RVC infections were readily missed when only a fast antigen detection test was applied. Indeed, on n=22 of 36 farms tested (61%), either RVA and/or RVC could be detected at high viral loads, whereas rotaviruses were only detected on 9 (25%) of the farms using a diagnostic strip. RT-qPCR was the most sensitive method to detect pig RVAs in fecal samples, but the diagnosis was missed in one fecal sample containing a G9P[7] strain. These findings emphasize that molecular diagnostic assays in use should still be monitored on a regular base, to make sure that they will pick up all circulating variants. The high viral loads detected in the current study are indicative for an important role of RVAs and RVCs in the pathogenesis of diarrhea. Nonetheless, care should be taken when interpreting the finding of rotaviruses at very low viral loads. These might be samples collected just before or after the peak of viral replication, but it may also be that these are viruses present in the gut but kept under control by maternal SIgA antibodies. Future studies should include non-diarrheic control pigs as well, to assess if similar high viral loads of these RVA and RVC strains can be isolated from non-diarrheic piglets as well. As demonstrated here, RVCs may contribute to the pathogenesis of piglet diarrhea, which was previously unreported in Belgium [8]. RVC infections in the absence of RVA were detected on 16.7% of the farms, whereas RVC may have contributed to the pathogenesis together with RVA on another 3 farms. Marthaler and colleagues also demonstrated a high burden of RVC infections in diarrheic suckling pigs in the United States, Canada and Mexico [12, 17]. Regarding the results from this and previous studies, routine diagnostic testing for the presence of RVA and RVC in diarrheic feces of suckling pigs should be routinely performed.

Most fecal samples were also analyzed for the presence of *Escherichia coli* and *Clostridium perfringens*. Many of these bacteriological diagnoses are only based on the detection of bacterial colonies after isolation under (an)aerobic conditions. Not surprisingly, non-hemolytic *E. coli*, which are also inhabitants of healthy intestines, were isolated from most of the samples tested. These are often considered as nonpathogenic forms of *E. coli*, but care should be taken when interpreting pathogenicity solely on the presence of hemolysis. Indeed, it was demonstrated for ETEC strains associated with postweaning diarrhea in Danish piglets that the detection of virulence factor encoding genes was a better predictor for ETEC virulence, and that 12.2% of non-hemolytic isolates may still be virulent [38]. Also in a study in Belgium 16.6 % of the non-hemolytic produced virulence factors (Coddens et al., unpublished results). In the present study, it was found that the expression of virulence factors, such as fimbriae and enterotoxins, is not routinely being diagnosed by veterinarians,

most likely due to the extra costs associated with these expensive analyses. Only on one farm, virulence factors F18⁺, LT and STb were detected in a diarrheic feces sample. However, F18⁺ ETECs are normally associated with postweaning diarrhea or edema disease, and are normally not frequently encountered in suckling pigs as F18-receptor expression is not fully complete in most unweaned piglets [26, 39, 40]. On several farms *Clostridium perfringens* infections may have contributed to the pathogenesis of diarrhea in suckling pigs as well. The majority of *Cl. perfringens* positive samples were free of RVA and RVC infections. However, the finding of the bacteria alone by isolation is insufficient for diagnosis as it is also a normal inhabitant of the intestine. The finding of high bacterial counts and/or the detection of bacterial toxins is confirmatory for a *Cl. perfringens* infection [28]. Two *Cl. perfringens* type A (alpha toxin) and one type C infections (beta toxin) were found in diarrheic suckling pigs in the present study. Many strains expressed the beta2 toxin, but its importance in the pathogenesis of diarrhea was recently questioned [41]. For reliable testing of toxins, correct storage and rapidly investigation of fecal samples should be warranted, as these toxins are highly susceptible to degradation by proteases [42, 43]. *Clostridium difficile* and coccidiosis were not routinely diagnosed in the present study, and were only detected in 1 and 2 cases, respectively. The occurrence of coccidial infections might have been underestimated here by the limited number of analyses requested by veterinarians. Studies performed in other countries demonstrated that *Isospora suis* was detected at higher prevalence rates in suckling pigs. As an example, 39% of diarrheic German suckling pigs from 8 to 14 days old were positive for *I. suis*. In Japan, 9.2% of diarrheic suckling pigs tested positive for this pathogen, and in many cases these samples were coinfecting with RVA as well [21, 22].

Regarding the above diagnostic findings, it seems difficult for veterinarians to come to sound conclusions and to setup effective prophylactic and therapeutic measures. The high prevalence of RVA and RVC infections detected in the present study indicates that rotaviruses are an attractive target for prophylaxis of diarrheic problems in young piglets. Nonetheless, specific control measures for rotavirus infections are not available. In view of future vaccine formulation, which should be administered to gilts and sows to boost lactogenic immunity, the genes encoding outer capsid proteins of circulating Belgian RVA and RVC strains were characterized as well. A large genetic diversity among genes encoding VP7 and VP4 proteins of RVA strains was noticed in the present study. Among the different G and P genotypes, G5, G4 and P[7] were most prevalent.

Here, the genetic characterization of pig RVC VP7 and VP4 genes is also being reported, which has only scarcely been studied before. An initial genotype classification system was established by Martella and colleagues, and recently further optimized by Marthaler and coworkers [12, 13]. The genetic diversity of pig RVC VP7 genes has been shown to be very high in comparison to the diversity observed for human RVC strains. However, until now most characterized RVC VP7 genes have been derived from pigs, which may bias these results. Still, it may be suggested that RVC strains from pigs are circulating already for a longer time in the population, or are being placed under a higher antigenic/genetic pressure than human RVCs. Most Belgian pig RVC strains belonged to genotype G6, which is currently the largest pig genotype. One Belgian strain belonged to genotype G1, which also contains RVC reference strain RVC/Pig-tc/USA/Cowden/1980/G1Px. Nonetheless, the diversity observed in the latter genotype was also rather large. The difficulty to grow RVC strains in cell culture makes it difficult to understand whether these different genotypes also represent different serotypes. Obtaining a better insight in the antigenic and serotypic diversity of these RVC strains, as well as for RVA strains, will be crucial for accurate formulation of future pig rotavirus vaccines. It was shown earlier that strains RVC/Pig-tc/USA/Cowden/1980/G1Px, RVC/Cow-tc/JPN/Shintoku/1991/G2Px, and RVC/Pig-tc/USA/HF/19xx/G3Px were serotypically distinct. Furthermore, strain Cowden was serologically related to a Japanese human strain [11]. Interestingly, strains Cowden, Shintoku and HF belong to 3 distinct large clades within the VP7 phylogenetic tree (Figure 1). Genotype G1, containing strain Cowden, clustered together with human G4 strains in one of these clades. It may be hypothesized that strains belonging to a specific phylogenetic clade are serologically related, but this should be further explored with *in vitro* experiments in pig and human enterocytes. Nonetheless, serological cross-reactions may be influenced by VP4 proteins as well.

Interestingly, all pig RVC genes encoding VP7 and VP4 proteins clustered distinctly from human and cow RVC strains. The pig strain Cowden uses sialic acid residues for infection of cell cultures and hemagglutination of red blood cells. Concerning the genetic conservedness observed among VP4 genes of human RVC strains, it may be hypothesized that human RVC strains use a single specific carbohydrate moiety for attachment to the intestinal surfaces of the human gut. Regarding the diversity among VP4 genes of animal RVC strains, it is very likely that pig rotavirus strains are capable of recognizing and using a wider scala of carbohydrate moieties for attachment. This first step in the viral replication seems to be a major host species barrier. Also for RVAs it is likely that the VP4 proteins encoded by the

P[6], P[7], P[13] and P[23] genotypes use different carbohydrate moieties for entrapment in mucus, or for attachment to enterocytes. This has already been described for several RVA strains with distinct VP4 genotypes [44, 45]. It is very likely that the susceptibility of certain pigs to rotaviruses infections is determined by factors such as the pig's race, age and histo-blood groups, which determine the exact specificities of carbohydrate moieties on the mucus and enterocytes. As P[6] genotypes are shared between human and pig RVAs, the interspecies transmission of such strains may occur more easily [8, 46].

As a general conclusion, RVA and RVC infections should be regularly diagnostically investigated in cases of diarrhea in suckling pigs. RT-qPCR is the best diagnostic technique. Isolation and detection of virulence genes should be assessed for a more accurate diagnosis of ETEC infections, whereas isolation, bacterial counting and toxinotyping can be advised for the detection of *Clostridium perfringens* infections [28, 38]. Furthermore, it is advised to include sedimentation and flottation in a routine diagnostic protocol as well, as coccidia have been detected at higher prevalence rates in other countries in the past [24, 25]. At short sight, it may seem an expensive approach to test for multiple pathogens, but a more accurate etiological diagnosis may enable the farmer to implement prophylactic measures in a more targeted manner, and will certainly deliver him a beneficial return on investment on the long term. Regarding the results of the present study, gilts and sows need to be vaccinated with an attenuated oral vaccine containing RVA and RVC, to boost lactogenic immunity and to protect suckling piglets. Still, it should be further investigated whether certain RVA and RVC induce a good heterogeneous protection under *in vivo* conditions. Future research is definitely needed, as such vaccines will likely not only reduce the burden of rotavirus infections in pigs, but might also lower the number of bacterial and parasitic co-infections, eventually leading to a reduced use of antimicrobial drugs as well.

3.2.5. Acknowledgments

The authors thank Ytse Noppe for her excellent technical assistance. ST was supported by a scholarship of the Special Research Fund of Ghent University. LMD was supported by a doctoral fellowship of The Research Foundation-Flanders (FWO Vlaanderen). MZ and IR were supported by a doctoral fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen).

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Chapter 3.3.

Successive subclinical group A rotavirus infections in pigs after weaning on a closed Belgian pig farm

Summary

Group A rotaviruses (RVA) and enterotoxigenic *Escherichia coli* (ETECs) are important causes of diarrhea in weaned piglets. However, our knowledge on the relative role of RVA in the pathogenesis of postweaning diarrhea and growth retardation on Belgian pig farms is scarce. In the present pilot study, 9 weaned piglets of a large closed Belgian pig farm were followed for their excretion of RVA and *E. coli* during the first 24 days postweaning. Weights of pigs at weaning, and daily weight gains of all piglets were highly variable during the study period. Unexpectedly, all pigs became successively infected with 2 different RVA strains (G5P[13] and G9P[23]). However, the second replication peak was less pronounced, indicating that a low level of cross-protective immunity might have been induced by the infection of the first strain. All pigs shed non-hemolytic *E. coli*, but hemolytic *E. coli* were only detected in a few piglets at scattered timepoints. The limited proliferation of bacteria may be the result of prophylactic treatments with colistine sulphate and zinc oxide. Consequently, explosive diarrheic problems were absent on this farm. Future studies on a larger number of pig farms will be expanded to suckling pigs, and piglets after weaning until they reach the prefattening stages, and the precise role of RVA infections in the pathogenesis of piglet diarrhea, and on subclinical growth retardation, will be further investigated. By this way, it will become clear if controlling RVA infections is a profitable manner to reduce postweaning diarrhea and growth retardation in young piglets.

3.3.1. Introduction

In the first weeks of life, piglets are very fragile and sensitive to numerous infections with viruses, bacteria and parasites. After birth, they are housed together with the sow and fed milk, which is rich in secretory immunoglobulin A (SIgA) antibodies, protecting the offspring against enteric infections. However, at the age of 3 to 4 weeks, pigs are weaned and lactogenic immunity rapidly disappears, often leading to the development of postweaning diarrhea (PWD). Rotavirus, a member of the *Reoviridae* family, is considered as an important cause of PWD, together with enterotoxigenic *Escherichia coli* (ETEC). Eight groups (A to H) of rotaviruses have been identified in many species, including humans and pigs, based on nucleotide sequence cut-off values of the VP6 encoding genes [1, 2]. Nonetheless, RVA is considered as epidemiologically and clinically most important. Rotavirus particles have a triple layered capsid, surrounding a double stranded RNA genome of 11 segments. These segments encode 6 structural (VP1-VP4, VP6 and VP7) and 6 non-structural (NSP1-NSP6) proteins. The capsid core (VP2) surrounds the genome and the viral enzymes VP1 and VP3, responsible for transcription and replication. The inner capsid layer consists of VP6 proteins, whereas the outer capsid layer is composed of VP7 and VP4. The latter proteins elicit neutralizing antibodies. Genes encoding VP7 and VP4 can be classified into distinct genotypes using a 80% nucleotide-identity cut-off value [3, 4]. To date, 27 G-genotypes (VP7) and 37 P-genotypes (VP4) have been identified in many species [5, 6]. In a recent study, six different G-types (G2, G3, G4, G5, G9 and G11) and five different P-types (P[6], P[7], P[13], P[23] and P[27]) were found in 12 different G/P combinations in feces of Belgian piglets [7]. Furthermore, RVA infections were detected at a high rate (84%) in diarrheic fecal samples of weaned pigs from the US, Canada and Mexico [8]. Thirty six percent and 26% of diarrheic and non-diarrheic fecal samples of weaned Slovenian piglets tested positive for RVA, respectively [9]. Fu and Hampson (1987) investigated RVA shedding of piglets during the first two months of life by daily fecal sampling and testing for RVA using an enzyme immunoassay. All piglets shed the virus before 40 days of age. In a small number of piglets, RVA was shedded repeatedly with intervals between 1 to 11 days [10]. More recently, a longitudinal study from birth to slaughter was conducted on a Japanese farm, where fecal samples of 10 pigs were collected with 3 to 14 days time-intervals and investigated for the presence of RVA using RT-PCR. It was demonstrated that during their lives pigs become repeatedly infected with RVAs bearing different G/P genotype combinations [11]. However, no quantitative data on RVA shedding were acquired in these studies.

ETECs on the other hand, express fimbriae, by which they attach to the intestinal epithelium. Those expressing F4 or F18 are most predominant after weaning, and after attachment they start colonizing the intestine, followed by expression of thermo-labile (LT) or thermo-stabile (STa or STb) enterotoxines and the development of diarrhea [12]. Moreover, there might exist a synergistic effect between RVAs and ETECs in the pathogenesis of PWD, in which RVAs may pave the way for ETEC infections [13, 14].

In Europe, there are no vaccines available for active immunization of weaning pigs against RVA and ETEC. Since 2006, the use of antimicrobial growth promotants is forbidden in the EU, but soon hereafter an increased incidence of PWD problems was noticed [12, 15]. Nowadays, other antimicrobial substances are prophylactically applied to help control PWD problems. In a study conducted on 50 Belgian pig farms, 93% of all antimicrobial group treatments were prophylactic. Polymyxins were one of the most frequently used classes of antibiotics, the main indication being the prevention of PWD [16]. However, prophylactic use of colistin or other classes of antimicrobial drugs will facilitate the selection of antimicrobial drug resistance, and is harmful for animal and public health on the long run [17]. As an example, acquired colistin resistance was detected in 10% of ETEC isolates from diseased Belgian pigs [18]. As an alternative, zinc oxide addition to the feed at therapeutic concentrations is now increasingly being used on Belgian pig farms during the first 2 weeks after weaning in attempt to prevent PWD. In a model of epithelial cells using the Caco-2 cell line, it was shown that high levels of zinc oxide decrease paracellular epithelial permeability, and reduce the expression of inflammatory cytokines. However, zinc oxide does not directly suppress the growth of ETECs, but may have an inhibitory effect on adhesion of ETEC to the epithelium. Nonetheless, the use of zinc oxide may have a pollutant effect on the environment as well [12, 19, 20].

In the present pilot study, it was aimed to longitudinally investigate the excretion pattern of RVA and *E. coli* on a large Belgian pig farm with a history of diarrheic problems. A better understanding of the excretion dynamics of RVAs in weaned pigs will offer valuable information to better understand the role of RVAs in the pathogenesis of PWD, their effects on piglet growth performances and possible synergisms with ETECs. A better understanding of RVA circulation and its significance in weaned pigs will allow us finally to develop more accurate strategies for diagnosis and durable prevention.

3.3.2. Materials and methods

3.3.2.1. Farm description

A farrow-to-finish farm with 1000 sows located in East-Flanders, Belgium, was visited by the first author prior to the start of the study in January 2014. A strict hygiene protocol was in use, including obliged showering and wearing of on-farm clothes. Information on management, housing, and health control strategies were obtained and facilities were inspected. An all-in-all-out 4 weeks management system was applied. Sows of the Yorkshire breed and Piétrain boars were used for breeding. Gilts were raised in quarantine on the farm. The average number of delivered litters per sow was 2.52, resulting in 38 farrowed pigs per sow/year. The mortality rate in the farrowing house was 12.2%. Gilts and multiparous sows were housed in a separate building. According to the information of the farmer, the most important health problems in the farrowing house were watery diarrhea with a peak incidence one week after birth, coughing and *Streptococcus suis* infections. To overcome diarrheic problems in the farrowing house, gilts and sows were vaccinated at days 60 and 95 of gestation with the inactivated antibacterial vaccine Suiseng (Hipra Lab). Gilts are often fed diarrheic feces five weeks before farrowing as an oral immunization strategy. Suckling pigs are being vaccinated against *Mycoplasma hyopneumoniae* at the age of 13 days. Weaning was performed at 3 weeks of age and piglets were moved to 5 different barns in a single stable (Figure 1). Each barn contained 8 to 16 pens housing 25 pigs per pen.

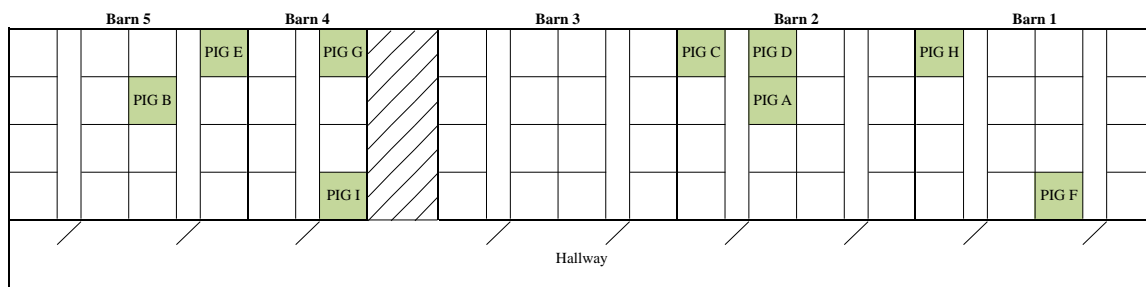


Fig. 1. Overview of the weaning shed and location of selected piglets (grey) in the different pens. The shed consisted of 2 sections (only one visualized), housing piglets of 2 production rounds, separated by a common hallway. Each section consisted of 5 different barns (thick borders), each composed of 8 or 16 pens (thin lines), each housing 25 pigs/pen.

The most important health problems in the weaning barn were bloody diarrhea, acute mortality, coughing and *Streptococcus suis* infections. According to the farmer's information, the average daily weight gain had lowered in the past 2 years. To control PWD,

zinc oxide at therapeutic concentrations was added to feed during the first 10 days after weaning. Furthermore, colistin sulphate (Promycine, VMD) was also added to the feed in the first week postweaning. Drinking water was acidified because of the high pH of drinkingwater.

3.3.2.2. Selection and follow-up of piglets

At the first farm visit, 9 pigs (one per litter), were randomly selected from litters of gilts and multipare sows. Background information about the litters of origin was written down, and is provided in Table 1.

Table 1. Background information of pigs included in the study.

Piglet	Sex	Weight at weaning (kg)	Parity of sow	Litter size	Diarrhea in nursery shed
A	M	5.2	5	19	Yes
B	F	5.8	2	14	Yes
C	F	8.1	4	12	No
D	M	7.2	2	18	No
E	M	5.9	2	19	No
F	F	6.2	1	16	Yes
G	F	4.1	1	16	Yes
H	F	4.2	1	13	No
I	M	3.1	1	16	No

Longitudinal follow-up started at the day of weaning (day 0), and was always executed by the first author. Pigs were weaned in the morning, and the first farm visit was executed in the late afternoon. The farm was visited every 2 days in the first 2 weeks postweaning, and next every 2 to 3 days until 24 days after weaning. Each time, pigs were weighed, alertness was scored, and a score was given based on the fecal consistency (Figure 2). A fecal score of 7 and 6 was considered normal. Two fecal specimens per pig were collected at each timepoint, using a dry cotton swab (Copan). The weight of one swab was determined before and after swabbing, and this swab was used for virological analysis. On arrival at the Laboratory of Virology, 1 ml of phosphate buffered saline containing 1000 U/ml penicillin (Continental Pharma, Puurs, Belgium), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/ml gentamicin (Gibco BRL, Merelbeke, Belgium) and 0.01% v/v Fungizone (Bristol-Myers Squibb, Braine L'Alleud, Belgium) was added to the swab collection tubes and placed on a shaker for 30 min at 4°C to release viral particles from the cotton swab. The fecal suspension was then brought into 1.5 ml eppendorfs and stored at -70°C. The second swab was transported to a private diagnostic laboratory (DGZ Vlaanderen, Torhout, Belgium) for bacteriological analysis.

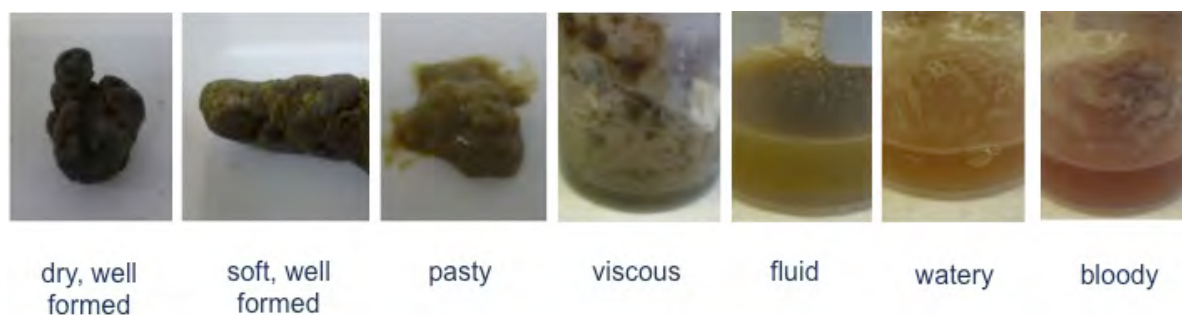


Fig. 2. Fecal scoring system. From left to right: dry (score 7) to bloody (score 1).

3.3.2.3. Quantification of RVA shedding

Viral RNA was extracted out of 140 μ l of fecal swab fluid using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Eluted RNA was stored at -70°C . The presence of RVA was quantified using RT-qPCR, according to previously described procedures [7]. Briefly, RNA was denatured at 95°C for 2 min and immediately brought on ice. RT-qPCR reaction mixtures (20 μ l/reaction) consisted of 10 μ l Precision OneStep qRT-PCR Mastermix with SYBR Green, ROX and Inert blue pipeting dye (Primerdesign, Southampton, United Kingdom), 400 nM (0.8 μ l) of forward primer (Integrated DNA Technologies, Iowa, USA), 50 nM (0.1 μ l) of reverse primer (IDT), 6.1 μ l of nuclease free water and 3 μ l template RNA. Water was used as a negative RT-qPCR control. Standard curves were designed using 10-fold serial dilutions of standard RNA with a range of 6 \log_{10} to 1 \log_{10} copies/reaction in duplicates. Reaction mixtures were brought into Brightwhite 96-well qPCR plates (Primerdesign). Reaction conditions were: 55°C for 10 min, 95°C for 8 min, 40 cycles of amplification (95°C for 10 sec and 60°C for 60 sec) and melt curve analysis. Samples were analyzed in an ABI StepOnePlus qPCR apparatus (Applied Biosystems). Baseline and threshold were set automatically and manually, respectively. Taking into account the weight of the collected feces, the number of RVA copies/g of feces was calculated for each pig at each time point. A sample was considered positive if specific amplification was observed for both repetitions. Samples were quantifiable when Cq values were lower than 30. Cq values between 30 and 35 were considered positive, but not quantifiable, and named as <LOQ.

3.3.2.4. Sequence analysis of VP7 and VP4 encoding genes

RVA strains circulating on the pig farm were characterized by sequencing of the partial VP7 and VP4 encoding genes using RNA extracted from fecal swabs collected at the peak of viral

shedding. RNA was denatured at 95°C for 2 min and chilled on ice. The Qiagen OneStep RT-PCR Kit (Qiagen) was used for amplification of the partial genes using primersets Beg9/End9 or Gen_VP7F/Gen_VP7R for VP7, and VP4_1-17F/VP4R_Deg for VP4, as described previously [7, 21, 22]. Each reaction (25 µl) consisted of 5 µl QIAGEN OneStep RT-PCR Buffer, 1 µl of dNTP Mix, 1 µl of QIAGEN OneStep-RT-PCR enzyme mix, 1.5 µl of forward and reverse primer (830 nM), 10 µl of nuclease free water and 5 µl of RNA template. Reaction conditions were as follows: 50°C for 30 min, 94°C for 15 min, followed by 35 cycles of amplification: 94°C for 30 sec, 48°C for 30 sec and 72°C for 2 min. A final elongation step was performed at 72°C for 10 min. Afterwards, 9 µl of PCR product was mixed with 1 µl of loading buffer and loaded on polyacrylamide gels. Electrophoresis was performed at 200V during 36 min, followed by ethidium bromide staining of the gels for detection of positive samples. Five µl of each positive PCR product was treated with 1 µl of USB ExoSAP-IT[®] PCR Product Cleanup (Affymetrix) and Sanger sequenced with the forward primer using the ABI Prism BigDye terminator cycle sequencing reaction kit (ABI Prism 3130xl). Sequences were corrected using 4Peaks software and RVA VP7 and VP4 genotypes were determined using BLAST and RotaC^{2.0}.

3.3.2.5. Bacteriological analysis of fecal samples

Diagnosis of *Escherichia coli* was performed at a private diagnostic laboratory (DGZ Vlaanderen, Torhout, Belgium). Fecal swabs were inoculated on MacConkey agars, followed by incubation at 37°C under aerobic conditions. A swab was also inoculated on a blood agar, to determine if *Escherichia coli* isolates were able to induce hemolysis. The number of colonies per plate was counted and scored as follows: <10 colonies/plate (1), 10-50 colonies/plate (2) or >50 colonies/plate (3).

3.3.2.6. Statistical analysis

To compare the viral shedding during 2 different viral infections, the area under the curves (AUC) were calculated. For each individual infection, the number of RNA copies/g at the moment of highest viral shedding (t_{max}), and viral loads at one timepoint before and on the timepoint after this t_{max} , were used to calculate AUC values by drawing trapezoids. The AUC for each trapezoid was calculated using the formula: $((y_1+y_2)/2) \times (t_2-t_1)$ in Excel (Microsoft). Non-log transformed viral loads (copies/g) were used for this analysis. A Wilcoxon matched-pairs signed rank test was used to analyze the difference between the two infections for all animals. A p-value <0.05 was considered significant. Furthermore, a correlation (Spearman's correlation) was investigated between the severity of viral

replication in the first week, defined as the AUC between days 2 and 8, and the daily weight gain of the pigs in this period. Graphs were generated using Graphpad Prism 6.

3.3.3. Results

3.3.3.1. Fecal score

In all pigs, dry (score 7) or soft (score 6), but well formed feces were predominantly observed throughout the whole study period (Figure 3). However, a limited number of episodes of reduced fecal consistency were observed in some pigs, but these lasted only 1 or 2 days and fecal scores were not drastically reduced. As such, explosive diarrheic problems were not observed at the herd level.

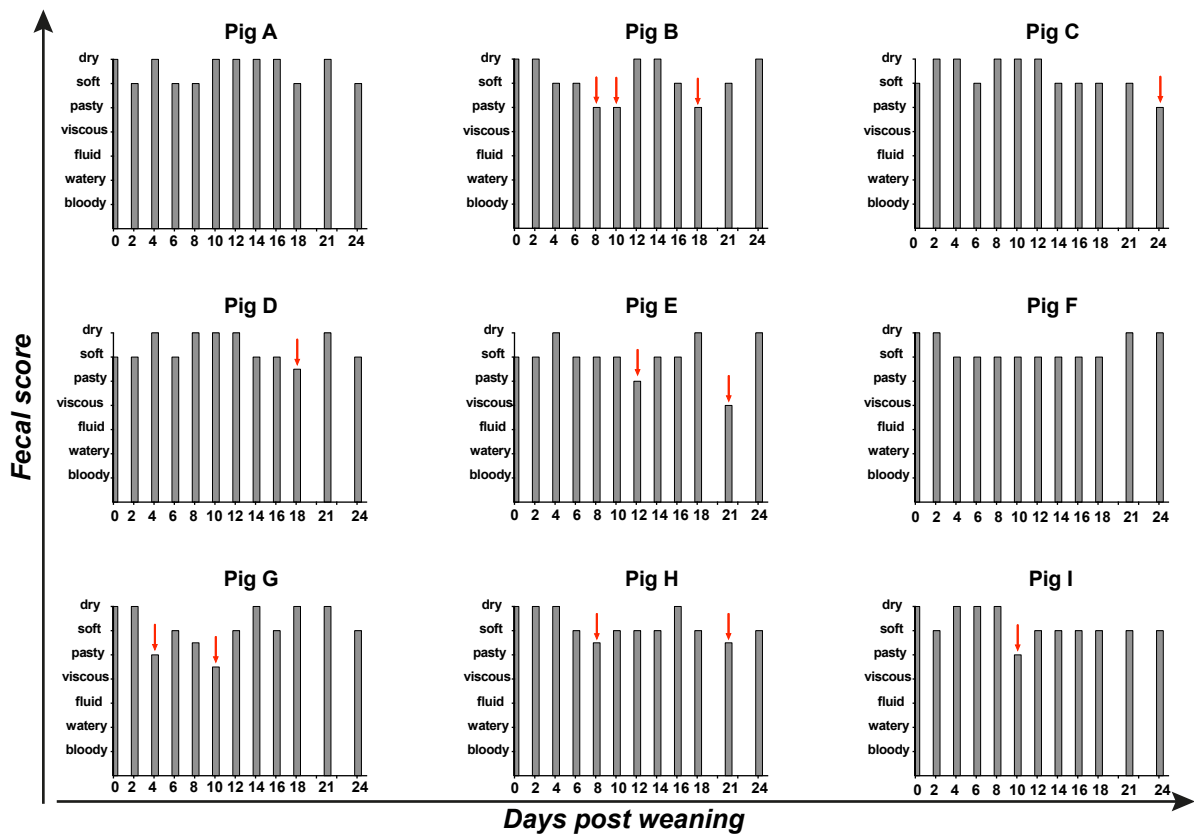


Fig 3. Fecal score. Arrows indicate episodes of reduced fecal consistency (score <6).

