Detection and characterization of potentially zoonotic enteric viruses in livestock and the influence of probiotics on their shedding

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Summary

Infections with a variety of enteric viruses can cause clinical disease in humans and livestock, leading to gastroenteritis, encephalitis or hepatitis. For some of these viruses, a zoonotic potential has been supposed, because of a close genetic relationship of strains detected in humans and animals. Therefore, the possibility of a direct or indirect transmission of those viruses between livestock and humans is suspected. Particularly, the foodborne transmission of potentially zoonotic viruses is of increasing interest, as the number of foodborne viral diseases in humans increased in the last decades according to the World Health Organization. However, the prevention and control of many foodborne viral infections is currently hampered by the lack of knowledge about the distinct transmission pathways of the respective viruses and the importance of specific reservoir hosts. In addition, strategies to prevent virus transmission from livestock to humans are currently based exclusively on general hygienic measures.

In this study, the distribution of selected enteric viruses with suspected zoonotic potential in pigs and poultry should be assessed. The presence of astrovirus (AstV), encephalomyocarditis virus (EMCV), norovirus genogroup II (NoV GII), group A rotavirus (GARV) and hepatitis E virus (HEV) should be analysed in pigs from Germany. The distribution of avian rotaviruses of groups A and D (AvRV-A and -D) should be assessed in poultry from different regions of the world. Sensitive detection systems had to be developed for these purposes. The detected viruses should be compared with known strains from humans and livestock in order to estimate their zoonotic potential. As one option to decrease enteric virus excretion, the effects of feeding pigs with the probiotic bacterium *Enterococcus faecium* (*E. faecium*) NCIMB 10415 should be investigated.

Sensitive and specific real-time RT-PCR assays were developed, which were able to detect human as well as porcine strains of the respective viruses. The detection limits of the assays ranged from 15 to 78 molecules per PCR reaction. For avian group A and D rotaviruses, real-time RT-PCR assays were developed here for the first time. Although these assays were shown to be more sensitive than conventional detection methods available for avian rotaviruses, their sensitivity was still low for some of the analysed strains.

The analysis of faecal samples from chickens and turkeys originating from different countries of Europe and from Bangladesh showed, that AvRV-A and -D are highly prevalent (58.8% and 65.9%, respectively) in the flocks. The investigation of faecal samples of pigs at slaughter age

originating from slaughterhouses of three different regions in Germany resulted in the detection of all viruses, which were tested for. Particularly, NoV GII and AstV were found with high detection rates in slaughter pigs (14.2% and 20.8%, respectively). GARV, EMCV and HEV were detected in only a small number of pigs at slaughter age (0.8%, 4.2% and 2.5%, respectively).

The phylogenetic analyses of genome parts of the detected pig viruses showed a high sequence identity of 91% and 90% to known human strains of HEV and GARV, respectively. This finding supports the zoonotic potential of these viruses. In contrast, the detected strains of AstV and NoV GII clearly clustered together with typical porcine virus strains, thus questioning their ability of zoonotic transmission to humans. For EMCV, no sequences could be generated for comparison.

In an experimental feeding trial with sows and their piglets, no significant differences were recorded in the excretion of HEV, EMCV and NoV GII between the *E. faecium* NCIMB 10415 fed group and the control group. However, AstV was only detected in the control group and GARV was shed significantly later and with lower amounts in the probiotic feeding group. An activation of specific T cell populations was found in the probiotic feeding group, which may explain some of the effects caused by feeding with *E. faecium* NCIMB 10415.

It can be concluded from the study, that potentially zoonotic viruses are present in faecal samples of pigs of different age in Germany, including pigs at slaughter. The detected GARV and HEV strains were closely related to human viruses, thus indicating a potential for their zoonotic transmission. Although the prevalence of these human-related viruses was rather low, the detection of those viruses in pigs at slaughter should raise awareness on the improvement of hygienic standards in the meat-producing and -processing industry. One way for decreasing excretion of some of the viruses may be the application of probiotics; however, further studies have to confirm the results and should aim to elucidate the distinct mechanisms of action. In addition, other potentially zoonotic viruses like kobuvirus and sapovirus should be included in future investigations. The detection of AvRV-A and -D with high prevalence in poultry worldwide, as shown here, should result in efforts to further characterize these viruses in more detail in order to estimate their zoonotic potential.

Zusammenfassung

Infektionen mit verschiedenen enteralen Viren können beim Menschen und Nutztier zu Gastroenteritis, Enzephalitis oder Hepatitis führen. Einige dieser im Mensch und Tier detektierten Viren zeigen eine enge genetische Verwandtschaft zueinander und stehen deswegen unter Verdacht ein zoonotisches Potential zu besitzen. Eine direkte oder indirekte Übertragung zwischen Tier und Mensch erscheint deshalb möglich. Die Übertragung von potentiell zoonotischen Viren durch Lebensmittel ist hierbei von besonderem Interesse, da die Zahl der lebensmittelbedingten Viruserkrankungen beim Menschen in den letzten Jahrzenten, Angaben der Weltgesundheitsorganisation zufolge, gestiegen ist. Die Prävention und Kontrolle von durch Lebensmittel übertragenen Virusinfektionen ist jedoch schwierig, weil die genauen Übertragungswege der Viren sowie die Bedeutung spezifischer Reservoirwirte oft nur wenig bekannt sind. Darüber hinaus sind Strategien zur Verhinderung der Virusübertragung vom Nutztier auf den Menschen derzeit ausschließlich auf allgemeine Hygienemaßnahmen beschränkt.

In dieser Studie sollte die Verbreitung ausgewählter enteraler Viren, für die ein zoonotisches Potential angenommen wird, in Schweinen und Geflügel ermittelt werden. Schweine aus Deutschland sollten auf Astrovirus (AstV), Enzephalomyokarditis Virus (EMCV), Norovirus Genogruppe II (NoV GII), Gruppe A Rotavirus (GARV) und Hepatitis E Virus (HEV) hin untersucht werden. Des weiteren sollte die Verbreitung aviärer Rotaviren der Gruppen A und D (AvRV-A und -D) in Geflügel aus unterschiedlichen Regionen weltweit untersucht werden. Zu diesem Zweck sollten sensitive Nachweismethoden entwickelt werden. Die detektierten Viren sollten mit bekannten Stämmen aus Mensch und Tier verglichen werden, um deren zoonotisches Potential einschätzen zu können. Als eine Möglichkeit, die Ausscheidung von Viren zu vermindern, sollte der Einfluss der Fütterung mit dem probiotischen Bakterium *Enterococcus faecium* (*E. faecium*) NCIMB 10415 im Schwein untersucht werden.

Sensitive und spezifische Real-time RT-PCR Assays zum Nachweis humaner sowie entsprechender porziner Virusstämme wurden entwickelt. Die Nachweisgrenzen der Assays lagen zwischen 15 und 78 Molekülen pro PCR-Ansatz. Im Rahmen dieser Studie wurden erstmals Real-time RT-PCR Assays zum Nachweise aviärer Rotaviren der Gruppen A und D entwickelt. Obwohl die Sensitivität dieser Assays im Vergleich zu konventionellen Methoden

zur Detektion aviärer Rotaviren nachweislich höher war, wurden einige der Stämme mit nur geringer Sensitivität detektiert. Die Untersuchung von aus unterschiedlichen Ländern Europas und aus Bangladesch stammenden Kotproben von Hühnern und Puten zeigte, dass AvRV-A und -D mit hoher Prävalenz (58,8 % und 65,9 %) in den Beständen vorkommen. Bei der Untersuchung von Kotproben von Schweinen aus drei unterschiedlichen Schlachthöfen in Deutschland konnten alle Viren, auf die die Proben untersucht wurden, nachgewiesen werden. Insbesondere NoV GII und AstV wurden mit hohen Detektionsraten in Schlachtschweinen vorgefunden (14,2 % und 20,8 %). GARV, EMCV und HEV hingegen wurden nur in einer geringen Anzahl von Schlachtschweinen detektiert (0,8 %, 4,2 % und 2,5 %). Anschließende phylogenetische Analysen von partiellen Genomsequenzen zeigten eine hohe Sequenzhomologie der detektierten HEV- und GARV-Stämme von jeweils 91 % und 90 % zu bekannten humanen Stämmen. Diese Ergebnisse stützen die Annahme eines zoonotischen Potentials dieser Viren. Im Gegensatz dazu waren die detektierten NoV GII- und AstV-Stämme jeweils nur entfernt mit humanen Viren verwandt und zeigten hohe Sequenzhomologien mit typisch porzinen Stämmen, so dass eine mögliche zoonotische Übertragung auf den Menschen fraglich erscheint. Für EMCV konnten keine Sequenzen für phylogenetische Analysen generiert werden.

In einem experimentellen Fütterungsversuch mit Sauen und deren Ferkeln konnten keine signifikanten Unterschiede der Ausscheidung von HEV, EMCV und NoV GII zwischen *E. faecium* NCIMB 10415-gefütterten Tieren und der Kontrollgruppe beobachtet werden. Hingegen wurde AstV nur in der Kontrollgruppe detektiert und GARV wurde von Tieren der Probiotika-gefütterten Gruppe signifikant später und in geringerer Menge ausgeschieden. In der Probiotika-gefütterten Gruppe konnte außerdem eine Aktivierung spezifischer T-Zell-Populationen nachgewiesen werden, die einige der durch die Fütterung mit *E. faecium* NCIMB 10415 verursachten Effekte erklären könnte.

Aus den Ergebnissen dieser Studien kann geschlossen werden, dass potentiell zoonotische Viren in deutschen Schweinen unterschiedlichen Alters, darunter auch bei Schweinen im Schlachtalter, nachgewiesen werden können. Die detektierten HEV- und GARV-Stämme waren eng mit humanen Viren verwandt, was für eine mögliche zoonotische Übertragung spricht. Auch wenn die Prävalenz der GARV- und HEV-Stämme eher niedrig war, sollte der Nachweis dieser Viren in Schlachtschweinen die Aufmerksamkeit auf verbesserte hygienische Maßnahmen in der Fleischproduzierenden und -verarbeitenden Industrie

lenken. Die Anwendung von Probiotika könnte eine Möglichkeit zur Verminderung der Ausscheidung einiger dieser Viren bieten. Diese Ergebnisse sollten jedoch in weiteren Untersuchungen bestätigt werden; außerdem sollte der genaue Wirkmechanismus des Probiotikums aufgeklärt werden. In zukünftigen Studien sollten auch weitere potentiell zoonotische Viren wie Kobuviren und Sapoviren eingeschlossen werden. Die hier ermittelten hohen Prävalenzen aviärer Rotaviren der Gruppen A und D im Geflügel sollten Ausgangspunkt für weitere Untersuchungen sein, um diese Viren zu charakterisieren und ihr zoonotisches Potential besser einschätzen zu können.

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List of Abbreviations

A		
	AstV	astrovirus
	Av	avian
	AvRV	avian rotavirus
B		
	bp	base pairs
C		
	cDNA	complementary DNA
D		
	DNA	desoxyribonucleic acid
	ds	double-stranded
E		
	E. faecium	*Enterococcus faecium*
	e.g.	for example (from Latin *exempli gratia*)
	ELISA	enzyme-linked immunosorbent assay
	EMCV	encephalomyocarditis virus
	et al.	and others (from Latin *et alii*)
	EU	European Union
F		
	FAO	Food and Agriculture Organization of the United Nations
	FRET	fluorescence resonance energy transfer
G		
	G	genogroup or genotype
	GARV	group A rotavirus
H		
	HEV	hepatitis E virus
I		
	ICTV	International Committee on Taxonomy of Viruses
	IEM	immune electron microscopy
	IgA	immunoglobulin A

	IgG	immunoglobulin G
K		
	kb	kilobase
N		
	NADRV	novel adult diarrhoea virus
	NoV	norovirus
	nm	nanometer
O		
	ORF	open reading frame
P		
	PAGE	polyacrylamide gel electrophoresis
	PCR	polymerase chain reaction
R		
	RdRp	RNA-dependent RNA polymerase
	RNA	ribonucleic acid
	RT	reverse transcription
	RT-PCR	reverse transcription polymerase chain reaction
	RV	rotavirus
S		
	sIgA	secretory immunoglobulin A
	ss	single-stranded
U		
	UK	United Kingdom
	USA	United States of America
	UTR	untranslated region
V		
	VLP	virus-like particle
	VP	viral protein
	VPg	viral protein genome-linked
W		
	WHO	World Health Organization

1 General Introduction

1.1 Enteric virus diseases in humans and animals

Enteric viruses are viruses that can be found in the intestinal tract of humans and animals. They are mainly associated with gastroenteritis, but can also lead to enterically transmitted hepatitis, as well as diseases with other organ manifestations after migration from the intestine (FAO/WHO, 2008). Most often, they are transmitted by the faecal-oral route and can spread easily by person-to-person contact, contaminated food, water and surfaces.

Diarrhoeal diseases are widely distributed in the industrialized world and are a leading cause of morbidity and mortality in developing countries (Atmar, 2010; Chen et al., 2012). While parasites and bacteria are known as cause of diarrhoeal disease since the 17th century, viruses were first suspected to induce diarrhoea in the 20th century. In 1972, Kapikian et al. discovered the so-called "Norwalk virus" in infectious, bacteria-free stool filtrates by immune electron microscopy (IEM) (Kapikian et al., 1972). This discovery represented a milestone in the history of viral gastroenteritis and allowed the identification of further gastroenteritis-causing viruses such as rotavirus and astrovirus in the following years (Bishop et al., 1973; Madeley and Cosgrove, 1975).

Epidemic as well as sporadic cases of hepatitis mainly occur in developing countries, but gain an increasing impact in industrial countries also. As early as 1931, Findlay, Dunlop and Brown postulated an "ultra-microscopic virus" to be the cause of epidemic jaundice (Findlay et al., 1931). But it took more than 40 years for discovery of hepatitis A and B virus by IEM, and another 20 years for hepatitis C, D and E virus (Choo, et al., 1989; Dane et al., 1970; Feinstone et al., 1973; Reyes et al., 1990; Rizzetto et al., 1977).

Enteric viruses also represent a major problem for farm animals (de Wit et al., 2011; Halaihel et al., 2010; Koenen et al., 1999). In addition to the animal diseases caused by them, some viruses are considered to have a zoonotic potential, thus constituting a risk for humans by direct transmission from animals or by meat products contaminated during the slaughtering process. This includes viruses associated with gastroenteritis like rotavirus (RV), astrovirus (AstV) and norovirus (NoV), as well as hepatitis E virus (HEV) and encephalomyocarditis virus (EMCV) (**table 1**). For AstV, NoV and EMCV a zoonotic potential is assumed because of a close relationship of human and animal virus strains. However, the epidemiological evidence

for a zoonotic transmission of these viruses is so far relatively low. In contrast, the zoonotic transmission has been proven for HEV and RV.

Table 1: **Enteric viruses in humans and livestock and their zoonotic potential**.

Virus	Disease in humans	Disease in livestock	Zoonotic potential
Astrovirus	Gastroenteritis/ asymptomatic	*Pig*: diarrhoea/ asymptomatic *Cattle*: asymptomatic *Poultry*: hepatitis/ interstitial nephritis	Unclear
Encephalomyocarditis virus	Fever/encephalitis/ neck stiffness/ aseptic meningitis/ neurological disorders/ asymptomatic	*Pig*: acute myocarditis/ reproduction disorders/ asymptomatic *Cattle*: unclear *Poultry*: -	Unclear
Hepatitis E virus	Hepatitis/ asymptomatic	*Pig*: subclinical *Cattle*: - *Poultry*: subclinical/ hepatitis-splenomegaly syndrome/ big liver and spleen disease	Proven
Norovirus	Gastroenteritis/ asymptomatic	*Pig*: diarrhoea/ asymptomatic *Cattle*: diarrhoea/ asymptomatic *Poultry*: -	Unclear
Rotavirus	Gastroenteritis/ asymptomatic	*Pig*: diarrhoea/ asymptomatic *Cattle*: diarrhoea/ asymptomatic *Poultry*: diarrhoea/ asymptomatic	Proven

-, virus until now not detected in this species.

For most of the viruses listed in **table 1**, the knowledge about clinical disease and epidemiology is well investigated for humans, while only limited data are available about the prevalence of those viruses in livestock. One exception is EMCV, for which information about the clinical impact of EMCV infections in humans is only scarcely available. Although EMCV is widespread in pig farms, also for pigs epidemiological data are mainly missing. For virus detection cell culture was the “gold standard” in the last century. However, it has been more and more replaced by molecular methods like real-time PCR in the last years (Leland and Ginocchio, 2007). Real-time PCR assays are not only sensitive and specific, but also enable an absolute quantification of viruses and therefore provide reliable and comparable diagnostic results.

Because enteric viruses have a major impact on illness in humans and livestock, strategies for prevention and control are of high importance. Maintenance of hygienic standards plays a key role in prevention and control of enteric virus infections, as antiviral drugs and vaccines are not available for most of these viruses. An additional approach to prevent and control viral diseases may be the use of probiotics, whose influence on severity of disease and shedding of virus has been shown *in vitro* and *in vivo* for some viral agents (Grandy et al., 2010; Munoz et al., 2011). In the European Union (EU), the probiotic bacterium *Enterococcus faecium* (*E. faecium*) NCIMB 10415 is an authorised feed additive for pigs (Cylactin®) (EFSA, 2013), as positive effects on pig performance have been shown in several studies (Zeyner and Boldt, 2006). However, its influence on enteric viruses has not been investigated so far.

In the next paragraphs, the viruses investigated in the study, the diseases caused by them as well as the current knowledge about their zoonotic potential will be shortly summarized. The background on the applied diagnostic methods and on the use of probiotics against viral diseases will be presented, before the aims of the study will be enrolled.

1.2 Viruses

1.2.1 Encephalomyocarditis virus (EMCV)

EMCV infections can cause neurological or myocardial diseases in a variety of animals and can lead to high losses in pig farms. In humans, infections with EMCV have been only scarcely characterized. A zoonotic potential is assumed but the evidence for a zoonotic transmission is low so far.

1.2.1.1 Taxonomy and phylogeny

A

Family: *Picornaviridae*

Genus: *Cardiovirus*

Species: *Encephalomyocarditis virus*

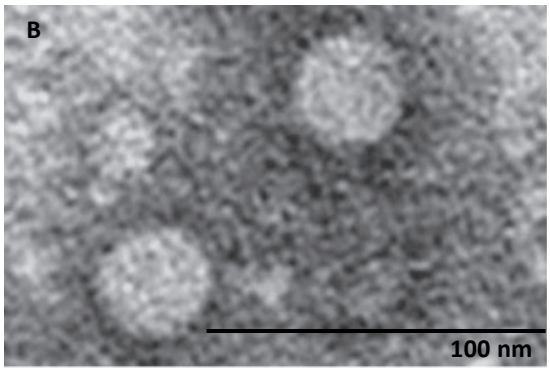

Figure 1: **Taxonomy and electron micrograph of EMCV**. **A** Taxonomic classification of EMCV. **B** Electron micrograph of EMCV obtained from a non-human primate by cell culture (Yeo et al., 2013). The length of the bar corresponds to 100 nm.

The encephalomyocarditis virus is classified into the family *Picornaviridae*, which also contains well-known human and animal pathogenic viruses, e.g. poliovirus of the genus *Enterovirus*, hepatitis A virus of the genus *Hepatovirus* and foot-and-mouth disease virus of the genus *Aphtovirus*. Within the genus *Cardiovirus* the species *Encephalomyocarditis virus* and *Theilovirus* are differentiated based on sequence homologies, the natural host spectrum and genome organization (**figure 1**) (Knowles et al., 2012). While the species *Theilovirus* comprises of several serotypes, only one serotype of the species *Encephalomyocarditis virus*, EMCV-1, was known until 2012. A second serotype, EMCV-2, was characterized very recently as an isolate from the wood mouse (*Apodemus sylvaticus*) showed a high divergence to all other isolates (Philipps et al., 2012).

1.2.1.2 Structure of virus and genome

EMCV is characterized by a non-enveloped, spherical, icosahedral capsid of 30 nm in diameter (**figure 1**). 60 protomers, consisting of the four capsid proteins VP1-VP4, build a protective protein layer around a linear, single-stranded RNA with positive polarity and a size of about 7.8 kb (Carocci and Bakkali-Kassimi, 2012). The structure of the viral genome is depicted in **figure 2**.

