Diploma thesis

Evaluation of a novel assay for detection of norovirus RNA based on a rapid amplification and detection protocol using two different extraction platforms

submitted by

Alexandra Carina Radler

for receiving an academic Medical Degree

Medical Doctor, MD

(Dr. med. univ.)

at the

Medical University of Graz

Institute of Hygiene, Microbiology and Environmental Medicine

First supervisor

Univ.-Prof. Dr.med.univ. Harald H. Kessler

Second Supervisor

Priv.-Doz.[In] Mag.[a] rer.nat. Dr.[in] scient.med. Evelyn Stelzl

Graz, 07.07.2020

I declare that I have written this diploma thesis independently, that I have not used other than the sources/resources cited, and that I have explicitly marked all material, which has been quoted either literally or by content from the sources used.

Graz, am 07.07.2020 Alexandra Carina Radler eh.

Abstract

Background: Noroviruses are significant pathogens causing infectious gastroenteritis. It is of utmost importance that patients infected with norovirus are identified as early as possible.

Objectives: The aim of this study was to compare results obtained by the Anchor Norovirus PCR Kit (Anchor Diagnostics) with the RIDA®GENE Norovirus assay (R-Biopharm).

Materials and Methods: The analytical performance of the Anchor Norovirus PCR Kit was assessed utilizing two international pilot study panels. The clinical performance was evaluated with 109 anonymized residual clinical stool samples. For detection of norovirus RNA, all samples were extracted on the EMAG® (bioMerieux) platform and the MagNA Pure 24 (Roche) instrument followed by amplification and detection using the Anchor Norovirus PCR Kit. The RIDA®GENE Norovirus assay in combination with the EMAG® platform served as reference test system. Amplification and detection were performed on the Light Cycler® 480 II (Roche) for all 3 test systems.

Results: When the analytical performance was assessed, all positive and negative panel members were identified as positive and negative, respectively. When the clinical performance was evaluated, 69 samples tested positive and 40 negative with the reference test system. When the Anchor Norovirus PCR Kit was used in combination with the EMAG® platform, all 69 positives tested positive, 36 of 40 negatives tested negative, and 4 samples showed inhibition; in combination with the MagNA Pure 24 platform, all 69 positives tested positive, 4 of 40 negatives tested negative, and 36 samples showed inhibition. All valid results were found to be concordant. The overall time required for an extraction of 8 samples on the EMAG® platform was 80 minutes, the hands-on time 20 minutes. Corresponding times when using the MagNA Pure 24 platform were 40 and 15 minutes, respectively.

Conclusions: Rapid detection of norovirus RNA in stool samples is advisable; however, the Anchor Norovirus PCR Kit in combination with the MagNA Pure 24

platform showed minor performance due to the significantly increased number of inhibitions. In combination with the EMAG® platform, significantly less inhibitions were observed. The Anchor Norovirus PCR Kit in combination with the MagNA Pure 24 platform showed a significantly shorter turn-around time; however, only 8 samples can be run in parallel.

Kurzfassung

Hintergrund: Noroviren sind hochinfektiöse Erreger und gelten als die häufigste Ursache für Gastroenteritis-Ausbrüche. Um eine zuverlässige Diagnose zu stellen und klinische Entscheidungen treffen zu können, ist ein schneller und exakter Nachweis von Norovirus RNA von größter Bedeutung.

Zielsetzung: Das Ziel dieser Studie war der Vergleich des Anchor Norovirus PCR Kits (Anchor Diagnostics) unter Verwendung von zwei verschiedenen Extraktionsplattformen mit dem RIDA®GENE Norovirus Assay (R-Biopharm).

Materialien und Methoden: Die analytische Leistung des Anchor Norovirus PCR Kits wurde mit zwei Pilotstudien eines internationalen Ringversuchsprogrammes bestimmt. Die klinische Leistung wurde anhand von 109 anonymisierten Stuhlproben von PatientInnen mit Gastroenteritis untersucht. Zum Nachweis der Norovirus RNA wurde der Anchor Norovirus PCR Kit nach Extraktion auf der EMAG® (bioMerieux) bzw. auf der MagNA Pure 24 (Roche) Plattform verwendet. Der RIDA®GENE Norovirus Assay in Kombination mit der EMAG® Plattform diente als Referenzsystem. Die Amplifikation und der Nachweis der Norovirus RNA wurden für alle 3 Testsysteme mit dem Light Cycler® 480 II (Roche) durchgeführt.