3.3.3.2. Pig growth

The absolute weights and daily weight gains of the pigs at different timepoints are demonstrated in Table 2. Weaning weight varied between 3.1 to 8.1 kg, and was 5.5 kg on average. These weight differences could still be observed at the end of the study period, with an average pig weight of 11.3 kg, varying between 7.7 to 16.8kg. Not surprisingly, daily

weight gain was lowest in the first week after weaning (average 89 g/day), but then gradually increased. However, average daily weight gain over the 24 study period varied largely between 125-358 g/day (average 241 g/day). Absolute individual growth curves of each pig are shown in Figure 4.

Table 2. Weights and daily weight gains of piglets.

Piglet	Weight of piglet (kg) at day				Daily weight gain (g/day) between days			
	0	6	14	24	0-6	6-14	14-24	0-24
A	5.2	4.7	5.5	8.2	-83	100	270	125
B	5.8	6.7	8.6	13.0	150	238	440	300
C	8.1	8.4	12.0	16.7	50	450	470	358
D	7.2	7.7	9.2	12.4	83	188	320	217
E	5.9	6.7	7.7	10.7	133	125	300	200
F	6.2	7.0	8.9	13.6	133	238	470	308
G	4.1	4.8	6.8	10.2	117	250	340	254
H	4.2	5.0	6.9	9.3	133	238	240	213
I	3.1	3.6	5.2	7.7	83	200	250	192
Average	5.5	6.1	7.9	11.3	89	225	344	241

3.3.3.3. RVA shedding by weaning pigs

Rotavirus excretion patterns are shown in Figure 4 for each individual pig. RVA shedding was observed in 6 pigs at the day of weaning, but at low viral loads (<LOQ to 5.91 log₁₀ copies/g feces). These positive piglets were nursed by gilts (piglet F to I) or high parity sows (piglet A and C). RVA shedding was not observed in the three pigs (pig B, D and E) farrowed by second parity sows. Rapidly after weaning, all pigs clearly excreted RVA in their feces. The peak of viral shedding was generally observed at 4 to 6 days postweaning, whereas in pig A this peak lasted until 8 days after weaning. RVA replication typically lasted until 8-12 days postweaning, but the duration of viral excretion was variable between pigs. All the RVA strains excreted in the beginning of the weaning period beared the G5P[13] genotype combination.

Shortly after the first infection wave, a second infection with RVAs was demonstrated in all pigs. The onset of the second replication gulf coincided with the end of the first replication wave. During this second replication phase, a G9P[23] strain was excreted by almost all pigs, except piglet D, which excreted a G5P[23] strain. The G-genotype and P-genotype of the strains shed by pigs H and B, respectively, could not be assessed. In some pigs, it seemed like a third replication wave was occurring, but RVA shedding was only observed at very low titers. The AUC of the first replication gulf was significantly ($p=0,0195$) higher than the

AUC of the second replication wave. Piglet A demonstrated the poorest growth and demonstrated the highest overall RVA replication. Nonetheless, taking into account all piglets, there was no statistical correlation between the daily weight gain (2-8 days) and the AUC of RVA shedding in this time interval.

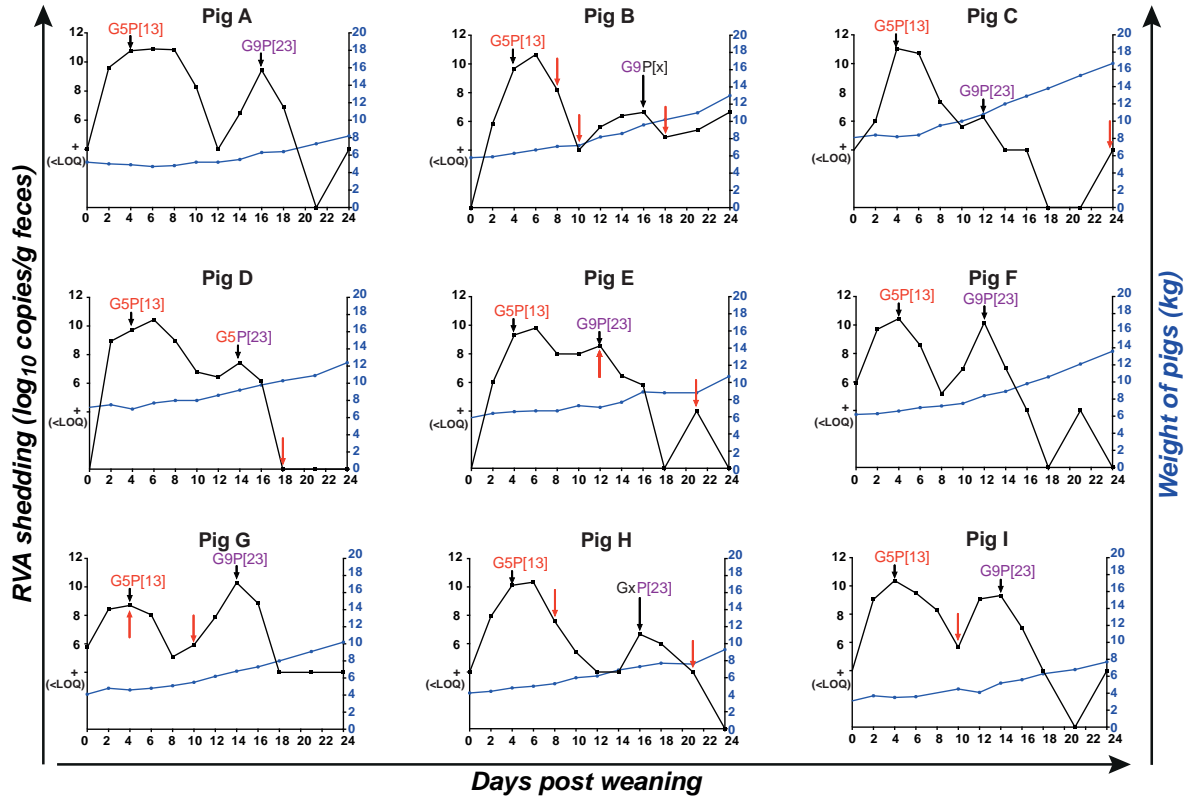


Fig. 4. Rotavirus shedding and absolute growth curves of individual pigs. Episodes of reduced fecal consistency are shown by a red arrow. The G/P genotype combination of the strain found during each infection is mentioned above the timepoint (black arrow) of which the sample was used for genetic characterization.

3.3.3.4. Shedding of *Escherichia coli*

Bacteriological analysis was performed on fecal samples collected during the first 2 weeks postweaning. Variable numbers of colonies of non-hemolytic *E. coli*'s could be isolated from the fecal samples of every pig at almost every timepoint. An exception was pig C, from which no bacteria could be isolated on day 4. Hemolytic *E. coli* infections were less frequently observed, and only occurred in a limited number of piglets (D, F and H). On day 2 postweaning, a small number of hemolytic *E. coli* colonies were isolated from feces of piglet D and F. A higher number of colonies could be isolated from pig H at day 2, but also at days 8 and 14 after weaning.

3.3.4. Discussion

Rotavirus and *Escherichia coli* are important causes of PWD, leading to reduced weight gain, increased mortality and increased administration of antimicrobial drugs. Here, a longitudinal study was conducted to investigate the role and importance of RVA infections in the pathogenesis of piglet diarrhea, and to study the existence of a possible synergistic effect between the virus and *E. coli*. A single relatively large farm housing 1000 sows was selected for longitudinal follow-up of weaned piglets. A thorough evaluation was conducted prior to the start of the study. Diarrheic problems and reduced growth in the weaning barns were relatively common. In an attempt to prevent the emergence of PWD, zinc oxide and colistin were administered prophylactically to the feed, and drinking water was acidified.

Using RT-qPCR, it was possible for the first time to quantitatively assess RVA shedding by weaned piglets under field conditions [7]. Nine piglets were selected from the maternity barns and initial follow-up was planned to be executed during the first 14 days after weaning. Eventually, it was demonstrated that pigs were still shedding the virus at day 14 postweaning, and therefore the follow-up period was extended until all piglets more or less stopped shedding virus. Surprisingly, six piglets were already shedding RVA at the day of weaning, albeit at relatively low viral loads (< LOQ to 5.91 copies/g). Most likely, these infections were obtained from the farrowing house. In general, lactogenic immunity provided by gilts is less well developed, as they generally have not been exposed to RVA infections regularly circulating in the farrowing house [23]. Indeed, four of these RVA positive piglets were farrowed by gilts, whereas the three RVA negative piglets were farrowed by second parity sows. Remarkably, 2 of the positive piglets were nursed by high parity sows. It could be hypothesized that piglets from sows with a very high parity (4 to 5) are also less well protected against RVA infections than piglets farrowed by second or third parity sows. If sows of second or third parity offer a good maternal protection, suckling piglets are better protected against RVA infections and overall incidence of RVA infections in these litters will be reduced. This may lead to a reduced number of reinfections for the sow as well, and consequently the sow's local intestinal immune system is not boosted by the virus and lactogenic immunity is hampered. Eventually, the offspring becomes more susceptible for RVA infections again. Nonetheless, this hypothesis should be investigated further in more pigs from more farms.

All piglets clearly demonstrated two waves of RVA replication during the 24 days follow-up period. Characterization of the genes encoding outer capsid proteins VP7 and VP4 of RVAs from the first replication wave demonstrated the circulation of a G5P[13] strain in all pigs.

The second replication wave was the result of an infection with another RVA strain bearing a G9P[23] combination. However, a G5P[23] strain was recovered from pig D at day 14 postweaning, which likely was an on-farm reassortant virus. Generally, reassortment is relatively uncommon for human RVAs, but the chance for successful formation of a reassortant RVA may be higher in pigs, as their production process is more intensive and they become repeatedly infected with different RVA strains during their lives [24-27]. Indeed, reinfections with genetically heterogeneous RVA strains have been observed on Japanese pig farms as well, and the occurrence of on-farm reassortant RVAs was also noticed [11, 28]. A significant difference between the amount of RVA excreted during these 2 separate replication waves in the individual pigs was noticed. In general, the infection with the second RVA strain was less pronounced and shorter than the first replication wave, suggesting that some cross-protection between both strains was already established shortly after infection with the first strain.

Weaning weights of the piglets were highly variable, which also had an impact on the daily weight gain during the first weeks after weaning, and weights reached by pigs at the end of the study. No statistical correlation between RVA shedding and the daily weight gain of the piglets could be identified in the present study. Still, it might be that a high level of RVA replication can have a negative impact on the growth of young piglets. As an example, piglet A experienced the highest RVA excretion of all pigs in the first week, and daily weight gain was clearly hampered in this pig. Nonetheless, it is generally observed that pigs show a reduced weight gain shortly after weaning. Important influencing factors are stress, reduced appetite, the change from a liquid to solid diet, and subclinical or clinical infections with pathogens. Indeed, negative effects on daily weight gain have also been described for porcine circovirus type 2 infections [29]. A more elaborate study will be conducted to further investigate the role of RVA infections on these reduced growth performances shortly after weaning.

Despite the clear replication of 2 different RVA strains in all piglets, no explosive diarrheic problems were noticed. Only a couple of pigs showed the excretion of hemolytic *E. coli*'s, whereas all piglets were shedding non-hemolytic *E. coli*'s. However, hemolysis is not a good predictor for pathogenicity of ETECs, and it would be better to investigate the expression of virulence factor encoding genes as well in future studies [30]. As an example, real-time PCR assays targeting genes encoding F4 and F18 fimbriae may be a good alternative to assess the virulence of ETEC infection in weaned piglets, and would directly allow to absolute quantify the number of excreted ETECs. The kinetics of RVA and ETEC excretion would facilitate to

investigate the existence of a possible synergism between both pathogens in the pathogenesis of PWD as well.

Despite the detection of RVA and *E. coli*'s in fecal samples of all pigs, the situation was kept well under control on the farm, which can be explained by several hypotheses. The use of colistin may have suppressed the proliferation of pathogenic ETECs, whereas zinc oxide may have had a beneficial effect on the permeability of the intestinal tract. Still, it remains remarkable that RVA replication itself did not induce severe enteritis. Moreover, the G9P[23] may have been more virulent than the first strain, but due to the rapid induction of protective immunity, the development of clinical symptoms could be prevented. It could also be that these G5P[13] and G9P[23] RVA strains were less virulent, although they have already been isolated from diarrheic piglets as well [7]. However, other viral proteins than those encoded by VP7 and VP4 genes may be involved in virulence as well.

It will be further investigated on more pig farms if different RVA strains can be assigned to different pathotypes, such as pathogenic (diarrheic), subclinical (growth reduction) or apathogenic (no symptoms), and if they behave synergistic with some ETEC strains in the pathogenesis of piglet diarrhea. This research will be expanded to other age groups as well, including suckling pigs and pigs after weaning until they reach the prefattening stages. Reducing the incidence of diarrhea and obtaining optimal growth performances is very important on pig farms, as piglets can be transmitted more easily and earlier to subsequent production stages. Controlling RVA infections may be a durable alternative to reduce the use of antimicrobial drugs and zinc oxide for the prevention of PWD and growth retardation after weaning. Therefore, more research to further sustain these hypotheses is needed, as it will benefit the pig and human health on the long term.

3.3.5. Acknowledgments

The authors are grateful to the farmer to allow us to collect samples from his pigs. Furthermore, the authors thank technician Ytse Noppe for here excellent technical assistance during the processing of the fecal samples. ST was supported by a fellowship of the Special Research Fund of Ghent University. M.Z. and I.D.M.R. were supported by doctoral fellowships from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). L.M.B.D. was supported by a doctoral fellowship from the Research Foundation-Flanders (FWO Vlaanderen).

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Chapter IV

Genetic characterization of Belgian pig rotaviruses and other viruses

4.1. Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotavirus (2015). Sebastiaan Theuns¹, Elisabeth Heylen², Mark Zeller², Lowiese M.B. Desmarets¹, Inge D.M. Roukaerts¹, Marc Van Ranst², Hans J. Nauwynck^{1*} and Jelle Matthijssens^{2*}. *Journal of Virology* **85** (2), 1043-1057

4.2. The fecal virome of a non-diarrheic Belgian piglet contains group A and C rotaviruses, and an astrovirus and enterovirus (2015). Sebastiaan Theuns¹, Nádia Conceição-Neto, Elisabeth Heylen, Mark Zeller, Inge D.M. Roukaerts, Lowiese M.B. Desmarets, Marc Van Ranst, Hans J. Nauwynck* and Jelle Matthijssens*. Manuscript in preparation

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Chapter 4.1.

Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses

Summary

Group A rotaviruses (RVAs) are an important cause of diarrhea in young pigs and children. An evolutionary relationship has been suggested to exist between pig and human RVAs. This hypothesis was further investigated by phylogenetic analysis of the complete genomes of six recent (G2P[27], G3P[6], G4P[7], G5P[7], G9P[13], and G9P[23]) and one historic (G1P[7]) Belgian pig RVA strains, and of all completely characterized pig RVAs around the globe. In contrast to the large diversity of genotypes found for the outer capsid proteins VP4 and VP7, a relatively conserved genotype constellation (I5-R1-C1-M1-A8-N1-T7-E1-H1) was found for the other 9 genes in most pig RVA strains. VP1, VP2, VP3, NSP2, NSP4 and NSP5 genes of porcine RVAs belonged to genotype 1, which is shared with human Wa-like RVAs. However, for most of these gene segments, pig strains clustered distantly from human Wa-like RVAs, indicating that viruses from both species have entered different evolutionary paths. However, VP1, VP2 and NSP3 genes of some archival human strains were moderately related to pig strains. Phylogenetic analysis of the VP6, NSP1 and NSP3 genes, as well as amino acid analysis of the antigenic regions of VP7 further confirm this evolutionary segregation. The present results also indicate that the species barrier is less strict for pig P[6]-strains, but that chances for successful spread of these strains in the human population are hampered by the better adaptation of pig RVAs to pig enterocytes. However, future surveillance of pig and human RVA strains is warranted.

4.1.1. Introduction

Enteric diseases in pigs are mostly encountered during two critical time-points, in the suckling period and after weaning. Several pathogens are frequently involved in the pathogenesis of piglet diarrhea, including rotavirus, porcine epidemic diarrhea virus, transmissible gastroenteritis virus, *Escherichia coli*, *Clostridium perfringens*, *Salmonella* sp., *Brachyspira* sp. and *Isospora suis*. Furthermore, co-infections between different rotaviruses and other pathogens can be found frequently in diarrheic pigs [1].

Rotaviruses are a major cause of diarrhea in many species, including pigs and humans. Five official rotavirus species (A to E) and 3 tentative species (F-H) have been established based on nucleotide similarities of the VP6 encoding genes [2]. Species A, B and C are frequently isolated from feces of diarrheic and non-diarrheic pigs, whereas rotavirus group E was only detected once in a pig fecal sample [3]. Rotavirus species H also seems to be epizootologically important in pigs, but its role in the pathogenesis of piglet diarrhea remains to be elucidated [4, 5]. Nevertheless, group A rotaviruses (RVAs) are considered as the most important rotavirus species in pigs and humans.

The RVA genome possesses 11 segments of dsRNA, encoding for 6 structural (VP1-VP4, VP6 and VP7) and 6 non-structural proteins (NSP1-6). Genes encoding outer capsid proteins VP7 and VP4 are of utmost importance, because these proteins can induce neutralizing antibodies [6-8]. For VP7 and VP4, 27 G- and 37 P-genotypes, respectively, have been recognized in many host species so far [8, 9]. In feces from pigs, 12 different G-genotypes (G1 to G6, G8 to G12 and G26) and 13 different P-types (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], P[34]) have been found worldwide [8, 10, 11]. Recently, the circulation of genotypes G2, G3, G4, G5, G9 and G11 in combination with P[6], P[7], P[13], P[23] or P[27], has been demonstrated in Belgium [1]. Characterization of the oldest Belgian pig RVA strain (RVA/Pig-tc/BEL/RV277/1977/G1P[7]) isolated in 1977, identified the rare G1P[7] genotype combination. This strain was isolated from a pool of watery feces of 3 piglets of 2 days old that were kept in isolation for experimental purposes. Four days after isolation, they showed symptoms of watery diarrhea, dehydration and finally died [1, 12]. The vast genetic diversity seen among genes encoding the outer capsid proteins of pig strains is in contrast with the relatively restricted number of G/P combinations found in human RVA strains.

Due to the segmented nature of the rotavirus genome, a full genome-based classification system has been established for RVA. As such, each gene is abbreviated by a letter followed by a number designating the genotype. The genotype constellation of each RVA strain can

be presented as follows: G_x-P_[x]-I_x-R_x-C_x-M_x-A_x-N_x-T_x-E_x-H_x, which stands for genotypes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 [7]. Complete genome analysis of ancient cell-culture grown pig RVA strains (RVA/Pig-tc/USA/OSU/1977/G5P[7], RVA/Pig-tc/USA/Gottfried/1983/G4P[6], RVA/Pig-tc/MEX/YM/1983/G11P[7], RVA/Pig-tc/IND/RU172/2002/G12P[7]), possessing different G/P combinations for VP7 and VP4, identified in most cases a I5-R1-C1-M1-A8-N1-T1-E1-H1 genetic backbone for the other viral genes [13, 14].

Most human RVA strains carry one of the 2 major genotype constellations, G_x-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1 or G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2, and are called Wa-like or DS-1 like strains, respectively [15]. Pig and human Wa-like RVA strains are suggested to possess a common evolutionary ancestor, because genotype 1 genes can be found in strains from both species [6]. However, the restricted number of complete genomes of contemporary wild-type pig RVA strains makes it difficult to come to sound conclusions. To obtain better insights in the evolutionary relationship between pig and human RVAs, more completely characterized pig RVA genomes are necessary. A number of pig RVA strains from Canada, Thailand, South Korea and Italy, possessing different VP7/VP4 genotype combinations, have been characterized to date. Most of these strains also possessed an I5-C1-R1-M1-A8-N1-T1-E1-H1 genotype constellation for the 9 remaining genes [16-19]. However, the T7 genotype for NSP3 has frequently been detected in these studies as well [17, 18]. A rarity was the detection of the dual I5+I14 genotype for VP6 in a G2P[34] strain from Canada, which also possessed the rarely detected E9 genotype for NSP4 [17]. VP7 and VP4 genes of Belgian strains have recently been characterized, but information about the genetic composition of the other 9 genes of these Belgian strains was lacking. One exception is the completely characterized genome of strain RVA/Human-wt/BEL/BE2001/2009/G9P[6] isolated from a Belgian child, which has been suggested to be a pig-to-human interspecies transmission event [20].

In the present study, it was aimed to characterize the complete genomes of a selection of Belgian pig RVAs possessing a wide diversity of VP7 and VP4 genotypes. In addition, historic strain RV277 (G1P[7]) was included. An increased availability of complete pig RVA genomes will not only provide useful information for the development of preventive measures against pig RVA infections, but will also lead to a better understanding of the evolutionary relatedness between pig and human RVAs. This will facilitate recognition of newly emerging or reassorted pig-derived strains in humans.

4.1.2. Materials and methods

4.1.2.1. Selection of rotavirus strains

Fecal samples from diarrheic and asymptomatic piglets were collected on Belgian pig farms in 2012, as reported before [1]. Six contemporary pig RVA strains were included for complete genome analysis in the present study (Table 1). In the present study we aimed to include strains with representative G/P combinations from the previous study, and to cover at least all unique VP4 and VP7 genotypes.

Five samples were obtained from diarrheic pigs (12R002, 12R005, 12R006, 12R041 and 12R0046), whereas one sample (12R022) was obtained from a non-diarrheic pig. From these, fecal suspensions were used as starting material for RNA isolation and RT-PCR. In addition, a historic Belgian pig RVA strain (isolated in 1977) was also completely characterized to analyze if this strain was genetically similar to contemporary pig strains. Cell culture supernatant of the first passage of strain RV277 in MA104 cells was used as starting material for RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and RNA was stored at -70°C.

Table 1. Genotypes of VP7/VP4, and Genbank accession numbers of pig strains analyzed in the present study.

	12R002 G5P[7]	12R005 G4P[7]	12R006 G3P[6]	12R022 G2P[27]	12R041 G9P[13]	12R046 G9P[23]	RV277 G1P[7]
VP1	KM820695	KM820696	KM820697	KM820698	KM820699	KM820700	KM820701
VP2	KM820702	KM820703	KM820704	KM820705	KM820706	KM820707	KM820708
VP3	KM820709	KM820710	KM820711	KM820712	KM820713	KM820714	KM820715
VP4	KM820716	KM820717	KM820719	KM820721	KM820718	KM820720	KM820722
VP6	KM820723	KM820724	KM820725	KM820726	KM820727	KM820728	KM820729
VP7	KM820730	KM820731	KM820732	KM820733	KM820734	KM820735	KM820736
NSP1	KM820737	KM820738	KM820739	KM820740	KM820741	KM820742	KM820743
NSP2	KM820667	KM820668	KM820669	KM820670	KM820671	KM820672	KM820673
NSP3	KM820674	KM820675	KM820676	KM820677	KM820678	KM820679	KM820680
NSP4	KM820681	KM820682	KM820683	KM820684	KM820685	KM820686	KM820687
NSP5	KM820688	KM820689	KM820690	KM820691	KM820692	KM820693	KM820694

4.1.2.2. Reverse transcriptase polymerase chain reaction

RNA was denatured at 95°C for 2 min and immediately chilled on ice. For the shorter gene segments (VP6, VP7, NSP2, NSP3, NSP4 and NSP5), reaction mixtures consisted of 5 µl RNA, 5 µl of 5x QIAGEN OneStep RT-PCR Buffer, 1 µl of dNTP Mix, 1.5 µl of forward and reverse primer (830 nM), 1 µl of QIAGEN OneStep RT-PCR enzyme mix, and nuclease

free water in a total volume of 25 μ l per reaction. Reaction volumes were upscaled to 50 μ l for the longer gene segments (VP1, VP2, VP3, VP4, and NSP1). Primers used for RT-PCR amplification of the complete gene segments can be made available upon request to the authors.

Reverse transcriptase was performed at 50°C for 30 min, followed by *Taq* polymerase activation at 94°C during 15 min followed by 35 cycles of amplification. For the longer fragments, denaturation was performed for 30 sec at 94°C, annealing for 30 sec at 50°C, and extension at 72°C during 6 min. For the shorter fragments denaturation was performed for 30 sec at 94°C, annealing for 30 sec at 47°C, and extension at 72°C during 2 min. A final extension step was performed for 10 min at 72°C. Afterwards, 9 μ l of PCR product was mixed with 1 μ l of loading buffer and loaded on polyacrylamide gels. Electrophoresis was performed at 200V during 36 min, followed by ethidium bromide staining of the gels for detection of positive samples.

4.1.2.3. Sanger sequencing

Five microliter of PCR positive samples was treated with 1 μ l of USB ExoSAP-IT[®] PCR Product Clean-Up (Affymetrix, Santa Clara, California, USA) and sequenced with an ABI Prism BigDye terminator cycle sequencing reaction kit (ABI Prism 3130xl, Applied Biosystems) using forward and reverse primers. Next, remaining parts of the coding regions were further covered by primer-walking sequencing. The 5' and 3' terminal sequences were obtained using a modified version of the single-primer amplification method [21]. Briefly, a modified amino-linked oligonucleotide (TGP-Linker, 5'-PO₄-TTCCTTATGCAGCTGATCACTCTGTGTCA-spacer-NH₂-3') was linked to the 3' end of both strands of denatured RNA using T4 RNA Ligase (Promega, Leiden, The Netherlands). Next, RT-PCR was performed using an internal gene-specific primer and primer TGP-3Out. First, reverse transcriptase was performed at 45°C for 30 min followed by PCR activation at 95°C for 15 min. During 45 min, temperature was gradually lowered from 83°C to 60°C, 10 min at 72°C, followed by 40 cycles of amplification at 94°C for 45 sec, 45°C for 45 sec and 70°C for 1 min. A final extension was performed at 70°C during 7 min. Products were separated by polyacrylamide gel electrophoresis during 18 min at 200V, followed by sequencing.

4.1.2.4. Phylogenetic analysis

Sequence analysis was performed using 4Peaks (Mekentosj, Amsterdam, The Netherlands), and genotypes for all genes were determined using BLAST and RotaC^{2.0} [22]. Multiple

sequence alignments were performed using the ClustalW plug-in in MEGA 5.2.2, followed by manual editing.

For each gene, maximum-likelihood phylogenetic trees were constructed and bootstrap-analysis was set at 500 replicates. Substitution models were determined for each gene separately in MEGA 5.2.2. Pairwise distances were calculated using the p-distance model in MEGA 5.2.2.

4.1.2.5. Genbank accession numbers

Nucleotide sequences were uploaded in Genbank and accession numbers are provided in Table 1.

4.1.3. Results

4.1.3.1. Genotype constellation of Belgian pig group A rotaviruses

The genotype constellations of all Belgian pig RVAs are shown in Table 2. Whereas a wide variety of G/P genotypes was detected for the VP7 and VP4 encoding genes, the following constellation was dominantly found for the other genes of Belgian pig rotaviruses: Gx-P[x]-I5-R1-C1-M1-A8-N1-T7-E1-H1. For the inner capsid protein of ancient strain RV277, an I1 genotype was encountered. Although the T7 genotype for NSP3 was found in the majority (n=4/7) of Belgian strains, the T1 genotype was also encountered in 3 out of 7 RVA strains. Furthermore, an E9 genotype was found for NSP4 in non-diarrheic strain 12R022.

4.1.3.2. Phylogenetic analysis of genes encoding structural proteins

4.1.3.2.1. Outer capsid proteins (VP7 and VP4)

A maximum-likelihood phylogenetic tree was constructed for VP7, using the general time reversible model with gamma distribution and invariant sites. Belgian pig strains of the present study were assigned to 6 different G-genotypes (G1, G2, G3, G4, G5 and G9) for the glycosylated outer capsid protein VP7 (Figure 1). Overall, a high genetic diversity was demonstrated by phylogenetic analysis of the VP7 encoding genes. Of interest, for genotypes which are shared between pig and human RVA strains, such as G1, G2, G3, G4 and G9, the genetic distance between pig and human strains was high. As an example, ancient strain RV277 clustered distantly from contemporary human G1 strains (84.7 to 86.8% nucleotide similarity). However, this strain was most closely related to the rare human G1P[6] strain RVA/Human-tc/JPN/AU-19/1997/G1P[6] (94.8%). This strain belonged to an animal-like

Table 2. Genotype constellation of completely characterized pig RVA strains^a.

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5	Ref
RVA/Pig-tc/BEL/RV277/1977/G1P[7]	G1	P[7]	I1	R1	C1	M1	A8	N1	T7	E1	H1	TS
RVA/Pig-wt/BEL/12R022/2012/G2P[27]	G2	P[27]	I5	R1	C1	M1	A8	N1	T7	E9	H1	TS
RVA/Pig-wt/BEL/12R006/2012/G3P[6]	G3	P[6]	I5	R1	C1	M1	A8	N1	T1	E1	H1	TS
RVA/Pig-wt/BEL/12R005/2012/G4P[7]	G4	P[7]	I5	R1	C1	M1	A8	N1	T7	E1	H1	TS
RVA/Pig-wt/BEL/12R002/2012/G5P[7]	G5	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1	TS
RVA/Pig-wt/BEL/12R041/2012/G9P[13]	G9	P[13]	I5	R1	C1	M1	A8	N1	T7	E1	H1	TS
RVA/Pig-wt/BEL/12R046/2012/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	TS
RVA/Pig-wt/CAN/F8-4/2006/G2P[6]/[7]	G2	P[6/7]	I5	R1	C1	M1	A8	N1	T7	E1	H1	[17]
RVA/Pig-wt/CAN/AB82/2006/G2P[34]	G2	P[34]	I5/I14	R1	C1	M1	A8	N1	T7	E9	H1	[17]
RVA/Pig-tc/VEN/A131/1988/G3P[7]	G3	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1	[6]
RVA/Pig-wt/THA/CMP29/08/2008/G3P[13]	G3	P[13]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[19]
RVA/Pig-wt/THA/CMP40/08/2008/G3P[23]	G3	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[19]
RVA/Pig-wt/THA/CMP48/08/2008/G3P[23]	G3	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[19]
RVA/Pig-tc/USA/Gottfried/1983/G4P[6]	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1	[13]
RVA/Pig-tc/USA/OSU/1977/G5P[7]	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1	[13]
RVA/Pig-tc/KOR/PRG9121/2006/G9P[7]	G9	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[16]
RVA/Pig-wt/CAN/F7-4/2006/G9P[7]/[13]	G9	P[7/13]	I5	R1	C1	M1	A8	N1	Tx	E1	H1	[17]
RVA/Pig-wt/THA/CMP45/08/2008/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T7	E1	H1	[19]
RVA/Pig-tc/KOR/PRG921/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[16]
RVA/Pig-tc/KOR/PRG9235/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[16]
RVA/Pig-tc/KOR/PRG942/2006/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[16]
RVA/Pig-wt/ITA/2CR/2009/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T7	E1	H1	[18]
RVA/Pig-wt/ITA/3BS/2009/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[18]
RVA/Pig-wt/ITA/7RE/2009/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[18]
RVA/Pig-tc/CHN/NMTL/2008/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[23]
RVA/Pig-tc/MEX/YM/1983/G11P[7]	G11	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1	[13]
RVA/Pig-tc/VEN/A253/1988/G11P[7]	G11	P[7]	I5	R1	C2	M1	A1	N1	T1	E1	H1	[6]
RVA/Pig-wt/CAN/F6-4/2006/G11P[13]	G11	P[13]	Ix	Rx	Cx	M1	A8	N1	T7	Ex	Hx	[17]
RVA/Pig-tc/IND/RU172/2002/G12P[7]	G12	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1	[14]

^aBelgian strains are shown in bold. Color code: blue, typical pig genotype; green, Wa-like genogroup; red, DS-1-like genogroup. TS is this study.

cluster within the G1 genotype, but still RV277 clustered distantly from other pig and bovine G1 strains (85.1 to 90.4%). Moreover, pig G2 strains clustered even more distantly from human strains (78.7 to 81.0%). One strain, RVA/Pig-wt/CAN/F8-4/2006/G2P[6]/[7], clustered in between pig (79.2 to 80.1%) and human RVA strains (80.4 to 81.5%). Strain 12R022 was most related to a Spanish G2 strain RVA/Pig-wt/ESP/34461-4/2003/G2P[23]

(93.0%). While G3 strains can also be encountered in different species, pig G3 strains were slightly more related to human G3 strains than to G3 strains from cats and horses (79.2 to 81.9%), albeit at a high genetic distance (86.1 to 88.8%). Different lineages within G4 have also been described, and contemporary human G4 strains have been shown to be genetically distinct from pig G4 strains. As an example, Belgian strain 12R005 clustered distinct from contemporary human G4 strains (83.0 to 83.7%). However, this strain clustered together with pig-like human G4P[6] RVA strains from Hungary (85.0 to 87.2%). The highest relatedness between pig and human strains was demonstrated within the G9 genotype. The two included pig G9 strains were 93.2% similar to contemporary human G9 strains from the major subcluster of lineage III. Both G9 strains were also highly related to the Belgian pig-like human strain RVA/Human-wt/BEL/BE2001/2009/G9P[6] (95.9 to 96.2%). Furthermore, genetic diversity within the G5 genotype was high and strain 12R002 clustered most closely to Korean strain RVA/Pig-wt/KOR/187-1/2006/G5P[7] (87.1%).