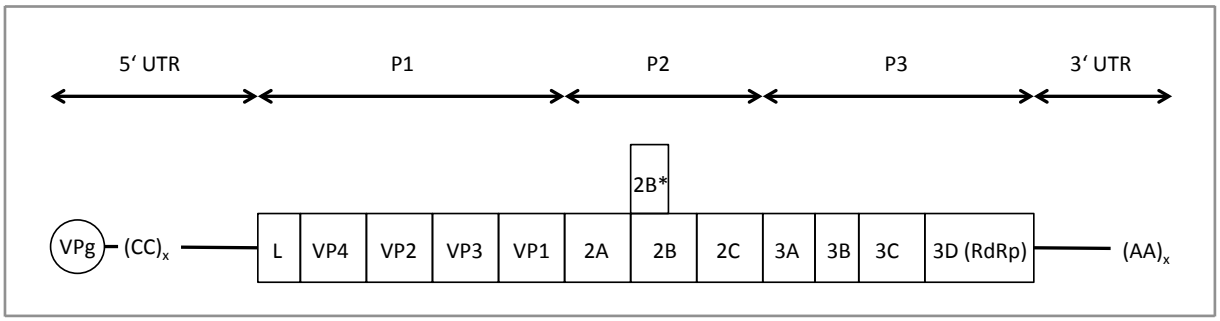

Figure 2: **Organization of the EMCV genome**. The RNA encodes a polyprotein, which is posttranslationally cleaved into the leader protein (L), the precursor P1 (capsid proteins VP1-VP4) and the precursors P2 and P3 (non-structural proteins 2A-3D). The predicted protein 2B* is translated via ribosomal frameshifting. ORF, open reading frame; UTR, untranslated region; VPg, viral protein genome-linked; $(CC)_x$, poly(C) tract; $(AA)_x$, poly(A) tail; RdRp, RNA-dependent RNA polymerase.

Framed by untranslated regions (UTRs) at the 5' and 3' end, the EMCV genome consists of one single open reading frame (ORF). This ORF encodes a polyprotein, which is posttranslationally cleaved into the structural proteins VP1-VP4 and the non-structural proteins 2A-3D (Carocci and Bakkali-Kassimi, 2012). Only recently, the existence of an additional frameshift was reported, indicating that EMCV at least contains two ORFs (Loughran et al., 2011). A special feature is the poly(C) tract at the 5' end of the genome, which is specific for EMCV and aphtoviruses (Carocci and Bakkali-Kassimi, 2012).

1.2.1.3 Pathogenesis and clinical features

The pathogenesis of infections with EMCV is largely unknown and is best characterized in rodents, pigs and primates (Carocci and Bakkali-Kassimi, 2012). Typically, cardioviruses replicate asymptomatically in the gastrointestinal tract after faecal-oral ingestion.

Some strains are able to replicate in the central nervous system and induce neurological disorders after intracerebral injection (Blinkova et al., 2009).
In infection experiments with pigs, virus was detected up to three days after challenge with EMCV in the blood, faeces and nasal excretions of the animals (Billinis et al., 2004). No data are available on the shedding of the virus in humans.

1.2.1.3.1 Humans

The clinical relevance of infections with EMCV in humans is only scarcely understood, as only few cases are documented. Mainly, the knowledge about EMCV infections in humans is based on studies from the middle of the last century. Infections are associated with fever, neck stiffness, lethargy, delirium and headache (Oberste et al., 2009; Smadel and Warren, 1947). Furthermore, cases of aseptic meningitis, encephalitis and other neurological disorders are reported (Bieling and Koch, 1952; Dick et al., 1948). No data on incubation period or duration of symptoms are available. It is assumed that EMCV infections in humans are common but are either asymptomatic or remain unrecognized (Czechowicz et al., 2011; Tesh, 1978).

1.2.1.3.2 Livestock

In livestock, infections with EMCV are reported in pigs and sporadically in cattle. Piglets usually suffer from an acute focal myocarditis, characterized by cardiac inflammation, cardiomyocyte necrosis and sudden death. The mortality rate in EMCV-infected pre-weaning piglets can reach up to 100%, but decreases with the age of the animals (Alexandersen et al., 2012). In sows, infection is characterized by reproduction disorders, including abortion, foetal death or mummification of piglets (Koenen et al., 1999). Further described symptoms in pigs include anorexia, apathy, trembling, paralysis or dyspnoea (Alexandersen et al., 2012). Frequently, infections in pigs are asymptomatic (Maurice et al., 2005).
Reports about EMCV infections in cattle are scarce. The virus has been isolated from a calf with myocardial lesions in the 1960ies in Florida and just recently from a splenectomised calf without clinical signs in Australia (Diallo et al., 2013; Gainer, 1967). However, the clinical impact of EMCV infections in cattle is unclear.

1.2.1.4 Host spectrum, geographical distribution and epidemiology

In contrast to other viruses of the family *Picornaviridae*, which are restricted to one or only few hosts, EMCV shows a broad host range and can be detected in various mammals, rodents, non-human primates and birds (Carocci and Bakkali-Kassimi, 2012). Several outbreaks with EMCV are reported in captive wildlife populations (Canelli et al., 2010) but also in free-ranging elephants (Grobler et al., 1995). In pigs, which are the most susceptible domestic animals, the virus has a worldwide distribution. The transmission of the virus is only poorly understood, but rodents are considered to be the natural reservoir of EMCV as they were associated with outbreaks in various animal species (Canelli et al., 2010; Grobler et al., 1995). Recent studies assume wild boars as another possible reservoir for EMCV (Billinis, 2009).

1.2.1.4.1 Humans

Epidemiological data on the prevalence of EMCV in humans are mainly restricted to studies on the prevalence of antibodies against EMCV in serum samples, which reach from 1% to 51% (**table 2**).

Table 2: **Prevalence of anti-EMCV antibodies in human serum**.

Country	Population group	EMCV prevalence (%)	Reference
Philippines	Army personnel with chills, fever, severe headache, neck stiffness and pleocytosis	38	Smadel and Warren, 1947
Mexico	Healthy population	7	Gajdusek and Rogers, 1955
Europe **Africa** **Uruguay**	Healthy population	3-8 18-44 5	Barski and Cornefert, 1957
USA **Peru**	Healthy population	4 10	Jonkers, 1961
Europe **Africa** **North America** **Southeast Asia**	Healthy population	1-5 10-51 5-8 3-27	Tesh, 1978
Austria	Hunters	15	Deutz et al., 2003
Austria	Zoo employees	5	Juncker-Voss et al., 2004
Peru	Patients with unspecific febrile illness	6-17	Czechowicz et al., 2011
Mexico	Swine-specialist veterinarians	47	Rivera-Benitez et al., 2014

Some studies from the middle of the last century report the isolation of EMCV from patients with encephalitis, aseptic meningitis and other unspecific diseases of the central nervous system (Bieling and Koch, 1952; Dick et al., 1948). However, viruses were isolated after inoculation in mice, so it cannot be excluded that the viruses originated from the mice and not from human clinical specimens. A case of proven virus detection from humans was published in 2009, where EMCV was directly isolated by cell culture from patients suffering from unspecific febrile illness with headache, dyspnoea and nausea (Oberste et al., 2009). In Germany, no data on the prevalence of EMCV or of antibodies against the virus are available.

1.2.1.4.2. Livestock

Rodents are supposed to play a crucial role in the transmission of EMCV in pig production. Cases of faecal-oral transmission by ingestion of infected carcasses as well as contaminated food or water are reported (Alexandersen et al., 2012; Koenen et al., 1999). Limited horizontal transmission from pig-to-pig and vertical (transplacental) transmission has been described (Billinis et al., 1999a; Koenen et al., 1994). In addition, Billinis et al. (1999b) report persistent EMCV infections in piglets that might play an important role in transmission of the virus.

Several studies on the prevalence of anti-EMCV antibodies in pigs are available ranging from 2% to 69% (**table 3**).

Table 3: **Seroprevalence of anti-EMCV antibodies in sera of pigs of different age**.

Country	EMCV prevalence (%)	Reference
UK	28	Sangar et al., 1977
Canada	10	Sanford et al., 1985
Italy	69	Gualandi et al., 1989
USA	15	Zimmerman et al., 1991
USA	38	Smith et al., 1992
Japan	26	Shibata et al., 1993
The Netherlands	9	Augustijn et al., 2006
France	2	Bakkali-Kassimi et al., 2006
Korea	9	An et al., 2009
China	52	Ge et al., 2010

The study of Maurice et al. (2005) implies that seroprevalence is varying, depending on the age of the animals, the location in an endemic or non-endemic area as well as the clinical history of EMCV infection on the farm. Furthermore, several studies indicate that antibody-seroprevalence increases with the age of the animals (An et al., 2009; Ge et al., 2010; Maurice et al., 2005; Shibata et al., 1993). In Germany, no studies on the prevalence of anti-EMCV antibodies in pigs were performed. Data on the prevalence of viral RNA are generally not available.

1.2.1.5 Zoonotic potential

A zoonotic potential of EMCV is assumed since the middle of the 20th century. In this period a number of cases of human infections with EMCV have been described, in which mice and primates were suspected as potential source of infection (Dick et al., 1948; Jungeblut and Dalldorf, 1943). Further studies report cases of human EMCV infection acquired in the proximity to pig farms with a high incidence of porcine EMCV, as well as high anti-EMCV antibody titres in sera of zoo employees, hunters and veterinarians (Deutz et al., 2003; Juncker-Voss et al., 2004; Kirkland et al., 1989; Rivera-Benitez et al., 2014). Outbreaks in zoos with different affected animal species with clinical symptoms of EMCV infection support the assumption of a zoonotic transmission among animals (Canelli et al., 2010). Especially during the period of significant advances on xenotransplantations (transplantation between different species) in the end of the 20th century, the assessment of the zoonotic potential of EMCV gained a higher attention. Because of anatomical and physiological similarities between pigs and humans, porcine tissues are suitable for xenotransplantations (Brewer et al., 2001). In the course of this, experiments were conducted, showing that EMCV can persist in the porcine myocard and that porcine EMCV are able to infect human cardiomyocytes *in vitro* (Brewer et al., 2001).

1.2.1.6 Prevention, control and vaccination

Strategies for prevention and control of EMCV infections have been developed only for pig farms so far. Until now, the main strategy for prevention is the elimination of rodents in the surrounding environment of farms, as the transmission by rodents seems to be the most

effective transmission pathway (Canelli et al., 2010; Koenen et al., 1999). No antiviral drugs are available against EMCV. In the USA, a vaccine for pigs, based on inactivated EMCV, is commercially available (Alexandersen et al., 2012). Several live and inactivated EMCV vaccines have been tested in wildlife species, however, with varying effectiveness (McLelland et al., 2005). In the last years a new vaccine based on virus-like particles (VLPs) was developed, which showed high levels of seroconversion *in vivo* but still needs to be tested in animal challenge experiments (Jeoung et al., 2011).

1.2.2 Hepatitis E virus (HEV)

The hepatitis E virus is the etiologic agent of hepatitis in humans. Large epidemic outbreaks of HEV have been reported from developing countries, whereas in industrial countries sporadic HEV cases are dominating. In the last years, the number of cases of autochthonous infections in industrialized countries increased constantly. The virus can be transmitted zoonotically by ingestion of raw or undercooked meat and meat products.

1.2.2.1 Taxonomy and phylogeny

A

Family: *Hepeviridae*

Genus: *Hepevirus*

Species: *Hepatitis E virus*

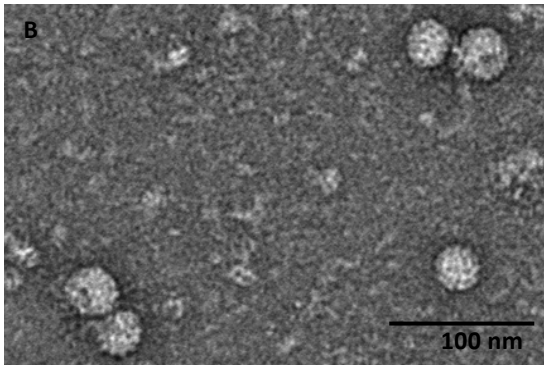

Figure 3: **Taxonomy and electron micrograph of HEV**. **A** Taxonomic classification of HEV. **B** Electron micrograph of HEV obtained from human specimen by cell culture (Dr. J. Reetz, BfR, Berlin). The length of the bar corresponds to 100 nm.

According to the 9th report of the International Committee on Taxonomy of Viruses (ICTV), *Hepevirus* is the sole genus in the 2006 newly established family *Hepeviridae* (Meng et al., 2012). The genus comprises only one species, the *Hepatitis E virus*. HEVs from humans, swine, mongoose and deer belong to this species. The status of avian HEV as well as HEV from rats, bats and other hosts is still tentative (Meng et al., 2012). The species *Hepatitis E virus* is divided into four known genotypes, G1-G4. Genotypes 1 and 2 exclusively comprise HEV from humans and are endemic in large parts of Asia, Africa and Mexico. Genotypes 3 and 4 can be detected in humans and animals and are prevalent in the industrialized countries of Europe, as well as in North America, Australia and Southeast Asia (Scobie and Dalton, 2013).

According to Lu et al. (2006) the four genotypes can be subdivided into 24 subtypes (1a-e, 2a-b, 3a-j, 4a-g). Recently, several studies proposed the existence of additional new genotypes (Smith et al., 2013).
All isolates described so far belong to one single serotype (Meng et al., 2012).

1.2.2.2 Structure of virus and genome

HEV is a non-enveloped virus with a spherical, icosahedral capsid. The capsid consists of capsomeres, which are build from homodimers of one single protein, and has a diameter of 27-34 nm. Inside the capsid, a linear, single-stranded RNA of positive polarity is located (Meng et al., 2012). The genome has a size of approximately 7.2 kb and contains three ORFs (**figure 4**).

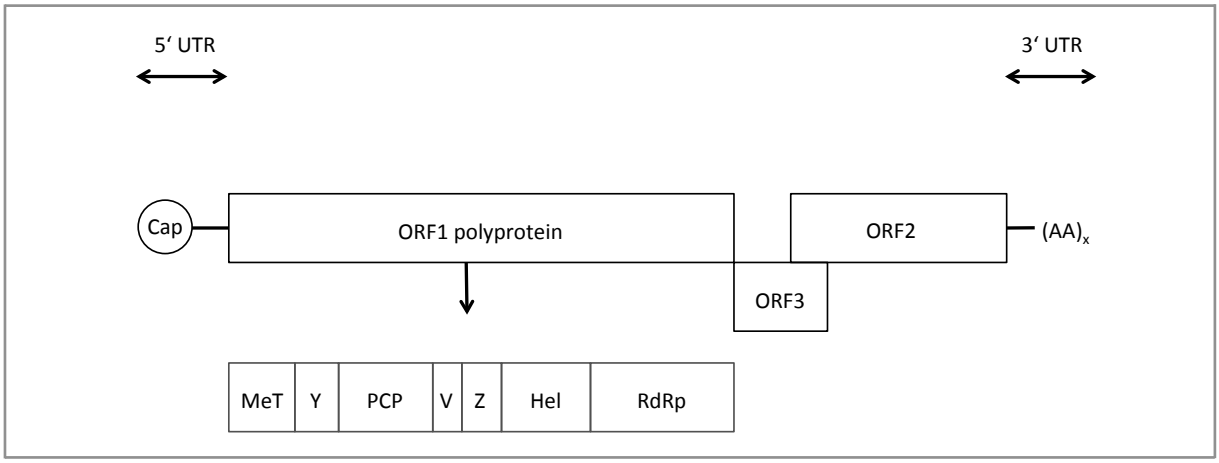

Figure 4: **Organization of the HEV genome**. ORF1 encodes a polyprotein, which is posttranslationally cleaved into non-structural proteins Met, Y, PCP, V, Z, Hel and RdRp. ORF2 encodes the capsid protein. ORF3 codes for a phosphoprotein, whose function is not yet fully understood. ORF, open reading frame; UTR, untranslated region; Cap, 5’ methylated cap structure; $(AA)_x$, poly(A) tail; RdRp, RNA-dependent RNA polymerase.

At the 5’ and 3’ end short untranslated regions are located. ORF1 codes for a polyprotein, which is posttranslationally cleaved into non-structural proteins, while ORF2 encodes the capsid protein. ORF3 starts between both ORFs and overlaps with ORF2. It codes for a small phosphoprotein, which is related to morphogenesis and pathogenesis of the virus (Teshale and Hu, 2011).

1.2.2.3 Pathogenesis and clinical features

The pathogenesis of HEV has been only poorly elucidated so far. After oral ingestion of the virus, primary replication is suspected to take place in the intestinal tract. From there, the virus is translocated to the blood and reaches the liver via the portal vein. There it replicates in the cytoplasm of the hepatocytes (Feng and Lemon, 2014). However, there is evidence that liver disease is rather immune-mediated than caused by viral damage of hepatocytes (Feng and Lemon, 2014). In humans, viraemia lasts four weeks on average, in pigs up to two weeks. The virus can be shed with the stool or faeces for several weeks after infection (Kasorndorkbua et al., 2005; Takahashi et al., 2007).

1.2.2.3.1 Humans

In humans, infection with HEV can induce a mild to moderate, self-limiting hepatitis, characterized by jaundice, malaise, anorexia, fever and hepatomegaly. Symptoms occur after an incubation period of 40 days on average. In pregnant women, infections with HEV genotypes 1 and 2 can cause loss of the foetus as well as death of the mother due to an increased incidence of acute liver failure and disseminated intravascular coagulation (Khuroo and Kamili, 2006). The overall mortality of HEV infections lies between 1% and 4% with exception of pregnant women and patients with chronic liver disease, which show an increased mortality rate (Khuroo and Kamili, 2006; Scobie and Dalton, 2013). Chronic infections with HEV can occur in immunocompromised patients after transplantations or in patients with HIV (Kamar et al., 2014). Also, cases of extrahepatic manifestations have been reported (Bhagat et al., 2008; Kamar et al., 2011). However, the majority of HEV infections are asymptomatic (Scobie and Dalton, 2013).

1.2.2.3.2 Livestock

Infections with HEV can be detected frequently in pigs. Infection is generally subclinical, irrespective whether infection occurred naturally or experimentally (Leblanc et al., 2007; Lee et al., 2009). In cattle, evidence for infections with HEV is only based on the presence of antibodies in animals without clinical signs (Arankalle et al., 2001; Geng et al., 2011).

In poultry, avian HEV infections can cause hepatitis-splenomegaly syndrome or big liver and spleen disease, both characterized by enlarged liver and spleen as well as slightly increased mortality (Huang et al., 2002; Meng, 2011) However, most infections with avian HEV also remain subclinical (Meng, 2011).

1.2.2.4 Host spectrum, geographical distribution and epidemiology

HEV shows a broad host spectrum and has been detected in several mammals as well as in birds and marine animals (Scobie and Dalton, 2013). In humans and animals, the virus occurs throughout the world, with a genotype-specific distribution in humans (**figure 5**). Generally, the virus is transmitted by the faecal-oral route.

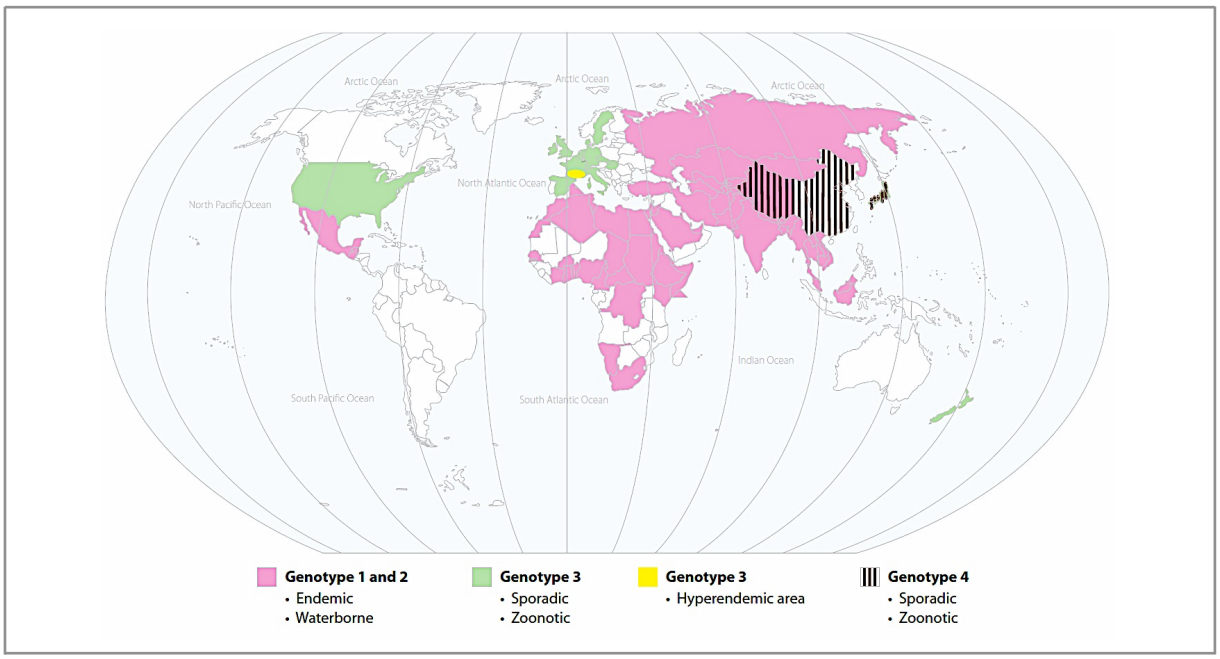

Figure 5: **Geographical distribution of HEV genotypes in humans** (Scobie and Dalton, 2013). Genotypes 1 and 2 are endemic in wide parts of Asia and Africa, while genotypes 3 and 4 occur sporadically in Europe, North America and Southeast Asia.