For construction of the VP4 maximum-likelihood tree, the Hasegawa-Kishino-Yano model with gamma distribution and invariant sites was used. Phylogenetic analysis of the encoding genes showed that some genotypes clustered more closely together than others (Figure 1). As an example, one cluster was composed of the major human genotypes P[4], P[8], and genotypes P[6] and P[19], which have been detected both in pigs and humans. Within P[6], Belgian strain 12R006 clustered distantly from the contemporary human P[6] strains (81.1 to 82.3%). In contrast, the Belgian pig strain was most closely related to pig-like human strains from Belgium and Hungary (90.6 to 97.0%). The other P-genotypes which are mainly encountered in animals, made up several other groups of genotypes. Three Belgian P[7] strains were included in the present study. Contemporary strains 12R002 and 12R005 clustered together (90.6%), but were distantly related to ancient strain RV277 (85.6 to 88.6%), which clustered most closely to ancient strain OSU and a Korean strain (95.0%). The genetic diversity in the P[13] genotype was apparent, with the presence of multiple lineages. Intra-genotypic heterogeneity was also high in genotype P[23]. Belgian strain 12R046 clustered most closely to strain RVA/Pig-tc/CHN/NMTL/2008/G9P[23] from China (93.2%). One P[27] strain, 12R022, was included in the present study, but only a partial VP8* sequence could be obtained. This strain was relatively closely (89.9%) related to strain RVA/Pig-wt/THA/CMP034/2000/G2P[27] from Thailand.

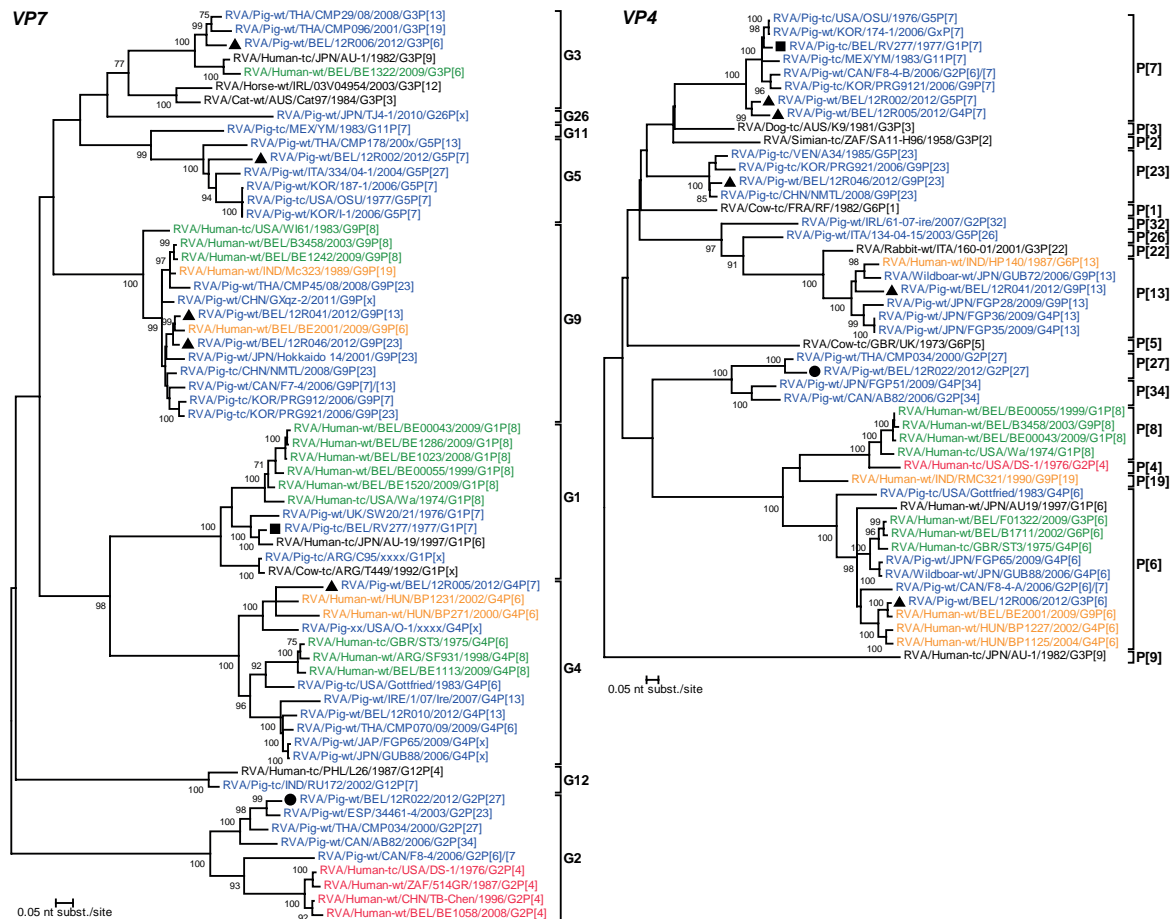


Figure 1. Maximum-likelihood phylogenetic trees based on coding sequences of VP7 and VP4 genes. Bootstrap values ($n=500$ replicates) lower than 70% are not shown. Pig strains are colored in blue, whereas human Wa-like strains are in green. Human DS-1-like strains are in red. Orange strains are suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with a triangle (contemporary and diarrheic), circle (contemporary and non-diarrheic) or square (diarrheic and historic).

4.1.3.2.2. Inner capsid protein (VP6)

A maximum-likelihood phylogenetic tree of VP6 encoding genes, constructed using the Tamura 3 model with gamma distribution and invariant sites, is shown in Figure 2. Whereas the I5 genotype almost exclusively contained pig RVA strains, the I1 genotype mainly contained human strains. Belgian pig I5 strains were highly related to each other (95.2 to 97.8%). An exception was strain 12R022, which was less related to other Belgian contemporary strains (93.3 to 94.1%), and clustered together with Thai strains (94.1 to 94.4%). RV277 and Gottfried clustered within the I1 genotype together with modern human, archival human, pig and pig-like human strains. RV277 clustered more closely to archival human strains from the USA (94.0 to 94.5%), a pig strain from China (93.1%), and pig-like

human strains from Hungary (93.3 to 93.6%), than to contemporary human strains (89.6 to 91.2%). The genetic distance between RV277 and Gottfried was relatively high (89.3%). Genotype specific amino acid mutations between I1 and I5 strains were mainly situated at the interaction site between the VP6 trimer and VP7 (Figure 3). No genotype I14 strains were encountered in Belgium during the present study.

4.1.3.2.3. Core scaffold protein (VP2) and viral enzymes (VP1 and VP3)

The maximum-likelihood trees for VP1 and VP2 were constructed using the general time reversible model with gamma distribution and invariant sites, whereas the Tamura 3 model with gamma distribution and invariant sites was applied for VP3.

Phylogenetic analysis of the genes encoding structural proteins VP1, VP2 and VP3 showed that all Belgian porcine RVA strains could be assigned to genotype 1 (Figure 2). However, multiple lineages existed within the genotype 1 of the corresponding genes, but porcine and human RVAs could mainly be assigned to typical porcine or human subclusters. However, the VP1 and VP2 genes of some archival human Wa-like strains clustered closely to those of pig strains.

Within the C1 genotype of VP2, Belgian strains 12R002, 12R006, 12R022, and 12R041 formed a subcluster (92.1 to 97.7%) together with pig-like human strain BE2001 (92.8 to 95.3%). In contrast, these Belgian strains were genetically more distinct from another Belgian strain 12R005 (90.8 to 92.1%). Remarkably, pig strain YM from Mexico was most related to several archival human Wa-like strains from the USA (94.7 to 95.4%). Nonetheless, historic strain RV277 was also closely related to archival human Wa-like strains RVA/Human-wt/USA/DC2106/1976/G3P[8] and RVA/Human-wt/USA/DC4772/1976/G3P[8] (93.0%). In contrast, contemporary human Wa-like strains clustered distinct from porcine strains. Furthermore, Belgian strain 12R046 clustered most closely to strain RVA/Human-wt/NCA/OL/2010/G4P[6] from Nicaragua (93.3%).

Genes encoding the viral RNA-dependent RNA-polymerase (VP1) were classified into two large subclusters within the genotype R1. Overall, a high genetic diversity was present among pig strains within this genotype, which was lower between contemporary human strains, and between most archival human strains. Belgian strains 12R002, 12R022, and 12R046 belonged to the first clade, but were genetically distinct since they all belonged to different subclusters (85.6 to 86.4%). Within the second clade, Belgian pig strains 12R005, 12R006, 12R041, and RV277 were closely related to each other (96.3 to 97.0%) and formed a subcluster with pig strains from Italy (93.7 to 95.4%), and pig-like human strain BE2001

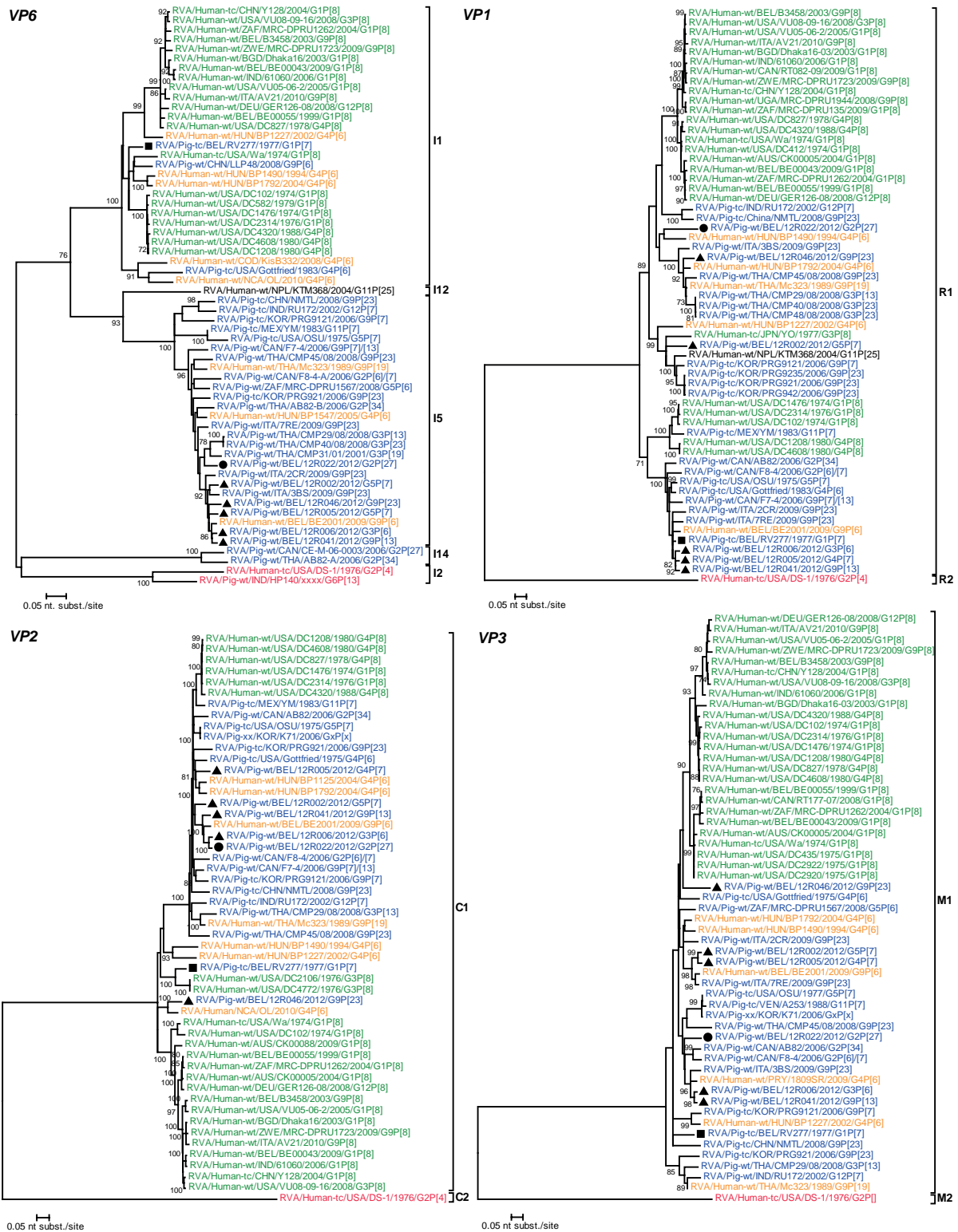


Figure 2. Maximum-likelihood phylogenetic trees based on coding sequences of VP6, VP1, VP2, and VP3 genes. Bootstrap values ($n=500$ replicates) lower than 70% are not shown. Pig strains are colored in blue, whereas human Wa-like strains are in green. Human DS-1-like strains are in red. Orange strains are suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with triangle (contemporary and diarrheic), circle (contemporary and non-diarrheic) or square (diarrheic and historic).

from Belgium (95.0 to 96.1%). Remarkably, pig strain YM clustered closely to some archival human Wa-like strains (92.4 to 93.9%).

For the viral guanylyl- and methyltransferase (VP3), all Belgian pig strains belonged to genotype M1, which was composed of different intra-genotypic lineages. Pig strains were distinct from contemporary and ancient Wa-like human rotaviruses. Strains 12R002 and 12R005 were genetically closely related to each other (97.5%), and formed a cluster with pig-like human strains, and pig strains from Italy (90.7 to 94.7%). Strains 12R006 and 12R041 formed a cluster with Italian and Canadian pig strains, and a pig-like human strain from Paraguay (90.7 to 95.2%). Historic strain RV277 was distantly related to the Korean strain RVA/Pig-tc/KOR/PRG9121/2006/G9P[7] (88.8%) and the ancient American pig strain Gottfried (88.8%).

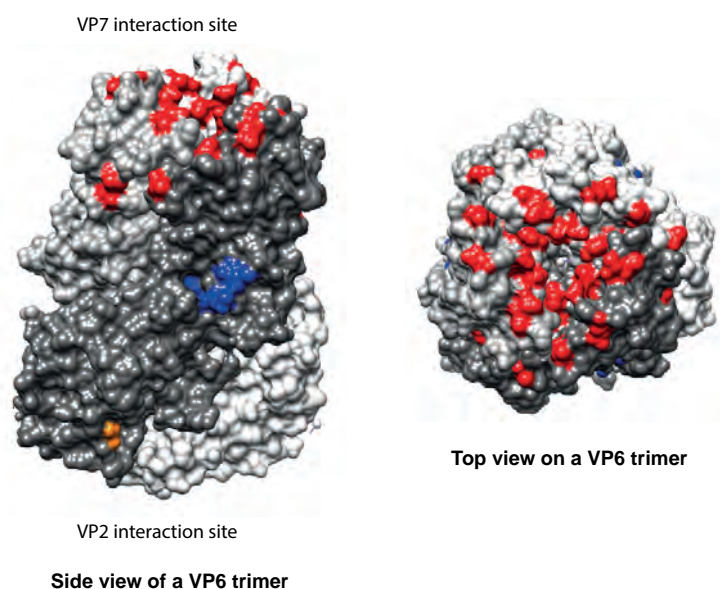


Figure 3. Three-dimensional representation of a VP6 trimer (PDB 1QHD) demonstrating genotype specific amino acid changes between strains of the I1 and I5 genotype. Mutations at the interaction site between VP6 and VP7 are in red. Mutations at the side of VP6 are in blue, mutations at the interaction site with VP2 are in orange.

4.1.3.3. Phylogenetic analysis of genes encoding non-structural proteins

4.1.3.3.1. Interferon antagonist (NSP1)

A general time reversible model was used for construction of the NSP1 maximum-likelihood phylogenetic tree. Analysis of the genes encoding the interferon antagonist NSP1 showed the clustering of all Belgian pig strains within the A8 genotype (Figure 4). Different

subclusters were demonstrated in this genotype, but even the genetic distances between strains from the same clusters were highly variable. Belgian strains 12R005, 12R006 and 12R046 formed a subcluster with pig-like human strains from Belgium and Hungary, and ancient strain Gottfried (89.2 to 97.9%). Furthermore, Belgian strains 12R041, 12R002 and 12R022 clustered together with the pig Italian strain RVA/Pig-wt/ITA/2CR/2009/G9P[23] (90.9 to 92.8%). Ancient strain RV277 clustered most closely to pig strains from Italy and Thailand (92.2 to 92.6%). All human and archival Wa-like strains clustered in genotype A1, distinct from some pig strains and human reference strain Wa.

4.1.3.3.2. *Viroplasm-associated proteins (NSP2 and NSP5)*

The Tamura 3 model with gamma distribution and invariant sites was used for construction of maximum-likelihood phylogenetic trees for NSP2 and NSP5. Analysis of the NSP2 genes demonstrates 2 clades within the N1 genotype of NSP2 (Figure 5). In one subcluster, Belgian strains 12R005 and 12R041 clustered together with pig strains from Canada and South Korea, and the rhesus monkey strain RVA/Rhesus-tc/USA/TUCH/2002/G3P[24] (93.6 to 94.5%). Within the other clade, ancient strain RV277 clustered together with pig strains from Thailand, Mexico and South Korea, and with the contemporary Belgian human strain RVA/Human-wt/BEL/B3458/2003/G9P[8] (94.3 to 96.1%). Strain 12R006 clustered most closely to pig-like Brazilian human strain RVA/Human-wt/BRA/HST327/1999/G4P[6] (93.2%). However, this strain was also distantly related to contemporary and archival human Wa-like strains (90.6 to 92.2%) and pig strain NMTL (91.3%). Another subcluster was formed by Belgian pig strains 12R002, 12R022 and 12R046, which were highly related to the Belgian pig-like human strain BE2001 (96.7 to 97.4%). Remarkably, modern and archival human Wa-like strains could be assigned to different subclusters, but were rather distinct from pig strains.

Phylogenetic analysis of the genes encoding NSP5 demonstrated an overall low genetic diversity within genotype H1 (Figure 5). Pig strain 12R002 clustered with pig strain RVA/Pig-wt/IND/HP140/xxxx/G6P[13] (97.9%), and pig-like human strain RVA/Human-wt/IND/RMC321/xxxx/G9P[19] (97.7%), but were more distantly related to archival human Wa-like strains and one modern Wa-like strain RVA/Human-wt/AUS/CK00005/2004/G1P[8] (95.4 to 95.7%). Belgian strains 12R005, 12R006, 12R022, 12R041, 12R046 and RV277 were more closely related to each other (96.4 to 98.9%), whilst being slightly more distantly related to strain 12R002 (95.6 to 96.4%). Belgian pig strains were relatively closely related to another cluster of contemporary and historic human Wa-like strains (94.5 to 98.2%).

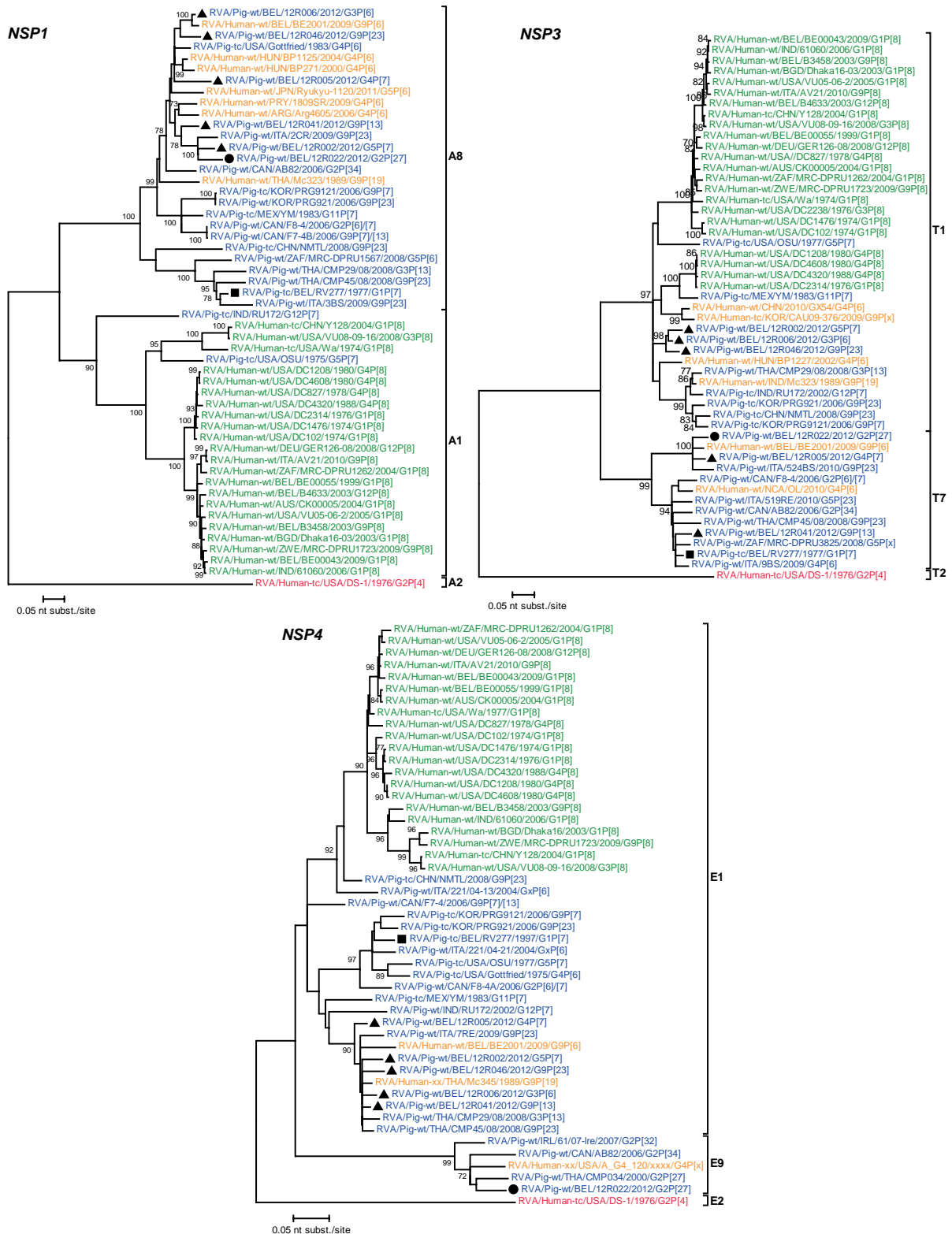


Figure 4. Maximum-likelihood phylogenetic trees based on coding sequences of NSP1, NSP3 and NSP4 genes. Bootstrap values (n=500 replicates) lower than 70% are not shown. Pig strains are colored in blue, whereas human Wa-like strains are in green. Human DS-1-like strains are in red. Orange strains are suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with a triangle (contemporary and diarrheic), circle (contemporary and non-diarrheic) or square (diarrheic and historic).

4.1.3.3.3. Translation enhancer (NSP3)

NSP3 maximum-likelihood trees were constructed using the Tamura 3 model with gamma distribution. Genes encoding the NSP3 proteins of Belgian pig RVA strains clustered in two genotypes: T1 or T7 (Figure 4). Within genotype T1, a subcluster was formed by Belgian strains 12R002, 12R006 and 12R046 (94.4 to 96.0%). Belgian pig strains within the T1 genotype were more distantly related to modern human Wa-like strains (87.2 to 91.6%), and a smaller cluster of human archival Wa-like strains from the USA (88.8 to 90.5%). Historic pig strains OSU and YM were relatively distantly related to strains of these modern (86.2 to 88.6%) and archival clusters (88.2 to 92.9%), respectively. Within the T7 genotype, two phylogenetic clusters were formed. One consisted of the Belgian strains 12R005, 12R022, BE2001 and Italian strain RVA/Pig-wt/ITA/524BS/2010/G9P[23] (93.1 to 95.2%), whereas the other cluster within T7 consisted of pig strains 12R041 and RV277, which were moderately related to pig strains from Canada, Italy, Thailand and South Africa (91.2 to 95.6%).

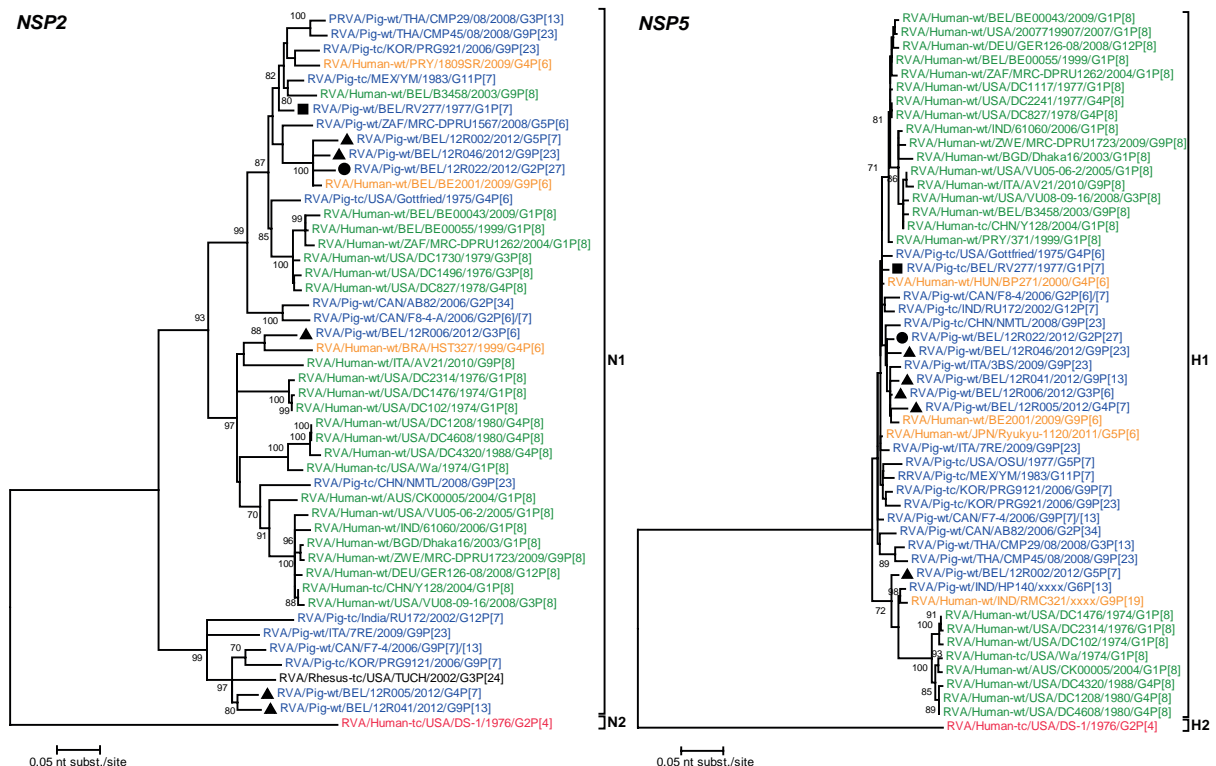


Figure 5. Maximum-likelihood phylogenetic trees based on coding sequences of NSP2 and NSP5 genes. Bootstrap values ($n=500$ replicates) lower than 70% are not shown. Pig strains are colored in blue, whereas human Wa-like strains are in green. Human DS-1-like strains are in red. Orange strains are suspected interspecies transmission events between pigs and humans. Belgian pig strains are marked with a triangle (contemporary and diarrheic), circle (contemporary and non-diarrheic) or square (diarrheic and historic).

4.1.3.3.4. Enterotoxin (NSP4)

For construction of NSP4 maximum-likelihood trees, the Tamura 3 model with gamma distribution was used. Most of the Belgian pig RVA strains clustered together within the E1 genotype of NSP4 (Figure 4). As such, strains 12R002, 12R005, 12R006, 12R041 and 12R046 formed a cluster with strains from Thailand, Italy and pig-like human strains BE2001 and Mc345 (93.7 to 97.9%). Strain RV277 was only distantly related to the other Belgian pig E1 strains (88.7 to 89.8%), and clustered together with ancient strains OSU and Gottfried, and pig strains from Canada, Italy and South Korea (93.1 to 94.6%). A major human Wa-like subcluster, containing modern and ancient strains, was demonstrated in the E1 genotype. Strain 12R022 belonged to a cluster of pig RVAs from Ireland, Thailand and Canada within the E9 genotype (90.8 to 92.9%). Only one human strain (RVA/Human-wt/USA/A_G4_120/xxxx/G4P[x]) possessing this genotype has been detected so far, which was relatively closely related to the porcine E9 strains (91.4 to 92.7%).

4.1.3.4. Analysis of the antigenic regions of VP7 of porcine and human strains

An amino acid (aa) analysis of the neutralization epitopes present on VP7 of Belgian pig RVA strains was performed. Amino acid residues were compared with corresponding residues of other pig strains and a selection of Belgian human RVA strains (Figure 6). For VP7, 3 neutralizing domains have been described in literature, namely 7-1a, 7-1b and 7-2 [24, 25]. Only four out of 29 amino acids (positions 98, 104, 201, 264) of these antigenic regions were conserved among all pig and human RVA strains included in the present study. Ancient strain RV277 and human strain AU-19 differed at 3 aa positions. An identical number of mutations was noticed between RV277 and the other pig G1 strains. RV277 was antigenically slightly more divergent from contemporary Belgian human G1 strains from lineage I and II, with 4 and 7 aa differences, respectively. Most variable positions were located in epitope 7-1a, but the single most variable residue was located in epitope 7-2 (aa 217). More antigenic differences were present on the heavily glycosylated VP7 proteins belonging to the G2 genotype. Up to 11 amino acid mutations were present between Belgian pig G2 strain 12R022 compared to contemporary human G2 strains. Multiple aa differences between pig and human G2 strains were located at possible glycosylation sites. Strain F8-4, which clustered phylogenetically in between pig G2 strains and human G2 strains, was different in 14 aa residues from strain 12R022. Within the G3 genotype, antigenic epitopes were relatively conserved among pig and human strains. Only one aa (aa 129) difference was observed between Belgian and Thai pig G3 strains. Three to five amino acid differences

were seen between strain 12R006 and Belgian human G3 strains. In contrast to pig strains, human G3 strains possessed a potentially extra N-linked glycosylation site at aa 238. Two to 10 aa differences were noticed between strain 12R005 and other pig G4 strains. Strain 12R005 was also clearly antigenically different from contemporary human G4 strains with up to 10 aa differences. Furthermore, pig G5 strains seemed to possess relatively conserved antigenic epitopes, with only two to five aa differences between 12R005 and other pig G5 strains.

The antigenic epitopes of Belgian pig G9 strains 12R041 and 12R046 were nearly identical with only one amino acid difference (aa 123). These strains were antigenically more distinct from Belgian strains 12R003 and 13R068, belonging to another phylogenetic lineage within the G9 genotype, with up to seven aa differences. Apparent was the loss of a possible N-linked glycosylation site in epitope 7-2 (aa 221) in pig strains 12R003 and 13R068, and in Belgian pig-like human strain BE2001. This N-linked glycosylation was also lacking in the contemporary Belgian G9 strains. Further analysis of the antigenic epitopes showed a high antigenic relatedness of pig G9 strains 12R041 and 12R046 and contemporary human G9 strains, with only one other mutation present in epitope 7-1a. Two possible N-linked glycosylation sites at aa 211 and 242 were conserved among all pig and human G9 strains.

	7-1a													7-1b										7-2									
	87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	230	242	143	145	146	147	148	190	217	221	284				
G1	T	N	N	G	E	W	K	D	Q	S	V	V	D	K	Q	N	V	D	N	T	K	D	Q	D	L	S	I	N	G				
RVA/Pig-w/BEL/RV277/1977/G1P[7]																																	
RVA/Pig-w/USA/019/1975/G1P[8]																																	
RVA/Pig-w/GBR/SW202/1/1976/G1P[7]																																	
RVA/Pig-w/ARG/C95xxxx/G1P[4]																																	
RVA/Human-w/BEL/BE1286/2009/G1P[8]																																	
RVA/Human-w/BEL/BE1126/2009/G1P[8]																																	
RVA/Human-w/BEL/BE1175/2009/G1P[8]																																	
RVA/Human-w/BEL/BE1280/2009/G1P[8]																																	
G2	T	N	S	T	E	W	T	N	Q	N	A	I	D	K	Q	E	V	N	S	S	R	Q	T	T	S	D	T	S	G				
RVA/Pig-w/BEL/12R022/2012/G2P[7]																																	
RVA/Pig-w/ESP/34461-4/2003/G2P[2]																																	
RVA/Pig-w/THA/CMP034/2008/G2P[2]																																	
RVA/Pig-w/CAN/AB22/2005/G2P[4]																																	
RVA/Pig-w/CAN/6-4/2006/G2P[7]																																	
RVA/Human-w/BEL/BE1141/2009/G2P[4]																																	
RVA/Human-w/BEL/BE1056/2006/G2P[4]																																	
RVA/Human-w/BEL/BE1251/2009/G2P[4]																																	
RVA/Human-w/BEL/BE1248/2009/G2P[4]																																	
G3	T	T	N	N	S	W	K	D	Q	D	A	I	D	K	Q	D	T	N	S	N	K	D	A	A	L	S	E	A	G				
RVA/Pig-w/BEL/12R006/2012/G3P[8]																																	
RVA/Pig-w/THA/CMP2906/2008/G3P[13]																																	
RVA/Pig-w/THA/CMP096/2001/G3P[19]																																	
RVA/Human-w/BEL/BE1214/2009/G3P[8]																																	
RVA/Human-w/BEL/BE1259/2009/G3P[8]																																	
RVA/Human-w/BEL/BE1322/2009/G3P[8]																																	
G4	A	T	G	N	E	W	K	D	Q	N	L	V	D	R	Q	N	T	G	D	A	R	I	A	G	E	S	M	N	G				
RVA/Pig-w/BEL/12R005/2012/G4P[7]																																	
RVA/Pig-w/USA/Gothard/1983/G4P[8]																																	
RVA/Pig-w/JPN/GP85/2006/G4P[4]																																	
RVA/Pig-w/JPN/GU885/2006/G4P[4]																																	
RVA/Pig-w/SAO-1/xxxx/G4P[4]																																	
RVA/Human-w/BEL/BE1126/2009/G4P[8]																																	
RVA/Human-w/BEL/BE1113/2009/G4P[8]																																	
G5	N	T	A	T	K	W	T	E	Q	D	A	V	E	R	Q	D	I	N	D	S	K	D	G	N	L	S	T	A	G				
RVA/Pig-w/BEL/12R002/2012/G5P[7]																																	
RVA/Pig-w/ITA/204/4/1/2004/G5P[7]																																	
RVA/Pig-w/KOR/1/2006/G5P[7]																																	
RVA/Pig-w/THA/CMP179/2005/G5P[13]																																	
G9	T	T	G	T	E	W	K	N	Q	N	A	I	D	K	Q	N	T	A	D	N	K	D	S	T	L	S	E	N	G				
RVA/Pig-w/BEL/12R041/2012/G9P[13]																																	
RVA/Pig-w/BEL/12R046/2012/G9P[23]																																	
RVA/Pig-w/BEL/12R003/2012/G9P[23]																																	
RVA/Pig-w/BEL/13R056/2013/G9P[7]																																	
RVA/Human-w/BEL/BE2001/2009/G9P[8]																																	
RVA/Pig-w/THA/CMP49/2005/G9P[23]																																	
RVA/Pig-w/KOR/PRG21/2006/G9P[23]																																	
RVA/Pig-w/JPN/Hokkaido/14/2003/G9P[23]																																	
RVA/Pig-w/ITA/ZC/2008/G9P[23]																																	
RVA/Human-w/BEL/BE1242/2009/G9P[8]																																	
RVA/Human-w/BEL/BE1119/2009/G9P[8]																																	
RVA/Human-w/BEL/BE1322/2009/G9P[8]																																	

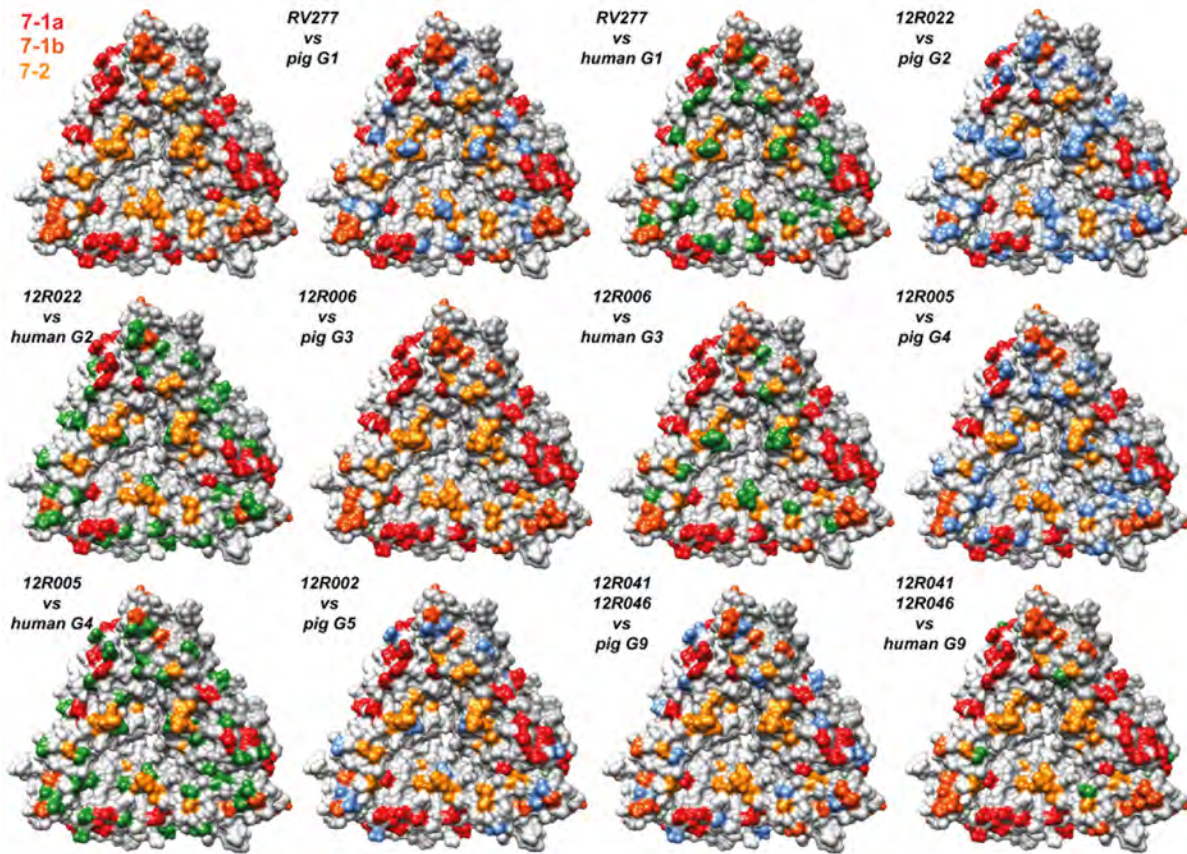


Figure 6. Amino acid residues of the main antigenic regions of genotypes G1, G2, G3 and G4, G5 and G9 of the glycoprotein VP7. Upper panel: amino acid mutations between Belgian pig strains from the present study (bold) and pig strains from other countries are in blue, whereas mutations between these Belgian pig strains and human strains are in green. Lower panel: the main antigenic regions demonstrated by shades of red and orange on a 3D structural model of the VP7 trimer (PDB 3FMG) (model in the upper left corner). The other models are a comparison between Belgian strains and pig/human strains belonging to the same genotype, respectively. Mutations are indicated with the same colors as in the upper panel.

4.1.3.5. Head-to-tail gene duplications in gene segment 11

Belgian pig strains 12R005, 12R006, 12R022, 12R041 and 12R046 possessed a gene duplication at the 3' end of gene segment 11 (Figure 7). The starting point of each duplication was nucleotide 326 of the parental strain. The duplications of strains 12R005, 12R006, 12R041 and 12R046 had a size of 300 nucleotides, whereas the duplication of strain 12R022 was 275 nucleotides in length. All duplications covered the 3' end of the NSP5 open reading frame. Overall, similarity between the coding sequences and duplications was relatively low, ranging from 87.0 to 90.5% identity.

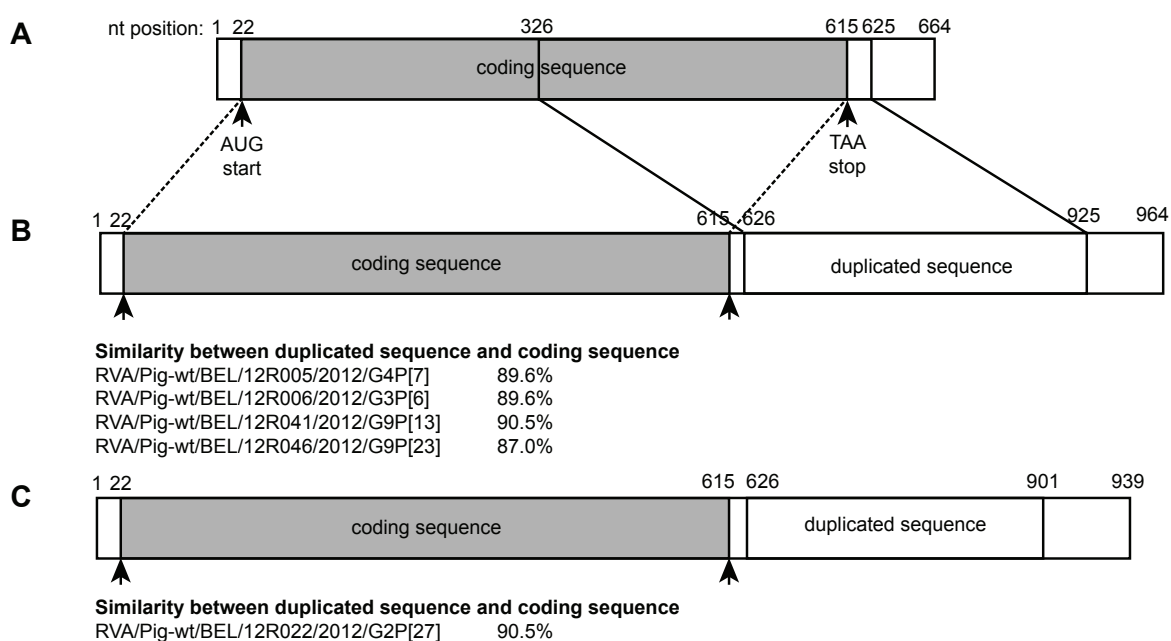


Figure 7. Representation of gene duplications present in the NSP5 gene of pig RVA strains. Panel A shows a normal NSP5 gene segment. Panel B and C demonstrates the NSP5 genes of Belgian pig strains containing gene duplications with a length of 300 and 275 bp, respectively. Start- and stopcodons are marked with arrows. Coding sequence for NSP5 protein is in gray, whereas non-coding sequences are white.

4.1.4. Discussion

Group A rotaviruses are considered as an important cause of diarrhea in suckling and weaned piglets, and is the leading cause of diarrhea in children under 5 years of age. An evolutionary relationship between pig RVAs and human Wa-like strains has been suggested to exist, but there are still many gaps in our understanding of the genetic constellations of pig RVAs [6]. Furthermore, it is not completely clear if pig RVAs form a considerable risk for spread in the human population after interspecies transmission, and if they can readily

reassort with human strains. In the present study, the genetic constellation of six contemporary Belgian pig RVAs and one historic RVA strain was assessed, and their evolutionary relationship with human and other pig RVAs was investigated.

Sequence analyses of the VP7 and VP4 genes of the Belgian strains included in this study showed a high genetic diversity in these genes [1]. Furthermore, many mutations were present in the antigenic regions of pig strains belonging to different genotypes of VP7. Whether these mutations also result in a reduced cross-protection between different genotypes should be further investigated by sero-neutralization assays using pig intestinal epithelial cells instead of MA104 cells, since the latter do not allow the propagation of all pig strains. VP7 genotypes shared between human and pig RVAs have clearly evolved into different evolutionary directions. Indeed, this is demonstrated by the phylogenetic distinction between pig and human strains in different subclusters within the same genotype. These conclusions can also be drawn upon analysis of the amino acid residues of the main antigenic regions, 7-1a, 7-1b and 7-2, present on this glycosylated outer capsid protein. Especially for G2, human and pig strains clustered phylogenetically separately and numerous amino acid differences were present in the main antigenic regions of their VP7 proteins. Similar findings were described by others, and it was suggested that pig G2 strains can be categorized as borderline G2 strains, which is here further confirmed [26, 27]. In contrast, contemporary pig and human G9 strains seem to be much more related than pig and human strains from other VP7 genotypes. This is also clear from the limited number of mutations present between antigenic regions of pig and human VP7 proteins. Pig G9 strains have been proposed as ancestors of currently circulating G9 strains in humans. Most likely, these strains have adapted to the human population after an interspecies transmission event that occurred relatively recent in the early 1990s [28]. This relatively short time has not enabled the human G9 strains to evolve far from their pig counterparts.

Phylogenetic analysis of the VP4 genes demonstrated the clustering of the major human VP4 genotypes P[8] and P[4] with genotypes P[6] and P[19], which are both shared between humans and pigs [29]. As shown in the phylogenetic trees of the present study, almost all pig-like human RVA strains possessed a P[6]-genotype for VP4. It can be suggested that pig strains bearing the P[6] genotype are crossing the species barrier more easily than strains bearing typical porcine VP4 genotypes, such as P[7]. The VP8* domain of the VP4 protein contains a lectin domain which is responsible for binding to carbohydrates present on mucus, and/or the enterocyte's surface. A plausible explanation for a seemingly possible weaker interspecies barrier of P[6] strains is the recognition of a common or similar carbohydrate

moiety on the pig and human enterocyte by P[6] strains. This might be the H type 1 antigen, which has been demonstrated to be recognized by P[6] *in vitro* [30]. In contrast, P[7] strains, described as sialidase sensitive, thus dependent on sialic acids for their attachment to the cell surface *in vitro*, do most likely not fit the carbohydrate moieties present in the human gut, and interspecies transmission events from these typical pig strains to humans are practically never seen [31].

Despite the high genetic diversity seen in the VP7 and VP4 genes, the genetic backbone of Belgian pig RVA strains was relatively conserved. A genetic backbone with a I5-R1-C1-M1-A8-N1-T7-E1-H1 constellation was mainly detected in Belgian pig RVAs, which is similar to genetic constellations detected in pig RVAs from other studies on different geographical locations (Table 2). This conserved backbone might be essential for efficient viral replication of pig RVAs in pig enterocytes. An even more conserved genetic backbone is seen in human RVAs. Two major genogroups of human RVAs circulate worldwide: Wa-like RVAs and DS-1-like RVAs. Whereas Wa-like RVAs predominantly possess internal genotype 1 genes (I1-R1-C1-M1-A1-N1-T1-E1-H1), the DS-1 like strains mainly possess internal genotype 2 genes (I2-R2-C2-M2-A2-N2-T2-E2-H2) [15]. Gene reassortments between Wa-like and DS-1-like RVAs have been detected, but not frequently, and it has been suggested that certain gene constellations are functioning best when kept together [15, 32, 33]. The classification of the pig rotavirus genes VP1, VP2, VP3, NSP2, NSP4 and NSP5 into genotypes R1, C1, M1, N1, E1 and H1, respectively, which also contain human Wa-like RVAs, is suggestive for an evolutionary relationship between pig and human RVAs. Nonetheless, the present results also indicate that pig and human Wa-like RVAs have entered distinct evolutionary routes. As demonstrated in the phylogenetic trees for VP3, NSP2, NSP4 and NSP5, genes of modern and archival human Wa-like strains are moderately to poorly related to those of pig strains, despite the fact that they belong to the same genotype. Exceptions in the NSP2 tree were two modern human Wa-like strains, B3458 and AV21, which clustered distinctly from other human strains, but slightly closer to pig strains. However, for the VP1, VP2 and NSP3 genes, some pig strains were moderately related to archival human Wa-like strains, which was also observed in other studies [34, 35]. Furthermore, the genetic diversity between pig genes is usually higher than between human Wa-like RVAs. It might be that pig RVAs have been circulating for a longer period of time in the pig population and that they have accumulated more mutations in their viral genes, in contrast to human RVA strains. However, whether pig RVAs were progenitors of human RVA strains or vice versa cannot be concluded from the present study, but the fact that both

species share a common ancestor is further confirmed by the phylogenetic relationships of most internal gene segments [6]. Whether pig and human Wa-like RVA strains are able to create more viable reassortants than Wa-like and DS-1-like human strains cannot be definitely concluded, but can be assumed given the lower genetic distance between pig and human Wa-like genes. Another explanation for the higher genetic diversity in the pig genes might be the existence of a stronger evolutionary drift in pig RVAs in comparison to human RVAs. This is not unlogical as pigs come repeatedly in contact with different rotavirus strains during the intense production stages [36, 37].

This stronger evolutionary drift may also be represented by the existence of the I5, A8 and T7 genotypes for VP6, NSP1 and NSP3, respectively. It is possible that proteins encoded by these genotypes allow for a better match with the cellular machinery in the porcine enterocyte during viral replication, but other discrete changes in other viral proteins may be involved in host range restriction as well. Nonetheless, these assumptions should be proven by *in vitro* infection experiments using (reassortant) pig and human RVA strains in relevant cell models of the pig and human enterocytes.

NSP1 has already been described to be likely involved in host range restriction [38, 39]. Amino acid analysis of NSP1 A1 and A8 strains, revealed that the N-terminal domain of the protein, which contains a zinc finger motif, was most conserved among both genotypes. This part of NSP1 functions as an E3 ligase, necessary for ubiquitination of interferon regulatory proteins such as IRF3, IRF5, IRF7 or β -TrCP. These regulatory factors are recognized by the C-terminal half of NSP1. Recognition of these regulatory factors by NSP1 results in ubiquitination of these proteins, followed by proteosomal degradation and suppression of the interferon response [32, 40]. However, the C-terminal half of NSP1 was highly variable between the 2 genotypes and it might be that A8 strains recognize and suppress different proteins, or species-variants of these proteins, that are involved in the interferon pathway than A1 strains *in vivo*.

While I5 is the dominantly detected genotype for VP6, the I1 genotype is only seldomly seen in pigs. In the present study, ancient strain RV277 from 1977 also possessed an I1 genotype for VP6. Furthermore, the I1 genotype has also recently been detected in pig-like human strains from Hungary and the Democratic Republic of Congo [41, 42]. Several amino acid changes were observed at the apical site of the VP6 trimer (Figure 3), known to interact with the VP7 outer capsid proteins [32]. It might be hypothesized that the I5 genotype encodes for a viral inner capsid protein that allows a better functional fit with the vast diversity of VP7 and VP4 proteins beared by pig RVA strains.

More and more T7 genotypes of NSP3 are being encountered in pigs nowadays, whereas in the past only T1 genotypes have been detected [17, 18, 20]. Genotype specific aa changes between T1 and T7 strains, but also species specific amino acid changes not related to the genotype, were observed. Most of these changes were located in the C-terminal region of NSP3, which is known to interact with eukaryotic initiation factor 4G [32, 43, 44]. These species and genotype specific changes may lead to a better recognition of human or pig eIF4G, thus likely suppressing host cell protein synthesis in favor of viral protein translation. Another interesting finding was the detection of an E9 genotype for non-diarrheic strain 12R022. Until now, almost all E9 genotypes have been encountered in pigs. An exception is the detection of an E9 genotype in A_G4_120, a human G4 strain of which the VP4 genotype was untypeable. Most likely this strain was the result of a pig to human interspecies transmission event. It might be interesting to further investigate and compare the biological functions of NSP4 proteins encoded by E9 and E1 genotypes in pig intestinal epithelial cells.

One of the most conserved genes in pig RVAs was NSP5. It is involved in the formation of the viroplasm, but its full repertoire of functions remains to be elucidated. Five Belgian RVA strains possessed a partial head-to-tail gene duplication, with the duplicated region starting at an identical location at the 3' end of gene segment eleven. An almost identical duplication was also noticed in the Belgian pig-like human strain BE2001 isolated from a child [20]. Most duplications had a size of 300 nt, but that of strain 12R022 had a size of 275 nt. The presence of these rearrangements behind the stop-codon results in an unaltered NSP5 protein. Most likely, these rearranged strains must have been circulating already for a long time in the Belgian pig population, since relatively low nucleotide identities between the coding sequences and their duplicated counterparts were observed. This suggests the accumulation of many mutations in these duplications over time. The exact function(s) of these duplications remain(s) elusive, but gene segments possessing duplications are thought to be preferentially incorporated in progeny virus. This principle has therefore been applied to improve rotavirus reverse genetics systems [45].

Regarding the future development of preventive measures against RVA infections in pigs, the high diversity seen for VP7 and VP4 might be a tremendous challenge at first sight. To protect offspring during the first weeks of life, neutralizing lactogenic antibodies directed against different serotypes of VP7 and VP4 are necessary. As such, the development of a multivalent vaccine covering the different G/P types circulating in the pig population might be necessary. Here, each individual strain will likely elicit a homotypic neutralizing antibody

response. Combining multiple strains will then result in a broad heterotypic lactogenic protection. In contrast, if a vaccine needs to be applied to young pigs to protect them actively against weaning diarrhea, it might be sufficient to include a monovalent attenuated vaccine, as it has been shown that subsequent rotavirus infections will induce protection against heterotypic strains in humans [46]. Internal RVA proteins such as VP6, VP2, NSP2 and NSP4 also induce non-neutralizing antibodies, but they might have a protective effect *in vivo* [47, 48]. VP6, for instance is one of the most immunogenic proteins in RVA and anti-VP6 antibodies may have intracellular neutralizing capacities [49]. As VP6, VP2, NSP2 and NSP4 are relatively conserved among pigs, the inclusion of a single strain in a weaning piglet vaccine might be sufficient to protect against strains bearing different VP7/VP4 combinations, but with a similar internal backbone. Further research will be executed to explore these hypotheses.

As a conclusion, the evolutionary relationship between pig and human Wa-like RVAs can be confirmed. However, it seems that viruses from both species have entered different evolutionary routes, likely resulting in a better adaptation to their host species. This evolutionary diversification might hamper efficient spreading of pig strains in the human population after interspecies transmission, and *vice versa*. Nonetheless, the first species barrier is the interaction of VP4 and carbohydrates on mucus and the surface of enterocytes. As until now almost all pig-like human strains contained a P[6] genotype, it can be concluded that the species barrier is less strict for this genotype. Future surveillance of pig and human RVAs is warranted to facilitate the detection of interspecies transmission events, the occurrence of gene reassortments and subsequent adaptation of pig RVAs to the human population.

4.1.5. Acknowledgments

MZ and IR were supported by a doctoral fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). LMD was supported by a doctoral fellowship of the Research Foundation-Flanders (FWO Vlaanderen).

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Chapter 4.2.

The fecal virome of a non-diarrheic Belgian piglet contains group A and C rotaviruses, and an astrovirus and enterovirus

Summary

The application of next generation sequencing to study the fecal virome of pigs is only poorly reported. In the present study, a fecal sample from a non-diarrheic piglet was analyzed using this technology, to not only reveal the complete genomes of a group A and C rotavirus present in this sample, but also that of other viruses. Here, the second complete genome of a pig group C rotavirus (RVC) is reported. This finding offered new insights into the relatedness of RVCs from pigs and other host species. The pig strain found here was only poorly related to RVCs from humans and cows. Remarkably, it was also only distantly related to the other completely characterized pig RVC strain, Cowden. The gene encoding the outer capsid protein VP7 belonged to the rare G3 genotype, which indicated that this strain might be serologically distinct from most other pig RVC strains. A putative new RVC VP6 genotype I8 was identified as well. The group A rotavirus strain present in this fecal samples contained the rare pig genotype combination G11P[27], but not all gene segments could be characterized. Still, typical pig RVA genotypes I5, A8, and T7 were found for the viral proteins VP6, NSP1, and NSP3. Unexpectedly, the fecal virome of this piglet also contained an astro- and enterovirus. Results of the current study indicate that many viruses may circulate in non-diarrheic piglets. Here, they could not be directly associated with clinical symptoms of diarrhea, but still they might have had a potential subclinical impact on pig performance. The fast evolution of next generation sequencing may form a powerful tool for future diagnostics in veterinary practice. Its application will certainly lead to better insights into the relevance of many (sub)clinical enteric viruses, that may have remained unnoticed using traditional diagnostic techniques. Eventually, the discovery of such viruses will stimulate the development of new and durable prophylactic measures to improve pig production.

4.2.1. Introduction

Diarrhea is one of the most important health problems in young pigs, and several enteric pathogens such as rotavirus, coronavirus, *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*, *Brachyspira* spp. and coccidiosis have been recognized as etiological causes [1]. Furthermore, the intestines of pigs may harbour many viruses, bacteria and parasites for which the significance or existence might be unknown. The entirety of viruses shed in feces is called the fecal virome, and its composition is currently being investigated by many researchers due to the availability of modern nucleotide sequencing technologies such as next-generation sequencing (NGS). Shan et al. (2011) reported the first use of NGS to investigate fecal viromes of healthy and diarrheic pigs on a high-density pig farm in Japan. On average, 4.2 and 5.2 mammalian viruses were shed by non-diarrheic and diarrheic piglets, respectively [2]. A process-controlled method, based on the addition of a defined amount of bacteriophages to allow for comparison between different samples, was applied by Sachsenröder et al. (2014) to investigate the fecal virome present in a pooled fecal sample of five 35 days old pigs. Most viruses detected could be classified as bacteriophages (73.9%), mammalian viruses (23.9%) or plant viruses (0.8%) [3]. Furthermore, this research group recently demonstrated that the pig fecal virome is not being influenced by feeding of the probiotic bacterium *Enterococcus faecium*, whereas it fluctuates with piglet age [4]. Zhang et al. (2014) reported the detection of 15 distinct mammalian viruses in a pooled fecal sample of 27 diarrheic piglets from China [5]. In general, most abundantly detected mammalian viruses in all these studies were kobuvirus, rotavirus, pig stool-associated ssDNA virus, astrovirus, sapovirus and enterovirus [2-4, 6].

Rotaviruses have been recognized as an important cause of diarrhea in young pigs and children. They belong to the family of the *Reoviridae* and possess a triple layered capsid, protecting the genome of eleven segments of double stranded RNA. The core (VP2) surrounds the genome, and also encloses the viral enzymes VP1 and VP3. VP6 proteins form the inner capsid layer, which is covered by the outer capsid proteins VP7 and VP4. The viral genome also encodes 6 different non-structural proteins (NSP1-NSP6). The genus *rotavirus* can be subdivided into 8 different species (A to H), based on nucleotide sequence identities of the genes encoding inner capsid protein VP6 [7]. Group A rotaviruses (RVA) have been considered as clinically and epidemiologically most important in humans and animals, including pigs. As such, most research has been conducted on RVAs. Due to its segmented genome, a full genome-based classification has been established for RVAs. In this system, each gene segment is abbreviated by a letter, followed by a number designating the

genotype. The resulting genotype constellation of an RVA strain is being presented as follows: G_x-P_[x]-I_x-R_x-C_x-M_x-A_x-N_x-T_x-E_x-H_x for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5, respectively [8, 9]. The outer capsid proteins VP7 and VP4 are very important in this classification system, as they can induce the formation of neutralizing antibodies. Many VP7 (n=27) and VP4 (n=37) genotypes have been detected in multiple species [10, 11]. In pigs, a large diversity of VP7/VP4 genotypes can be encountered [12-17]. As an example, six different G-genotypes for VP7 (G2, G3, G4, G5, G9 and G11), and five different P-genotypes for VP4 (P[6], P[7], P[13], P[23] and P[27]) were recently encountered in 12 different G/P combinations in feces from Belgian diarrheic and non-diarrheic piglets [18]. Overall, these pig RVA strains possessed a relatively conserved pig genetic backbone for the other 9 gene segments, namely I5-R1-C1-M1-A8-N1-T7-E1-H1, which demonstrated a clear evolutionary relationship with genes of human Wa-like RVA strains [9, 19].