1.2.2.4.1 Humans

In humans, the main transmission route as well as the epidemiology of the virus are dependent on the genotype. In the developing world, where genotypes 1 and 2 are prevalent, transmission is mainly waterborne causing large outbreaks. Especially young

adults between 15 and 30 years of age develop clinical hepatitis (Scobie and Dalton, 2013). Mortality rates up to 25% are reported in pregnant women infected with HEV genotypes 1 and 2 (Scobie and Dalton, 2013). In contrast, genotypes 3 and 4, which are prevalent in industrialized countries, are mainly transmitted zoonotically either by direct contact to infected animals or by eating uncooked or undercooked meat or meat products (Scobie and Dalton, 2013). HEV genotypes 3 and 4 show a high infection rate in middle-aged and elderly persons. Moreover, the infection shows a gender specific prevalence as more men than women become infected (Dalton et al., 2008). An increased mortality rate in pregnant women, as for genotypes 1 and 2, has not been observed. Furthermore, HEV can be transmitted by blood transfusions and also transplacental transmission from mother to child has been reported (Khuroo and Kamili, 2006; Vollmer et al., 2012).

Studies on the seroprevalence of anti-HEV antibodies show a broad distribution in humans worldwide, with a prevalence of up to 23% (**table 4**). Only few studies detected even higher seroprevalences (Mansuy et al., 2011). However, the seroprevalences may vary due to the application of serological methods with differing sensitivity and specificity. Generally, seroprevalence increases with increasing age of the individuals (Johargy et al., 2013; Xu et al., 2013). Individuals highly exposed to pigs or wild boars show higher seroprevalences than the general population (de la Caridad Montalvo Villalba et al., 2013; Krumbholz et al., 2012). In Germany, several studies on HEV seroprevalence have been conducted, showing varying results between 6% and 16% (Krumbholz et al., 2012).

Table 4: **Seroprevalence of anti-HEV IgG antibodies in sera of human blood donors and general population**.

Country	HEV prevalence (%)	Reference
France	3	Boutrouille et al., 2007
UK	16	Dalton et al., 2008
Bangladesh	23	Labrique et al., 2009
Japan	3	Takeda et al., 2010
France	52	Mansuy et al., 2011
Germany	16	Krumbholz et al., 2012
Germany	6	Vollmer et al., 2012
Saudi Arabia	19	Johargy et al., 2013
Iran	7	Ehteram et al., 2013
Ghana	5	Meldal et al., 2013
USA	19	Xu et al., 2013

1.2.2.4.2 Livestock

In pigs, HEV is transmitted most likely by the faecal-oral route (Kasorndorkbua et al., 2005). The virus can be detected in all age groups. Most studies detected higher prevalences of viral RNA in faeces of pigs <4 months of age compared to older pigs (de la Caridad Montalvo Villalba et al., 2013; Leblanc et al., 2007; McCreary et al., 2008). However, some studies reported even high HEV RNA prevalences in fattening pigs and pigs at slaughter (**table 5**).

Table 5: **HEV RNA prevalence in faeces of pigs >4 months of age**.

Country	Age of pigs	HEV prevalence (%)	Reference
Canada	22-29 weeks	41	Leblanc et al., 2007
Italy	8-9 months	27	Di Bartolo et al., 2008
Canada	Pigs at slaughter*	14	Leblanc et al., 2010
Italy	9 months	9	Di Martino et al., 2010
The Netherlands **Belgium**	5-6 months	15 7	Hakze van der Honing et al., 2011
Italy **Spain** **Czech**	Pigs at slaughter *	41 38 3	Di Bartolo et al., 2012
Cuba	20-28 weeks	30	de la Caridad Montalvo Villalba et al., 2013

*, no further details on the age of the pigs.

A comparative study in Europe revealed an HEV RNA prevalence from 8% to 30% in weaners, from 20% to 44% in growers and from 8% to 73% in fatteners (Berto et al., 2012). In Germany, no studies on the prevalence of HEV RNA in pigs were performed. However, in porcine liver samples an RNA prevalence of 13.5% has been detected in German pigs (Baechlein et al., 2013). In studies from other countries prevalences of up to 11.5% were detected in liver samples (Feagins et al., 2008). Seroprevalence of anti-HEV IgG antibodies in pigs is very high and can reach over 90% in pigs at slaughtering age (Di Bartolo et al., 2011).

In cattle, antibodies against HEV have been detected in serum samples with prevalences of approximately 6% in India (Arankalle et al., 2001) and up to 25% in China (Geng et al., 2011). One single study reported the detection of HEV RNA in cattle (Hu and Ma, 2010). However, the finding needs to be confirmed.

Avian HEV can be detected in chickens worldwide. Seroprevalence shows an age-dependent distribution of approximately 17% in chickens <18 weeks of age and around 36% in adult chickens (Huang et al., 2002).

1.2.2.5 Zoonotic potential

Shortly after HEV has been recognized as viral agent of non-A, non-B hepatitis cases in humans, successful experimental infection of pigs raised awareness to a possible zoonotic potential of the virus (Balayan et al., 1990). Furthermore, in 1997 an HEV was isolated from pigs, which was closely related to human HEV (Meng et al., 1997). In the following years, HEV was shown to be able to cross the species barrier under experimental conditions (Meng et al., 1998) confirming the results of Balayan et al. (1990) and underlining the risk of a zoonotic transmission between humans and pigs. After HEV RNA was detected in commercially sold pig livers (Yazaki et al., 2003) sporadic cases of acute hepatitis have been linked to the consumption of contaminated meat and meat products (Colson et al., 2010; Takahashi et al., 2004). Today, HEV is recognized as a zoonotic virus with pigs and probably other animals as reservoirs (Meng, 2011).

1.2.2.6 Prevention, control and vaccination

As hepatitis E is usually a moderate and self-limiting disease, in most cases a special treatment is not indicated. However, in severe cases, hospitalization and liver transplantation can be necessary (Teshale and Hu, 2011). Antiviral drugs for treatment of acute hepatitis are not available so far. The main strategies for prevention of the disease are hygienic measures and the access to clean water (Teshale and Hu, 2011). Furthermore, by heating of meat and meat products prior to consumption, the risk of zoonotic fooborne HEV transmission can be reduced (Feagins et al., 2008).

A vaccine against HEV is in development for years. Promising candidates were tested in phase I and II clinical trials, but only one vaccine based on VLPs has been shown highly effective in a phase III trial (Teshale and Hu, 2011; Zhu et al., 2010). Hecolin® is distributed commercially in China and negotiations with the WHO are currently being held to register the vaccine for worldwide commercialization (Park, 2012).

1.2.3 Norovirus (NoV)

Noroviruses are the leading cause of gastroenteritis in adults worldwide and cause estimated 200,000 deaths of children in developing countries per year (Atmar, 2010). In livestock, infections with NoV can be associated with diarrhoea. The zoonotic character of NoV infections is not proven until now, but is assumed as strains of humans and pigs belong to the same genogroup.

1.2.3.1 Taxonomy and phylogeny

A

Family: *Caliciviridae*

Genus: *Norovirus*

Species: *Norwalk virus*

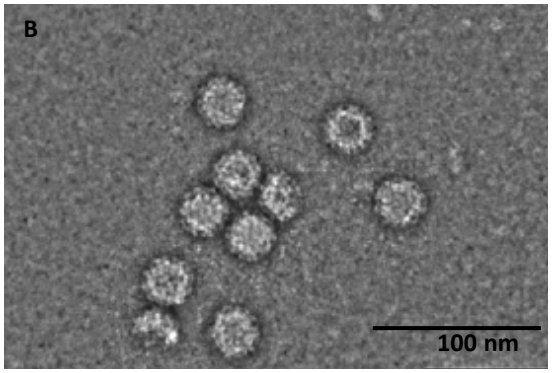

Figure 6: **Taxonomy and electron micrograph of NoV**. **A** Taxonomic classification of NoV. **B** Electron micrograph of NoV GII obtained from human stool (Dr. J. Reetz, BfR, Berlin). The length of the bar corresponds to 100 nm.

The genus *Norovirus* belongs to the family *Caliciviridae*, together with four other genera (**figure 6**). The species *Norwalk virus* shows a high genetic heterogeneity and is divided into different genogroups and genotypes by means of sequence identities. Determination of genogroups is based on sequence similarities in the ORF2 and enables a classification into the five genogroups GI-GV (Clarke et al., 2012). Noroviruses of genogroups I and IV are found exclusively in humans, while genogroup II NoV can be detected in humans and pigs. Genogroup III and V NoV have been detected in cattle and mice, respectively. A further proposed genogroup, GVI, has been detected in humans and dogs. However, it is until now not officially approved (Mesquita et al., 2013). Genotypes can be determined by sequence similarity of ORF2 (capsid genotype) or ORF1 (polymerase genotype), although the designation to capsid genotypes is more common. Overall, the five genogroups can be

subdivided into more than 30 genotypes, with the highest diversity within genogroup II (Hoa Tran et al., 2013).

A classification of serotypes is not possible for NoV so far because of the lack of an *in vitro* model for the propagation of NoV (Zheng et al., 2006).

1.2.3.2 Structure of virus and genome

The capsid of NoV is non-enveloped and shows a spherical, icosahedral structure. Depending on pH, the diameter ranges between 27 and 35 nm (**figure 6**). The virus capsid is build by one single protein, VP1, which forms a shell of 90 dimers with protruding capsomeres. This special arrangement leads to 32 cup-shaped depressions, which can be observed by electron microscopy, and is responsible for the designation of the family (calici from Greek *kalyx*, cup) (Clarke et al., 2012). The genome consists of a linear, single-stranded RNA with positive polarity and a size of approximately 7.5 kb. It has three ORFs, with ORF1 coding for non-structural proteins, ORF2 for the capsid protein and ORF3 for a small structural protein, which occurs in only one or two copies per virion. The precise function of ORF3 is not known, but it is suggested to play a role in the encapsidation of the viral genome (Karst, 2010). Short UTRs at the 5' and 3' end flank the three ORFs (**figure 7**).

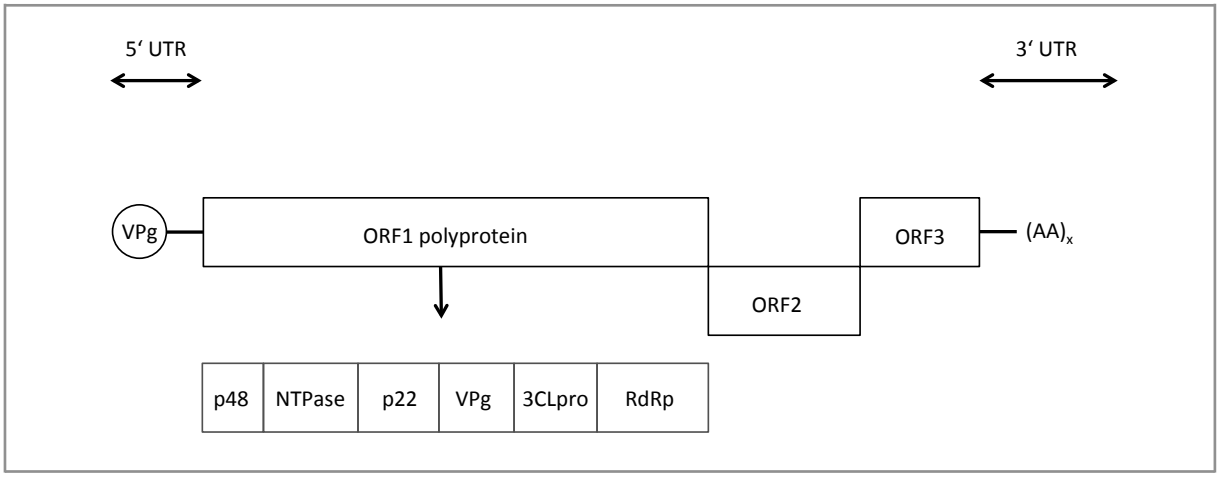

Figure 7: **Organization of the NoV genome**. ORF1 encodes a polyprotein, which is posttranslationally cleaved into non-structural proteins p48, NTPase, p22, VPg, 3CLpro and RdRp. ORF2 and ORF3 code for the capsid protein and a protein of unknown function. ORF, open reading frame; UTR, untranslated region; VPg, viral protein genome-linked; $(AA)_x$, poly(A) tail; RdRp, RNA-dependent RNA polymerase.

1.2.3.3 Pathogenesis and clinical features

The knowledge about NoV pathogenesis is limited because of the lack of appropriate animal models. The virus replicates in the intestine and causes there histological alterations (Karst, 2010). Until recently, the infection was thought to be restricted to the gastrointestinal tract but recent studies were able to detect NoV in serum samples (Takanashi et al., 2009).
NoV are shed in high concentrations of up to 10^{11} genome copies per gram stool from infected individuals (Atmar et al., 2008). The virus can be shed up to several weeks after infection also from asymptomatic individuals (Atmar et al., 2008; Karst, 2010). Especially in immunosuppressed individuals, infections with NoV can become chronic leading to virus shedding for months (Saif et al., 2011).

1.2.3.3.1 Humans

Infections with NoV are characterized by a short incubation period of 24 hours on average, followed by sudden onset of vomiting and diarrhoea (Karst, 2010; Lee et al., 2013b). The disease can be accompanied by nausea, fever, and abdominal cramping pain (Karst, 2010). Symptoms usually persist for one or two days; however, in elderly and immunocompromised patients as well as in children disease up to seven days and longer are common (Atmar, 2010; Karst, 2010). NoV can be detected in all age groups. However, infections occur more frequently in the elderly and children under five years of age (Atmar, 2010; Bernard et al., 2014). In those age groups, as well as in immunocompromised individuals, more severe diseases with increased mortality are described (Bernard et al., 2014; Karst, 2010). The NoV genotype seems to have an influence on the appearance of clinical symptoms as infections with genotype GII.4 cause more often clinical symptoms than infections with other genotypes (Friesema et al., 2009). However, asymptomatic infections are also common (Atmar, 2010). Cases of extraintestinal manifestation were reported in the last years (Nakajima et al., 2012).

1.2.3.3.2 Livestock

In cattle, infections with NoV may be associated with diarrhoea, while NoV infections in swine frequently remain asymptomatic. Whereas most studies described NoV only in healthy finisher pigs, one recently performed study reported symptomatic NoV infections in piglets (Shen et al., 2012). No NoV has been detected in poultry so far.

1.2.3.4 Host spectrum, geographical distribution and epidemiology

Noroviruses have a rather narrow host spectrum. Specific NoV groups can be found in humans, swine, cattle, mice and dogs. The virus occurs worldwide and is transmitted via the faecal-oral route (Atmar, 2010).

1.2.3.4.1 Humans

In humans, the main transmission route is by direct contact from human-to-human by excretions. Infections frequently occur in closed facilities like nursing homes, daycare centres, hospitals, cruise ships and military camps (Mathijs et al., 2012). NoV gastroenteritis outbreaks are also often linked to the consumption of fresh food, like raspberries, lettuce and mussels as well as processed products in restaurants, canteens and catering services, thus pointing out a foodborne transmission route (Mathijs et al., 2012). Nosocomial infections with NoV are common and also cases of airborne transmission are reported (Atmar, 2010).
In developing countries, infections with NoV account for around 200,000 deaths of children every year (Atmar, 2010). However, high NoV prevalences can be detected worldwide in faeces of children with diarrhoea (**table 6**). In Germany, only few studies on the prevalence of viral NoV RNA were performed, detecting high prevalences of over 30% (Oh et al., 2003; Wiegering et al., 2011).

Table 6: **Prevalence of NoV in faeces of children admitted to hospital with acute gastroenteritis**.

Country	NoV prevalence (%)	Reference
Russia	13	Podkolzin et al., 2009
South Africa	14	Mans et al., 2010
UK	16	Cunliffe et al., 2010
Germany	31	Wiegering et al., 2011
Korea	17	Park et al., 2011
Japan	34	Kawada et al., 2012
Brazil	37	Siqueira et al., 2013
Iran	13	Najafi et al., 2013
USA	21	Payne et al., 2013
India	10	Menon et al., 2013b

With a detection rate of 96%, genogroup II is the most prevalent genogroup detected in humans throughout the world (**figure 8**). Generally, infections with genogroups I and IV occur rather seldomly. However, in some countries like Egypt, Yemen or Madagascar, a NoV GI prevalence of up to 30% was observed. The most prevalent genotype in genogroup II is GII.4 followed by GII.3 (Hoa Tran et al., 2013).

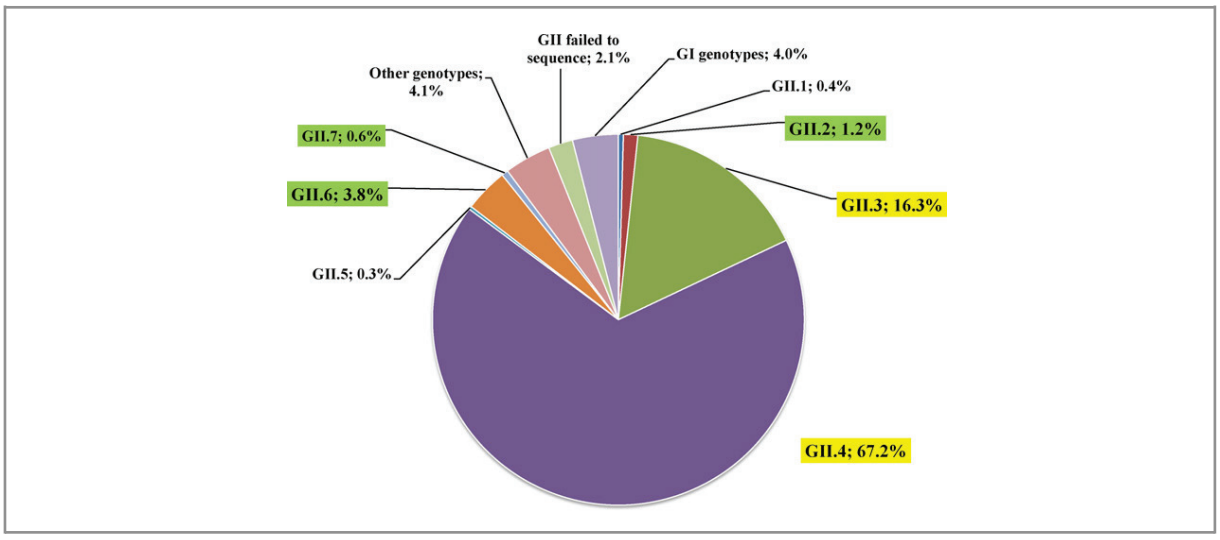

Figure 8: **NoV genogroup and capsid genotype prevalence in sporadic cases worldwide** (Hoa Tran et al., 2013). Worldwide, GII.4 is the predominant genotype in sporadic cases of NoV-induced gastroenteritis, followed by GII.3.

Infections with NoV occur all over the year, but are in general more common during winter (Ahmed et al., 2013). Antibodies are mostly acquired during childhood and the prevalence can reach over 90% in adult individuals, against NoV GII as well as GI (Menon et al., 2013a).

After introduction of a rotavirus vaccine programme, NoV became the leading cause of hospitalized cases of gastroenteritis in some countries (Payne et al., 2013).