RVCs are also being considered as an etiological cause of diarrhea in young pigs, but the virus has also been detected in fecal samples of non-diarrheic piglets at a lower prevalence rate [20-23]. A genotype classification system for RVC VP7 encoding genes has been established by Marthaler et al. (2013). Pig RVC VP7 genes were mainly assigned to genotypes G1, G3, G5, G6 to G9, whereas human and cow RVCs were grouped in genotypes G4 and G2, respectively [24]. Recently, Suzuki et al. (2015) identified a nucleotide sequence identity cut-off value for the classification of VP4 genes into 6 distinct genotypes [25]. Furthermore, a tentative genotype classification system has been assigned to VP6 encoding genes [26]. Only a limited number of complete genomes of RVC strains isolated from a pig, cows, humans and other species is available at the moment, which limits our understanding of RVC evolution [27-30].

In a previous study, we identified a low RVA load (4.79 log₁₀ copies/g feces) in a fecal sample from a non-diarrheic Belgian piglet, collected in 2012. This strain contained the rare pig VP7/VP4 genotype combination G11P[27] [18]. We also identified a much higher load of an RVC strain (9.56 log₁₀ copies/g feces) in this sample. Therefore, it was decided to unravel the entire fecal virome of this interesting fecal sample by means of NGS. We aimed to investigate the evolutionary relationship between the RVC strain from the pig and those of other species to assess better the risk for future interspecies transmission and reassortment events between pig and other RVC strains. Furthermore, the future application of NGS in veterinary diagnostics is being discussed.

4.2.2. Materials and methods

4.2.2.1. Processing of fecal sample

A twenty percent fecal suspension of sample 12R021 was made in phosphate buffered saline containing 1000 U/ml penicillin (Continental Pharma, Puurs, Belgium), 1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/ml gentamicin (Gibco BRL, Merelbeke, Belgium) and 0.01% v/v Fungizone (Bristol-Myers Squibb, Braine L'Alleud, Belgium) [18]. For Sanger sequencing, RNA was extracted from the fecal suspension using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and store at -70°C.

For Illumina sequencing, the fecal suspension was filtered twice using 0.8 µm and 0.45 µm membrane filters. Two microliter of Benzonase Nuclease (Novagen), 1 µl of Micrococcal Nuclease (New England Biolabs) and 1 µl of NEBNext® RNase III RNA Fragmentation Module (New England Biolabs) in 7 µl of homemade buffer (1 M Tris, 100 mM CaCl₂ and 30 mM MgCl₂) were added to 140 µl of fecal filtrate, and incubated for 2 hours at 37°C to destroy free and bacterial DNA/RNA. Next, seven microliter of EDTA was added to the sample for enzyme inactivation. Extraction of viral RNA was performed using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions, but without using carrier RNA.

4.2.2.2. RT-PCR of RVA gene segments

Sanger sequencing was applied in an attempt to obtain the complete genome of RVA/Pig-wt/BEL/12R021/2012/G11P[27]. RNA was denatured at 95°C for 2 min and immediately chilled on ice. For RT-PCR amplification of short gene segments (VP6, VP7, NSP2, NSP3, NSP4 and NSP5), reaction mixtures consisted of 5 µl RNA, 5 µl of 5x QIAGEN OneStep RT-PCR Buffer, 1 µl of dNTP Mix, 1.5 µl of forward and reverse primer (830 nM), 1 µl of QIAGEN OneStep RT-PCR enzyme mix, and nuclease free water in a total volume of 25 µl per reaction. Reaction volumes were upscaled to 50 µl for the longer gene segments (VP1, VP2, VP3, VP4, and NSP1). Primers used for RT-PCR amplification of the complete gene segments can be made available upon request.

Reverse transcriptase was performed at 50°C for 30 min, followed by *Taq* polymerase activation at 94°C during 15 min followed by 35 cycles of amplification. For the longer fragments, denaturation was performed for 30 sec at 94°C, annealing for 30 sec at 50°C, and extension at 72°C during 6 min. For the shorter fragments denaturation was performed for

30 sec at 94°C, annealing for 30 sec at 47°C, and extension at 72°C during 2 min. A final extension step was performed for 10 min at 72°C. Afterwards, 9 µl of PCR product was mixed with 1 µl of loading buffer and loaded on polyacrylamide gels. Electrophoresis was performed at 200V during 36 min, followed by ethidium bromide staining of the gels for detection of positive samples. If RT-PCR was unsuccessful, two RNA extracts were combined and precipitated using 70% ethanol. Concentrated RNA and DNA was diluted in nuclease free water and used to repeat the RT-PCR.

4.2.2.3. Sanger sequencing and nucleotide sequence analysis

Five microliter of PCR positive samples was treated with 1 µl of USB ExoSAP-IT[®] PCR Product Clean-Up (Affymetrix, Santa Clara, California, USA) and sequenced with an ABI Prism BigDye terminator cycle sequencing reaction kit (ABI Prism 3130xl, Applied Biosystems) using forward and reverse primers. If possible, remaining parts of the coding regions were further covered by primer-walking sequencing.

Analysis of nucleotide sequences obtained by Sanger sequencing was executed using the 4Peaks software (Mekentosj, Amsterdam, The Netherlands). RVA genotypes were determined using BLAST and RotaC^{2.0} [31].

4.2.2.4. Preparation of nucleic acids for Illumina sequencing

Total RNA was amplified using the Whole Transcriptome Amplification Kit (Sigma Aldrich). Therefore, 0.5 µl Library Synthesis Solution was added to 2.82 µl of RNA, followed by denaturation for 2 min at 95°C. RNA was cooled to 18°C and 0.5 µl Library Synthesis Buffer, 0.4 µl Library Synthesis Enzyme and 0.78 µl of water was immediately added to the reaction. The mixture was subjected to the following temperature profile: 18°C, 25°C, 37°C, 42°C and 70°C for 10, 10, 30, 10 and 20 minutes respectively. Samples were cooled down to 4°C followed by a brief centrifugation step. A mastermix containing 60.2 µl of nuclease free water, 7.5 µl of Amplification Mix, 1.5 µl of WTA dNTP mix and 0.75 µl Amplification Enzyme was added to the sample and incubated as follows: 94°C for 2 min and 30 cycles at 94°C for 30 sec and 70°C for 5 min. WTA products were purified with the MSB[®] Spin PCRapace kit (Strattec) according to the manufacturer's instructions and prepared for Illumina sequencing using the KAPA Library Preparation Kit (Kapa Biosystems), according to the instructions of the manufacturer.

4.2.2.5. Illumina sequencing and nucleotide sequence analysis

Fragments ranging from 350-600 bp were selected using the BluePippin (Sage Science) according to the manufacturer's instructions. Sequencing of the samples was performed on a HiSeq 2500 platform (Illumina) for 300 cycles (150 bp paired ends). Raw reads were trimmed for quality and adapters, and were *de novo* assembled into contigs using SPAdes [32]. Scaffolds were classified using a tBLASTx search against all complete viral genomes in Genbank using an e-value cut-off of 10^{-10} . Scaffolds with a significant tBLASTx hit were retained and used for a second tBlastx search against the Genbank nucleotide database using an E-value of 10^{-4} [33].

4.2.2.6. Phylogenetic analysis

RVC genotypes for VP7 were assigned using the nucleotide identity cut-off values proposed by Marthaler and colleagues [24]. Multiple sequence alignments were performed using the ClustalW plug-in in MEGA 5.2.2, followed by manual editing. For each RVC gene segment, maximum-likelihood phylogenetic trees were constructed and bootstrap-analysis was set at 500 replicates. Substitution models were determined for each gene segment separately. Pairwise distances were calculated using the p-distance model with 500 bootstrap replicates.

4.2.3. Results

4.2.3.1. Genetic constellation of RVA/Pig-wt/BEL/12R021/2012/G11P[27]

Only the partial genome of pig strain RVA/Pig-wt/BEL/12R021/G11P[27] could be determined using Sanger sequencing, and a further attempt to obtain the remaining parts of the genome using NGS was unsuccessful, due to the low viral load in the sample. The VP7 and VP4 genotypes were already disclosed in a former study and were G11 and P[27], respectively [18]. Here, only the genes encoding VP6, NSP1, NSP2 and NSP3 could be characterized and possessed the typical pig I5, A8, N1 and T7 genotypes, respectively.

4.2.3.2. Genome composition of RVC/Pig-wt/BEL/12R021/2012/G3Px

In contrast to the RVA strain, the entire genome of the RVC strain could be revealed using NGS. Overall, this pig RVC strain was genetically poorly related to genes of previously characterized pig, cow and human RVC strains, which is demonstrated in Table 1.

Table 1. Nucleotide similarities between pig RVC strain 12R021 and other RVC strains.

Strain	Nucleotide similarities (%) between genes of strain 12R021 and other strains										
	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVC/Pig-tc/USA/Cowden/1980/G1Px	72.6	74.5	85.4	88.5	86.8	87.4	73.2	81.3	76.8	82.6	85.4
RVC/Cow-wt/JPN/Toyama/2010/G2Px	71.2	73.6	81.4	79.7	78.8	78.1	68.8	80.8	77.2	66.4	75.7
RVC/Cow-wt/JPN/Y/08/2008/G2Px	71.7	73.6	81.8	79.6	79.4	78.2	68.7	80.4	77.0	67.8	75.4
RVC/Human-wt/GBR/Bristol/1988/G4Px	72.8	71.0	83.0	84.6	83.9	83.3	65.5	80.8	78.2	68.3	74.8
RVC/Human-wt/BGD/BS347/2005/G4Px	72.5	71.0	82.9	84.7	83.6	82.7	64.1	80.3	77.5	68.5	74.9
RVC/Human-wt/CHN/Wu82/2001/G4Px	72.8	70.8	83.1	84.8	83.3	86.4	65.7	80.9	77.4	68.5	74.4
RVC/Human-wt/KOR/CAU 10-312/2010/G4Px	72.6	70.9	82.5	84.7	83.7	82.4	63.9	79.8	77.3	68.3	74.3

4.2.3.2.1. Genes encoding outer capsid proteins VP7 and VP4

As shown in Figure 1, the genes encoding the RVC outer capsid protein VP7 were subdivided into 9 distinct genotypes (G1 to G9). Pig RVC strains were present in genotypes G1, G3, G5, G6, G7, G8 and G9. Genotype G4 is formed by a cluster of highly related human RVC strains in between genetically diverse pig RVC genotypes G1, G5 and G7 and G9. Furthermore, the G2 genotype consisted of only cow RVCs, genetically distinct from pig and human RVC strains. The Belgian pig RVC strain 12R021 belonged to genotype G3, which clustered distinctly from all other genotypes. Nonetheless, our Belgian strain showed only 88.1 to 88.9% nucleotide similarity with the only 2 other described pig G3 strains from the USA (88.1% to 88.9%).

The genes of the outer capsid protein VP4 could be phylogenetically divided into two major clusters (Figure 2). One contained genetically conserved human RVC VP4 genes, whereas the other consisted of genetically diverse pig and cow RVC strains. Belgian strain 12R021 clustered together with RVC strains RV0143 and P141 from the United States and the Czech Republic (84.9 to 87.0%), but was only poorly related to bovine and other pig strains from the Czech Republic.

4.2.3.2.2. Genes encoding structural VP6

A tentative genotype classification system for inner capsid protein VP6 was proposed by Suzuki and colleagues [26]. A major cluster within the VP6 phylogenetic tree (Figure 3) consisted of all the pig RVC strains divided over five different genotypes (I1, I4-7), whereas cow and human RVC strains belonged to genetically distinct genotypes I3 and I2, respectively. Belgian strain 12R021 clustered at a relatively high genetic distance in between the pig genotypes I1 (84.2 to 85.6%) and I7 (86.0 to 87.7%).

4.2.3.2.3. Genes encoding structural proteins VP1, VP2 and VP3, and non-structural proteins

For the other gene segments of RVC strains, only a limited number of nucleotide sequences are available in Genbank. The majority of RVC genes encoding proteins VP1, VP2, NSP1, NSP4 and NSP5 clustered together according to the host species of origin, with specific human, cow and pig subclusters (Figures 2 and 4). For all but one gene segment, pig RVC strains 12R021 and Cowden clustered in between two larger subclusters of human and cow RVC strains (Figures 2 and 4). Furthermore, for VP3, the two pig RVCs clustered in between 2 distinct human subclusters (Figure 2).

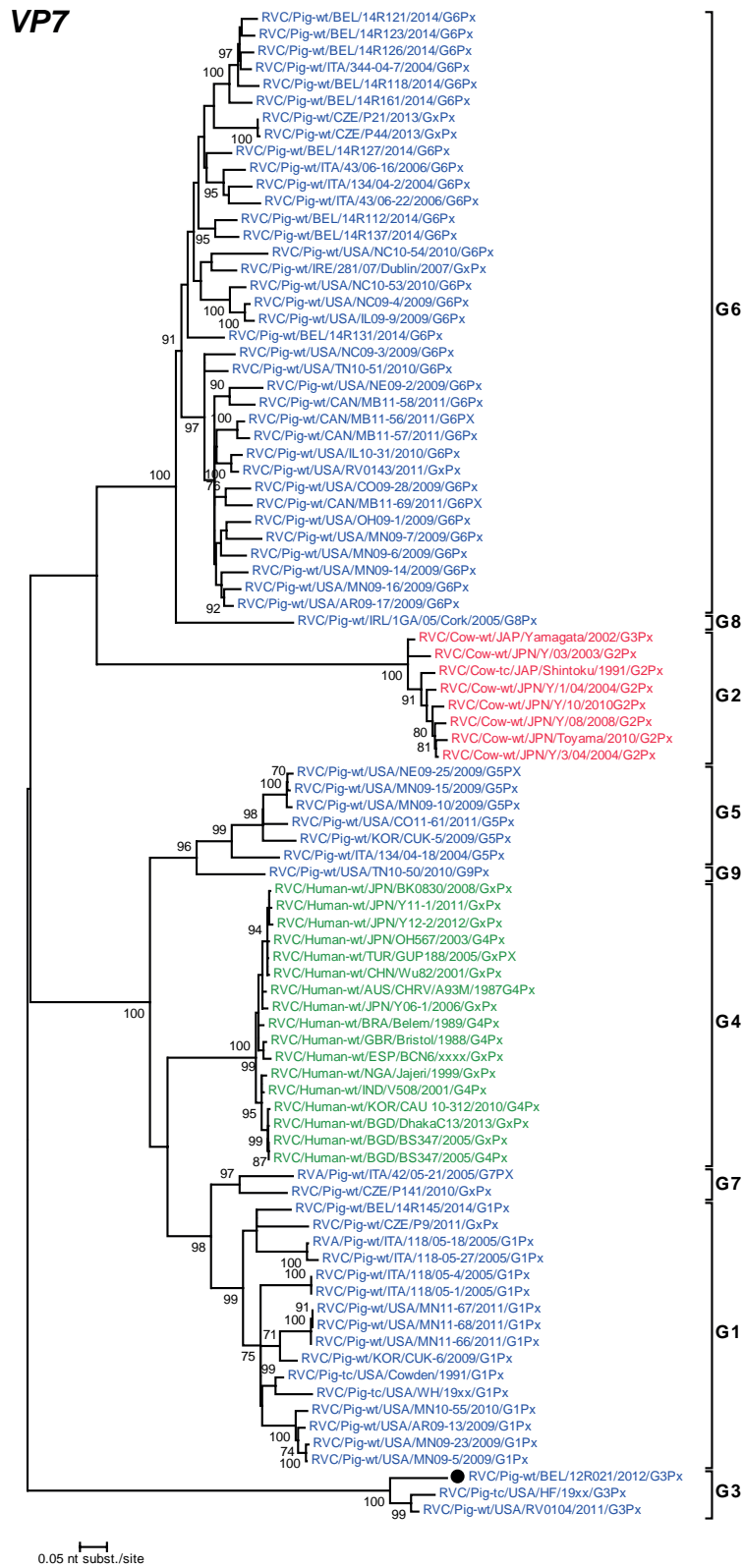


Figure 1. Maximum-likelihood phylogenetic tree based on the coding sequences of RVC VP7 genes. Bootstrap values (n=500 replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3Px is marked with a circle.

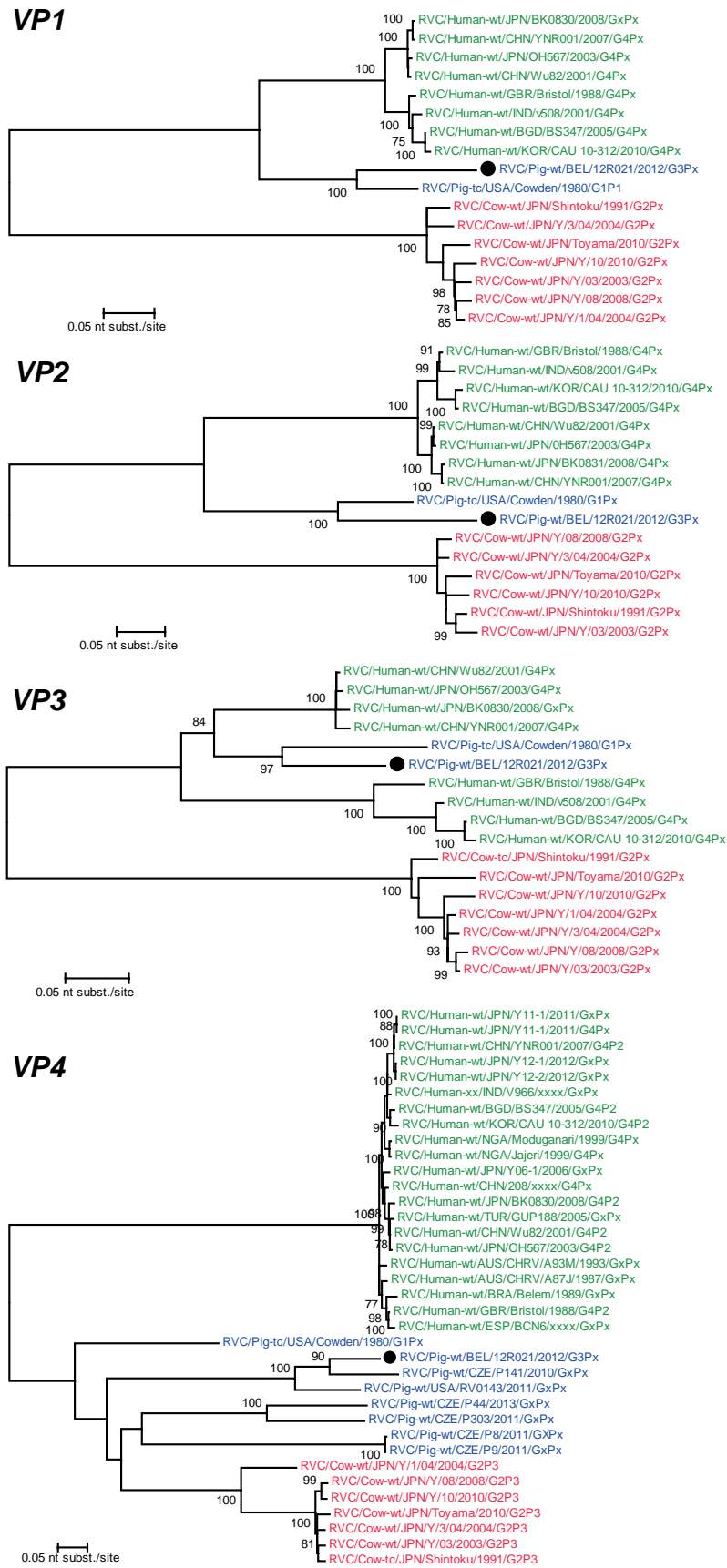


Figure 2. Maximum-likelihood phylogenetic trees based on the coding sequences of RVC VP1, VP2, VP3 and VP4 genes. Bootstrap values (n=500 replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3Px is marked with a circle.

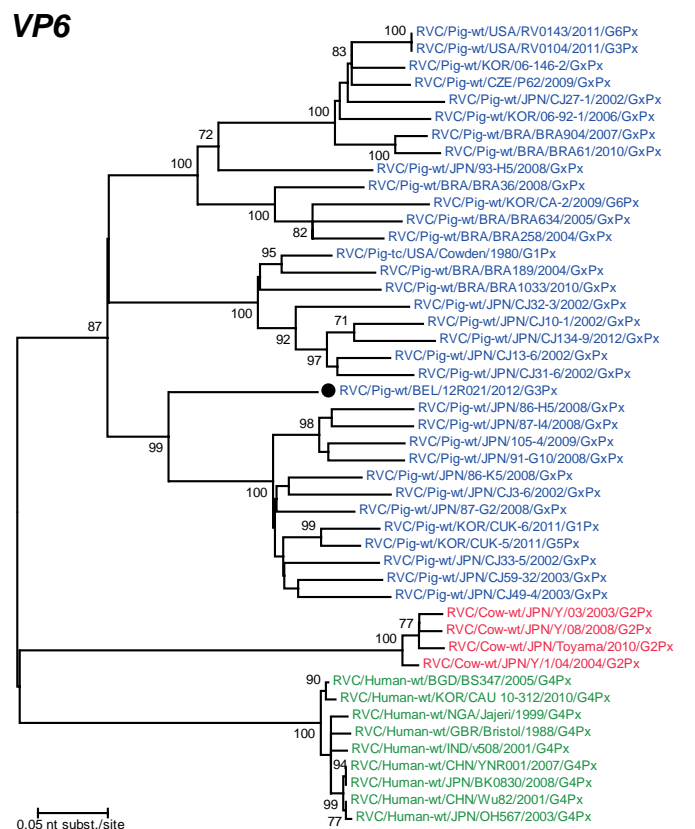


Figure 3. Maximum-likelihood phylogenetic tree based on the coding sequences of RVC VP6 genes. Bootstrap values ($n=500$ replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3Px is marked with a circle.

Interestingly, VP1, VP2, VP3 and VP6 encoding genes of strain 12R021 were slightly more related to those of human RVCs than those of cow RVCs (Table 1 and Figures 2 and 3). For genes encoding nonstructural proteins, the genetic divergence between pig RVCs and human or cow RVCs was even more pronounced, whereof the gene encoding NSP1 was the most variable (Table 1 and Figure 4). Slightly more nucleotide sequences of genes encoding enterotoxin protein NSP4 have been deposited into Genbank. The Belgian strain 12R021 formed a cluster together with strains from the United States and the Czech Republic at a high genetic distance (81.7 to 88.1%). In contrast, NSP4 genes of cow and human RVC strains were relatively more conserved than those found in pig RVCs.

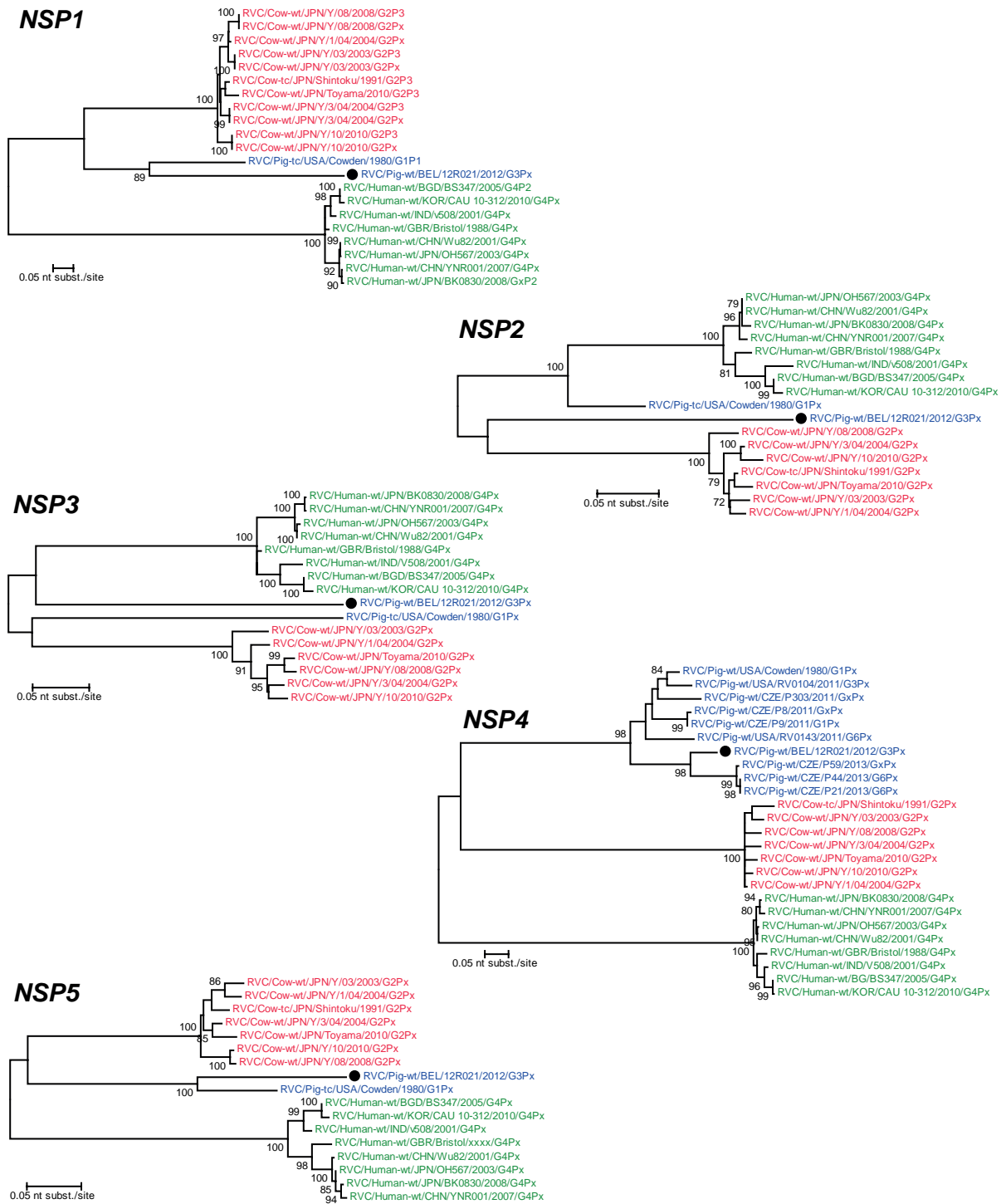


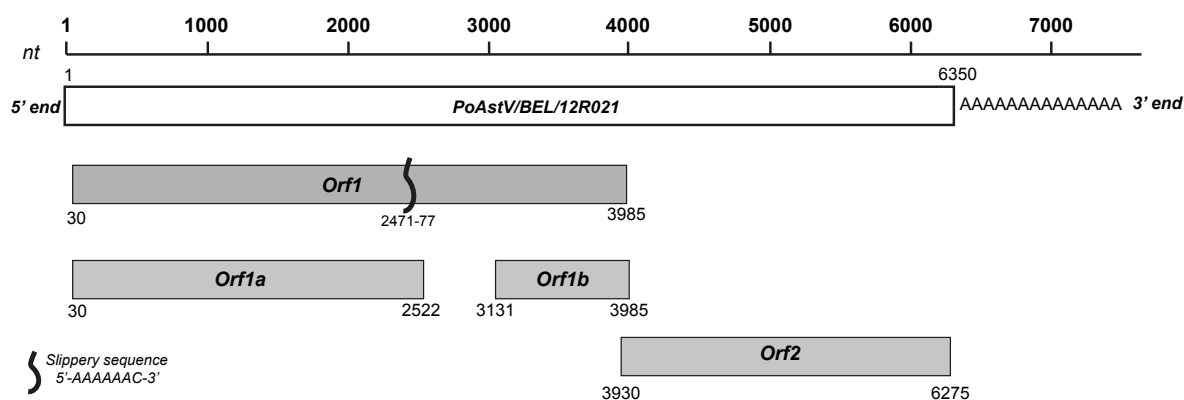
Figure 4. Maximum-likelihood phylogenetic tree based on the coding sequences of available RVC NSP1-NSP5 genes. Bootstrap values (n=500 replicates) lower than 70% are not shown. Pig, cow and human RVC strains are colored blue, red and green, respectively. Belgian strain RVC/Pig-wt/BEL/12R021/2012/G3Px is marked with a circle.

4.2.3.3. Other viruses present in the fecal virome

A porcine astrovirus (PoAstV) with a genome size of 6350 nt (ssRNA) was recovered from the fecal virome as well. The genome organization of PoAstV-BEL-12R021 is presented in Figure 5a. Briefly, 3 major open reading frames were identified: ORF1a (2493 nt), ORF1b (855 nt) and ORF2 (2346 nt). A slippery sequence (5'-AAAAAAC-3') was present between nucleotides 2471-2477, which is responsible for a ribosomal frameshift. As such, the entire ORF1 had a size of 3954 nucleotides. Furthermore, a poly(A) tail was present at the 3' end of the viral genome. BLAST analysis of the entire genome demonstrated that this strain was most closely related (79%) to pig strain PoAstV2-US-IA122 from the United States (Genbank JX556690) [34].

Furthermore, the fecal virome also contained an enterovirus, which had a ssRNA genome containing a single open reading frame of 6507 nt (Figure 5b), preceded by a relatively large untranslated 5' region. The single ORF encodes for a P1 protein that can be subdivided into 4 different structural (VP1 to VP4) and 7 non-structural proteins (2a-2c, 3a-3d). BLAST analysis of the enterovirus genome revealed the highest relatedness (80%) to pig strain swine/K23/2008/Hun from Hungary (Genbank HQ702854) [35].

A. Astrovirus genome organization



B. Enterovirus genome organization

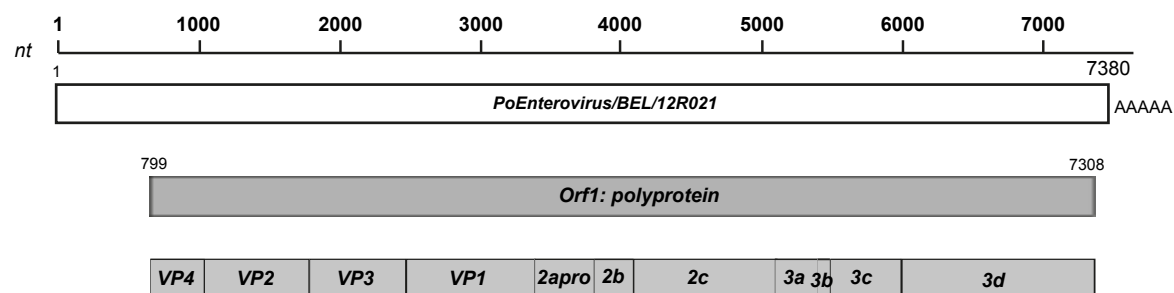


Figure 5. Genome organization of pig (A) astro- and (B) enterovirus isolated from fecal sample 12R021. The complete genomes are marked in white, and open reading frames are marked in gray.

4.2.4. Discussion

The fecal virome of piglets can harbour a wide diversity of viral species, including bacteriophages, mammalian and plant viruses. Among the mammalian viruses, genetic material of kobu-, rota-, corona-, astro-, sapo- and enteroviruses were most frequently encountered in recent virome studies [2-5]. Here it was reported that the fecal virome of a Belgian non-diarrheic piglet contains a group A and C rotavirus, an astrovirus and an enterovirus. First of all, rotaviruses have been identified as important causes of diarrhea in children and many animal species, including pigs. Especially in pigs, RVAs harbour a wide diversity of heterogeneous VP7 and VP4 encoding genes, resulting in antigenic variation of outer capsid proteins. Recently, six different G-genotypes (G2, G3, G4, G5, G9 and G11) for VP7 and five different P-genotypes for VP4 (P[6], P[7], P[13], P[23] and P[27]) in 12 different G/P combinations have been identified in fecal samples of diarrheic and non-diarrheic Belgian piglets [18]. Despite the large genetic/antigenic variation observed for the outer capsid proteins, the genetic constellation of the other 9 gene segments (I5-R1-C1-A8-N1-T7-E1-H1) was relatively conserved in these Belgian pig RVAs and pig strains from around the globe. In contrast, genetic diversity is less apparent for human RVA VP7 and VP4 genes, and most human strains possess either a Wa-like I1-R1-C1-A1-N1-T1-E1-H1 backbone, or DS-1-like I2-R2-C2-A2-N2-T2-E2-H2 backbone. It was hypothesized earlier that an evolutionary relationship between pig and human Wa-like RVAs, and between cow and human DS-1-like RVAs might exist, whereof the first relation has recently been confirmed by the analysis of more complete pig RVA genomes [9, 19]. An RVA strain (RVA/Pig-wt/BEL/12R021/2012/G11P[27]) in a non-diarrheic fecal sample possessed a rare pig G11P[27] genotype combination, but was only present at a low viral load (4.79 log₁₀ copies/g feces). Here it was attempted to reveal the entire genome of this strain, which was only partially successful. This was most likely due to the low viral load of the RVA strain in the fecal sample. Indeed, for successful RT-PCR amplification of some gene segments, further concentration of the RNA extracts using ethanol precipitation was necessary. Still, typical pig genotypes I5, A8 and T7 could be assigned to genes encoding the inner capsid protein (VP6), the interferon antagonist (NSP1) and the translation enhancer (NSP3), respectively. Similar pig genotypes have been disclosed from other pig RVA strains around the world, and have recently also been detected in Belgian pig strains [9, 19, 36-42]. It was suggested that proteins encoded by these pig genotypes may allow for a better match with the cellular machinery of the pig intestinal epithelial cells during viral replication [19]. Furthermore, an N1 NSP2 genotype was harboured by this strain as well, further confirming

the evolutionary relationship between pig RVAs and human Wa-like RVAs, and suggesting that this unusual G11P[27] virus also contained a typical pig genotype constellation [9, 19]. Our knowledge on the evolution of group A rotaviruses is increasing, as more complete genomes of human and animal RVA strains are being disclosed. This is in large contrast with the knowledge available on the genetics of RVCs, and especially that of pig RVCs. Only one ancient pig RVC strain (RVC/Pig-tc/USA/Cowden/1980/G1Px) has been characterized completely to date [28]. The non-diarrheic fecal sample also contained a high load ($9.56 \log_{10}$ copies/g feces) of an RVC strain. Using Illumina sequencing, it was possible to obtain the complete genome sequence of this RVC strain, but not that of the RVA strain. An accurate serotype classification system does not exist for RVCs, due to the difficulty to grow the virus in cells, but this is definitely needed in view of future RVC vaccine development. A genotype classification system has only been developed for a limited number of RVC gene segments, including those encoding VP7 and VP6, and recently also for VP4 [24-26]. The VP7 encoding gene of RVC strain 12R021 belonged to genotype G3, which was until now only discovered in 2 other pig RVC strains from the United States. Tsunemitsu et al. (1992) demonstrated that one of these G3 strains (HF), likely belonged to another serotype as strain Cowden (genotype G1) and Shintoku (genotype G2). This may indicate that strain 12R021 is not only genetically, but also serologically distinct from most other RVC strains isolated to date [43].

Here, a large diversity among VP4 encoding genes of pig and cow RVC strains was observed, which was in large contrast to the high relatedness between human RVC VP4 genes. Recently, Suzuki et al. (2015) proposed a VP4 genotype classification system (P1 to P6) based on a nucleotide sequence identity cut-off value of 80%. In this system, VP4 genes of human and cow RVC strains were classified in the genotypes P2 and P3, respectively, whereas pig VP4 genes belonged to genotypes P1, and P4 to P6. Still, care should be given when using this classification system, as only a limited number of VP4 nucleotide sequences from few host species (pig, cow and human) have been included [25]. It has been demonstrated for the RVA VP4 proteins that the highly variable VP8* domain possesses the capacity to recognize different carbohydrate moieties dependent on the genotype. As an example, typical pig genotype P[7] recognizes sialic acids, whereas differences in carbohydrate recognition have been observed between important human RVA genotypes P[6] and P[8] [44-46]. RVCs also possess a highly variable VP8* domain. The dependence on sialic acids for binding and infection of cells, and for hemagglutination of human type O, guinea pig and mouse red blood cells was demonstrated for pig RVC strain Cowden [47].

Still, it might be that the diversity observed among genes encoding VP4 proteins of contemporary pig RVCs results in the capacity to recognize and bind a wider scala of carbohydrate moieties for attachment to mucus and enterocytes. In contrast to the high diversity of VP4 genes of pig RVCs, it is more likely that human RVCs characterized to date only use a single conserved type of carbohydrate for attachment, as they are genetically much more conserved.

In contrast to human and cow RVC strains the diversity among known pig RVC VP6 genes is rather large. Using the proposed RVC VP6 nucleotide identity cut-off value of 87%, the VP6 gene of strain 12R021 could not be classified completely into one of the existing genotypes [26]. As such, 12R021 can be seen as a border-line I7 or a tentative new genotype I8. For pig RVAs, and for human RVAs from different genogroups, it was described that genotype-specific mutations were present at the VP7 interface of the VP6 proteins [19, 48]. The diversity observed among pig RVCs might have similar purposes. First, it may allow for a better recognition of different VP7/VP4 proteins encoded by different genotypes, resulting in a better structural fit in mature particles. Second, the high diversity observed in VP6 might be the cause of immunological selection pressure as well. Indeed, VP6 proteins have an important immunological function which has long been underestimated. For RVAs it was demonstrated that most antibodies are elicited against VP6, and unexpectedly less against the outer capsid proteins VP7 and VP4 [49]. Immunoglobulin A (SIgA) antibodies are internalized at the basal side of enterocytes before transcytosis, followed by excretion in the lumen. During this transcytosis, antibodies against the RVA VP6 proteins are thus able to intracellularly neutralize viral replication by sterical blockade of type I channels, thereby hampering RNA egress during transcription [50]. The availability of RVC susceptible cells will allow us to further investigate these hypotheses.

Moreover, gene segments encoding the remaining viral proteins of pig RVC strains clearly clustered in between genes of human and cow RVC strains. Unfortunately, only 2 pig RVC strains have been characterized to date, and both are genetically very divergent from each other, a characteristic which is less present in human and cow RVC strains [27-30]. It might be that pig RVC strains are thus circulating for a longer time in the population, or are being put under stronger immunological pressure due to repeated contacts with the virus in different production rounds. Yet, it is not possible to conclude for any ancestral roles between pig, cow and human RVCs. Analysis of more complete genomes of RVC strains from different host species should elucidate these questions. However, at least for RVC VP3

genes, a closer relationship between genes from pig and human RVCs can be suggested, as pig RVCs clustered in between 2 human VP3 subclusters [27].

Finally, results from this study also highlight that many viral species may reside in the intestine of piglets without even being noticed [2-5]. Most of these viral species are not routinely diagnostically investigated in cases of diarrhea on pig farms. Here, a porcine astrovirus (PoAstV), member of genus *Mamastrovirus* within the family *Astroviridae* was detected. Cross-neutralization assays have been applied to classify astroviruses into different serotypes, but this has been hampered by the lack of ability to grow all astrovirus strains in cell culture. Nowadays, classification is more often performed using genetical analysis of ORF2, which is the most variable astrovirus gene [51, 52]. A consensus classification system for porcine astrovirus is not available, as there is a vast genetic heterogeneity among strains recently detected. Still it is difficult to associate these infections with disease, as they may be isolated from healthy pigs as well [34, 52, 53]. This is in contrast to humans, where they have been associated with the pathogenesis of diarrhea [52]. Also for enteroviruses in piglets their role in the development of diarrhea is being questioned, and they are more frequently associated with neurological problems and abortion in pigs [54]. Enteroviruses belong to the family *Picornaviridae*, genus *Enterovirus G*. This genotype currently contains eleven types (EV-G1 to EV-G11) [55]. The Belgian enterovirus strain 12R021 was most closely related to a Hungarian strain, which was genetically distinct from the former prototype enterovirus strains PEV9 and PEV10, now called EV-G1 and EV-G2, respectively [35]. Further analysis of the genome will allow to further type this strain. Interestingly, The long 5' non-coding sequence is helping the virus for initiation of translation, by forming a secondary structure [56]. Still, these subclinical infections may have an impact on pig growth, which has been ascribed for instance to porcine circovirus infections [57]. Nonetheless, the detection of all these viruses provided only a picture of one time-point, and the history and outcome of these infections is unknown. In view of future studies in the field, analyses of the fecal viromes of diarrheic and non-diarrheic piglets will likely enable to discover new viruses, which might contribute to subclinical and clinical diseases in young piglets, but have remained unnoticed because of the lack of virus-specific diagnostic tests. Together with the availability of susceptible enterocyte cell lines for pigs, this will open a completely new era of research, which will eventually lead to innovative diagnostic protocols and control measures of (sub)clinical enteric diseases in the young pigs.

4.2.5. Acknowledgments

ST was supported by a scholarship of the Special Research Fund (BOF) of Ghent University. NCN, MZ and IDMR were supported by a doctoral fellowship of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). LMD was supported by a doctoral fellowship of The Research Foundation-Flanders (FWO Vlaanderen). The authors are grateful to DGZ Vlaanderen for supplying fecal material.

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Chapter V

General discussion

Diarrhea is one of the most important health problems affecting young piglets, which can be the consequence of non-infectious factors, such as feed changes and unavailability of maternal milk, and infectious causes, such as infections with viruses, bacteria and/or parasites. Diarrheic problems result in reduced weight gain, increased mortality and an elevated use of antimicrobial drugs. In humans, diarrhea is a major problem in young children and attributes significantly to the more than 6 million children that yearly pass away before reaching the age of 5 years. Rotavirus is considered as the most important cause of childhood diarrhea, resulting each year in 450.000 fatal cases, mainly in developing countries of Africa and Asia [1]. In contrast to their significant importance in humans, the forthcoming of rotavirus infections in young piglets has remained relatively unheeded, especially in Belgium in the past decades. Nonetheless, soon after the discovery of pig rotaviruses, their ability to induce diarrhea in young piglets was demonstrated by *in vivo* studies [2-4]. Moreover, rotaviruses can cross the species barrier and the existence of an evolutionary relationship between pig and human rotaviruses has been suggested [5].

In this doctoral thesis, it was aimed to develop new diagnostic tools to study the occurrence of rotavirus infections in Belgian piglets. Rotaviruses may form an attractive target for new and durable preventive measures to control diarrheic problems in young piglets. Therefore, Belgian rotavirus strains were thoroughly characterized in view of future vaccine formulation. It was also aimed to extend our knowledge on the evolutionary relationship between pig and human rotaviruses, as this increases our understanding of the risk for interspecies transmission events of rotaviruses between pigs and humans, in both directions.

5.1. Rotavirus infections in Belgian piglets

5.1.1. Improvement of diagnostics for pig RVA

Since the discovery of pig rotaviruses in the feces of diarrheic piglets in the 1970s, their ability to cause diarrhea in young pigs was demonstrated by *in vivo* experiments [2-4]. Thereafter, little attention was paid to the circulation of this virus in the Belgian swine population. In contrast, prevalence studies in many countries around the world reported that RVA infections were relatively frequently occurring [6-12]. Therefore, it was hypothesized that the diagnosis of pig RVAs in Belgium was mainly hampered by the lack of available sensitive diagnostic assays. In routine veterinary practice, fast antigen detection tests are mainly being used to diagnose RVA infections in pigs. Indeed, there is no golden standard diagnostic test available to detect RVA infections in piglets, and also in many different prevalence studies, several diagnostic assays with unequal levels of sensitivity have been applied. Nowadays, molecular diagnostic techniques based on reverse transcriptase polymerase chain reaction are being applied more frequently. One of the major challenges associated with the design of such assays is the need for specific primers targeting conserved genomic regions.

In the present thesis, a novel RT-qPCR assay was designed against the conserved gene segment 11 (NSP5) of pig RVA strains. SYBR[®] Green was selected as fluorescent dye to detect RT-qPCR amplicons, because it is generally cheaper than fluorescently labeled hydrolysis probes. By the use of 10-fold dilution series of an *in vitro* generated dsRNA fragment, it was possible to make a standard curve for absolute quantification and to take into account the efficiency of the reverse transcriptase reaction at high and low concentrations of RNA template, since this is the most fragile step in the whole RT-qPCR procedure [13, 14]. In contrast, most other RVA qPCR assays use plasmids or PCR products to setup a standard curve [9, 15-20]. The linear dynamic range of the new assay was between 7 log₁₀ and 1 log₁₀ copies per reaction. Furthermore, the sensitivity of the assay could be drastically improved by brief denaturation of RNA templates prior to adding them to the mastermix. RVA loads as low as 10^{4.35} copies/g feces could be quantified, whereas specific signals beneath the lowest point in the standard curve were considered as positive, but below the limit of quantification (<LOQ). In Chapter 4.1. of this thesis, gene duplications were encountered at the 3' end of NSP5 genes of some of the Belgian pig RVA isolates. These gene rearrangements initiated from nucleotide position 326 (using the Gottfried strain as a reference) while our RT-qPCR spanned a target region between nucleotides 117 and 232. As such, these duplications could not have led to an overestimation of our quantification results.

To validate the performance of the newly established RT-qPCR, 34 fecal samples mainly from weaned piglets, were analyzed for the presence of RVA. RT-qPCR was far more sensitive than fast antigen detection tests and virus isolation in MA104 and IPEC-J2 for the detection of RVA infections in piglets, and it has the advantage of giving quantitative data. Using conventional RT-PCR, it was also possible to detect RVA in most of the RT-qPCR positive samples. However, several primer sets were needed, and a new reverse primer was to be designed to successfully amplify the partial VP4 genes of most isolates. As such, RT-PCR was far more labour-intensive than RT-qPCR, and is less suitable for routine diagnostics or prevalence screening.

In Chapter 3.2, another forty five diarrheic fecal samples from pigs less than 2 weeks old from 36 Belgian pig farms were collected at a private diagnostic laboratory. It was shown that RVA infections on Belgian pig farms were often missed when only a fast antigen detection test was applied. In contrast with the study from Chapter 3.1, where most of the samples were analyzed using an antigen ELISA, all samples tested in Chapter 3.2 were analyzed using a strip, which was, remarkably, unable to detect RVA in several samples with a high viral load. Not unlogically, these results might indicate that the more labour-intensive antigen ELISA is more sensitive than the fast and convenient immunochromatographic strip for diagnosis of RVA infections in pigs. Still, it should be noted that in one diarrheic sample the diagnosis of an RVA infection was missed by our RT-qPCR, whereas it was detected using the strip and confirmed using RT-PCR and sequencing. This indicates that surveillance and sequencing of circulating RVA strains also must be conducted, in order to keep current rotavirus diagnostic tools up to date. This has also been reported for another RT-qPCR against the RVA NSP3 gene segment, which is frequently being used to detect human RVA strains [21]. The original primer set was not useful to detect all human RVA strains, because of the presence of 3 nucleotide mismatches in the forward primer region. Addition of a new forward primer increased the sensitivity of the assay [22].

5.1.2. Genotyping of Belgian pig RVA strains

Having optimized the diagnostics for pig RVA infections in Belgian pigs, it was aimed to genotype these positive isolates. Therefore, the genotypes of the genes encoding outer capsid proteins VP7 and VP4 of RVA positive isolates were characterized. The outer capsid proteins induce neutralizing antibodies and characterization of the genes encoding these proteins provides useful information for future vaccine formulation. In Chapter 3.1, twenty six different RVA isolates from diarrheic (n=23) and non-diarrheic (n=3) piglets were genotyped using the established 80% nucleotide sequence identity cut-off value to

discriminate VP7 and VP4 genotypes [5, 23]. Surprisingly, even in this relatively small sample size, a high genetic heterogeneity among the VP7 and VP4 genes was encountered. Six different G-genotypes (G2, G3, G4, G5, G9 and G11) for VP7 and 5 different P-genotypes (P[6], P[7], P[13], P[23] and P[27]) for VP4 were found in 12 different G/P combinations. G5P[7] was the most predominant G/P genotype combination. Strains bearing the combinations G4P[13], G2P[27] and G11P[27] were only isolated from piglets that were not diarrheic at the moment of sample collection. In Chapter 3.2., 17 other isolates from diarrheic Belgian suckling piglets less than 2 weeks old were genotyped. Similar genotypes were identified here, but G2, G11 and P[27] could not be detected. It was not possible to characterize 2 strains. In Table 1, the results of both studies are summarized, resulting in a total of 43 RVA Belgian isolates of which we were able to genotype the outer capsid proteins VP7 and VP4.

Table 1. Genotype distribution among Belgian pig group A rotaviruses.

	G2	G3	G4	G5	G9	G11	Gx	Total
P[6]		1 (2.3%)	5 (11.6%)	2 (4.7%)				8 (18.6%)
P[7]		1 (2.3%)	1 (2.3%)	8 (18.6%)	4 (9.3%)			14 (32.6%)
P[13]			<i>1 (2.3%)</i>	3 (7.0%)	3 (7.0%)			7 (16.3%)
P[23]			2 (4.7%)		5 (11.6%)			7 (16.3%)
P[27]	<i>1 (2.3%)</i>					<i>1 (2.3%)</i>		2 (4.7%)
P[x]			2 (4.7%)	1 (2.3%)			2 (4.7%)	5 (4.7%)
Total	1 (2.3%)	2 (4.7)	11 (25.6%)	14 (32.6%)	12 (27.9%)	1 (2.3%)	2 (4.7%)	43 (100%)

Italic: strains isolated from non-diarrheic piglets

In summary, no less than 14 different G/P combinations were encountered, confirming that the genes encoding the outer capsid proteins of pig RVAs are genetically heterogeneous. Among all characterized Belgian RVA strains, genotypes G5 (32.6%) and P[7] (32.6%) were most predominant for VP7 and VP4, respectively. These were also the most frequently detected genotypes among pigs worldwide [24]. Furthermore, the genotype combination G5P[7] was also most prevalent (37.3%) worldwide, which was also the case here in Belgium (18.6%) [24]. Nonetheless, geographic differences in strain predominance have been described as well. Indeed, G4P[6] RVAs were most dominant in studies in the United Kingdom and Vietnam, whereas G9P[13] RVAs were mostly isolated in Ohio, United States [6, 25, 26].

In the present thesis, the first Belgian pig RVA isolate RV277 was also genotyped. This strain was isolated in 1977 from a pool of watery diarrhea of 3 pigs, 4 days after removal

from the sow at 2 days of age [4]. Whereas for VP4 a typical pig P[7]-genotype was detected, a G1 genotype was disclosed for VP7. In general, this VP7 genotype is a rare finding in pigs, and animals in general, but is the most important genotype in humans.

5.1.3. RVC infections in Belgian piglets

So far, non-group A rotaviruses, such as RVB, RVC, RVE and RVH, have been encountered in feces of piglets around the world. In the present thesis, it was aimed to obtain better insights in the occurrence of RVC infections in Belgian piglets as well. However, in routine veterinary practice, pig RVCs cannot be detected because diagnostic tests are not available. Soon after the publication of our NSP5 assay, Marthaler and colleagues published a new set of primers and hydrolysis probes for detection of pig RVA, RVB and RVC in feces of piglets [9]. In that study, primers were designed against the VP6 encoding genes of each rotavirus species. In Chapter 3.2, the RT-qPCR from the aforementioned study was optimized for use in a SYBR[®] Green format. A 10-fold dilution series of a synthetic dsRNA template was included to setup a standard curve, which allowed for absolute quantification of RVC loads in fecal samples. The presence of RVC was demonstrated in 29% (n=13) of 45 fecal samples collected from Belgian diarrheic suckling pigs and viral loads ranged between $10^{5.40}$ and $10^{11.63}$ copies/g. In the United States, the importance of RVC was already demonstrated previously, and most infections with RVC only were detected in neonatal piglets with an age of less than 3 days old [9, 27]. Combined with the findings reported in the present thesis, this led us to conclude that RVC diagnosis should be routinely performed in cases of diarrhea among suckling piglets.

The Belgian pig RVC strains isolated from diarrheic suckling pigs were also characterized for their outer capsid proteins VP7 and VP4 (Chapter 3.2.). A nucleotide identity cut-off value of 85% was established to classify the VP7 encoding genes of RVC strains in 9 different genotypes (G1 to G9) [27]. All Belgian RVC isolates from diarrheic suckling pigs belonged to pig genotype G6, except one strain, which possessed the pig genotype G1. Interestingly, in Chapter 4.2 a high viral load of RVC was encountered in a fecal sample of a non-diarrheic weaned piglet as well. The VP7 gene of this strain belonged to the relatively rare pig genotype G3. We also characterized the VP4 genes of RVC isolates from Belgian diarrheic suckling piglets. A genotype classification system for RVC VP4 genes was lacking until recently. First, Suzuki and co-workers reported a nucleotide cut-off value of 80% upon analysis of human, cow and pig RVC strains. By using this classification system, RVC VP4 genes can be assigned to 6 distinct genotypes (P1 to P6) [28]. However, shortly thereafter, Jeong and colleagues identified a nucleotide cut-off value of 83% to assign RVC VP4 genes

into 7 different P-genotypes (P1 to P7). Using the cut-off value proposed by Jeong and colleagues, the VP4 genes of our Belgian RVC isolates were classified in the pig genotype P5 [29]. However, this genotype classification is still relatively premature, as only a limited number of RVC VP4 sequences are available. Consensus on this VP4 genotype classification will likely be reached after discussion in the Rotavirus Classification Working Group.

At this moment it remains unclear to which extent RVB, RVE and RVH are circulating in the Belgian swine population. Diagnostic assays for these viral species will be setup in the near future as well, and their occurrence and pathogenic importance will be further exploited in longitudinal field studies in Belgian suckling piglets and piglets after weaning. As an example, RVB infections have been found most frequently in diarrheic feeder piglets (>55 days) in the United States [9, 30]. More recently, RVH has been recovered from feces of diarrheic piglets in Brazil and the United States, underlining their potential importance in the pathogenesis of piglet diarrhea. Most of the samples analyzed in the US were collected from diarrheic piglets after weaning (n=111) and 18% of these tested positive [31, 32]. Given the occurrence of this rotavirus species in other countries around the world, these species are probably also prevalent on Belgian pig farms.

5.1.4. What about co-infections between rotaviruses and other pathogens?

In Chapters 3.1 and 3.2, the results of diagnostic tests for other enteric pathogens were provided by the diagnostic laboratories, if available. In general, diagnostic testing for the presence of *Escherichia coli* in diarrheic samples of young piglets is a routine practice. These findings let us to hypothesize that in some cases RVAs and *E. coli* strains may act synergistically in the pathogenesis of piglet diarrhea, as demonstrated hereafter in the hypothetical model shown in Figure 1.

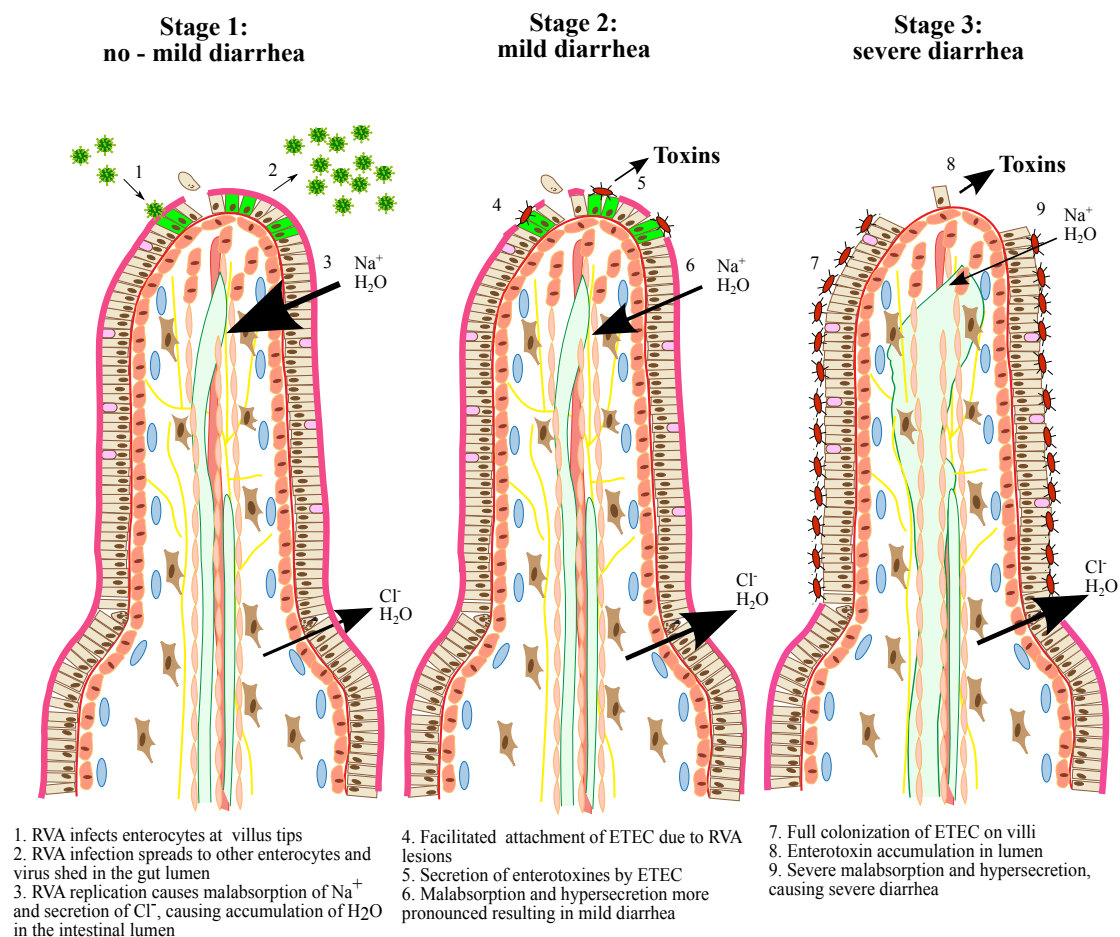


Fig 1. Hypothetical synergistic model between RVA and some ETEC strains in the pathogenesis of piglet diarrhea.

First RVA will pass the mucus layer and infect enterocytes at the villous' tips. Replication of RVA will induce mild to moderate malabsorption and hypersecretion, causing accumulation of fluid in the lumen, which results in a mild diarrhea. The replication of RVA likely also causes small lesions in the mucuslayer, which allows bacteria to attach to the surface of the underlying enterocytes. Bacteria will start to colonize further the epithelial surface of the intestinal villi and will release enterotoxins which exacerbates malabsorption and hypersecretion, finally resulting in severe diarrhea and dehydration.

A synergism between RVA and *E. coli* was already suggested earlier in the 1980s, soon after the discovery of RVAs. Indeed, Bohl and colleagues suggested that some ETEC infections may be secondary to RVA infections [2]. Lecce and colleagues weaned piglets under two different experimental conditions, namely a "sanitary" and "unsanitary" system. RVA diarrhea was induced approximately 16 days postweaning in the sanitary system, and hemolytic *E. coli* was not detected in these piglets. In contrast, in an unsanitary system piglets demonstrated RVA diarrhea 3 days after weaning and after a couple of days the

bacterial flora of these diarrheic piglets shifted from non-hemolytic to hemolytic *E. coli*. After recovery, the microbiota shifted back to non-hemolytic *E. coli* [33]. Still, care should be taken when interpreting the finding of non-hemolytic *E. coli* isolates, because in a study in Denmark it was demonstrated that many of these isolates may still express virulence factors and can thus be pathogenic even if they do not cause hemolysis in aerobic cultures [34]. This emphasizes that the isolation of a bacterial species is most often not enough to presume that it was the cause of the diarrheic problems, which was also demonstrated in Chapter 3.2. It is advised to detect virulence factors, such as fimbriae and enterotoxins, of *E. coli* isolates in order to assess their pathogenicity. Also for *Clostridium perfringens*, the isolation of the bacteria alone is not sufficient, as *Clostridium perfringens* type A is also a member of the normal microbiota. Shedding of a high number of bacteria and detection of the alpha and beta toxins are indicative for an etiological cause of the bacterium in the pathogenesis of diarrhea [35]. The relevance of the finding of the beta2 toxin was recently questioned [36]. However, due to the high costs associated with extra analyses to identify virulence factors, these assays are often not executed in routine veterinary practice, making it often difficult to come to sound diagnostic conclusions and to install effective therapeutic and prophylactic measures.