1.2.3.4.2 Livestock

Studies on the prevalence of NoV in cattle are only scarce, but a prevalence of up to 44% has been determined in European countries (Milnes et al., 2007). In the USA, bovine NoV was detected in cattle with a prevalence of up to 80% (Wise et al., 2004).

In swine, NoV are usually found in healthy finisher pigs without clinical signs. However, recent studies report the detection of NoV from other age groups and from piglets with diarrhoea (Chao et al., 2012; Mijovski et al., 2010; Shen et al., 2012). Several studies on the prevalence of NoV in faeces of pigs have been published, but no data are available for Germany so far (**table 7**). One study is available on the seroprevalence of anti-NoV antibodies in pigs, which determined a prevalence of 71% in the USA and 36% in Japan (Farkas et al., 2005).

Table 7: **Prevalence of NoV in faeces of pigs of different age**.

Country	NoV prevalence (%)	Reference
The Netherlands	2	van Der Poel et al., 2000
USA	2	Wang et al., 2005
USA	20	Wang et al., 2006
Canada	25	Mattison et al., 2007
Belgium	5	Mauroy et al., 2008
Canada	20	L'Homme et al., 2009a
Korea	2	Keum et al., 2009
Japan	18	Nakamura et al., 2010
Korea	1	Song et al., 2011
USA	19	Scheuer et al., 2013

1.2.3.5 Zoonotic potential

A zoonotic transmission of NoV was first suspected after an outbreak of gastroenteritis in a retirement home, which could be linked to a diseased dog (Humphrey et al., 1984). A zoonotic potential of NoV was further supported at the end of the 20th century, when NoV were detected in pigs, which were genetically related to human genogroup II NoV (Sugieda and Nakajima, 2002; Wang et al., 2005). Furthermore, NoV of genotype GII.4, the most

predominant genotype in humans, were reported to be present in faeces of livestock and GII.4-like noroviral RNA was also found in retail meat samples (Mattison et al., 2007). Under experimental conditions it was shown, that human NoV were able to induce diarrhoea in calves, and infected piglets shed the virus with their faeces (Cheetham et al., 2006; Souza et al., 2008). Additionally, antibodies against bovine NoV were detected in humans with a higher prevalence in veterinarians (28%) than in control individuals (20%) (Menon et al., 2013a; Widdowson et al., 2005). However, all these hints only suggest a zoonotic potential of NoV, whereas their zoonotic transmission is not convincingly proven so far.

1.2.3.6 Prevention, control and vaccination

Because of the rather mild clinical course of infection, no special therapy is indicated in most cases. In serious cases, the supply of isotonic liquids may be necessary in order to counteract dehydration. The main strategy for control of the disease is the prevention of virus transmission. Furthermore, attainment of high standards of hygiene, especially in the food industry, is a key factor in the prevention and control of NoV disease (Atmar, 2010).

The main challenge about the vaccination against NoV infections is their poorly understood immunity. The lack of a cell culture model for the propagation of NoV further complicates the clarification of the role of neutralizing antibodies during infection. The high heterogeneity of NoV additionally hampers the development of a vaccine (Vinje, 2010). However, some experimental vaccines based on VLPs have been developed (Vinje, 2010).

1.2.4 Rotavirus (RV)

Rotaviruses are the leading cause of severe gastroenteritis in children worldwide and account for more than 600,000 deaths every year (Martella et al., 2010). In livestock, infections with RV can cause great losses due to diarrhoea and dehydration. A zoonotic transmission is possible and can occur by direct contact with animals or indirect by contaminated surfaces, food and water.

1.2.4.1 Taxonomy and phylogeny

A

Family: *Reoviridae*

Genus: *Rotavirus*

Species: *Rotavirus A-E*

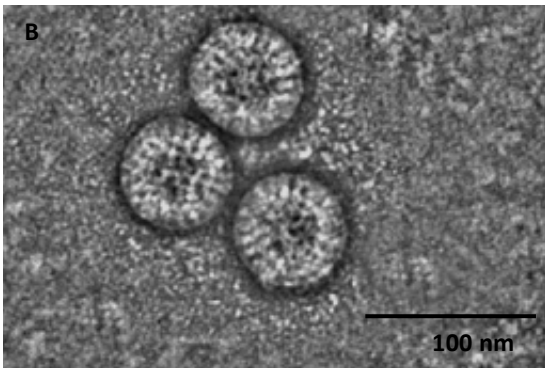

Figure 9: **Taxonomy and electron micrograph of GARV. A** Taxonomic classification of GARV. **B** Electron micrograph of GARV obtained from human stool (Dr. J. Reetz, BfR, Berlin). The length of the bar corresponds to 100 nm.

Rotavirus is a genus of the family *Reoviridae*, consisting of five officially approved species (*Rotavirus A, B, C, D, E*) and three additional viruses that have not been classified until now (Rotavirus group F, G and NADRV) (**figure 9**) (Attoui et al., 2012). The RV species are also designated as RV groups. Criteria for demarcation of species are based on the sequence of the VP6 gene, as well as on their ability to exchange genome segments, which is only possible among RV of the same species (Attoui et al., 2012). Rotaviruses of groups A (GARV), B and C are able to infect humans as well as various animal species. GARV are the leading cause of severe episodes of viral gastroenteritis in humans and animals all over the world, whereas non-GARVs are considered to have less public health importance (Martella et al., 2010). Rotaviruses of groups D, F and G are specific for poultry, while rotaviruses of group E have been detected only once in pigs. The novel adult diarrhoea virus (NADRV), which was

identified during human gastroenteritis outbreaks in China, seems to represent an additional species.

GARV are furthermore classified into so called G- and P-types based on their VP4 and VP7 genes, respectively. G- and P-types can be determined either by cross-neutralization (serotypes) or by sequence comparison (genotypes), whereby genotype determination is more common nowadays. While G-types are mostly similar, whether determined by neutralization or sequence comparison, P-types differ depending on the method used. Therefore, a dual nomenclature is common for P-types: the P-serotype is indicated by a number and the P-genotype is indicated by a number in square brackets. So far, 27 G-types and 35 P-types are known (Matthijnssens and Van Ranst, 2012). A new classification system is based on all 11 genome segments. The genotype configuration is Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (respectively to VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 encoding genes), whereby x is replaced by Arabic figures. A recently published review lists 16 I, 9 R, 9 C, 8 M, 16 A, 9 N, 12 T, 14 E and 11 H types (Matthijnssens and Van Ranst, 2012). For a long time, grouping of avian rotaviruses (AvRV) was mainly based on distinct electrophoretic migration patterns in polyacrylamide gel electrophoresis (PAGE). However, recently sequences from group D, F and G AvRV have been published, showing a clustering of groups A, C, D and F separated from groups B, G and NADRV (Johne et al., 2011).

1.2.4.2 Structure of virus and genome

RV are non-enveloped viruses, consisting of three concentric protein layers. The inner layer is build by VP2 and contains the genomic RNA, as well as VP1 and VP3. The latter proteins are organized in up to 12 complexes bound to the inner surface of the VP2 layer. VP6 builds the second layer, which is surrounded by the outer layer, consisting of VP4 and VP7. VP7 is arranged as trimers building the surface of the layer and giving the particle a diameter of 70 nm. VP4 forms 60 trimeric spikes, which enlarge the diameter to 100 nm (**figure 9**) (Attoui et al., 2012). The genome consists of 11 linear segments of a double-stranded RNA with a size of about 18.5 kb (**figure 10**). With exception of segment 11, all genes are monocistronic. The genome codes for six structural proteins and five to six non-structural proteins (Attoui et al., 2012). All segments are 5'-capped and share conserved 5' and 3' termini.

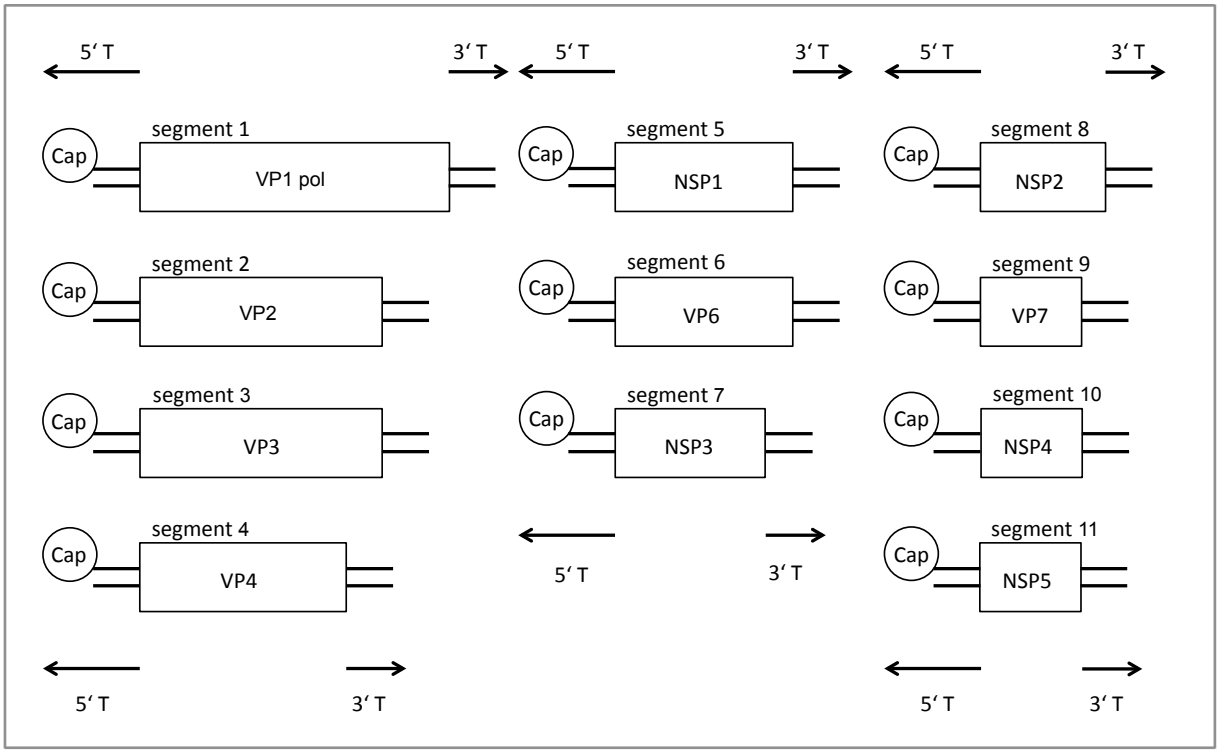

Figure 10: **Organization of the GARV genome**. The genome consists of 11 segments, which are transcribed into six structural proteins (VP1-VP6) and five to six non-structural proteins (NSP1-NSP5). T, terminus; Cap, 5' methylated cap structure.

1.2.4.3 Pathogenesis and clinical features

The knowledge about the pathogenesis of RV is mainly based on *in vitro* studies and experiments in animal models. RV replicate in the small intestine and usually cause severe histological changes (Ramig, 2004). The virus is shed in high concentrations of more than 10^{11} particles per gram stool of infected individuals for up to several weeks (Anderson and Weber, 2004; Chen et al., 2012).

1.2.4.3.1 Humans

The symptoms of RV-induced disease can vary between an asymptomatic course, mild diarrhoeal disease and severe episodes of gastroenteritis with dehydration and death. After an incubation period of approximately 48 hours, illness sets on with nausea, fever and diarrhoea and usually lasts for three to eight days (Chen et al., 2012; Lee et al., 2013b). Non-compensated dehydration and the resulting electrolyte imbalance can cause a vast

number of deaths in children, especially in developing countries. Chronic infections with GARV occur in immunocompromised individuals. Additionally, in some cases, extraintestinal virus was detected in the liver and other organs (Medici et al., 2011; Ramig, 2004). Mortality due to rotavirus gastroenteritis varies between industrialized countries, where frequent infections are common but show a low mortality and developing countries, where GARV infections are one of the main reasons for life-threatening diarrhoea (Chen et al., 2012). Severe episodes of gastroenteritis usually affect children between six months and two years of age. Overall, children under five years of age are highly affected by GARV infections (Chen et al., 2012). Infections in adults are less common, but can also cause severe gastroenteritis (Anderson and Weber, 2004).

1.2.4.3.2 Livestock

In cattle, GARV are the leading cause of neonatal diarrhoeal disease and have a great economic impact due to a high mortality or reduced weight gain. Asymptomatic infections are rather uncommon in young animals but usually occur in older animals (Alfieri et al., 2006; Steyer et al., 2008).

Also in pigs, GARV have an economic impact, as GARV infection is associated with weaning and post-weaning enteritis in piglets. However, asymptomatic infections are also reported frequently (Midgley et al., 2012; Steyer et al., 2008).

In poultry, infections with rotaviruses of groups A and D are common in young birds and can be associated with mild to severe cases of diarrhoea, depression, reduced weight gain, runting and stunting syndrome and increased mortality (Martella et al., 2010; Trojnar et al., 2010). Also, asymptomatic and subclinical infections are reported (Villarreal et al., 2006). The knowledge about the clinical impact of infections with group F and G AvRV is only poorly elucidated. However, AvRV-F and -G have been detected in chickens with runting and stunting syndrome, diarrhoea and growth depression (Kindler et al., 2013; Otto et al., 2006).

1.2.4.4 Host spectrum, geographical distribution and epidemiology

RV have a wide host range and can be detected in various mammals and birds worldwide. The main transmission route is faecal-orally (Martella et al., 2010).

1.2.4.4.1 Humans

In humans, transmission from human-to-human and fomites occurs most commonly. In industrialized countries, nosocomial transmission also plays an important role (Anderson and Weber, 2004). Other possible transmission pathways are via respiratory droplets, contaminated food and water. Zoonotic transmission by direct contact with animals is also possible (Chen et al., 2012; Levy et al., 2009; Martella et al., 2010).

A seasonality of rotavirus infections is not clearly proven. In temperent regions, infections are more common in the winter months, with varying infection peaks between autumn and spring (Chen et al., 2012; Levy et al., 2009). A strict winter-seasonality is only described for the USA (Levy et al., 2009). In tropical regions, infections occur throughout the year with several peaks. Surveillance studies indicate that the most prevalent strains worldwide are G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]; however, strain prevalence changes year-by-year (Martella et al., 2010). Generally, RV infections are very widespread among the human population. Studies on the seroprevalence of anti-RV antibodies show, that until the age of five years seroprevalence of anti-RV group A antibodies reaches 90-95% (Cox et al., 1998). Despite the introduction of rotavirus vaccines in several countries, GARV prevalences are high in faeces of children admitted to hospital due to acute gastroenteritis (**table 8**) (Cunliffe et al., 2010; Mathew et al., 2014). This also includes children from Germany, where a prevalence of 41% was detected recently (Wiegering et al., 2011).

Table 8: **Prevalence of GARV in faeces of hospitalized children with acute gastroenteritis**.

Country	GARV prevalence (%)	Reference
Russia	44	Podkolzin et al., 2009
South Africa	24	Mans et al., 2010
UK	38	Cunliffe et al., 2010
Germany	44	Wiegering et al., 2011
Korea	18	Park et al., 2011
Japan	36	Kawada et al., 2012
USA	12	Payne et al., 2013
Iran	24	Najafi et al., 2013
India	36	Mathew et al., 2014
Ghana	50	Enweronu-Laryea et al., 2014

1.2.4.4.2 Livestock

In pigs GARV are highly prevalent and several studies investigated a high prevalence of GARV in faeces of pigs (**table 9**).

Table 9: **Prevalence of GARV in faeces of pigs of different age**.

Country	Health status of pigs	GARV prevalence (%)	Reference
Germany	Diarrhoeic	4	Wieler et al., 2001
Japan	Diarrhoeic	66	Katsuda et al., 2006
Slovenia	Mostly non-diarrhoeic	20	Steyer et al., 2008
Spain	Diarrhoeic/non-diarrhoeic	37	Halaihel et al., 2010
India	Diarrhoeic	26	Kusumakar et al., 2010
Korea	Diarrhoeic	38	Kim et al., 2010
Japan	Diarrhoeic/non-diarrhoeic	18	Miyazaki et al., 2012
Denmark	Diarrhoeic	10	Midgley et al., 2012
Hungary	Diarrhoeic/non-diarrhoeic	4	
Slovenia	Diarrhoeic/non-diarrhoeic	20	
India	Diarrhoeic/non-diarrhoeic	10	Dubal et al., 2013
USA	Diarrhoeic/non-diarrhoeic	12	Amimo et al., 2013

According to most of the studies, prevalence is increased in piglets between three and eight weeks of age (Halaihel et al., 2010; Miyazaki et al., 2012; Steyer et al., 2008). Studies on the prevalence of GARV in German pigs are scarce. Only one study is available, which detected RV in 4% of faecal samples by electron microscopy (Wieler et al., 2001). The most prevalent G-type in swine worldwide is G5, followed by G3 and G4. For P-types, P[7] is the most prevalent followed by P[6] and P[13] (Papp et al., 2013). In swine, up to 100% of adult animals are seropositive for anti-GARV antibodies (Prabha and Verghese, 2009).

In poultry, AvRV of groups A and D are highly prevalent, especially in young animals. The prevalence of AvRV-A can reach up to 70%, while AvRV-D was detected with a prevalence of up to 53% (Bezerra et al., 2012; Moura-Alvarez et al., 2013; Otto et al., 2006; Pantin-Jackwood et al., 2008). In contrast, AvRV of groups F and G are only scarcely detected in poultry (Ahmed and Ahmed, 2006; Otto et al., 2006).

1.2.4.5 Zoonotic potential

There is experimental as well as epidemiological evidence for zoonotic transmission of RV. Heterologous infection of piglets with human and bovine strains was successful, resulting in disease (Azevedo et al., 2005; Park et al., 2013). Furthermore, rhesus rotavirus replicated efficiently in experimentally infected rabbits, and was even transmitted horizontally (Ciarlet et al., 2000). In addition, direct interspecies-transmission under natural conditions between human and animal strains is reported (Matthijnssens et al., 2006). In some cases, symptomatic infections of humans by animal RV have been described (Esona et al., 2009). RV are therefore suspected to be zoonotically transmitted by contaminated surfaces and food, or by direct contact with animals (Martella et al., 2010; Steyer et al., 2008).

1.2.4.6 Prevention, control and vaccination

No etiologic therapy exists for RV disease, as the disease is usually self-limiting. However, severe cases of diarrhoea may need hospitalization with parenteral dehydration. Especially in developing countries, dehydration causes a high mortality in children due to limited access to medical care, poor sanitation standards and malnutrition (Chen et al., 2012).
Two human vaccines were successfully developed in the end of the 20th century (Rotarix®, GSX; Rotateq®, Merck), which were approved after extensive clinical trials (Chen et al., 2012). By the end of 2012, vaccination was part of the national immunization programme in 41 countries (WHO, 2013). Since the use of oral rotavirus vaccines, the prevalence of RV in children hospitalized with acute gastroenteritis decreased in most countries (Chen et al., 2012). However, high prevalences of RV still can be detected in children until the age of five years, in low-income countries, countries without a national rotavirus immunization

programme, as well as in countries after introduction of rotavirus vaccine (see section 1.2.4.4.1, **table 8**).

For pigs and cattle, vaccines against RV are available (Papp et al., 2013). However, the pig vaccine is only available in the USA. In Germany, several combined vaccines against RV and other diarrhoeal pathogens like coronavirus and *Escherichia coli* are admitted for cattle (Paul-Ehrlich-Institut, 2014). For avian species, no vaccine is available so far.

1.2.5 Astrovirus (AstV)

Astroviruses are usually enteropathogenic agents, which are associated with gastroenteritis in humans and animals. However, extraintestinal manifestations of AstV infections are common in birds. Together with rota- and noroviruses, astroviruses are considered as the leading cause of gastroenteritis in children worldwide. A zoonotic potential is assumed but not definitively proven so far.