5.1.5. Successive subclinical rotavirus A infections in pigs after weaning

We aimed to further investigate the shedding of RVA and *E. coli* in piglets after weaning in a pilot study on a large closed Belgian pig farm. All nine piglets that were followed during 24 days after weaning started shedding RVA at the moment of weaning, or shortly hereafter. The outer capsid proteins of these strains were genotyped, and all strains possessed the G5P[13] genotype specificities. It was suggested that this RVA infection was acquired during the stay in the farrowing house, but kept under control by the lactogenic immunity. However, piglets farrowed by gilts and old sows were shedding the virus already at the moment of weaning, albeit at very low titers. This was not the case for piglets farrowed by second parity sows. Most likely, the milk of gilts and old sows contained lower concentrations of SIgA antibodies than that of second parity sows, as the latter come more in contact with RVA due to a higher incidence of infections in previous litters, which results in a boost of lactogenic immunity. The peak of RVA shedding was generally observed at 4 to 6 days after weaning, and replication of the G5P[13] strain lasted around 8 to 12 days. Surprisingly, immediately after the infection of this RVA strain, all piglets became infected with a second RVA strain, characterized as G9P[23]. This led us hypothesize that weaned piglets are successively being infected with heterotypic RVA strains, as shown in Figure 2.

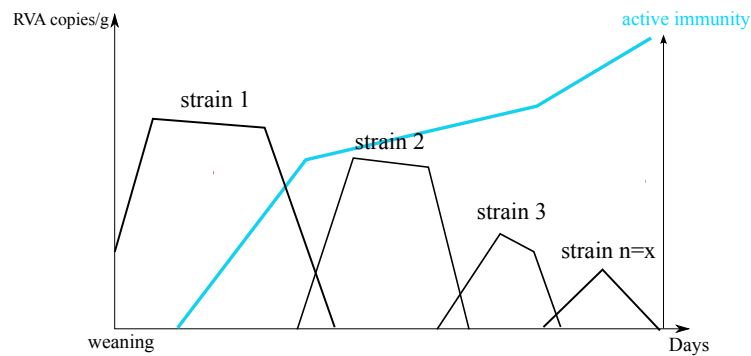


Fig. 2. Successive RVA infections in piglets after weaning.

Nonetheless, the extent of viral replication of this latter strain was significantly lower than that of the first replication wave with the G5P[13] strain. In some piglets it appeared that even a third replication wave started to take place, which in his turn was also lower than the second replication wave. Therefore, it appears that shortly after RVA infection some level of cross-protection is being induced against successive infections with different heterotypic strains. The finding of such waves of RVA replication let us to assume that successive RVA infections may also take place in the farrowing house and in older piglets after weaning, or even in fattening pigs.

Indeed, recurrent infections with different RVA strains throughout the production process from birth to slaughter have been demonstrated using conventional RT-PCR in a Japanese study. In that study, pigs were infected with around 5-6 strains throughout their lives [37]. However, as piglets become older they will likely develop local immunity which rapidly clears these infections and as such minimizes the outcome of these infections: e.g. less/no diarrhea, no growth retardation. Most piglets in the current pilot study were also colonized with non-hemolytic *E. coli*, whereas hemolytic *E. coli* was only detected at scattered time-points in some piglets. No explosive diarrheic problems were seen on this pig farm. Indeed, many prophylactic control measures were in use, such as addition of colistin and zinc oxide to the feed, and addition of organic acids to drinking water. Most likely, these measures helped to control the *E. coli* infections, and prevented enterotoxigenic *E. coli* strains to adhere to and colonize small lesions induced by the replication of the RVA strains. Nonetheless, in future studies it would be more useful to quantify the *E. coli* shedding in the fecal samples as well, like we did here now for the first time for RVA. New RT-qPCR assays against the conserved genes *FaeG* and *FedF* will be used to quantify excretion of F4⁺ and F18⁺ *E. coli* strains in future longitudinal field studies on more farms. Another interesting hypothesis resulting from the present study was that RVA strains may induce growth retardation in piglets as well. Interestingly, the piglet demonstrating the highest RVA

replication in the first week after weaning, represented by the area under the curve, showed the poorest weight gain. Therefore in future studies on more pig farms, not only the role of rotavirus replication on the pathogenesis of piglet diarrhea (clinical outcome), but also its impact on growth retardation (subclinical outcome) will be investigated. The occurrence of other important pathogens will be investigated as well, as they may also influence the observed outcome. Furthermore, the follow-up of piglets will be extended to suckling piglets in the farrowing house and piglets after weaning until they reach the prefattening stages.

In view of this hypothesis, targeting RVA infections might be a useful strategy to control the pathogenesis of piglet diarrhea and may result in a better growth of young piglets. As an example, vaccination of pregnant gilts and sows with an oral attenuated rotavirus vaccine will boost the lactogenic immunity of the sow and will protect suckling piglets against RVA infections in the farrowing house. Next, piglets need to be vaccinated at the moment of weaning to actively induce immunity when lactogenic protection is abruptly stopped. Therefore, prevention of RVA infections may be an alternative to the prophylactic use of antibiotics or zinc oxide in feed after weaning. When RVA cannot damage the epithelium, it likely becomes more difficult for *E. coli* to colonize the intestine and to cause severe diarrhetic symptoms. Eventually, strategies to prevent RVA infections may lead to a reduced consumption of antimicrobial drugs and lowering of the chances for antibiotic resistance selection, which will be beneficial for pig and human health on the long term.

5.1.6. Next-generation sequencing as a diagnostic tool for pig farms: a dream?

Still, in veterinary diagnostics and field studies, we are looking to specific pathogens, of which their pathogenicity or importance was previously demonstrated. The availability of next generation sequencing allows us to look further than are current knowlegde is reaching, and allows us to identify new enteric pathogens, which may exacerbate clinical disease (diarrhea), or pathogens which have a subclinical impact on the growth performances of piglets. In Chapter 4.2, next generation sequencing was initially applied to disclose the genomes of rotaviruses present in the fecal sample of a non-diarrhetic piglet, but interestingly, this analysis also revealed the presence of a pig astro- and enterovirus in the feces of that piglet. The entirety of viruses shed in feces is called the fecal virome. Only a limited number of studies have applied next generation sequencing to analyze the fecal viromes of piglets [38-41]. In a study from Sachsenröder and colleagues around three quarters of all viruses detected in the feces of piglets were bacteriophages, whereas one quarter of all viruses were of mamalian origin. Only a small minority of the viruses present in feces were of plant origin [40]. In general, most abundantly detected mammalian viruses

in all these studies were kobuvirus, rotavirus, pig stool-associated ssDNA virus, astrovirus, sapovirus and enterovirus [38, 40-42]. As further longitudinal studies will be conducted on more farms to investigate the excretion of rotaviruses and bacteria from birth to the prefattening stages, it will be interesting to analyze these samples with next generation sequencing as well. This may reveal new links between completely new or poorly characterized viruses and the pathogenesis of piglet diarrhea or subclinical growth retardation. Nowadays, we are mostly looking for one specific pathogen that was the cause of the disease, but it is more likely that co-infections of viruses, bacteria and parasites operate together in the pathogenesis of piglet diarrhea. These could even be viruses of which we are still not aware of. The availability of susceptible pig intestinal epithelial cell lines would enable us to study the replication of such viruses *in vitro*, and to develop new diagnostic and prophylactic measures against these new pathogens. Considering the moderately high costs (€200 per sample for consumables) and relatively long time it requires to prepare and analyze the samples (2 working days), it seems not feasible at the moment to include this method in routine veterinary diagnostic analyses, where quick and cheap assays are desired.

5.2. The evolutionary relationship between pig and human rotaviruses

5.2.1. Complete genome characterization of recent and ancient Belgian pig RVAs

After having genotyped the outer capsid proteins of Belgian pig RVA strains in Chapter 3, it became evident that the genes encoding these proteins were genetically very heterogeneous. Therefore, it was interesting to further investigate the complete genome constellation of a representative selection of Belgian pig RVAs. First, only a small number of pig RVAs ($n \approx 20$) have been sequenced entirely to date. In contrast, around 800 complete human RVA genomes were available in Genbank in 2014. Second, the existence of an evolutionary relationship between pig and human RVAs was suggested earlier, but our understanding was hampered by the lack of completely characterized, representative pig RVA genomes [5]. Finally, characterization of pig RVAs may facilitate the selection of strains for the development of new pig RVA vaccines, as such a vaccine strain should possess a genome constellation which is representative for the contemporary situation.

A selection of 7 recent and 1 ancient Belgian pig RVA strains was made for further complete genome characterization in Chapter 4. These strains bore representative G/P genotype combinations and covered at least all unique VP7 and VP4 genotypes found among pigs in

Chapter 3.1. The contemporary strains beared G2P[27], G3P[6], G4P[7], G5P[7], G9P[13], G9P[23] and G11P[27] genotypes, and the ancient strain RV277 possessed a G1P[7] genotype combination. The nucleotide sequences were obtained by RT-PCR amplification of the eleven gene segments of each strain, followed by Sanger sequencing. Using BLAST and the RotaC^{2.0} online webtool, genotypes could be assigned to each individual gene segment, based on the nucleotide sequence identity cut-off values established by Matthijssens and colleagues [5, 43]. Despite the large genetic heterogeneity among the genes encoding the outer capsid proteins VP7 and VP4, the genotypes of the 9 other gene segments of Belgian pig RVA strains were more conserved (Table 2).

Table 2. Genotype constellation of Belgian RVA strains.

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/Pig-tc/BEL/RV277/1977/G1P[7]	G1	P[7]	I1	R1	C1	M1	A8	N1	T7	E1	H1
RVA/Pig-wt/BEL/12R022/2012/G2P[27]	G2	P[27]	I5	R1	C1	M1	A8	N1	T7	E9	H1
RVA/Pig-wt/BEL/12R006/2012/G3P[6]	G3	P[6]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-wt/BEL/12R005/2012/G4P[7]	G4	P[7]	I5	R1	C1	M1	A8	N1	T7	E1	H1
RVA/Pig-wt/BEL/12R002/2012/G5P[7]	G5	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-wt/BEL/12R041/2012/G9P[13]	G9	P[13]	I5	R1	C1	M1	A8	N1	T7	E1	H1
RVA/Pig-wt/BEL/12R046/2012/G9P[23]	G9	P[23]	I5	R1	C1	M1	A8	N1	T1	E1	H1
RVA/Pig-wt/BEL/12R021/2012/G11P[27]	G11	P[27]	I5	Rx	Cx	Mx	A8	N1	T7	Ex	Hx
Consensus constellation of pig RVAs	Gx	P[x]	I5	R1	C1	M1	A8	N1	T7	E1	H1

x, unable to identify genotype

These genotype constellations were also similar to those of other pig RVA strains characterized around the world and it was concluded that Gx-P[x]-I5-R1-C1-M1-A8-N1-T7/T1-E1-H1 is the consensus pig genotype constellation for the viral proteins VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5.

Interestingly, human RVA strains can generally be classified in two major and one minor genogroups or genotype constellations, which are demonstrated hereafter in Figure 3. Human genogroup 1 strains or “Wa-like RVAs”, possess overall genotype 1 genes (I1-R1-C1-M1-A1-N1-T1-E1-H1), mostly in combination with the G1P[8], G3P[8], G4P[8], G9P[8] genotypes for the outer capsid proteins. On the other hand, genogroup 2 RVAs or “DS-1-like RVAs” mainly possess genotype 2 genes (I2-R2-C2-M2-A2-N2-T2-E2-H2), mostly combined with G2P[4]. The minor genogroup 3 (I3-R3-C3-M3-A3-N3-T3-E3-H3) contains the so-called “AU-1-like RVAs”, but are infrequently being detected in the human population. Earlier, it was suggested that human Wa-like RVAs and pig RVAs, and human DS-1-like and cow RVAs, possess a common ancestor [5]. Figure 3 demonstrates that

genotype constellations of pig and human Wa-like RVAs, and cow and human DS-1-like RVAs, are overall shared. These findings suggest that there is an evolutionary relationship between these animal and human RVAs.

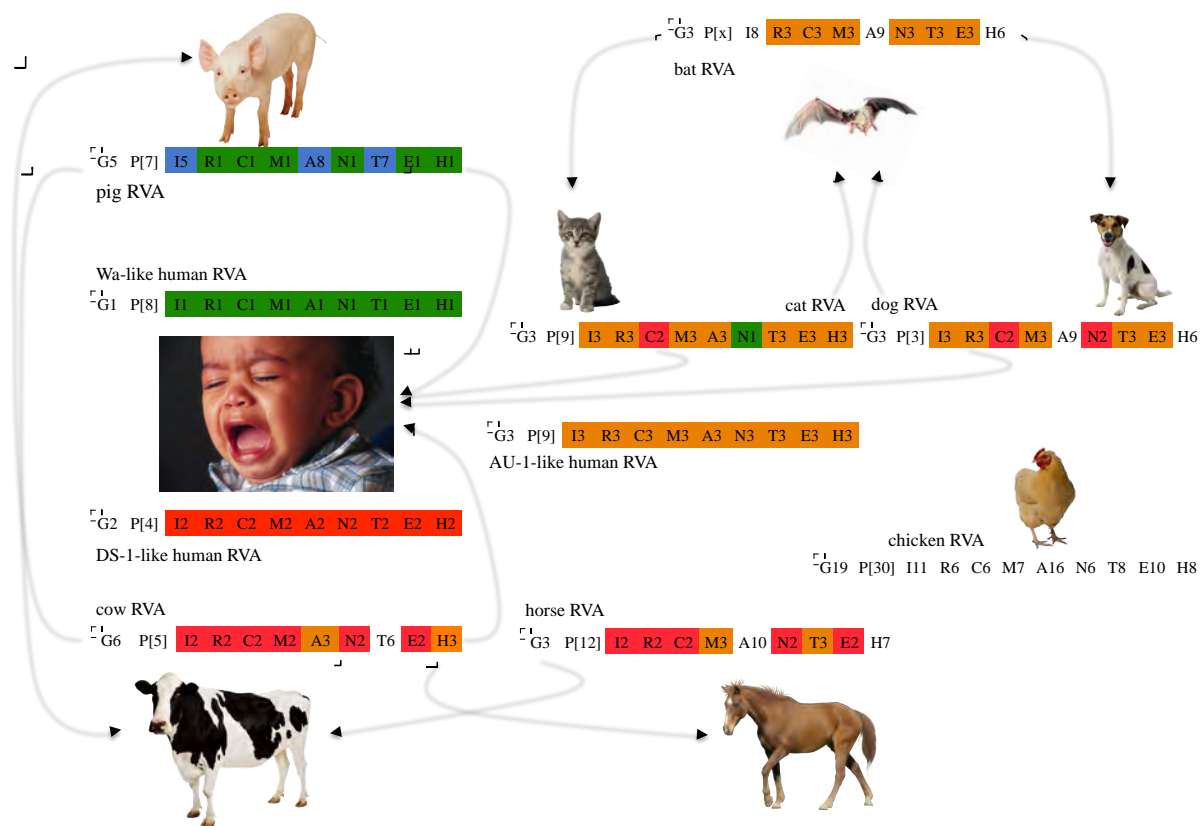


Fig. 3. Genetic relationship between RVAs from different host species.

However, it seems that there also exists some level of relatedness between RVAs from other host species (Figure 3), such as between RVAs from *Artiodactylae* and horses, and between RVAs from cats, dogs and bats. RVAs from birds typically are not closely related to RVAs found in mammals, which is demonstrated by their unique genotype constellation. As human and pig RVAs are evolutionary related, do they really form a threat for interspecies transmission, in both directions? Given the fact that interspecies transmission events are not very frequently observed, and that most of these species crossings are dead-end infections, it can be suggested that transmission between host species and subsequent spreading of the foreign virus in the new host species is likely hampered by the existence of one or more species barriers. To answer this question a closer look on the complete genome sequences of the Belgian pig RVAs was taken, and their relationship with human Wa-like RVAs examined hereafter. It should be noted that the hypotheses that are presented hereafter should

still be proven by means of *in vitro* experiments using pig and human RVA strains in relevant cell lines of the pig and human enterocyte, which are still not available.

5.2.2. VP4 and VP7 proteins: host species barriers or sources of antigenic diversity?

5.2.2.1. The viral lectin domain of VP4 as a host species barrier

One of the first barriers for interspecies transmission of RVA strains seems to be the VP4 protein, which protrudes from the viral surface (Figure 4.a.). Indeed, this protein is able to bind carbohydrate moieties present on mucus and the surface of enterocytes, by means of its lectin domain on the globular VP8* subunit. With the diversity observed among VP4 encoding genes of RVAs isolated from different host species, viral attachment seems to be one of the key host species determinants.

Several animal RVA strains were considered as “sialic acid dependent” for attachment and infection of continuous cell lines, based on their reduced infection in cells treated with bacterial sialidases. Sialic acids were indeed major attachment factors for RVA strains from pigs, cows and dogs, possessing the P[1], P[2], P[3] and P[7] VP4 genotypes. In contrast, human RVA strains, and most other animal RVA strains were considered to be “sialic acid-independent”, because sialidase treatment did not influence infection in cell cultures. Nevertheless, it was demonstrated later that “sialic acid independent” human RVA strains do make use of sialylated glycans for infection of cells. However, these sialic acid residues were located more internally on the sugar tree and could not be removed by the commonly used bacterial sialidases in the former experiments [44-46].

Liu and colleagues identified that VP4 genotypes of all RVA strains could be classified into 5 large groups (P[I] to P[V]), based on similarities between VP8* amino acid sequences. It was suggested that VP8* proteins belonging to the same group, possessed binding-specificities for the same or similar carbohydrates. As an example, the sialidase-sensitive genotypes P[1], P[2], P[3] and P[7] belonged to group P[I], which also contained other RVA genotypes from animal origin, including pig genotypes P[13], P[23], P[26], P[27], P[32] and P[34]. Nonetheless, genotypes P[26], P[32] and [34] were not isolated from Belgian pigs here. The VP4 phylogenetic analyses in the present thesis confirmed the close clustering of the sialidase-sensitive genotypes P[1], P[2], P[3] and P[7] in the phylogenetic tree of VP4 genes. Furthermore, we also demonstrated that pig genotype P[23] clustered closely together with these sialidase-sensitive genotypes (Figure 4.b.), and we conclude here that P[23] strains are likely also dependent on sialic acids for infection of cells. This vision is supported

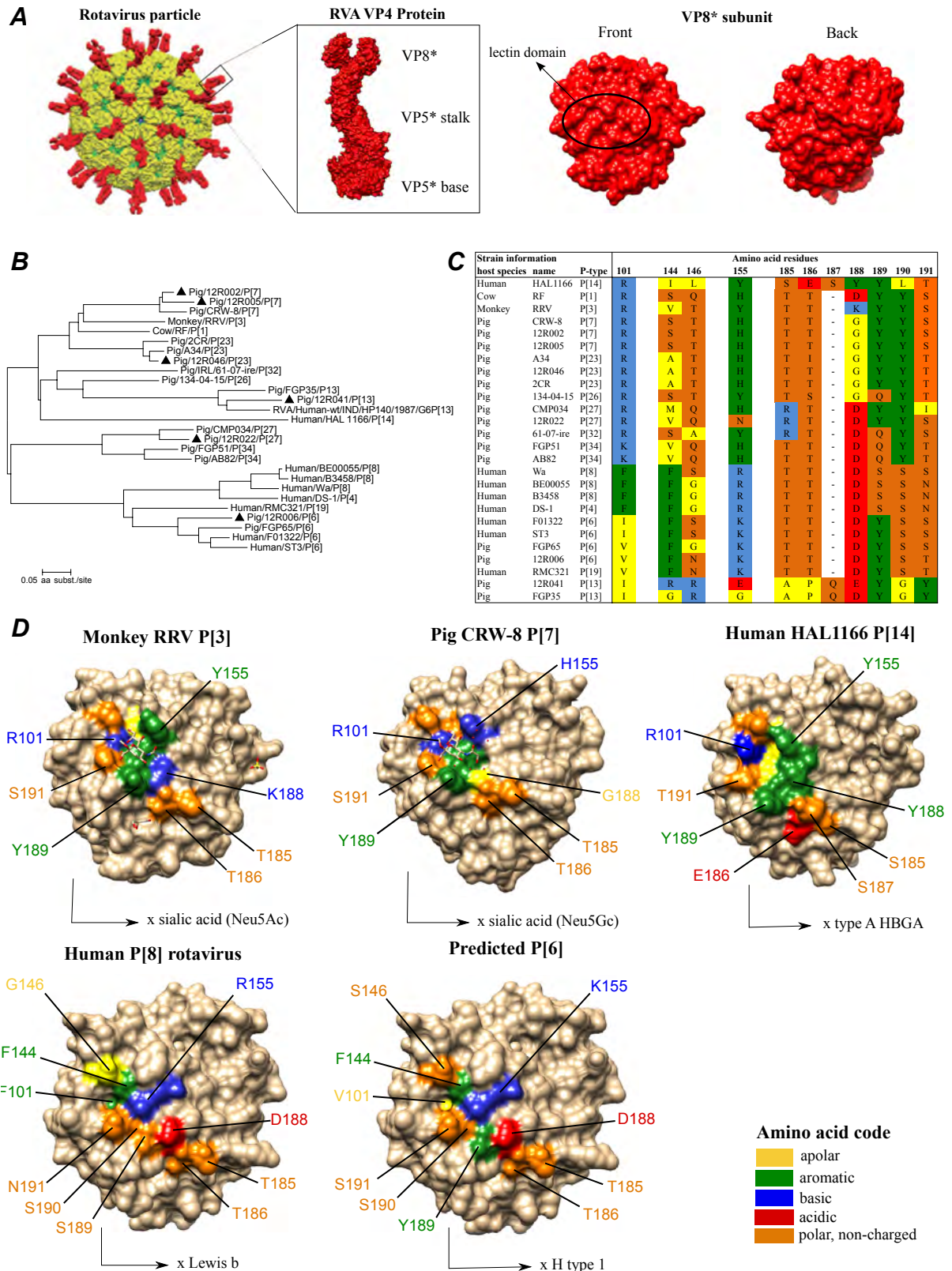


Figure 4. Analysis of the VP8* lectin domains from rotaviruses with different P-genotypes. A. Rotavirus and VP4 and VP8* structure and location of the carbohydrate binding domain in the VP8 subunit; B. Phylogenetic relationship of VP8* amino acid sequences of different rotavirus strains; C. Amino acid residues interacting with carbohydrates of strains with distinct genotypes; D. Lectin domains on different VP8* structures.

by the ability of P[23] strains to replicate efficiently in MA104 cell cultures, which was also observed for the sialidase-sensitive pig P[7] strains (Chapter 3.1). A structural model of the VP8* subunits of the sialidase-sensitive rhesus monkey strain RRV (P[3]) and pig strain CRW-8 (P[7]) was generated earlier and the key amino acid residues involved in the interaction with sialic acids were identified (Figure 4.c. and 4.d). The sialidase-sensitive pig strains possess a simple glycine at aa position 188, which causes less steric hindrance and allows binding with a sialic acid residue. Furthermore, the amino acid residues involved in attachment to carbohydrates are fairly similar between P[7] and P[23] strains (Figure 4.c.), indicating that they both use similar or identical sialylated carbohydrates for attachment and infection of MA104 cells, and probably also in enterocytes. Furthermore, it was demonstrated that sialidase-sensitive strains bind to Neu5Gc sialylated carbohydrates, whereas they did not bind to Neu5Ac glycans [47]. Neu5Gc residues have been encountered in many animal species and primates, but not in humans, whereas Neu5Ac is more commonly found on the surface of human cells [48]. This difference in sialic acid composition may explain why infections with typical pig P[7] and P[23] are almost never observed in humans. Indeed, the only reported case of an interspecies transmission event of a G5P[7] pig strain was detected in a Cameroonian child [49].

Nonetheless, differences between the carbohydrate interacting amino acids of VP8* proteins coded by genotypes P[26], P[27], P[32] and P[34] were also seen (Figure 4.c.). Interestingly, P[13] strains were most divergent from all other pig RVAs, and it might be that these pig strains use other attachment factors. These findings indicate, but do not confirm that pig RVA strains with different P-genotype specificities may bind to distinct carbohydrate residues for attachment to mucus or the enterocyte's surface, which will be investigated in the near future.

It was also demonstrated that genotypes P[9], P[14] and P[25] which clustered together in the group [III] according to Liu and colleagues, were able to bind type A histobloodgroup antigens (HBGAs) (Figure 4.d.). P[14] strains infect both humans and members of the *Artiodactylae* family, whereas P[9] strains mainly infect cats. On the other hand, group [II] contained the major human genotypes P[4] and P[8], and genotypes P[6] and P[19], which are shared between humans and pigs. Also in our phylogenetic analyses, these genotypes clustered closely together (Figure 4.b.), indicating the existence of a close evolutionary relationship between these four genotypes. Especially P[8] and P[4] are highly related genotypes, and serologically they are seen as subtypes of serotype 1, namely 1A and 1B. In previous studies, it was demonstrated that P[4] and P[8] strains use histobloodgroup antigens

(HBGAs) like the H-type 1 antigen and Lewis^b antigen for attachment to cells. On the other hand, for P[6] strains it was only observed that these are able to bind H-type 1 antigens [50]. Recently, P[8] strains have been shown to infect only Lewis- and secretor-positive children. On the other hand, P[6] strains mainly infected children with the Lewis-negative phenotype [51]. Only 4 to 6% of the Caucasian population possess the Lewis negative phenotype, whereas it can be higher than 30% in African populations. Indeed, these findings might explain why P[8] strains are generally more frequently observed in Caucasian individuals, whereas P[6] infections are readily more affecting African ethnical populations. In the most recently published paper on this topic, Böhm and colleagues (2015) confirmed that P[14] and P[9] strains were indeed able to bind type A HBGAs. Controversially, it was demonstrated that human RVA strains (P[4], P[6] and P[8]) did not bind to Lewis^b and H-type 1 antigens, contrasting the earlier findings described above. Instead, it was discovered that P[4] and P[6] strains, but not P[8] strains, do recognize and bind type A HBGAs, like it was reported for the genotypes P[9] and P[14]. As a result the dependency of rotavirus strain susceptibility on Lewis or secretor phenotypes remains to be further investigated [52].

Still, it was interesting to observe that P[6] and P[19] genotypes are shared between pigs and humans, which suggests that both species must possess a shared or similar carbohydrate on the surface of their enterocytes, making them susceptible for these strains. Indeed most interspecies transmission events from pigs to human detected so far were cases of P[6] strains. Examples of these are the transmission of the G9P[6] pig strain BE2001 to a Belgian child, causing diarrhea. Also in Hungary, evidence of interspecies transmission events from pigs were sporadically detected over a period of 15 years [53]. However, only blood groups A and O are expressed in pigs [54]. The lectin domain of these P[6] strains would fit better together with type A HBGAs or the H-type 1 antigen, as the presence of an aromatic amino acid (Y189) in the lectin domain would generate less space to interact with more complex sugars like Lewis^b (Figure 4.d.). On the other hand, P[4] and P[8] strains possess a smaller polar S189, which would enable to fit the Lewis^b antigen, as observed earlier.

Nonetheless, most researchers only talk about interspecies transmission between humans and animals in one direction. However, transmission can also occur from humans to pigs, but is infrequently detected because pig RVA surveillance is infrequently being conducted, in comparison to humans. As an example, a G5P[8] strain was detected in the feces of diarrheic pig in the United Kingdom. Whereas G5 is a common pig genotype, P[8] is a typical human genotype. The VP4 sequence of this P[8] strain was fairly identical to an Irish human P[8] isolate [25]. Likely the human VP4 gene segment had reassorted with a pig RVA strain after

a case of interspecies transmission from a human to a pig. These findings demonstrate that interspecies transmission can occur between pigs and humans, in both directions. We also suggest that these species crossings are more likely to occur with strains bearing the relatively closely related genotypes P[4], P[6], P[8] or P[19].

Interestingly, similar observations have been done in the research field of norovirus, a member of the family of the *Caliciviridae*. This virus is considered to be the second most important cause of childhood diarrhea, and is responsible for the majority of foodborne gastrointestinal disorders [55]. The virus has been classified into 5 genogroups, GI to GV [56]. In humans, genogroup GI, GII and GIV strains are found, whereas GII strains have also been detected in pigs. Within the genogroups, each strain is assigned a genotype, because a serological classification system is lacking through the inability to grow this virus in cell cultures. Strains bearing genotype 4 within genogroup II, briefly GII.4, are responsible for the majority of all human norovirus infections [55, 56]. In humans, it was seen that the host susceptibility to different norovirus strains is genetically determined. Non-secretor (*FUT2*) phenotypes were resistant to infections with GI.1 norovirus (e.g. Norwalk virus) [57]. An extensive overview of norovirus and its dependency on histoblood group antigens is beyond the scope of this discussion, but can be found in an excellent review from Ruvoën-Clout and colleagues [58]. Interestingly, it was also demonstrated that human norovirus strains belonging to GI and GII.4 were able to bind to secretor positive piglets, expressing type A or H antigens. Furthermore, after inoculation with a human GII.4 strain, a higher number of secretor-positive (A or H positive) than non-secretor piglets shed norovirus and seroconverted [59, 60]. These findings indicate that also in pigs genetic factors may play a role in norovirus susceptibility, which might also be the case for rotavirus infections. Furthermore, these findings indicate that type A and H antigens expressed in pigs are identical or similar to those found in humans. Interestingly, some human norovirus strains (GII.1 and GII.3) with Le^b binding specificities were able to bind to type A positive pig tissues. However, the *FUT3* gene, which determines the Lewis phenotype, has not been detected in other host species than humans. These findings suggest that a Lewis-like gene product might play a role in attachment of these human norovirus strains in pigs [59].

5.2.2.2. Antigenic differences between VP7 proteins of pig and human RVAs

By undergoing interspecies transmission, RVAs from a foreign host species may introduce antigenic/genetic diversity in a new host species population. By introducing new antigenic variants in the population, it may be that the population is naïve to such strains, which can potentially result in their rapid spreading in the new host species. Indeed, it was shown

earlier that human G9 RVA strains were the result of interspecies transmission events from pigs to humans. After several reassortment events with human Wa-like RVAs, these G9 strains were able to spread rapidly in the human population, and G9P[8] strains are now being considered as the fifth most important human RVA strain around the world [61]. Analysis of the antigenic regions (7-1a, 7-1b and 7-2) of the VP7 proteins of human and pig G9 strains, showed that only a very small number of amino acid mutations were present, further confirming their close evolutionary relationship.

It became also evident that many other VP7 genotypes, namely G1, G2, G3, and G4, were also shared between pig and human RVAs. However, human and pig G1, G2 and G4 VP7 genes were only poorly related to each other, and formed genetically distinct subclusters according to the host species of origin [62]. This was also demonstrated by comparison of the antigenic regions of the VP7 glycoproteins of Belgian pig RVA strains with their human counterparts and other pig strains (Chapter 4.1.). Several amino acid mutations were demonstrated between human and pig RVAs belonging to the same genotype. These findings indicate, that these viruses are not only genetically, but also antigenically distinct. Given the poor antigenic relationship between most pig and human VP7 proteins, it can be considered that pig VP7 proteins may form a potential source of antigenic diversity in the human population, and *vice versa*, and it might be that the vaccinated human population is not or only partially protected against such antigenically distinct animal strains.