1.2.5.1 Taxonomy and phylogeny

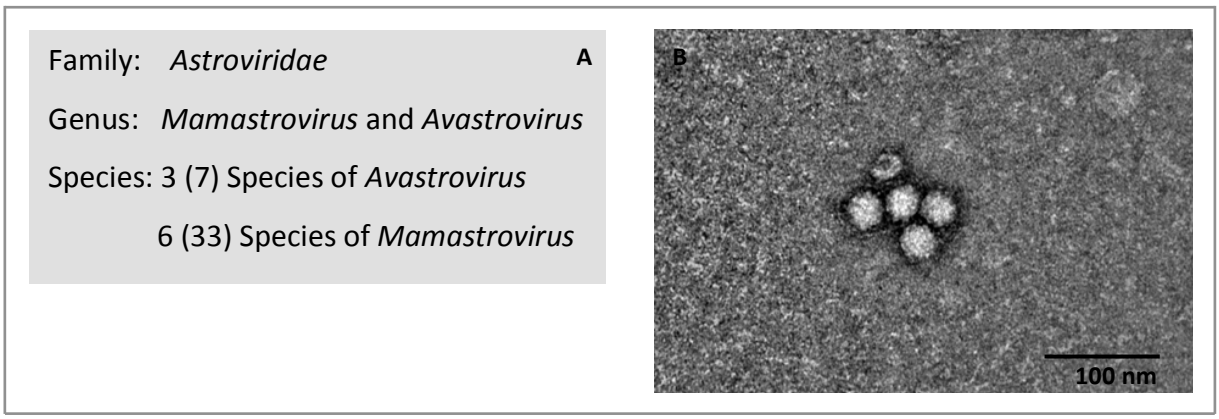

Figure 11: **Taxonomy and electron micrograph of AstV**. **A** Taxonomic classification of AstV. **B** Electron micrograph of avian AstV obtained from the intestinal content of a broiler chicken (Dr. J. Reetz, BfR, Berlin). The length of the bar corresponds to 100 nm.

The family *Astroviridae* is divided into two genera: the genus *Avastrovirus*, which comprises three species of avian astroviruses and the genus *Mamastrovirus* comprising six species of human and mammalian astroviruses (**figure 11**). For a long time classification of AstV was based exclusively on the host, from which the virus was isolated. According to the Astroviridae Study Group, species demarcation is now based on both, host range and genetic differences, thereby classifying AstV in 33 species of *Mamastrovirus* and seven species of *Avastrovirus* (**figure 11**) (Guix et al., 2013). On the basis of antigenic criteria, AstV are classified into serotypes and most studies show a high correlation to serotypes determined by sequence analysis (Guix et al., 2013). In humans, eight classic serotypes of AstV are known, which are designated as huAstV 1-8. New viruses have been detected in humans showing more sequence similarity to AstV from rat, mink and sheep than to human viruses

(AstV MLB and HMO-AstV). In pigs, five distinct genetic lineages are known based on sequence analysis of ORF2 (Mor et al., 2012; Xiao et al., 2013).

1.2.5.2 Structure of virus and genome

AstV are non-enveloped viruses with a spherical, icosahedral capsid of 28-30 nm in diameter (**figure 11**). Approximately 10% of virions show a five- or six-pointed starlike appearance under the electron microscope, which led to the designation astrovirus (astro from Greek *astron*, star). The virus capsid consists of at least three small viral particles, which are derived from one single precursor protein by proteolytic processing (Bosch et al., 2012). The genome of AstV is a linear, single-stranded RNA with positive polarity and a size of approximately 6.4-7.7 kb. The structure of the genome resembles that of *Caliciviruses* and consists of three ORFs and short UTRs at the 5' and 3' end (**figure 12**). ORF1a and ORF1b encode non-structural proteins, while ORF2 codes for the capsid protein precursor (Bosch et al., 2012).

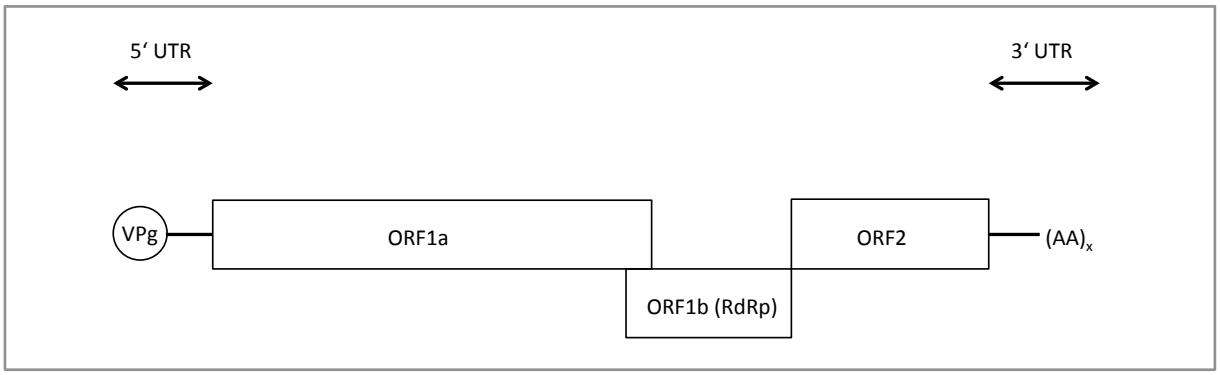

Figure 12: **Organization of the AstV genome**. ORF1a and ORF1b encode non-structural proteins. ORF2 encodes the capsid protein precursor. ORF, open reading frame; UTR, untranslated region; VPg, viral protein genome-linked; $(AA)_x$, poly(A) tail; RdRp, RNA-dependent RNA polymerase.

1.2.5.3 Pathogenesis and clinical features

Fundamental insights into the pathogenesis of AstV were gained in studies with sheep and turkeys, whereas an appropriate rodent model is still missing. AstV are able to infect cells in the small intestine, but clinical symptoms are not accompanied by obvious histological changes. Furthermore, inflammatory responses could not be observed (Moser et al., 2007).

The mechanisms, by which AstV cause diarrhoea is mainly unknown. However, there is evidence that an increase of the intestinal barrier permeability might be a contributor to diarrhoea (Moser et al., 2007). In humans, AstV is usually shed up to 14 days after infection (Kurtz et al., 1979; Shastri et al., 1998). However, prolonged faecal shedding for up to 17 weeks was also reported (Maldonado et al., 1998). In faeces of infected individuals AstV are shed in high concentrations of up to 10^{13} genome copies per gram (Bosch et al., 2013).

1.2.5.3.1 Humans

Human infections with AstV are characterized by self-limiting diarrhoea, which usually presents milder than diarrhoea caused by rotavirus. Diarrhoea can be accompanied by vomiting, nausea, headache, fever and dehydration (Moser and Schultz-Cherry, 2005). In volunteer studies and in naturally occurring outbreaks due to AstV, symptoms occurred three to five days after infection and lasted for about two to three days (Lee et al., 2013b; Moser and Schultz-Cherry, 2005). Young children are most likely to develop clinical illness, but infections also affect the elderly, immunocompromised patients and healthy adults (Moser and Schultz-Cherry, 2005). Recently, AstV could be associated with encephalitis in a 15-year-old boy and was furthermore detected in other organs like brain, kidney and heart in severely immunocompromised children (Quan et al., 2010; Wunderli et al., 2011). Frequently, infections with AstV remain asymptomatic (Maldonado et al., 1998; Mendez-Toss et al., 2004).

1.2.5.3.2 Livestock

Infections with AstV usually cause diarrhoea in several mammalian animal species, however, asymptomatic infections are also common. The clinical significance of AstV infections of pigs is unclear. In some studies, infection could be associated with diarrhoea, in other studies, the infection remained asymptomatic (Luo et al., 2011; Mor et al., 2012; Xiao et al., 2013).
In cattle, AstV infections are mostly asymptomatic (Tse et al., 2011). However, recently AstV were correlated with neurological disease in cattle (Li et al., 2013).
Avian AstV infections frequently occur with extraintestinal manifestation like hepatitis and interstitial nephritis (De Benedictis et al., 2011; de Wit et al., 2011). Additionally, AstV have

been associated with enteric disorders, tenosynovitis and arthritis in chickens and turkeys (de Wit et al., 2011).

1.2.5.4 Host spectrum, geographical distribution and epidemiology

AstV have been detected in a wide spectrum of hosts including diverse mammalian species and birds (De Benedictis et al., 2011). The virus is distributed all over the world and is transmitted by the faecal-oral route.

1.2.5.4.1 Humans

Different studies in adult volunteers show, that the transmission of AstV occurs by the faecal-oral route (Kurtz et al., 1979; Moser and Schultz-Cherry, 2005). Although only a limited number of reports are available, contaminated food also seems to play a role in transmission of AstV (Moser and Schultz-Cherry, 2005; Oishi et al., 1994). Several studies detected AstV in oysters and mussels with prevalences of up to 50% (Le Guyader et al., 2000). During several gastroenteritis outbreaks, AstV and other enteric viruses have been repeatedly detected in sewage plants, urban surface water and drinking water (He et al., 2011).

Epidemiological studies show that infections with AstV predominantly occur in children up to four years of age (Ayolabi et al., 2012; Guix et al., 2002). A prevalence of AstV shedding in hospitalized children between 1% and 9% in industrialized as well as developing countries was reported (De Benedictis et al., 2011). However, some studies have determined a prevalence of up to 40% in developing countries (**table 10**). In Germany, only one study is published, showing an AstV prevalence of 4% (Oh et al., 2003).

Sporadical outbreaks in elderly people (Marshall et al., 2007), military camps (Belliot et al., 1997) and immunocompromised patients (Wunderli et al., 2011) as well as nosocomial infections have been reported (Shastri et al., 1998).

AstV infection rates have been reported to be higher in the cold, dry winter months in temperent regions, while in tropical regions the AstV prevalence shows a peak in the rainy season (Bosch et al., 2013; Jeong et al., 2012; Maldonado et al., 1998).

Table 10: **Prevalence of AstV in faeces of children hospitalized with diarrhoea**.

Country	AstV prevalence (%)	Reference
Germany	4	Oh et al., 2003
Brazil	30	Resque et al., 2007
Thailand	18	Malasao et al., 2008
Russia	1	Podkolzin et al., 2009
South Africa	7	Mans et al., 2010
UK	5	Cunliffe et al., 2010
Italy	4	De Grazia et al., 2011
Korea	2	Park et al., 2011
Nigeria	40	Ayolabi et al., 2012
Iran	2	Najafi et al., 2013

The prevalence of the different serotypes varies depending on the region and can change in one region during the seasons (Bosch et al., 2013) Globally, serotype 1 is predominant, followed by serotypes 2-5 and 8, whereas serotypes 6 and 7 occur rather sporadically (Guix et al., 2002; Jeong et al., 2012; Mendez-Toss et al., 2004). Mixed infections with rotaviruses and other pathogens are common (Guix et al., 2002; Maldonado et al., 1998; Mendez-Toss et al., 2004).

Data about the seroprevalence of anti-AstV antibodies indicate, that approximately 75% of children in the age between five and ten years have developed antibodies against AstV. Studies from the UK report 90% seropositivity in children of five years of age and studies from Japan even assessed up to 100% seropositivity in the age of three years (Kobayashi et al., 1999; Kriston et al., 1996). Also for the newly detected types of human AstV, high antibody prevalences have been determined (Burbelo et al., 2011).

1.2.5.4.2 Livestock

Data about the prevalence of AstV in farm animals are mainly available for poultry. There, prevalences are high and can reach up to 85% in turkeys and 96% in chickens (Moura-Alvarez et al., 2013; Pantin-Jackwood et al., 2008; Smyth et al., 2009). Some recent studies investigated the prevalence of AstV in pigs, showing prevalences of up to 80% (**table 11**). However, no study has been published from Germany.

No studies on the prevalence of antibodies against AstV in pigs are known to the author.

For cattle, one study determined a prevalence between 60% and 100%, and another one only 2% (Bridger et al., 1984; Tse et al., 2011).

Table 11: **Prevalence of AstV in faeces of pigs of different age**.

Country	Health status of pigs	AstV prevalence (%)	Reference
Colombia	Diarrhoeic	24	Ulloa and Gutierrez, 2010
Canada	Healthy	80	Luo et al., 2011
USA	Diarrhoeic	62	Mor et al., 2012
USA	Mainly diarrhoeic (96% of samples)	64	Xiao et al., 2013
South Korea	Diarrhoeic/ non-diarrhoeic	20 21	Lee et al., 2013a

1.2.5.5 Zoonotic potential

Because of high sequence similarities between animal and human strains, AstV have been frequently supposed to be potential zoonotic agents. This assumption is strengthened by the detection of well-documented recombination events between human or animal AstV (Tse et al., 2011). For example, an AstV has been detected in a child containing sequences of multiple human astrovirus serotypes (Wolfaardt et al., 2011). Also, possible recombination events between human and porcine AstV have been reported (Ulloa and Gutierrez, 2010). The discussion about a zoonotic potential of AstV was encouraged after new strains have been found in human stool, which are closer related to AstV from rat, mink and sheep than to human AstV (Chu et al., 2010; Kapoor et al., 2009).

1.2.5.6 Prevention, control and vaccination

As AstV-induced diarrhoea is mostly mild and self-limiting in humans, a therapy is not indicated (Moser and Schultz-Cherry, 2005). Hospitalization with intravenous rehydration is not necessary in most cases. The interruption of the transmission chain and the maintenance of hygienical standards are the key elements to prevent further infections (Jeong et al., 2012). A vaccine against AstV is not available so far.

1.3 Detection of viruses

1.3.1 Overview

After the discovery of the first virus in the late 19th century by Loeffler and Frosch, several methods for the detection of viruses have been developed in the last century.
For a long time, cell culture was the "gold standard" for virus diagnostics and a large number of viruses have been discovered by their isolation in cell culture (Leland and Ginocchio, 2007). However, the method is time-consuming and not all viruses are able to be grown in cell culture. Therefore, other techniques like electron microscopy became essential methods for the detection of viruses. Primarily imaging techniques like immune electron microscopy (IEM) also led to the discovery of many viral agents of gastroenteritis and hepatitis (Bishop et al., 1973; Dane et al., 1970; Feinstone et al., 1973; Kapikian et al., 1972; Madeley and Cosgrove, 1975). However, the development of more sensitive and specific techniques replaced cell culture-based and electron microscopy methods during the last decades. Namely, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) techniques are currently among the most important methods in virus diagnostics (Leland and Ginocchio, 2007).
ELISA is a diagnostic tool based on the detection of antigens or antibodies in a liquid sample. By the use of a fluorogenic or chemiluminescent reporter, qualitative as well as quantitative results can be obtained. PCR techniques are used for the detection of viral genomes in clinical samples. The real-time reverse transcription (RT)-PCR, which enables quantification of genomes of viruses with RNA genomes, is mainly used in this study and is therefore explained in more detail.

1.3.2 Real-time RT-PCR

1.3.2.1 Principle

The real-time RT-PCR is a molecular technique, which creates and amplifies DNA molecules and simultaneously detects and quantifies the generated products (Watzinger et al., 2006). In a series of different steps, a DNA template is generated from an RNA template by reverse

transcription, which is subsequently exponentially amplified in the presence of enzymes, primers and a buffer solution. The detection of the PCR product in real-time is realized by the use of fluorescent dyes.

The real-time RT-PCR consists of three main steps (Mackay et al., 2002):

1. Reverse transcription of an RNA template into a complementary DNA (cDNA)
2. Amplification of cDNA
3. Detection and quantification of amplified DNA in real-time.

For the detection of PCR products, non-specific or specific fluorescent dyes are used. The use of a non-specific fluorescent dye is the simplest option for real-time RT-PCR. SYBR Green is the most widely used DNA-specific dye, which intercalates into double-stranded DNA. The measurable fluorescence intensity is directly proportional to the amount of generated PCR products. However, as the detection is based on intercalation, specific as well as non-specific products generate a signal, thus, false positive signals need to be verified by an additional melting curve analysis (Watzinger et al., 2006).

Specific fluorescent dyes are based on fluorescent probes, which only detect DNA of a specific nucleotide sequence; therefore, leading to highly specific results. Widely used probes are TaqMan® probes, which consist of a single-stranded oligonucleotide of the target-specific sequence, with a binding site between the used primer pair. The probe is labelled with a reporter fluorescent dye at the 5’ end and a quencher dye at the 3’ end. Based on the fluorescence resonance energy transfer (FRET), fluorescence is greatly reduced as long as reporter and quencher are in close proximity. The disruption of the probe by the 5’ to 3’ exonuclease activity of the polymerase leads to emission of fluorescence, which increases proportionally to the amount of PCR products generated (Watzinger et al., 2006) (**figure 13**).

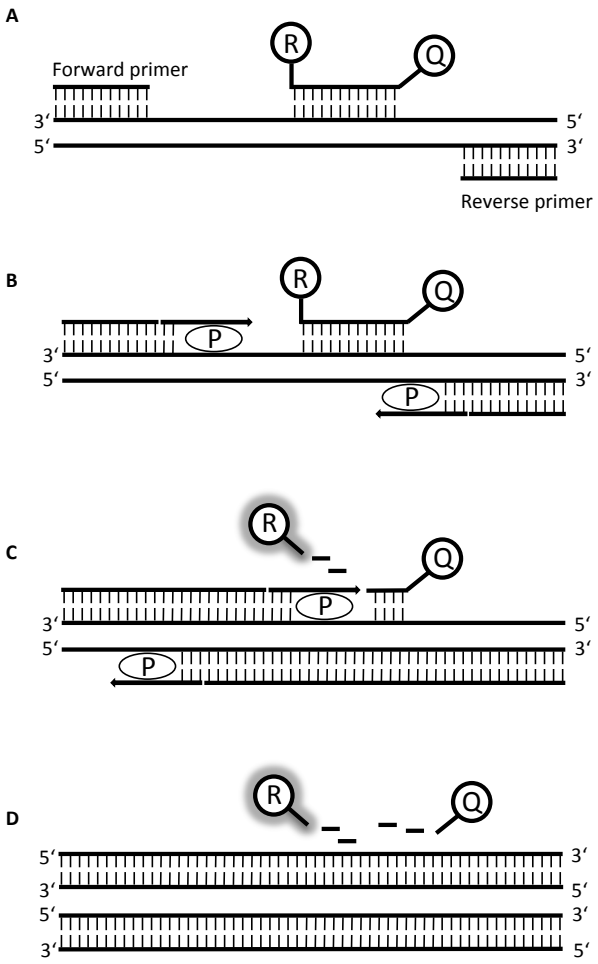

Figure 13: **Principle of TaqMan®-based real-time RT-PCR**. **A** Primers and probe bind to the nucleic acid template. **B** Polymerisation of a new DNA strand by an RNA-dependent DNA polymerase. **C** and **D** The 5' to 3' exonuclease activity of the polymerase degrades the probe and separates the reporter dye from the quencher dye, resulting in fluorescence. R, Reporter; Q, Quencher; P, RNA-dependent DNA polymerase.

1.3.2.2 Quantification

By means of real-time RT-PCR, quantification of the amplified DNA can be either relative or absolute. Relative quantification is easy to perform by comparison of the fluorescence signal of a test sample with that of a control sample. This method may be used for the investigation of changes in gene expression levels.

Absolute quantification relates the PCR signal to the input copy number (Watzinger et al., 2006) and can determine the number of viral genomes present in a given sample. By using external standard calibration curves from a dilution series of template RNA, this method is highly sensitive, specific and reproducible.

1.4 Probiotics and their possible interactions with viruses

1.4.1 Definition and characteristics

Since for many viral infections no antiviral drugs or vaccines are available, prevention strategies play a key role in their control. One approach, which may be used for enteric virus infections, is the modulation of the intestinal microbiota by the use of probiotics.

The intestinal microbiota consist of a diversity of bacteria, fungi, bacteriophages and viruses. Studies in germ-free animals indicated, that the microbiota have influence on health by alteration of metabolic, physiological, nutritional and immunological processes (Kotzampassi and Giamarellos-Bourboulis, 2012; Power et al., 2014).

Probiotics are live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). In many studies, an essential role of probiotics in the modulation of immunological, respiratory and gastrointestinal functions was observed (FAO/WHO, 2001; Fontana et al., 2013). Furthermore, probiotics have been shown to minimize the risk, the duration or the severity of infections (Fontana et al., 2013).

Bacteria need to fulfil certain criteria to be used as probiotics (Fontana et al., 2013). Among these criteria are:

- Acid and bile stability to survive the transit through the stomach and small intestine
- Ability to colonize the gastrointestinal tract and to retain functional health characteristics
- Safety by assessment of the genetic stability, the deleterious metabolic activities and the pathogenic potential of the bacterium.

The majority of bacteria used as probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*. These are gram-positive bacteria, which exist in large numbers in the intestinal tract. However, other bacteria like *Bacillus* or *Enterococcus* species, as well as non-pathogenic strains of *Escherichia coli* (*E. coli*) have been used or investigated as probiotics (Kotzampassi and Giamarellos-Bourboulis, 2012).

1.4.2 Mode of action of probiotics

The health-beneficial effect as well as the mode of action of probiotics seem to be unique for each individual strain. Furthermore, the effect is depending on the administered dose, the route of application and the frequency of intake (Kotzampassi and Giamarellos-Bourboulis, 2012). There is no single mode of action of probiotics, but three mechanisms are considered to explain the influence of probiotics on the health status (Kotzampassi and Giamarellos-Bourboulis, 2012).

1. Probiotics can change the gut ecology by reduction of luminal pH, production of antimicrobial substances, competition for limiting resources or blocking the adherence of pathogens (Kotzampassi and Giamarellos-Bourboulis, 2012; Power et al., 2014).
2. Probiotic bacteria can enhance the gut mucosal barrier, which is disrupted in enteric infections. This can be done by stimulation of the innate immune activity, by increase of the production of mucus or through modulation of tight junction protein phosphorylation (Kotzampassi and Giamarellos-Bourboulis, 2012; Power et al., 2014).
3. Probiotics are able to modulate the adaptive immune response through the interaction with the gut-associated immune system. Probiotic bacteria can enhance the humoral and cellular immune response of the host by increasing the production of B and T lymphocytes and natural killer cells. Furthermore, they are able to regulate the production of pro- and anti-inflammatory cytokines (Fontana et al., 2013; Kotzampassi and Giamarellos-Bourboulis, 2012).

1.4.3 Influence of probiotics on enteric viral infections

Diarrhoea is one of the most studied symptoms, for which clinical benefits of probiotics have been described. Various *in vitro* studies were conducted showing effects of probiotics against viral replication. An increased resistance of epithelial cells against virus-induced lysis, a blocking of viral attachment or a secretion of compounds that partially protect epithelial cells from infection have been demonstrated after treatment with probiotics (Colbere-Garapin et al., 2007). In particular, positive effects of probiotics on rotavirus-induced gastroenteritis have been observed in various studies, including several *in vitro* studies

(Munoz et al., 2011; Seo et al., 2010). Also, significant effects of probiotics on RV-induced gastroenteritis have been observed *in vivo*. Fang et al. (2009) showed, that administration of *Lactobacillus rhamnosus* 35 led to the shortening of the course of acute diarrhoea in children in a dose-dependent manner. Furthermore, a study conducted by Basu et al. (2009) demonstrated, that the frequency and duration of diarrhoea, the requirement for intravenous rehydration as well as the duration of the hospital stay were significantly decreased in children, which received *Lactobacillus rhamnosus* GG. Similar results were observed in other studies using *Saccharomyces boulardii*, *Bifidobacterium lactis* or a mix of different probiotic bacteria (Erdogan et al., 2012; Grandy et al., 2010).

A pig animal model has been widely used for studying human rotavirus infection, as gnotobiotic colostrum-deprived piglets show clinical symptoms after infection with human RV (Bridger et al., 1975; Vega et al., 2012). Based on this model, diverse studies were performed showing a positive effect of different *Lactobacillus* species on RV infection by modulation of immunological responses (Azevedo et al., 2012; Wen et al., 2012). Furthermore, it was shown that piglets receiving *Bifidobacterium lactis* HN019, *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb12 had milder diarrhoea and reduced virus shedding as compared to the untreated group (Chattha et al., 2013; Shu et al., 2001).

Studies on the influence of probiotic bacteria on other enteric viruses are scarce.
A few studies investigated the influence of probiotic bacteria on liver diseases, in which mostly beneficial effects were observed (Imani Fooladi et al., 2013). Investigations on human viral hepatitis were performed for hepatitis C virus and hepatitis B virus infections, showing that the administration of probiotics improved the laboratory markers of hepatitis or reduced virus replication (Lee do et al., 2013; Loguercio et al., 2005).
One single study has been published for NoV, in which the oral administration of milk, containing *Lactobacillus casei* strain shirota reduced the duration of fever during norovirus disease in humans (Nagata et al., 2011).
Neither for AstV nor for EMCV data from studies on the influence of probiotic bacteria are available.

2 Aims of the study

According to the World Health Organization (WHO), the number of foodborne viral infections has increased over the last decades (FAO/WHO, 2008). Some of those viral infections may be due to zoonotic viruses, which are transmitted from animals to humans by contaminated food. Potentially zoonotic enteric viruses include HEV, RV, AstV, NoV and EMCV. Pigs are known to harbour a variety of these enteric viruses in their gastrointestinal tract, and some of them show a high sequence similarity to human viruses. However, the knowledge about most of those viruses is rather scarce, especially about their occurrence in Germany. Furthermore, different groups of avian RV have been detected in poultry, whose zoonotic potential has not been assessed until now.

Against this background, a major aim of the study was to gain more insight into the prevalence of these potentially zoonotic enteric viruses in livestock. As a variety of zoonotic pathogens in pigs are known, the study mainly focused on the analysis of enteric viruses of pigs. Contamination of meat during the process of slaughtering represents a probable scenario of virus transmission to food. Therefore, faeces of pigs at slaughtering should be investigated for the presence of selected enteric viruses, and quantitative assessment of viruses shed in the faeces of pigs at slaughtering should help to assess the risk of foodborne human infections with those viruses. The characterization of the detected porcine viruses by sequencing of genome parts and subsequent comparison with the respective human viruses should help to estimate their zoonotic potential.

In addition to the pig samples, faeces of poultry were also included in the study, as poultry meat has increasing importance worldwide. Most of the enteric viruses known in poultry are only distantly related to human viruses, thus excluding a zoonotic potential. One exception are RV, which are known to exist in a broad genetic diversity in poultry, including the zoonotic group A rotaviruses. As a first step in assessing their risk to be transmitted to humans, the distribution of RV in poultry should be analysed here.

As a prerequisite for all of the investigations, sensitive and specific detection systems for the viruses should be developed here. Real-time RT-PCR systems were available for some of the viruses, but for others new primer/probe systems had to be developed. The quantitative assays should be able to detect both, the respective animal and human viruses.

A possible intervention strategy, which could prevent the zoonotic virus transmission, is to decrease their faecal excretion by the pigs. One possibility of modulation of infectious diseases and excretion of enteric pathogens could be the feeding with probiotics. Therefore, the influence of a probiotic bacterium on the excretion of the above mentioned viruses in experimentally raised pigs should be investigated here.

The generated data should help to assess the risk of foodborne human infections with zoonotic viruses and demonstrate the effectivity of a possible strategy to prevent their transmission.

3 Detection of avian rotaviruses of groups A, D, F and G in diseased chickens and turkeys from Europe and Bangladesh

Peter H. Otto; Muzahed Uddin Ahmed; Helmut Hotzel; Patrycja Machnowska; Jochen Reetz; Bernhard Roth; Eva Trojnar; Reimar Johne.

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3.1 Summary of Paper 1

Rotaviruses (RV) of groups A, D, F and G are known to occur in poultry and cause disease with symptoms of diarrhoea, growth retardation or the runting and stunting syndrome. However, sensitive PCR methods for the detection are mainly restricted to rotaviruses of group A. Furthermore, the prevalence of RV groups in different geographical regions is only scarcely known.

In this study, the presence of different RV groups in chickens and turkeys was assessed by analysing faecal samples from Europe and Bangladesh. Two studies were performed: In study A, faecal samples were analysed by conventional RT-PCR for the presence of avian rotaviruses (AvRV) of groups A and D and additionally by polyacrylamide gel electrophoresis (PAGE), which enables detection of non-A or -D AvRV strains. In study B, more sensitive real-time RT-PCR methods were applied in order to estimate the prevalence of AvRV-A and -D. To this end, sensitive real-time RT-PCR assays were established and RNA standards for the absolute quantification of viral genome copies were generated.

In 46.2% of faecal samples from study A RV were detected. In detail, 16.1%, 39.2% and 11% were tested positive for AvRV-A, -D or both, respectively. Rotaviruses of groups F and G were detected with only low prevalence of 2% and exclusively in faecal samples from chickens. In faecal samples from study B, analysed by real-time RT-PCR, RV were prevalent in 85% of the samples. Generally, high detection rates were observed in all geographical regions tested. In detail, 58.8% were tested positive for AvRV-A, 65.9% for AvRV-D and 38.9% for both. The differences in the detection rates between study A and study B seem to be mainly due to the different applied assays. In quantitative testings, the real-time RT-PCR showed a considerable higher sensitivity than the conventional RT-PCR and PAGE.

In conclusion, sensitive real-time RT-PCR assays for the detection of AvRV of groups A and D were established. Analysis of faecal samples indicated, that RV of both groups are highly prevalent in chickens and turkeys from commercial flocks in Europe and Bangladesh. In future, sensitive methods for the detection of AvRV-F and -G need to be established. Studies on the prevalence of AvRV of group A in chicken meat should be followed in order to estimate the risk of transmission to humans.

3.2 Key messages of Paper 1

- First report about sensitive real-time RT-PCR assays for the detection of AvRV of groups A and D
- AvRV-A and -D are highly prevalent in chickens and turkeys from commercial flocks in Europe and Bangladesh
- Co-infections with AvRV-A and -D are frequently detected
- AvRV of groups F and G seem to be only rarely present in poultry

3.3 Own contribution to Paper 1

I established the real-time RT-PCR assays for the detection of AvRV of groups A and D. For quantification, I generated RNA standards by cloning and *in vitro* transcription, which were used for comparative assessment of the detection limits of real-time as well as classical RT-PCR assays. Additionally, I wrote the respective parts of the manuscript and was engaged in critical reading and revision of the whole manuscript.

4.1 Summary of Paper 2

Detection and characterization of potentially zoonotic viruses in faeces of pigs at slaughter in Germany

Patrycja Machnowska; Lüppo Ellerbroek; Reimar Johne.

Veterinary Microbiology 2014; 168:60-8. http://dx.doi.org/10.1016/j.vetmic.2013.10.018

In addition to bacteria and parasites, pigs harbour a variety of viruses in their gastrointestinal tract. Some of those viruses are supposed to have a zoonotic potential and might therefore pose a risk for human health. Transmission of those viruses may occur by direct contact with the animals or by indirect pathways, such as faecal contamination of meat during the slaughtering process. Those viruses include astrovirus (AstV), norovirus GII (NoV GII), rotavirus group A (GARV), hepatitis E virus (HEV) and encephalomyocarditis virus (EMCV). However, data about the prevalence of those viruses in faeces of pigs are scarce, especially for Germany.

In this study, 120 faecal samples from fattening pigs at slaughter were derived from three slaughterhouses in the German federal states of Lower Saxony, North-Rhine Westphalia and Baden-Wuertemberg. All of the samples were screened for the presence of RNA of AstV, NoV GII, GARV, HEV and EMCV. By means of real-time RT-PCR, the samples were analysed and the number of viral copies per gram faeces was calculated using a standard curve method. Positive samples were re-tested by RT-PCR and sequencing and were used for phylogenetic analyses.

All five viruses were detected with varying detection rates. Except for HEV and GARV, viruses were detected in samples from all three geographical regions. The most prevalent viruses were AstV in 20.8% and NoV GII in 14.2% of the samples. The high detdection rates determined in this study are consistent with the results obtained in previously performed studies in other countries. EMCV, HEV and GARV were found only sporadically in 4.2%, 2.5%, 0.8% of the samples, respectively. As HEV and GARV usually affect pigs younger than four months of age, our results might reflect this age-dependence. To our knowledge, there are

no data available for comparison of the prevalence of EMCV RNA in faeces of pigs. Phylogenetic analyses showed that the detected AstV and NoV GII strains represent typical pig strains, which are only distantly related to human virus strains. In contrast, HEV and GARV strains found in this study, closely clustered with human strains and therefore might be able to be transmitted zoonotically to humans. For EMCV, no sequence could be obtained, probably due to very low amounts of viruses present in the samples.

It can be concluded that at the time of slaughter, pigs may harbour potentially zoonotic viruses in their gastrointestinal tract. By contamination of pig meat these viruses might get entry into the food chain. AstV and NoV GII are highly prevalent in the German pig population; however, the phylogenetic analyses suggest only a low zoonotic potential of these viruses. In contrast, GARV and HEV are less frequent at the age of slaughter, but are highly similar to human viruses. Higher sample numbers, more geographical regions and pig meat should be investigated in future to assess the risk of zoonotic virus transmission in more detail.

4.2 Key messages of Paper 2

- AstV and NoV GII are highly prevalent in faeces of German pigs at slaughtering age (20.8% and 14.2%, respectively)
- EMCV, HEV and GARV were also detected in faeces of slaughter pigs, although with lower detection rates (4.2%, 2.5% and 0.8%, respectively)
- Detected HEV and GARV strains clustered closely with human virus strains, while AstV and NoV GII strains represent typical pig strains
- First data on the prevalence of EMCV RNA in faeces of German slaughter pigs have been generated

4.3 Own contribution to Paper 2

I contributed to the paper by homogenisation and subsequent total nucleic acid extraction of the faecal samples. All samples were tested by myself for the presence of the mentioned viruses using real-time RT-PCR assays, which had been developed by me in an earlier study (see Paper 3). I analysed all data obtained after real-time RT-PCR and calculated the amount

of genome equivalents prevalent in all faecal samples. In addition, I developed the conventional RT-PCR assays for the specific viruses, purified the PCR products for subsequent sequencing and performed the phylogenetic analyses. Furthermore, the major part of the manuscript was written and revised by myself.

5.1 Summary of Paper 3

Feeding of the probiotic bacterium *Enterococcus faecium* NCIMB 10415 differentially affects shedding of enteric viruses in pigs

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Viral gastrointestinal diseases affect pigs of all ages and can cause high losses in piglets due to increased mortality. However, no antiviral therapy exists against virus infections of pigs and vaccines are only available for few viruses. In several studies, probiotics have been shown to be effective in the treatment of virus-induced diarrhoea in humans as well as in animal models.

In this study, two groups of sows and their piglets were fed with or without feed supplementation of the probiotic bacterium *Enterococcus faecium* (*E. faecium*) NCIMB 10415 and samples were taken at different time points. Blood and tissue samples from piglets were analysed for the presence of T and B cell populations by flow cytometry. Faecal samples from sows and piglets were tested for the presence of viral RNA of astrovirus (AstV), norovirus genogroup II (NoV GII), rotavirus group A (GARV), hepatitis E virus (HEV) and encephalomyocarditis virus (EMCV) by quantitative real-time RT-PCR. The presence of anti-rotavirus (RV) IgG and IgA antibodies in serum samples was analysed by means of ELISA methods.

T cell and B cell populations significantly differed between piglets of both groups. In ileal lymph nodes, B cells were less frequent in piglets of the probiotic fed group, while in the blood a significant increase of cytotoxic T cells in piglets from the probiotic feeding group at 12 days of age was observed. Furthermore, an increase of $CD4^+$ T helper cells in ileal lymph nodes of piglets from the probiotics fed group at 54 days of age was observed. Anti-RV IgG and IgA antibodies were detected in sows and piglets from both groups, although the

number of antibody-positive animals tended to be higher in the control than in the probiotic feeding group.

For the detection and quantification of viruses in faecal samples from pigs, highly sensitive real-time RT-PCR assays could be developed, which are capable of the detection of human and porcine strains of the five enteric viruses. No correlation between virus detection and membership to control or probiotic feeding group was detected for HEV, EMCV and NoV GII. In contrast, GARV was detected with significant difference in both groups; it was shed later and with lower amounts in piglets from the probiotic fed group. AstV was found exclusively in sows and piglets from the control group.

The results indicate a positive effect of supplementation with *E. faecium* NCIMB 10415 on RV. The distribution of AstV between both groups could also be explained by a different exposure to the virus prior to the beginning of the study. The increase of cytotoxic T cells in piglets of the probiotic feeding group might indicate an early immune stimulation, which might be advantageous against infections with RV. Further studies should aim on the confirmation of the observed effect in experimentally RV-infected piglets.

5.2 Key messages of Paper 3

- Sensitive and quantitative real-time RT-PCR assays for the broad detection of human and porcine strains of five enteric viruses were established
- Shedding of GARV occurred later and with lower amounts in piglets of the probiotic fed group, indicating a positive effect of *E. faecium* NCIMB 10415 supplementation on RV infections
- The positive effect of *E. faecium* NCIMB 10415 on the shedding of GARV seems to be caused by enhancement of cellular immunity

5.3 Own contribution to Paper 3

For this study, I collected faecal samples from sows and their piglets at different time points and extracted RNA from the homogenates. I established sensitive real-time RT-PCR assays for the detection of five enteric viruses. To enable absolute quantification, I have generated RNA standards for each virus by RT-PCR and subsequent *in vitro* transcription. I used 10-fold

dilutions of the appropriate RNA standard to assess the sensitivity and efficiency of each real-time RT-PCR assay. All faecal samples were tested by me using these methods. The amount of genome equivalents prevalent in each sample was calculated by myself, after analysing the obtained data. Furthermore, I tested serum samples from sows and piglets for the presence of RV-specific antibodies by means of ELISAs. Using a previously defined cut-off value, I assessed the antibody status for each sample. Finally, I wrote the corresponding parts of the paper and was involved in critical reading and revision of the whole manuscript.

6 General Discussion

6.1 Background

Farm animals are known to harbour a variety of potentially pathogenic agents in their gastrointestinal tract. These include bacteria, parasites and viruses, which can threaten animal and human health (Ziemer et al., 2010). Some of the viruses are closely related to human viruses and are therefore suspected to have a zoonotic potential. They might be transmitted from infected animals to humans by direct contact or indirectly through the contamination of meat with infected faeces during the slaughtering process. These viruses include AstV, HEV, NoV GII, RV and EMCV. While there is evidence for zoonotic transmission of HEV and RV, a zoonotic potential of AstV, NoV GII and EMCV has been suspected but until now not proven (Brewer et al., 2001; De Benedictis et al., 2011; Martella et al., 2010; Mathijs et al., 2012; Meng, 2011). Furthermore, the investigation is hampered by the lack of broad and sensitive detection methods for these viruses and therefore data on the prevalence of them are limited, especially in Germany. Additionally, strategies for the prevention and control of transmission of enteric viruses are mainly missing, as antiviral drugs do not exist and vaccines are available only for a few viruses. Therefore, alternative strategies may be necessary, of which the use of probiotics might represent a promising approach (Fang et al., 2009; Grandy et al., 2010; Seo et al., 2010).

In order to assess the prevalence of enteric viruses in livestock, faecal samples from poultry and pigs, originating from Germany, but also other European countries and Bangladesh, were tested by newly established real-time RT-PCR assays for the detection of enteric viruses. Detected viruses were further characterized and phylogenetic analyses were performed to assess the zoonotic potential. Furthermore, by analysis of pigs at slaughtering age, the risk of an indirect transmission of enteric viruses should be estimated and may help to raise awareness on the prevention and control of enteric virus infections. The results obtained from a pig feeding trial with the probiotic bacterium *E. faecium* NCIMB 10415 should help to assess the influence of probiotics on the shedding of enteric viruses.

6.2 Development of diagnostic tools

6.2.1 Real-time RT-PCR

For a long time, cell culture has been the method of choice for the diagnosis of viral infections. However, besides the fact that it is a laborious and time-consuming method, for a number of enteric viruses an efficient cell culture system could not be developed so far (Chapron et al., 2000). Furthermore, in the last years PCR techniques replaced cell culture as the "gold standard" in virus diagnostics. At the beginning of the project, conventional RT-PCR methods were still available for a variety of enteric viruses from diverse hosts including AstV, HEV, EMCV, NoV GII and GARV. However, real-time RT-PCR has been proven to be more sensitive than conventional RT-PCR, making real-time RT-PCR a more powerful tool in the detection of viruses (Mackay et al., 2002). Moreover, real-time RT-PCR provides an easy way for absolute quantification of viral genome copies in a given sample by the use of external calibration curves, with which highly specific and reproducible data can be generated. In addition, real-time RT-PCR reduces the risk of laboratory contaminations, which can lead to false-positive results, because the PCR products are always contained within the reaction vessel. In contrast, the vessel, which may contain a high amount of virus-specific DNA, has to be opened for analysis of conventional PCR products, which may lead to laboratory contaminations.

At the beginning of this study, sensitive real-time RT-PCR assays for the detection of potentially zoonotic enteric viruses in livestock were scarce or even completely missing (**table 12**).

The detection of avian rotaviruses by sensitive PCR methods was for a long time hampered by the lack of genomic sequences for these viruses. Therefore, no RT-PCR assays for AvRV-A and -D were available in 2010. The recently first published genome sequences of group A and D AvRV, however, enabled the development of real-time RT-PCR assays for those viruses in this study (Schumann et al., 2009; Trojnar et al., 2010).