Genotypes G5 and G11, which are closely phylogenetically related to each other and also detected here among Belgian piglets, are normally not found in the human population. Exceptions were sporadic cases, such as an epidemic with G5P[8] strains in South America in the 1980s, which was most likely the result of a reassortment event between a pig G5-strains and a human RVA strain [63-66]. All these results indicate that, whether or not pig RVAs possess VP7 genes that are similar or distinct to human VP7 genes, they are likely capable of crossing the host species barrier to infect humans. Most of these infections result in dead-end infections, and the potential mechanisms which may cause the inefficient spreading of pig and human RVAs in a new host species after interspecies transmission, are discussed hereafter.

5.2.3. Other viral proteins that could hamper efficient interspecies transmission

Earlier we concluded that pig RVA strains possess a consensus Gx-P[x]-I5-R1-C1-M1-A8-N1-T7/T1-E1-H1 pig genotype constellation. Even though, while several genes of pig and human Wa-like RVAs share the genotype 1, phylogenetic analysis revealed that genes encoding VP1, VP2, VP3, NSP2, NSP3, NSP4 and NSP5 of pig and contemporary human

Wa-like RVAs were only moderately to poorly related. Remarkably, VP1, VP2 and NSP3 genes of some pig RVAs were moderately related to historic human Wa-like RVAs [42, 67, 68]. These findings indicate that although most of the genes of pig and human RVAs belong to the same genotype 1, they readily segregated into different evolutionary directions.

Interestingly, for genes encoding VP6, NSP1, and NSP3, typical pig genotypes I5, A8 and T7 were disclosed, which are not shared with human Wa-like RVAs. Most likely, these pig genotypes played a key role in the adaptation of pig RVA strains to pig enterocytes. However, whether pig RVAs were progenitors of human RVA strains, or *vice versa*, cannot be definitely concluded based on the data available here. However, we carefully hypothesize that human RVAs might have been the progenitors for pig RVAs. As an example, the first Belgian pig RVA isolate contained a human G1 genotype for VP7 and possessed an I1 genotype for VP6, which is shared with human Wa-like RVAs. Other ancient strains such as OSU and Gottfried, possessed typical human Wa-like genes for NSP1 (A1) and VP6 (I1), respectively [69]. Also the VP1 and VP2 genes of historic human RVA strains were closely related to pig RVAs. However, there is a big gap of knowledge on pig RVAs missing, and if more ancient strains would have been available for characterization, it might have been easier to determine any ancestral role for human or pig RVAs. However, the hypothesis that human RVAs might be the ancestor for RVAs in pigs is in contrast with the observation that the genetic diversity among pig RVAs is generally much higher than that observed among human RVAs. This would indicate more in the direction of an ancestral role of pig RVAs for human RVAs. Still, the high genetic diversity among pig RVAs might be explained by the fact that the selection pressure is much higher in the pig population than in the human population. Indeed, the turnover in pig production is very high and pigs come into contact with different environments and many other pigs through the entire production process.

Despite a common origin of the progenitor human and pig RVA strains, it seems that currently there exist some species barriers which hamper the efficient spreading of RVA strains in a foreign host species. Otherwise, pig and human RVAs would be more frequently detected in the human and pig population, respectively. The first discussed barrier hampering interspecies transmission was the VP4 protein and its interaction with carbohydrates, but another protein with a key function in host species restriction is NSP1, an interferon antagonist. NSP1 plays a key role in avoiding the host's antiviral response. It was hypothesized in the present thesis that human strains encoding an A1 NSP1 protein replicate better in human enterocytes, whereas strains with an A8 NSP1 proteins better replicate in

pigs, because of a better match of the respective NSP1 proteins with their respective host species.

A highly conserved zinc finger or RING domain, containing cysteine and histidine residues, is present at the N-terminal domain of NSP1 [70]. Amino acid analysis of NSP1 A1 and A8 strains demonstrated that this region was indeed conserved between both genotypes. It was later demonstrated that this region functions as an E3 ligase, which cooperates with an E1 activating enzyme and E2 conjugating enzyme to ubiquitinate proteins to target them to the proteasome. On the other hand, the C-terminal half of NSP1 recognizes and binds different factors involved in the regulation of interferon responses, such as interferon regulatory factor 3 (IRF3), IRF7, IRF9 and β -TrCP [71-75]. By analysis of the amino acid sequences of pig and human RVA strains, it was shown in the present thesis that this region was highly variable between proteins coded by A1 and A8 genotypes. Therefore, it was suggested that A8 proteins might recognize and suppress different proteins, or species variants of proteins that are recognized by NSP1 proteins encoded by A1 genotypes. As such, it can be posed that pig RVA strains are less capable of suppressing the innate immune responses in human enterocytes, and *vice versa*.

Upon infection or by stimulation with cytokines, an interferon response can be triggered in the cell by activation of the NF- κ B pathway. In uninfected cells, NF- κ B is present in the cytoplasm and I κ B prevents its translocation to the nucleus. Some pathogen-associated molecular patterns and cytokines activate I κ B kinase (IKK), which phosphorylates two serine residues on I κ B. These serine residues are part of a phosphodegron motif, DSG Φ xS (wherein Φ is a hydrophobic amino acid). The phosphorylated I κ B is then recognized by β -TrCP, which is part of the complex SCF- β -TrCP with E3 ubiquitin ligase activities. Consequently, I κ B is ubiquitinated and targeted to the proteasome for degradation, enabling NF- κ B to translocate to the nucleus and trigger the expression of interferon regulatory genes, resulting in an interferon response.

Recently, Morelli and colleagues analyzed the nucleotide sequences of all NSP1 genotypes found in different host species. The NSP1 genes with A1, A2 and A8 genotypes from humans and pigs clustered closely together, distinct from all other NSP1 genotypes. At the C-terminal end of each NSP1 protein, there was a conserved DSGIS motif present, which was also present in our Belgian pig RVA isolates. Interestingly, this motif resembles the phosphodegron-motif present on I κ B. It was demonstrated that the NSP1 protein of pig strain OSU (genotype A1) is able to degrade β -TrCP, which is concerted by NSP1. The viral protein recognizes β -TrCP at its phosphodegron motif, and subsequently ubiquitinates it by

the E3 ligase activity of the C-terminal half of NSP1. As a consequence, I κ B degradation and NF- κ B translocation the nucleus is prevented and thereby the interferon response blocked [76]. However, these findings describe the actions of NSP1 proteins with A1 specificities, which are normally not found in pigs, except for OSU.

Di Fiori and colleagues recently also demonstrated that other human (A1) and pig (A8) RVA strains are also capable of suppressing the NF- κ B pathway by targeting β -TrCP to the proteasome. Interestingly, they also discovered that human RVA strains, but not porcine RVA strains, are able to reduce IRF7 levels [77]. As such, this might explain why porcine RVA replication is likely more effectively blocked in human enterocytes, and why many interspecies transmission events are dead-end infections. Still, NSP1 proteins can also block the interferon response at many other levels, which may in their turn function as a species barrier [78].

Another protein that may be involved as a barrier for interspecies transmission is NSP3. Here, the porcine origin of the T7 genotype of NSP3 was confirmed. The majority of Belgian pig RVA strains beared this genotype, and it is more and more frequently being detected among other pig RVA strains around the globe. However, another considerable fraction of pig RVA strains bears the genotype T1, which is also evolutionary related to human T1 NSP3 proteins. Most of the mutations between proteins encoded by T1 and T7 genotypes were located at the C-terminal region of the NSP3 protein, which interacts with the eukaryotic initiation factor 4G (eIF4G). These species- and genotype-specific changes may lead to a better recognition of human or pig eIF4G, thus likely better suppressing host cell protein synthesis in favor of viral protein translation [79-81].

While I5 is the dominantly detected genotype for VP6, the I1 genotype was seldomly seen in pigs. In the present study, ancient strain RV277 from 1977 also possessed an I1 genotype for VP6, like it was also shown for ancient strain Gottfried. Furthermore, the I1 genotype was also recently detected in pig-like human strains from Hungary and the Democratic Republic of Congo [53, 82]. Several amino acid changes were observed at the apical site of the VP6 trimer, which is known to interact with the VP7 outer capsid protein [81]. It might be hypothesized that the I5 genotype encodes a viral inner capsid protein that allows a better structural fit with the vastly diverse VP7 and VP4 proteins borne by pig RVA strains. So it might be that both variants (I1 and I5) are still circulating in the pig population, but that there is now a tendency for selection toward the I5 genotype, because of the aforementioned advantage.

As a conclusion, despite the close evolutionary relationship between pig and human Wa-like RVAs, it seems that both viruses have entered different evolutionary paths, making it more difficult for these strains to spread efficiently in a foreign host species after an interspecies transmission event. However, there is a risk that gene segments of pig RVAs are incorporated in human Wa-like RVAs, and *vice versa*, by reassortment after interspecies transmission and successful reassortment. For human RVA strains it was demonstrated that reassortment events between different human Wa-like RVAs are not very frequently occurring [42, 83]. Furthermore, reassortment between Wa-like and DS-1 like human RVAs have been detected, but very infrequently, and most of them ceased. Indeed, it was suggested that certain gene constellations function better when kept together, because they encode proteins that match better to each other [81]. Whether pig and human Wa-like RVA strains are able to create more viable reassortants than Wa-like and DS-1-like human strains cannot be definitely concluded but can be assumed given the shorter genetic distance between pig and human Wa-like genes. As such, surveillance of RVA strains in the human and pig population needs to be warranted, in order to detect emerging reassortant strains.

In the current thesis, the second complete RVC genome of a pig was also characterized, which showed a poor relatedness to another ancient pig RVC strain, Cowden, and RVC strains isolated from humans and cows. More research is needed to further explore the evolutionary relatedness among RVC strains isolated from different host species.

Finally, the insights from the current thesis open new opportunities to study the relatedness between rotaviruses from different host species and existence of rotavirus species barrier in the future. The availability of susceptible intestinal epithelial cell lines from pigs and humans will therefore be of major importance, and are currently being developed. Furthermore, the importance of rotavirus infections in the pathogenesis of piglet diarrhea and the economical impact will be assessed in the near future, and strategies will be developed to be able to prevent such infections on affected pig farms.

5.3. References

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Chapter VI

Summary - Samenvatting

Summary

Diarrhea is one of the most important health problems affecting young piglets. In humans, rotaviruses have been considered as an important cause of diarrhea in children under 5 years of age. In contrast, the situation in young piglets has remained unheeded, especially in Belgium in the past decades.

In **Chapter 1** of this thesis, the differential diagnosis of diarrhea in young piglets is being described. The focus in this introduction mainly lies on rotaviruses of pigs and humans. Hereafter, a short description of currently available measures to treat and prevent diarrheic problems in piglets is given.

In **Chapter 2**, the aims of this doctoral research are defined.

Chapter 3 describes the circulation of rotaviruses in Belgian suckling and weaned piglets. In **Chapter 3.1** the diagnostics for pig group A rotavirus (RVA) infections were improved by the development of a new quantitative real-time PCR assay (RT-qPCR). This assay was applied on a set of 28 diarrheic and 6 non-diarrheic fecal samples, most of them collected from weaned piglets, and was shown to be more sensitive than the routinely used antigen detection tests and virus isolation in MA104 and IPEC-J2 cell lines. Twenty six RVA positive isolates were genotyped for the genes encoding outer capsid proteins VP7 and VP4. Six different G-genotypes were detected for VP7 (G2, G3, G4, G5, G9 and G11), and five different P-genotypes were found for VP4 (P[6], P[7], P[13], P[23] and P[27]) in a total of 12 different G/P genotype combinations. These findings demonstrated that RVA strains circulating in Belgian piglets were highly genetically heterogeneous. Furthermore, almost all RVA positive samples were also co-infected with enteric bacteria.

In **Chapter 3.2**, a closer look was given to the importance of RVA and RVC infections in the pathogenesis of diarrhea in Belgian suckling piglets less than two weeks old. Therefore, 45 fecal samples from 36 different pig farms were collected at a private diagnostic laboratory. On 22 of 36 farms tested (61%), high viral loads of RVA) and/or RVC were detected. In contrast, rotavirus infections could only be detected on 9 of 36 pig farms (25%) when a commercial antigen detection test was applied. These findings are indicative for an important role for both rotavirus species in the pathogenesis of diarrhea in suckling pigs. Seventeen RVA isolates were also genotyped for their outer capsid proteins VP7 and VP4.

Similar genotypes as in chapter 3.1 were identified here. All characterized RVC strains belonged to genotype G6 (VP7), except for one strain possessing the G1 genotype. VP4 genes of Belgian RVC strains were genetically highly heterogeneous. Most rotavirus positive samples were also infected with *Escherichia coli*, whereas *Clostridium perfringens* infections were mainly detected in rotavirus negative samples. These results offered better insights in the importance of RVA and RVC infections in the pathogenesis of diarrhea in Belgian suckling piglets.

Eventually, in **Chapter 3.3** the shedding of RVA and *Escherichia coli* was investigated during a pilot study on a large closed Belgian pig farm in 9 piglets during the first 24 days after weaning. All piglets became successively infected with 2 heterogeneous RVA strains (G5P[13] and G9P[23]). However, the second replication peak was less pronounced, indicating that a low level of cross-protective immunity might have been induced upon infection with the first strain. All pigs shed non-hemolytic *E. coli*, but hemolytic *E. coli* were only detected in a few piglets at scattered timepoints. The limited proliferation of bacteria may be the result of prophylactic treatments with colistine sulphate and zinc oxide. Consequently, explosive diarrheic problems were absent on this farm. However, the present results do suggest that RVA infections might be able to induce subclinical growth retardation, as piglets with the highest RVA replication demonstrated the poorest weight gain. This study opened new hypotheses for further interesting research.

In **Chapter 4**, rotaviruses isolated from Belgian piglets and other enteric viruses were completely genetically characterized. First, in **Chapter 4.1** the complete genomes of a selection of six recent (G2P[27], G3P[6], G4P[7], G5P[7], G9P[13], and G9P[23]) and one historic (G1P[7]) Belgian pig RVA strains were assessed using Sanger sequencing, and phylogenetically analyzed together with all other globally available pig RVA genomes to investigate their evolutionary relationship with human RVAs. In contrast to the large diversity of genotypes found for the outer capsid proteins VP4 and VP7, a relatively conserved pig genotype constellation (I5-R1-C1-M1-A8-N1-T7/T1-E1-H1) was found for the other 9 genes in most strains. VP1, VP2, VP3, NSP2, NSP3, NSP4 and NSP5 genes of porcine RVAs belonged to genotype 1, which is shared with human Wa-like RVAs. For most of these gene segments, pig strains clustered distantly from human Wa-like RVAs, indicating that viruses from both species have entered different evolutionary paths. Phylogenetic analysis of the VP6, NSP1 and NSP3 genes, as well as amino acid analysis of the antigenic regions of VP7 further confirmed this evolutionary segregation. The results

also indicated that the species barrier is less strict for pig P[6]-strains, but that chances for successful spread of these strains in the human population are likely hampered by the better adaptation of pig RVAs to pig enterocytes.

Finally, in **Chapter 4.2** next generation sequencing was applied to study the fecal virome of a non-diarrheic piglet that was coinfecting with RVA and RVC. This study reports the second complete genome of a pig RVC strain, and offered new insights into the relatedness between RVCs from pigs and other host species. The pig strain found here was only poorly related to RVCs from humans and cows, and was only distantly related to the other completely characterized pig RVC strain Cowden. The gene encoding outer capsid protein VP7 belonged to the rare G3 genotype, which might also be serologically distinct from most other pig RVC strains. A putative new RVC VP6 genotype I8 was identified as well. The RVA strain present in this fecal samples contained the rare pig genotype combination G11P[27], but not all gene segments could be characterized. Still, typical pig RVA genotypes I5, A8, and T7 were found for the viral proteins VP6, NSP1, and NSP3. Unexpectedly, the fecal virome of this piglet also contained an astro- and enterovirus. These findings indicated that many viruses may circulate in non-diarrheic piglets. Here, they could not be directly associated with clinical symptoms of diarrhea, but still they might have had a potential subclinical impact on pig performance. The fast evolution of next generation sequencing may form a powerful tool for future diagnostics in veterinary practice.

In **Chapter 5**, the results of the present thesis are placed in a broader perspective. In the first part, the occurrence of RVA and RVC infections in Belgian piglets is being discussed and it was concluded that RT-qPCR assays are essential tools for rotavirus diagnostics in piglets. An overview of all genotyped strains is given. It was shown that most rotavirus positive samples were also co-infected with one or more enterica bacteria, and an hypothetical pathogenesis model is presented. Furthermore, the importance of successive RVA infections after weaning is being discussed. Finally, the possibilities of next generation sequencing in veterinary diagnostics and future research are being discussed.

In a second part, the evolutionary relationship between pig and human RVAs is being discussed. While RVA strains from both host species are related, it seems that they have entered different evolutionary paths and that interspecies transmission is hampered by one or more barriers.

Samenvatting

Diarree is één van de meest belangrijke gezondheidsproblemen bij jonge biggen. Bij de mens worden rotavirussen als een belangrijke oorzaak van diarree bij kinderen onder de 5 jaar oud beschouwd. In tegenstelling hiermee staat dat de situatie bij biggen weinig gerapporteerd is, voornamelijk in België tijdens de voorbije decennia.

In **hoofdstuk 1** van deze thesis wordt de differentiaal diagnose van diarree bij jonge biggen besproken. De focus is voornamelijk gericht op porcine en humane rotavirussen. Hierna volgt een korte beschrijving van de huidige beschikbare middelen om diarreeproblemen bij jonge biggen te behandelen en te voorkomen.

In **hoofdstuk 2** worden de doelstellingen van het huidige doctoraat gedefinieerd.

Hoofdstuk 3 beschrijft het voorkomen van rotavirussen bij zuigende en gespeende Belgische biggen. In **hoofdstuk 3.1** werd de diagnostiek voor porcine groep A rotavirus (RVA) infecties verbeterd door de ontwikkeling van een nieuwe kwantitatieve real-time PCR (RT-qPCR) test. Deze test werd gebruikt om 28 diarree- en 6 niet-diarreestalen te screenen, waarvan de meeste afkomstig waren van gespeende biggen. De RT-qPCR test bleek gevoeliger dan de routinematig gebruikte antigen detectietesten en virusisolatie in de cellijnen MA104 en IPEC-J2. Zesentwintig RVA positieve isolaten werden gegenotypeerd voor de genen die coderen voor de buitenste kapsiedeiwitten VP7 en VP4. Zes verschillende G-genotypes (G2, G3, G4, G5, G9 and G11) werden gedetecteerd voor VP7 en 5 verschillende P-genotypes (P[6], P[7], P[13], P[23] en P[27]) werden teruggevonden in 12 verschillende G/P genotype combinaties. Deze resultaten toonden aan dat de circulerende Belgische RVA stammen genetisch zeer heterogeen zijn. Verder bleken bijna alle RVA positieve meststalen één of meerdere enterische bacteriën te bevatten.

In **hoofdstuk 3.2** werd het belang van groep A en groep C rotavirussen in de pathogenese van diarree bij zuigende biggen jonger dan 2 weken oud bestudeerd. Hiervoor werden 45 fecale stalen gecollecteerd van 36 verschillende bedrijven aan een privaat diagnostisch laboratorium. Op 22 van de 36 bedrijven die getest werden (61%) konden hoge hoeveelheden viraal RNA van RVA en/of RVC gekwantificeerd worden met RT-qPCR. Wanneer gebruik gemaakt werd van een snelle antigen detectietest kon het rotavirus maar

op 9 van de 36 bedrijven (25%) gedetecteerd worden. Deze bevindingen zijn indicatief voor een belangrijke rol van RVA and RVC in de pathogenese van diarree bij zuigende biggen. Zeventien RVA positieve isolaten werden ook gegenotypeerd voor de buitenste kapsiedeiwitten VP7 en VP4. Gelijkaardige genotypes als in hoofdstuk 3.1 werden teruggevonden. Alle gekarakteriseerde RVC stammen bezaten het G6 genotype voor VP7, behalve één stam die een G1 genotype bezat. De VP4 genen van de Belgische RVC waren genetisch sterk verschillend. De meeste RVA positieve stalen bevatten ook *Escherichia coli*, terwijl *Clostridium perfringens* voornamelijk gedetecteerd werd in RVA negatieve stalen. Deze resultaten brachten betere inzichten in het belang van RVA and RVC infecties in de pathogenese van diarree bij zuigende biggen.

Finaal werd in **hoofdstuk 3.3** de uitscheiding van RVA en *Escherichia coli* bestudeerd in een pilootstudie op een groot, gesloten Belgisch varkensbedrijf bij 9 biggen tijdens de eerste 24 dagen na spenen. Alle biggen werden opeenvolgend geïnfecteerd met 2 heterogene RVA stammen (G5P[13] en G9P[23]). Hoewel, de tweede replicatiegolf minder uitgesproken was dan de eerste replicatiegolf, hetgeen er kan op wijzen dat er een zekere mate van kruisbescherming geïnduceerd werd tijdens de eerste infectiegolf. All biggen scheidde ook niet-hemolytische *E. coli* uit, terwijl hemolytische isolaten slechts op een aantal tijdstippen bij een paar biggen werden gedetecteerd. De beperkte proliferatie van de bacteriën is mogelijk te verklaren door het profylactische gebruik van colistine sulfaat en zinkoxide. Bijgevolg werden dan ook geen explosieve diarreeproblemen waargenomen. Niettegenstaande suggereerden de huidige resultaten dat RVA infecties ook een mogelijke impact hebben op subklinische groeivertraging, aangezien de biggen met de ergste RVA vermeerdering de slechtste gewichtstoename na het spenen kenden. Deze studie opende nieuwe hypothesen voor verder interessant onderzoek.

In **hoofdstuk 4** werden Belgische varkensrotavirus isolaten en andere enterische virussen genetisch gekarakteriseerd. Eerst werden in **hoofdstuk 4.1** de complete genomen van 6 recente (G2P[27], G3P[6], G4P[7], G5P[7], G9P[13] en G9P[23]) en één oud historisch (G1P[7]) Belgisch RVA isolaat blootgelegd door middel van Sanger sequencing. Deze genomen werden fylogenetisch geanalyseerd met alle beschikbare varkens rotavirus genomen om hun evolutionaire relatie met humane RVA stammen te onderzoeken. In contrast met de hoge mate aan genetische diversiteit die aanwezig was voor de genen die coderen voor VP7 en VP4, bleken bijna alle stammen een relatief geconserveerde varkens genotype constellatie (I5-R1-C1-M1-A8-N1-T1/T7-E1-H1) voor de overige 9 gensegmenten

te bezitten. VP1, VP2, VP3, NSP2, NSP3, NSP4 en NSP5 genen van porciene RVA stammen behoorden tot het genotype 1, dat gedeeld wordt met humane Wa-achtige RVA stammen. Voor de meeste van deze gensegmenten clusterden de varkensstammen op een relatief grote afstand van de humane stammen, hetgeen aanwijst dat virussen van beide species een aparte evolutionaire weg hebben ingeslagen. Fylogenetische analyse van de VP6, NSP1 en NSP3 genen, alsook een aminozuuranalyse van de antigene regio's van VP7b bevestigde deze evolutionaire segregatie. De resultaten van dit hoofdstuk toonden ook aan dat de species barriere voor porciene P[6] stammen vermoedelijk minder strikt is, maar dat de kans op succesvolle spreiding in de humane populatie belemmerd is doordat porciene RVA stammen vermoedelijk beter geadapteerd zijn aan de cel machinerie van varkensenterocyten.

Finaal werd in **hoofdstuk 4.2** gebruik gemaakt van next generation sequentie analyse om het fecale viroom van een gespeend big zonder diarree dat geïnfecteerd was met een RVA en RVC, te onderzoeken. Deze studie rapporteert slechts het tweede complete genoom van een porcien RVC isolaat en bracht ons nieuwe inzichten over de evolutionaire relatie tussen RVC stammen van varkens en deze van andere gastheerspecies. De varkensstam die hier werd teruggevonden was echter maar beperkt gerelateerd aan de andere volledig gekarakteriseerde RVC stam, Cowden genaamd. Het gen coderend voor het buitenste kapsiedeiwit VP7 behoorde tot het genotype G3 en is vermoedelijk ook serologisch verschillend van de meeste andere varkens RVC stammen. Een vermoedelijk nieuw VP6 genotype I8 werd ook geïdentificeerd. De RVA stam in dit fecale staal bezat een rare varkens genotype combinatie voor VP7 en VP4, namelijk G11P[27], maar niet alle gensegment konden succesvol geamplificeerd worden. Toch werden de typische varkensgenotypes I5, A8 en T7 teruggevonden voor de virale proteïnen VP6, NSP1 en NSP3. Onverwacht bevatte dit meststaal ook een astro- en enterovirus. Deze bevindingen tonen dat verschillende virussen kunnen circuleren bij biggen zonder klinische diarreesymptomen. Het is niet uit te sluiten dat deze wel een subklinische impact hebben op de biggegezondheid. De snelle evolutie van next generation sequencing kan mogelijk een krachtig hulpmiddel vormen voor toekomstig diagnostisch onderzoek in de diergeneeskunde.

In **hoofdstuk 5** werden de resultaten van de huidige thesis in een breder perspectief geplaatst. In het eerste deel wordt het voorkomen van RVA en RVC infecties bij Belgische jonge biggen besproken en werd geconcludeerd dat het gebruik van RT-qPCR resulteerde in

een aanzienlijke verbetering van de rotavirus diagnostiek. Een overzicht van alle genotypeerde rotavirusstammen werd vervolgens besproken. Er werd ook getoond dat de meeste rotavirus geïnfekteerde biggen ook een co-infectie met één of andere bacterie vertoonden en een hypothetisch pathogenetisch model werd voorgesteld. Verder wordt het belang van opeenvolgende RVA infecties na het spenen bediscussieerd en worden de mogelijkheden voor next generation sequencing in de veterinaire diagnostiek en toekomstig onderzoek geëvalueerd.

In een tweede deel wordt het evolutionaire verband tussen porcine en humane RVA stammen bediscussieerd. Hoewel RVA stammen van beide species verwant zijn, lijkt het erop dat beiden een aparte evolutionaire weg zijn ingegaan en dat interspecies transmissie geremd wordt door één of meerdere barrières.

Curriculum Vitae

Sebastiaan Theuns was born in Lier on the 26th of December 1986. He followed his high school at Sint-Jan Berchmanscollege in Antwerp and finished his studies in Latin-Mathematics in 2004. Next, he started the Bachelor studies for Veterinary Medicine at the University of Antwerp, and acquired his final Master degree in Veterinary Medicine (specialisation research), with distinction, at Ghent University in 2010.

Immediately hereafter, he started his PhD research to investigate the forthcoming of rotavirus infections in Belgian piglets. This research was performed at the Laboratory of Virology from the Department of Virology, Parasitology and Immunology, Ghent University. During his PhD, he setup a collaboration with the Laboratory for Clinical and Epidemiological Virology from the Rega Institute for Medical Research, KU Leuven, to investigate the evolutionary relationship between pig and human rotaviruses, and to analyze the fecal viromes of piglets. This PhD research was executed under guidance of the promoters Prof. Dr. Hans Nauwynck and Prof. Dr. Jelle Matthijnsens, and was funded by a grant from the Faculty Commission for Scientific Research of the Faculty of Veterinary Medicine, and a grant from the Special Research Fund (BOF) of Ghent University.

Publications

Publications in international journals

1. **Sebastiaan Theuns**[#], Nádia Conceição-Neto[#], Isaura Christiaens[#], Mark Zeller, Lowiese MB Desmarets, Inge DM Roukaerts, Delphine D Acar, Elisabeth Heylen, Jelle Matthijnsens*, Hans J Nauwynck* (2015). Complete genome sequence of a porcine epidemic diarrhea virus from a novel outbreak in Belgium, January 2015. *Genome Announcements* 3 (3):e00506-15. doi:10.1128/genomeA.00506-15
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Abstracts

1. **Theuns S**, Heylen E, Zeller M, Roukaerts ID, Desmarets LM, Van Ranst M, Nauwynck HJ*, Matthijnsens J*. Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses. 6th European Rotavirus Biology Meeting, 2015, Dijon, France
2. **Theuns S**, Vyt P, Desmarts LM, Roukaerts ID, Heylen E, Zeller M, Van Ranst M, Matthijnsens J, Nauwynck H. Circulation of pig group A and C rotaviruses in Belgian diarrheic suckling piglets and its impact on veterinary diagnostic analyses. 6th European Rotavirus Biology Meeting, 2015, Dijon, France
3. **Theuns S**, Heylen E, Zeller M, Roukaerts ID, Desmarets LM, Van Ranst M, Nauwynck HJ* Matthijnsens J*. Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses. Belgian Society for Virology: second annual meeting, 2014, Brussels, Belgium.
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14. **Theuns S**, Desmarets LM, Heylen E, Zeller M, Dedeurwaerder A, Van Ranst M, Matthijnsens J, Nauwynck HJ. Diagnosis of porcine rotavirus infections by RT-qPCR and RT-PCR reveals a possible synergism between enteric bacteria and genetically heterogenous porcine rotaviruses in Belgium. 5th European Rotavirus Biology Meeting, 2013, Valencia, Spain.
15. **Theuns S**, Heylen E, Zeller M, Van Ranst M, Matthijnsens J, Nauwynck HJ. Complete genomic analysis of Belgian porcine rotaviruses reveals a rather conserved porcine-like genotype constellation, despite large genetic diversity of VP7 and VP4. 5th European Rotavirus Biology Meeting, 2013, Valencia Spain.
16. Desmarets LM, **Theuns S**, Olyslaegers DA, Dedeurwaerder A, Vermeulen BL, Dewerchin HL, Nauwynck HJ. Contrasting replication of feline enteric coronavirus and feline infectious peritonitis virus in newly established feline intestinal epithelial cell cultures. Positive Strand RNA Viruses Symposium, 2013, Boston, Massachusetts, USA.

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Oral presentations

1. **Theuns S**, Heylen E, Zeller M, Roukaerts ID, Desmarets LM, Van Ranst M, Nauwynck HJ*, Matthijnsens J*. Complete genome characterization of recent and ancient Belgian pig group A rotaviruses and assessment of their evolutionary relationship with human rotaviruses. 6th European Rotavirus Biology Meeting, 2015, Dijon, France
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4. Theuns S. Virale problematiek in kennels. *In*: Kennelproblematiek, Institute for Permanent Education, Faculty of Veterinary Medicine, Ghent University, March 27, 2012, Merelbeke, Belgium.

Awards

1. Travel grant from the Organizing Committee of the 6th European Rotavirus Biology Meeting, 2015, Dijon, France, covering registration and accommodation.
2. International Mobility Credit from the Research Foundation - Flanders (FWO), covering transport costs to attend the 6th European Rotavirus Biology Meeting, 2015, Dijon, France.
3. Travel fellowship from the Organizing Committee of the 12th International Double Stranded RNA virus symposium, October 2015, Goa India, covering registration, accommodation and transport.

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