In comparison to this, some real-time RT-PCR methods for the detection of potentially zoonotic viruses in pigs were available in 2010, but these assays were mainly designed for the detection of human virus strains. Some of those assays have been shown to be able to

Table 12: Situation of real-time RT-PCR assays for the detection of potentially zoonotic enteric viruses in poultry and pigs.

Virus	Real-time RT-PCR assays until 2010	Reference
AvRV-A	No assay available	x
AvRV-D	No assay available	x
AstV	No assay available	x
EMCV	No assay available	x
HEV	Few assays for the broad detection of human and pig HEV available	Jothikumar et al., 2006 Gyarmati et al., 2007 Ward et al., 2009
NoV GII	Few assays for the broad detection of human and animal NoV available	Kageyama et al., 2003 Wolf et al., 2007 L'Homme et al., 2009b
GARV	One assay for the detection of human, porcine and bovine GARV available	Gutierrez-Aguirre et al., 2008

also detect strains of respective animal viruses in subsequent studies (Jothikumar et al., 2006; Kageyama et al., 2003; L'Homme et al., 2009b; Ward et al., 2009). However, as the used primers and probes were mainly based on sequences from human isolates, mismatches in porcine strains cannot be excluded. Therefore, multiple sequence alignments based on sequences from human and porcine virus strains were performed in this study, and all previously published RT-PCR assays for the detection of AstV, HEV, GARV, EMCV and NoV GII were re-investigated here. It could be shown, that the real-time RT-PCR assays designed by Jothikumar et al. (2006), Loisy et al. (2005), Pang et al. (2004) and Pinto et al. (2009), which were originally designed for the detection of human HEV, NoV GII, GARV and murine mengovirus, respectively, matched with porcine virus sequences. Therefore, they could be used in this study with original primer/probe sequences or only slight modifications (see Paper 3, table S2). In contrast, none of the assays designed for the detection of human AstV matched with respective porcine sequences. Therefore, a new assay had to be established, taking into account the diversity of human and porcine AstV sequences using a set of three primers and two probes.

RNA standards for use in quantification were produced for all assays by cloning of RT-PCR products and subsequent *in vitro* transcription. In a majority of published studies, DNA-based calibration curves have been used for the quantification of virus genome copy numbers. However, the use of DNA standards might be problematic as they are subject to

the PCR step only and do not take into consideration the reverse transcription step. But this step is especially influenced by various factors like priming efficiency, secondary and tertiary RNA structures and the efficiency of the reverse transcriptase used (Stahlberg et al., 2004; Watzinger et al., 2006). Therefore, the reverse transcription efficiency is not constantly 100% as assumed by using DNA standards, but rather between 30% and 40%, and the quantification results for the DNA standard and the initial RNA in a biological sample are not comparable (Stahlberg et al., 2004). Hence, RNA standards are more appropriate to enable quantification of RNA samples. Nevertheless, even by using RNA standards, the obtained results may differ between the RNA standard and the target RNA, as RT-PCR inhibitors might be present in the biological sample. Faecal samples are known to contain a variety of inhibitors like polysaccharides, bile salts, lipids and urea, which can influence the PCR by disturbing the function of the reverse transcriptase or DNA polymerase or by interference with fluorescent probes (Schrader et al., 2012).

Another aspect influencing the accuracy of quantification is the applied method for measurement of the RNA concentration of the RNA standard. The determination of the exact concentration of RNA in the standard is necessary for the calculation of the number of RNA molecules in each dilution of the standard, which is used to generate the standard curve (Lu et al., 2008). A common way to determine the RNA concentration is to measure its absorbance at 260 nm (A_{260}) in a nanospectrophotometer device (e.g. NanoDrop™, ThermoFischer). However, the method is vulnerable to contamination with DNA, proteins, phenol and contaminants commonly found in nucleic acid preparation kits like salts, chloroform or urea causing higher absorbance. While contamination with DNA can be easily removed by treatment with DNase, contamination with other substances is more difficult. Recently developed fluorescent dyes (e.g. RiboGreen®, Invitrogen) enable a much more sensitive and accurate way to quantitate RNA, as most substances, which might be present in the RNA preparation, do not interfere with the measurement (Rio et al., 2010). However, in this study the RNA concentration was determined in a nanospectrophotometer device. Therefore, it has to be taken into account that the number of viral genome copies quantified might slightly differ from the actual number of genomes in the sample due to the presence of inhibitors in the faecal samples and the applied method used for measuring the RNA concentration in the standard.

The newly developed assays for the detection of avian group A and D rotaviruses have been shown to be both reproducible and linear over a wide range with R^2 values of 0.99, which confirm the high linearity. However, the sensitivity and efficiency of both assays are rather low, with 2.65 E+02 and 84% for group A and 3.38 E+03 and 81.5% for group D avian rotavirus, respectively (**table 13**). The low efficiency of the assays may be caused by a poor primer/target binding efficiency. As the primers/probes of both assays were originally designed based on only a few available AvRV genomic sequences, a poor binding to the majority of other strains may have been resulted. This problem became also evident when cell culture supernatants of a turkey and a chicken strain of group A rotavirus were tested with the real-time AvRV-A assay, resulting in a much more reduced sensitivity of the assay for the turkey than the chicken strain. By the use of modified primers and probe, the turkey strain could be detected with high sensitivity (see Paper 1). Indeed, more recent sequence alignments of AvRV-A show that chicken and turkey strains are separated into two different phylogenetic clusters, indicating that it might be necessary to use different primers and probes for the detection of group A RV from different avian hosts (Schumann et al., 2009). The situation is even more complicated for group D rotaviruses, where only very few genomic sequences are available from different avian hosts. Especially, the samples of turkeys tested positive for AvRV-D in our study should be sequenced in order to obtain more sequence information on turkey rotaviruses. Summarizing these findings, the PCR assays described here enable for the first time the real-time RT-PCR detection of AvRV-A and -D. Although the sensitivity of the assays are shown to be higher than traditionally applied assays (PAGE), further development of the real-time RT-PCRs should be performed when more genomic sequences of AvRV isolates are available.

Table 13: **Detection limit and efficiency of real-time RT-PCR assays for the detection of potentially zoonotic enteric viruses in poultry and pigs**.

Real-time RT-PCR assay for the detection of	Efficiency (%)	Minimum number of detected copies/reaction
AvRV-A	84	2.65 E+02
AvRV-D	82	3.38 E+03
AstV	102	4.48 E+01
EMCV	98	2.00 E+01
HEV	91	6.80 E+01
NoV GII	92	7.80 E+01
GARV	101	1.54 E+01

In comparison to the avian rotaviruses, much more genomic sequences were available for AstV, HEV, EMCV, GARV and NoV GII, facilitating the performance of multiple sequence alignments and the design of sensitive primers and probes. Furthermore, primers and probes for distinct viruses have already been published and could therefore be used in this study (see section 6.2.1). In fact, all five assays have been shown to be reproducible, highly sensitive and efficient with detection limits between 15 and 78 viral copies per reaction and efficiencies between 91% and 102% (**table 13**). Furthermore, the assays are linear over a wide range and show R^2 values of 0.99. However, the performance of the assays should be further evaluated by a higher number of field samples. In this study, the cross-reactivity with virus isolates of different hosts and with related viruses could not be sufficiently tested due to the lack of archived samples. Hence, those tests should be performed to ensure the exclusivity and inclusivity of the developed assays. Also the applicability for different sample types like serum, blood or tissue samples should be analysed in future.

A recent study published by Garson et al. (2012) indicated, that the sensitivity of the real-time RT-PCR assay for the detection of HEV by Jothikumar et al. (2006) could be further increased by using a minor groove binder (MGB)-modified probe. MGB ligands provide the advantage to enable the use of shorter probes with higher melting temperatures than conventional TaqMan® probes (Yao et al., 2006). It might be therefore useful to test, if the performance of an MGB-modified probe with the RNA standard from this study leads to a further increase of the specificity and sensitivity of the assay. Recently, also a real-time RT-PCR assay for the detection of porcine EMCV was developed (Wang et al., 2012). However, its performance was evaluated only with limited samples from pigs experimentally challenged with EMCV. Furthermore, just recently a real-time RT-PCR assay for the detection of porcine AstV has been published (Xiao et al., 2013). However, as primers and probes were designed based on porcine sequences exclusively, the assay will probably not be able to broadly detect porcine and human strains of AstV.

6.2.2 Conventional RT-PCR

To characterize the detected viruses and to assess their zoonotic potential, conventional RT-PCRs were performed in order to obtain products for sequencing and phylogenetic analysis. The products generated with primers used for real-time RT-PCR could not be used

for this purpose, as they were too short to obtain reasonable results in a subsequent phylogenetic analysis. The RT-PCR assays used were either already published or newly designed during this study (see Paper 2, table 1). Unfortunately, only for a subset of positive samples sequences could be successfully generated by conventional RT-PCR. One possible reason might be the amount of viral RNA in the faecal samples, as there was a correlation between successful amplification by conventional RT-PCR and amount of virus genomes quantified by real-time RT-PCR. As real-time RT-PCR is much more sensitive than conventional RT-PCR, low levels of virus might fail to be detected by a conventional assay (Mackay et al., 2002). This might explain the inability to generate sequences for EMCV although diverse primer combinations have been tested. EMCV was found in only low amounts in the samples, whereas for AstV, which was found in rather high amounts, several sequences could be obtained. This assumption is also supported by the fact that all assays used were able to amplify the positive controls, which usually were extracted RNA from cell culture supernatants containing high amounts of virus. However, another explanation might be the presence of inhibitors in the faecal samples in comparison to the cell culture supernatants. Furthermore, the RNA in the samples might be partially degraded, enabling the amplification of shorter fragments by real-time RT-PCR but not longer fragments by conventional RT-PCR. Moreover, although the primers were designed for a broad detection of the viruses, it cannot be excluded that the primer combinations were not able to detect the specific virus strains present in the samples.

6.3 Prevalence of enteric viruses in livestock

6.3.1 Prevalence of rotaviruses in poultry

It is suspected that rotaviruses are highly distributed in poultry flocks all over the world and that infections are often associated with diarrhoea or runting and stunting syndrome. While rotaviruses of groups A and D have been frequently detected, rotaviruses of groups F and G are rather uncommon (Ahmed and Ahmed, 2006; Otto et al., 2006; Savita et al., 2008). However, investigation on the prevalence of different AvRV groups was hampered by the lack of sensitive detection methods, as only for group A rotaviruses RT-PCR assays were available. Studies performed in chicken and turkey flocks from the USA and Brazil using

classical RT-PCR showed, that AvRV-A are highly prevalent in poultry with rates of 46.5% in US-chickens, between 30.6% and 69.7% in US-turkeys and 51.8% in turkeys from Brazil. (Jindal et al., 2010; Jindal et al., 2012; Moura-Alvarez et al., 2013; Pantin-Jackwood et al., 2008). The results obtained in our study confirm the high prevalence of group A rotaviruses in chickens and turkeys as 58.8 % of all tested samples were tested positive by AvRV-specific real-time RT-PCR. In contrast, only 16.1% were tested AvRV-A-positive by conventional RT-PCR. The main reason for the difference is a different sensitivity of both assays, as it could be shown that the real-time RT-PCR assay was 1000 times more sensitive than the RT-PCR assay. In contrast to other studies, the detection rates of AvRV-A were lower in turkeys than in chickens. However, this is probably due to the relative low number of turkeys tested in this study (33 in study A; 18 in study B) in contrast to chickens (166 in study A; 375 in study B). In addition, a difference in sensitivity of the assays for chicken and turkey rotaviruses (see Paper 1) may distort the results.

In this study, faecal samples from poultry were analysed for the first time by sensitive PCR methods in order to determine the prevalence of AvRV-D. This resulted in 39.2% and 65.9% of samples tested positive by RT-PCR and real-time RT-PCR, respectively. Data for comparison of the results are scarce, as only one recently performed study for the detection of group D rotavirus using RT-PCR is available (Bezerra et al., 2012). With a prevalence of 53% in faecal samples from chickens from Brazil, those data are rather similar to the results obtained in our study. The high prevalence of both AvRV-A and -D in flocks from different countries indicates a high distribution of RV in poultry, as other studies have previously assumed.

Furthermore, rotaviruses of groups F and G were detected in 2% of the samples in our study using PAGE analysis. The low prevalence confirms the data from previously performed studies, indicating that AvRV-G and -F infections occur only rarely (Ahmed and Ahmed, 2006; Otto et al., 2006). However, it has to be taken into consideration that due to the lack of sequence information on those rotavirus groups, no sensitive detection methods have been developed so far. Therefore, the low detection rate might be explained by limitations of the detection methods used resulting in an underestimation of the frequency of AvRV-F and -G in poultry. The availability of sequence information obtained recently for group F and G rotaviruses will enable the development of sensitive PCR methods for the detection of those

viruses (Johne et al., 2011). The application of those methods in future will contribute to the knowledge about the prevalence and clinical relevance of AvRV-F and -G in poultry flocks.

6.3.2 Detection rate of enteric viruses in the German pig population

The availability of prevalence data for enteric viruses in pigs is strongly dependent on the specific virus studied. Investigations on HEV have been increased in the last years, since cases of human hepatitis could be linked to the consumption of raw or undercooked meat from pigs, wild boar and deer (Colson et al., 2010; Takahashi et al., 2004). Diverse studies tested porcine faecal, blood and liver samples for the presence of HEV RNA leading to a wealth of available prevalence data. Also some data on the prevalence of NoV GII and GARV in pigs are available. In contrast to this, the number of studies analysing the prevalence of AstV and EMCV in pigs are rather low. For Germany, data on the prevalence of HEV, AstV, NoV GII, EMCV and GARV in pigs are scarce or missing.

In our studies, the detection rates of the above mentioned viruses were determined in sows and their piglets from a feeding trial as well as from pigs at slaughter. However, especially the number of analysed sows and piglets was low (13 and 26, respectively). Therefore, the detection rates obtained from these animals are not representative for the German pig population and only allow an imprecise assessment of the prevalence of AstV, EMCV, HEV, NoV GII and GARV in German pigs.

The investigations performed in our studies show that all five enteric viruses can be detected in faecal samples from pigs of different age in Germany (**table 14**).

Especially, AstV and NoV GII have been determined with high detection rates in German pigs with 20.8% and 14.2% at slaughtering age, 34.6% and 19.2% at up to eight weeks of age and 30.8% and 0% in sows about one year of age, respectively (**table 14**). However, the data for piglets and sows have to be interpreted with care, as only a low number of pigs from an experimental feeding trial have been analysed. Neither for AstV nor for NoV GII prevalence data from Germany are available. Previously performed studies on porcine AstV detected high prevalences in pigs in the USA (between 62% and 64%) and Canada (79%), while the prevalences obtained from South Korea and Colombia are much lower with 19.4% and 23.8% (Lee et al., 2013a; Luo et al., 2011; Mor et al., 2012; Ulloa and Gutierrez, 2010; Xiao et al., 2013).

Table 14: Detection rates of potentially zoonotic enteric viruses in German pigs of different age. Faecal samples of 13 sows and 26 piglets from an experimental feeding trial, as well as 120 samples from fattening pigs from three German slaughterhouses have been analysed for the presence of AstV, EMCV, HEV, NoV GII and GARV.

Virus	Detection rate (%)		
	Piglets (12-54 days of age)	Pigs at slaughter (6-9 months of age)	Sows (around 1 year of age)
AstV	34.6	20.8	30.8
EMCV	23.1	4.2	7.7
HEV	0.0	2.5	53.8
NoV GII	19.2	14.2	0.0
GARV	19.2	0.8	7.7

In most studies, AstV were detected in pigs of all ages, with a tendency of a lower prevalence in mature pigs than in piglets and finisher pigs (Lee et al., 2013a; Xiao et al., 2013). In our study, the detection rates were comparable in pigs of all ages. However, comparison of the prevalence data from the different studies is difficult as most of the studies investigated faecal samples from diarrhoeic pigs, while in our studies most pigs showed no signs of diarrhoea. Luo et al. (2011) published a study presenting prevalence data of porcine AstV from healthy pigs. They detected AstV in 79% of faecal samples from healthy finisher pigs originating from three abattoirs in the province of Quebec. These results are much higher than the results obtained in our study, where the prevalence was determined to be 20.8% in pigs at slaughtering age. The different prevalence rates might be due to geographical differences or the difference in the time point of sampling. While Luo et al. (2011) collected faecal samples between 2005 and 2007, in our study samples were taken only at one time point in April and May 2011.

Prevalence data for porcine NoV are available from different countries. However, the data differ significantly and range from 1% to 25% (see section 1.2.3.4.2, **table 7**). The varying results might be explained by different sensitivities of the used RT-PCR methods. Generally, studies performed with primer pairs for the broad detection of caliciviruses or for the detection of human NoV resulted in lower positivity rates than studies performed with specific primers for porcine NoV (Mattison et al., 2007; Mauroy et al., 2008; Wang et al., 2005; Wang et al., 2006). The detection rate of NoV GII in fattening pigs and sows obtained in our study is comparable with the results obtained in studies, which used sensitive NoV-specific primers. By this, it is shown, that the prevalence of NoV in pigs was underestimated for a long time due to the lack of sensitive and specific detection methods. However, the

clinical relevance of NoV in pigs is still unclear. Although in experimental infections, gnotobiotic piglets have been shown to be susceptible to human NoV and furthermore developed signs of diarrhoea, until now NoV were detected almost exclusively in healthy finisher pigs. Only one single study detected NoV in two piglets suffering from diarrhoea (Shen et al., 2012). In our study, NoV have been found in young piglets between 12 and 54 days of age confirming the study of Shen et al. (2012), and showing that NoV also might occur in younger piglets. However, more studies are needed using highly sensitive and specific PCR assays to obtain data on the prevalence and to assess the clinical relevance of NoV infections in piglets.

GARV and EMCV were detected in our study in piglets, sows and pigs at slaughtering age. The detection rate was low in pigs at slaughtering age and in sows, ranging from 0.8% to 7.7% for both viruses (**table 14**). In contrast, the detection rate in faecal samples from piglets was much higher, with 19.2% and 23.1% for GARV and EMCV, respectively. Several studies investigated the prevalence of GARV in pigs of different age. However, the data are highly variable between different countries but sometimes also within the same country (see section 1.2.4.4.2, **table 9**). Most studies show, that GARV usually affect young pigs under the age of two months. In our study, the age-dependent pattern of GARV was confirmed, as the detection rate was higher in piglets than in fattening pigs and sows. Studies on the prevalence of GARV in pigs older than four months are rare. Only one study analysed the prevalence of GARV in faeces of post-weaning pigs and detected the virus in 16% of the faecal samples (Steyer et al., 2008). The study performed by us confirmed the occurrence of GARV infections in older pigs, although the prevalence was very low in pigs at slaughter. However, both studies show that GARV can be detected in pigs of all ages and further studies should be performed analysing the possible role of pigs in transmission of GARV to humans.

For EMCV, no data are available for comparison as this is the first study to determine the prevalence of EMCV RNA in faecal samples of pigs. Prevalence data for porcine EMCV have been mainly assessed by measurement of anti-EMCV antibodies in serum samples of pigs. These studies show that antibodies against EMCV can be detected in pigs of all ages indicating that infections with EMCV are common in pigs (An et al., 2009; Maurice et al., 2005). Especially in piglets, infections with EMCV may show a high mortality due to acute myocarditis. However, in our study, 23.1% of tested piglets were tested positive for EMCV

without showing any clinical signs. This might indicate that asymptomatic EMCV infections might occur frequently in piglets.

In this study, HEV was detected exclusively in pigs at slaughtering age and in sows, whereas no piglets have been tested positive. While in the sows the detection rate was high with 53.8%, the prevalence in pigs at slaughtering age was rather low with 2.5% (**table 14**). Several studies have been performed investigating the prevalence of HEV in pigs of different age. In general, pigs between two and four months of age are most frequently affected by infections with HEV. Maternal antibodies are transmitted to the piglets by the intake of colostrum and can persist in the piglets up to nine weeks of age (Pavio et al., 2010). This might explain the low prevalence in younger piglets, which is consistent with the result obtained in this study. However, a number of studies also detected a high RNA prevalence of up to 53% in piglets until eight weeks of age (Leblanc et al., 2007; McCreary et al., 2008). In a high number of studies, HEV was also detected in fattening pigs at slaughter age. Several studies were performed in Europe and up to 41% of healthy pigs at slaughter have been tested positive for HEV excretion (Di Bartolo et al., 2012). Other studies from Europe detected lower prevalence rates between 0% and 15% (Di Martino et al., 2010; Hakze van der Honing et al., 2011). This is the first report confirming the presence of HEV in faeces of German pigs at slaughtering age. Although the detection rate of 2.5% is low, it shows that pigs at slaughtering age can harbour potentially zoonotic viruses in their faeces. In most studies, the prevalence rates of HEV in sows are low (Berto et al., 2012; McCreary et al., 2008). However, Fernandez-Barredo et al. (2006) detected HEV RNA in 22% of faeces from sows. In our study, an even higher HEV detection rate of 53.8% was determined in faeces of sows. It might be possible, that sows are more susceptible to HEV infections due to stress during farrowing and suckling (Fernandez-Barredo et al., 2006). However, this needs to be confirmed in further studies analysing a large number of faecal samples from sows. Interestingly, a recently performed study indicates a seasonal pattern of HEV excretion in pigs, with the time point of the year influencing the presence of HEV (Lu et al., 2013). Therefore, samples taken at different time points of the year might be not directly comparable, which might explain the difference between the results.

6.4 Zoonotic potential of enteric viruses of animal origin in Germany

A major part of viral zoonotic agents is found in wildlife species. However, farm animals are also known to harbour zoonotic viruses e.g. Rift Valley fever virus, Orf virus or Influenza virus. Furthermore, a series of viruses with high sequence similarities between animal and human virus strains can be found in livestock. Such viruses are suspected to have a zoonotic potential and might be transmitted by direct or indirect contact from animals to humans. Of special interest are enteric viruses with a zoonotic potential, as they might enter the food chain by contamination of meat during slaughtering and therefore might represent a risk for human health.

There is strong evidence that GARV and HEV are zoonotic agents. In addition, for HEV contamination of food seems to be the main route for zoonotic transmission. HEV has been isolated from cases of human hepatitis, which have been linked to the consumption of raw or undercooked meat and meat products from pigs, wild boar and deer. Those isolates mainly belonged to genotype 3 (Colson et al., 2010; Takahashi et al., 2004). In our study, an HEV strain detected in faecal samples from pigs at slaughter showed the highest sequence similarity to a human and a wild board HEV strain from Germany of genotype 3i (91% and 90%, respectively). Strains of genotype 3i are increasingly found in humans, wild boars and pigs in Europe and recently also in different countries of South America (Mirazo et al., 2013). The possibility of a zoonotic transmission of this genotype is suspected (Baylis et al., 2012; Schielke et al., 2009). It should be therefore concluded, that HEVs present in German pigs have a high zoonotic potential and efforts should be done to prevent contamination of meat during slaughtering.

The strain of GARV obtained in our study showed a high sequence similarity to porcine but also human strains of GARV genotype G3 (94% and 90%, respectively). For genotype G3, a very broad host range has been described and diverse studies reported that human and porcine G3 genotypes share a high degree of sequence identity (Martella et al., 2010; Martinez-Laso et al., 2009). Furthermore, RV of this genotype have re-emerged during the last years (Martinez-Laso et al., 2009). The results of our study show therefore, that RV with a high zoonotic potential may be excreted by pigs at slaughter in Germany; thus, transmission due to contamination of meat should be avoided. Though it is known that also AvRV might have a zoonotic potential, no phylogenetic analyses were performed in our

study to further characterize the detected group A and D avian rotaviruses. However, the probability of a zoonotic transmission of AvRV is considered to be low although it cannot be completely excluded (Trojnar et al., 2010; Trojnar et al., 2013). As AvRV-D are exclusively found in birds, their zoonotic transmission seems to be of low probability. However, sequence analyses of the detected chicken and turkey viruses would be useful in future to further assess the potential of a zoonotic transmission between poultry and humans.

For AstV, EMCV and NoV GII the assumption of the zoonotic potential is not proven until now. The assessment of the zoonotic potential of EMCV is difficult as sequence information is mainly available for porcine EMCV strains only. However, recently a sequence of a human EMCV strain was published enabling a detailed comparison of the human strain with pig strains (EU979548, Nix et al., 2009, unpublished). Unfortunately, in our study, no sequence of the detected EMCV could be obtained. As the virus was detected in samples from pigs at slaughtering age, it would be of great interest to obtain sequence data to assess the zoonotic potential and the risk of a zoonotic transmission to humans. Further efforts should be done in future to develop a sensitive RT-PCR assay for the characterization and phylogenetic analysis of those viruses.

For both, NoV GII and AstV, several studies showed a relative high degree of sequence similarity between isolates from humans and animals (Kapoor et al., 2009; Mattison et al., 2007; Ulloa and Gutierrez, 2010). However, no consistent evidence of a zoonotic transmission of these viruses has been shown so far. All sequences obtained in our study from AstV and NoV GII clustered together with typical pig virus strains and were only distantly related to human viruses. Therefore, it has to be concluded that the zoonotic potential of porcine NoV and AstV in Germany is low.

6.5 Influence of the probiotic bacterium *Enterococcus faecium* NCIMB 10415 on enteric viruses

Most of the enteric viruses are shed in high concentrations with the faeces of infected individuals. Combined with a low minimal infectious dose of less than 100 particles and the high environmental stability of those viruses, an efficient spread of the viruses can be expected (Atmar, 2010; Chen et al., 2012). In our studies, viruses were found to be shed in high concentrations of up to 10^9 particles per gram faeces from piglets and up to 10^7

particles per gram faeces from pigs at slaughter age. These results are indicative for raising awareness on the control and prevention of enteric viral infections in meat production, especially as some of the detected viruses have a zoonotic potential. According to the WHO, food is regarded as the most common source of zoonotic disease in public health (FAO/WHO, 2001). Viruses introduced into a pig herd may pose a risk for humans in direct contact with infected animals like breeders, veterinarians and slaughterers. However, indirect transmission of viruses to food handlers and consumers also may occur due to contaminated meat and meat products. Strategies to prevent the transmission of viruses might include the use of specific-pathogen free herds or the application of vaccines or probiotics (**figure 14**).

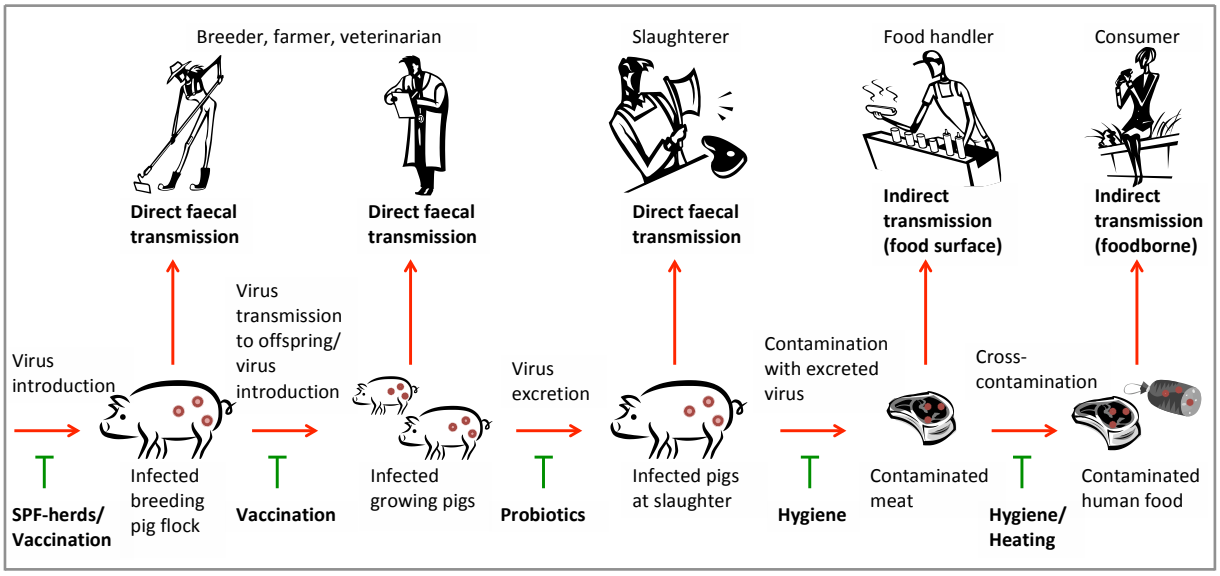

Figure 14: **Theoretical routes of transmission of zoonotic enteric viruses from pigs to humans and strategies of intervention**. Zoonotic enteric viruses might be transmitted by direct contact of humans with infected animals as well as indirectly by contaminated meat and meat products. Strategies for prevention of virus transmission may be the use of vaccines or probiotics and the maintenance of hygienical standards. Transmission routes are indicated in red. Intervention strategies are indicated in green. SPF, specific-pathogen free.

However, until now, strategies to prevent and control viral infections are mainly limited to the application of good hygiene practice. Antiviral drugs are not available for food-producing animals so far and although vaccines have been developed for RV and EMCV, they are not available for pigs and poultry in the EU. As it had been shown in several studies, that

probiotics can positively influence viral infections, their use might be a promising approach for the prevention and control of viral infections (Fang et al., 2009; Grandy et al., 2010).

In our study, the influence of the probiotic bacterium *Enterococcus faecium* NCIMB 10415 on naturally acquired infections with AstV, HEV, NoV GII, GARV and EMCV in pigs was investigated.

E. faecium is a normal inhabitant of the enteric microflora in humans and animals. In Europe, the specific strain NCIMB 10415 is widely used as feed additive (Cylactin®) in the pig industry (EFSA, 2013). In several studies, positive effects of *E. faecium* NCIMB 10415 on gastrointestinal diseases have been observed (Buydens and Debeuckelaere, 1996; Zeyner and Boldt, 2006). However, its influence on viral infections has not been investigated so far.

No effect of *E. faecium* NCIMB 10415 supplementation was observed on infections with HEV, EMCV and NoV GII in our study. However, the results indicate a positive influence of the used *E. faecium* strain on infections with GARV and AstV as three main findings have been observed:

1. The infection with GARV occurred later in piglets fed with the probiotic bacterium (day 34 and 54) than in piglets fed without *E. faecium* NCIMB 10415 (day 26 and 34).
2. Shedding of GARV was reduced by two orders of magnitude in piglets fed with *E. faecium* NCIMB 10415 than in piglets fed without the probiotic bacterium.
3. AstV were exclusively detected in piglets from the control group.

Different probiotic bacteria like *Lactobacillus rhamnosus*, *Bifidobacterium longum* and *Lactobacillus reuteri* were shown to have a positive effect on rotavirus infections in humans and in animal models (Fang et al., 2009; Munoz et al., 2011, Seo et al., 2010). However, the involved mechanisms are only poorly understood. Several studies indicate that the humoral immunity, which is mainly mediated by IgA, plays a crucial role in the protection against RV infections (Blutt et al., 2012). Studies in piglets observed a direct correlation between serum and intestinal secretory IgA (sIgA) and protection from disease (Azevedo et al., 2004). Also, in mice and humans levels of intestinal and serum IgA correlated with protection against RV infection (Blutt et al., 2012; O'Neal et al., 2000). Furthermore, in several studies the application of probiotics was correlated with an increase of IgA levels and a reduction of the duration of RV infection or of RV shedding in children (Majamaa et al., 1995). In some studies, this correlation was also observed for levels of IgG, although a protective effect of

IgG seems to be rather of minor importance in comparison to IgA (Westerman et al., 2005). Moreover, studies showed that B cell knockout mice shed RV chronically indicating a crucial role of B cells for RV immunity (Franco and Greenberg, 1999).

In contrast, in our study, the levels of serum RV-specific IgA were slightly higher in pigs of the control group than in pigs of the probiotic feeding group. Also, RV-specific IgG has not been detected in piglets of the probiotic fed group, but in piglets of the control group. In addition, B cells were less frequent in piglets of the probiotic fed group. Taken together, all these results indicate that the positive effect of *E. faecium* NCIMB 10415 is not caused by an enhancement of the humoral immunity. However, levels of serum IgA and intestinal sIgA may differ, and intestinal sIgA is considered to be more important for the clearance of RV (Blutt et al., 2012; O'Neal et al., 2000). As only serum samples were analysed for RV-specific antibodies in our study, an increase of the sIgA level in the intestine cannot be excluded.

Some authors assume, that antibodies are more critical for the protection from infection with RV, while the crucial factor for the clearance of RV infections are $CD8^+$ lymphocytes (Franco and Greenberg, 1999; McNeal et al., 1997). Indeed, several studies indicate that cytotoxic T cells are critical for the control and limitation of virus shedding even if antibodies are not present. In B cell deficient mice, depletion of $CD8^+$ cells prior to oral infection with murine rotavirus leads to persistent shedding of the virus (Franco and Greenberg, 1999; McNeal et al., 1997). Also, in fully immunocompetent mice $CD8^+$ T cell depletion results in a prolonged RV shedding (Franco and Greenberg, 1999). In our study, a significant increase of cytotoxic $CD8ß^+$ lymphocytes was observed in piglets from the probiotic feeding group at 12 days of age. This result indicates an early immune stimulation, which might explain the later and less severe shedding of RV in piglets from the probiotic feeding group.

The increase of cytotoxic T cells might also play a role in the clearance of AstV infections, as in this study AstV was exclusively detected in pigs from the probiotic feeding group. However, the positive effect of *E. faecium* NCIMB 10415 on AstV infections observed in our study must be interpreted with caution, as it simply may be that pigs from the control and the probiotic fed groups had a different exposure to the virus at the beginning of the study. The applied random assignment of the pigs to the groups with an unknown infection status at this time point could theoretically result in creation of an AstV-uninfected group, which would correspond to the observed exclusive detection of AstV in the control group. This situation is different for GARV, which was detected at the beginning of the study in both

groups in comparable amounts. Future investigations should include targeted infection experiments of known RV-free of AstV-free piglets in order to exclude random effects of field origin infections.

The mode of action of probiotics has not been sufficiently elucidated and also in our study, no clear mechanism for the positive effect of *E. faecium* NCIMB 10415 on infections with RV (and AstV) could be recognized. Further studies are necessary to assess the influence of *E. faecium* NCIMB 10415 on viral infections. As probiotics act in a dose-dependent manner, it might be useful to test different concentrations of the probiotic in future. To elucidate the influence of *E. faecium* NCIMB 10415 on RV immunity, colostrum-deprived piglets should be challenged with RV and the influence of *E. faecium* NCIMB 10415 or other probiotic bacteria should be monitored by analysis of clinical, pathological and immunological parameters.

Although strains of *E. faecium* are widely used as probiotics, there is concern regarding the safety of this probiotic bacterium. Enterococci are low pathogenic and are generally regarded as safe (GRAS). However, in the last years Vancomycin-resistant enterococci (VRE) of the genus *E. faecium* are increasingly the cause of nosocomial infections in humans which are resistant to standard antibiotic treatment. Studies have shown that the vanA gene cluster, responsible for the vancomycin resistance, can be transmitted to probiotic *E. faecium* strains *in vitro* (Lund and Edlund, 2001). Furthermore, clinical isolates of *E. faecium* contain several virulence factors (FAO/WHO, 2001) whose presence needs to be excluded prior to the use of an *E. faecium* strain as probiotic bacterium. Further investigation on the safety of *E. faecium* NCIMB 10415 should be therefore performed. Alternatively, there are various other probiotic bacteria such as *Bifidobacterium*, which completely lack pathogenicity and have also been shown to have positive effects on human and animal health (Chattha et al., 2013; Kotzampassi and Giamarellos-Bourboulis, 2012).

6.6 Conclusion and future prospects

The studies described here show, that several enteric viruses can be detected in German pigs and poultry of different age. Especially the detection of potentially zoonotic viruses in faeces of pigs at slaughtering age should raise awareness on a possible introduction of the viruses into the food chain. According to the WHO, in the past decades, foodborne disease incidence has been increased, which was mainly caused by microorganisms (FAO/WHO, 2008). The risk of foodborne viral illness is considered to be largely underestimated due to the lack of surveillance systems. The results of our studies may contribute to the knowledge about the prevalence of enteric viruses in faeces of livestock. However, the number of field samples and geographical regions should be increased in future investigations to gain broader insights into the prevalence and distribution of enteric viruses in German livestock. Furthermore, these studies were limited to only a low number of enteric viruses. There are several other viruses, which are known to cause disease in humans and animals and have been also assumed to have a zoonotic potential. An overview about selected viruses, which are candidates for future investigations, is presented in **table 15**.

Other food-producing animal species should also be considered for the investigation on zoonotic enteric viruses. Faecal samples from cattle are especially of interest, as the prevalence of enteric viruses in cattle is also mainly underinvestigated. Just recently, after its first detection in the 1960ies, natural EMCV-infection has again been detected in a calf and also a novel AstV, associated with neurological disease in cattle, has been identified (Diallo et al., 2013; Li et al., 2013). Furthermore, anti-HEV antibodies have been detected in cattle, while evidence for bovine HEV RNA is still insufficiently (Geng et al., 2011; Hu and Ma, 2010). All these findings indicate that cattle are also a source for potentially zoonotic viruses and should be monitored for their presence.

Table 15: **Enteric viruses with zoonotic potential causing disease in humans and livestock**.

Virus	Disease in humans	Disease in livestock	Zoonotic potential
Sapovirus (Calicivirus)	Gastroenteritis/ asymptomatic	*Pig*: asymptomatic/ diarrhoea *Cattle*: diarrhoea *Poultry*: asymptomatic/ runting and stunting syndrome	Unclear
Rotavirus group B	Gastroenteritis	*Pig*: diarrhoea *Cattle*: diarrhoea *Poultry*: -	Unclear
Rotavirus group C	Gastroenteritis	*Pig*: diarrhoea/ asymptomatic *Cattle*: asymptomatic/ diarrhoea *Poultry*: -	Proven
Enterovirus	Respiratory or gastrointestinal illness/ meningitis/ encephalitis	*Pig*: asymptomatic/ polioencephalomyelitis/ enteric disease/pneumonia *Cattle*: asymptomatic *Poultry*: runting and stunting syndrome/ respiratoy disease	Unclear
Kobuvirus	Gastroenteritis	*Pig*: diarrhoea/ asymptomatic *Cattle*: diarrhoea/ asymptomatic *Poultry*: -	Unclear

-, virus until now not detected in this species.

Studies on the influence of probiotic bacteria are so far mainly restricted to rotavirus. However, also norovirus and astrovirus are leading causes of viral gastroenteritis worldwide. As gnotobiotic piglets have been shown to be susceptible to human NoV they may also be used for studying the effects of probiotics on NoV infections (Cheetham et al., 2006; Jung et al., 2012). Unfortunately, until now no such animal model exists for AstV, thus hampering the investigation on possible effects of probiotics on this virus.

Although a positive effect of *E. faecium* NCIMB 10415 on RV infections was observed, negative effects of this probiotic bacterium on infections with *Salmonella enterica* have been reported recently (Siepert et al., 2014). Therefore, *E. faecium* NCIMB 10415 should be reconsidered for its use as a probiotic in pigs. Other probiotic organisms should be tested in future in order to assess their potential to positively affect viral enteric infections of livestock.

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I Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die Arbeit selbstständig und ausschließlich unter der Verwendung der angegebenen Mittel verfasst habe. Die verwendeten Quellen und Hilfsmittel sind vollständig angegeben. Ich versichere, dass diese Arbeit in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt wurde.

Ort, Datum

Unterschrif

II List of Publications

2014 **Machnowska P**, Ellerbroek L, Johne R. 2014. Detection and characterization of potentially zoonotic viruses in faeces of pigs at slaughter in Germany. Vet Microbiol 168:60-8.

2013 Johne R, Reetz J, Ulrich RG, **Machnowska P**, Sachsenroeder J, Nickel P, Hofmann J. 2013. An ORF1-rearranged hepatitis E virus derived from a chronically infected patient efficiently replicates in cell culture. J Viral Hepat [Epub ahead of print].

2012 Kreuzer S*, **Machnowska P***, Aßmus J, Sieber M, Pieper R, Schmidt M, Brockmann G, Scharek-Tedin L, Johne R. 2012. Feeding of the probiotic bacterium Enterococcus faecium NCIMB 10415 differentially affects shedding of enteric viruses in pigs. Vet Res 43:58.
*both authors contributed equally to the study

Otto P, Ahmed M, **Machnowska P**, Reetz J, Roth B, Trojnar E, Johne R. 2012. Detection of avian rotaviruses of groups A, D, F and G in diseased chickens and turkeys from Europe and Bangladesh. Vet Mircobiol 156:8-15.