Ergebnisse: Bei der Beurteilung der analytischen Leistung wurden alle Proben vom Anchor Norovirus PCR Kit in Kombination mit beiden Extraktionsverfahren korrekt identifiziert. In Bezug auf die klinische Leistung ergab der Referenztest 69 positive und 40 negative Ergebnisse. Bei der Extraktion mit der EMAG® Plattform lieferte das Anchor Norovirus PCR Kit 69 positive, 36 negative und 4 inhibierte Ergebnisse. In Kombination mit der MagNA Pure 24 Plattform waren 69 Proben positiv, 4 negativ und 36 inhibiert. Alle validen Ergebnisse stimmten überein. Die Gesamtzeit für eine Extraktion von 8 Proben mit der EMAG® Plattform war 80 Minuten, mit der MagNA Pure 24 Plattform 40 Minuten. Die manuelle Arbeitszeit betrug 20 bzw. 15 Minuten.

Fazit: Der schnelle Nachweis von Norovirus RNA ist von Vorteil, jedoch zeigte der Anchor Norovirus Kit in Kombination mit der MagNA Pure 24 Plattform eine signifikant erhöhte Zahl an Inhibierungen. In Kombination mit der EMAG® Plattform

wurden mit dem Anchor Norovirus Kit signifikant weniger Inhibierungen beobachtet. Die Gesamtzeit der Testung ist mit der MagNA Pure 24 Plattform signifikant kürzer, jedoch können damit nur acht Proben gleichzeitig bearbeitet werden.

Table of contents

Abstract ..... **III**

Kurzfassung ..... **V**

Table of contents ..... **VII**

1. Introduction **1**

1.1 Epidemiology and transmission 2

1.2 Genome 4

1.3 Genogroups and genotypes 5

1.4 Infection 5

1.4.1 Symptomatic infection 5

1.4.2 Asymptomatic infection 5

1.4.3 Sequelae 6

1.5 Diagnostic approaches 8

1.6 Prevention and Therapy 9

2. Objectives **10**

3. Materials and Methods **11**

3.1 Study design 11

3.1.1 Analytical performance 11

3.1.2 Clinical performance 12

3.1.3 Lab flow analysis 12

3.2 Methods 13

3.2.1 Extraction of nucleic acids 13

3.2.2 Amplification and detection 14

4. Results **16**

4.1 Analytical performance 16

4.2 Clinical performance 18

4.3 Lab flow analysis 19

5. Discussion **20**
6. References **23**
7. List of figures **25**
8. List of tables **26**

1. Introduction

Norovirus is an enteric pathogen causing a syndrome of sudden-onset, self-limiting emesis and diarrhea. The first illness described due to norovirus was in 1929 by Zahorsky, named “Hyperemesis hemis” or “winter vomiting disease”, since the outbreaks peaked in colder seasons (1). In 1968, an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, and the subsequent studies provided for the first time an accurate picture of a norovirus infection. About half of the school’s students were affected, suffering from vomiting, diarrhea and fever. The incubation period as well as the duration of symptoms took 48 hours each (2). The etiologic agent of this outbreak was unknown until 1972, when a 27-nm virus-like particle was discovered in an infectious stool filtrate by use of immune electron microscopy. The particle was given the name “Norwalk virus” and seen as the protoype agent of the genus norovirus (3).

1.1 Epidemiology and transmission

Norovirus infections are found worldwide. The most severely affected regions are developing countries with millions of hospitalizations and deaths each year (4,5). In Austria, the incidence rate in 2017 was 13.4/100,000 with a total of 1,172 reported cases (Fig. 1). Since patients with mild symptoms may not seek medical advice, the estimated number of unrecorded cases is high (2).

At least 95% of epidemic viral gastroenteritis outbreaks and more than 50% of all epidemic gastroenteritis outbreaks worldwide are caused by norovirus. These outbreaks involve people of all ages and occur primarily in closed settings, for example in health-care facilities, restaurants, schools and cruise ships (6-8). In 2017, 21 outbreaks were registered in Austria (Fig. 2).

The three main ways for human transmission are foodborne, waterborne and person-to-person contact. Community outbreaks tend to be associated with contaminated food, especially with raw fruits, vegetables, seafood, and water. Asymptomatic viral shedding of food service workers and infected persons in pre- and post-symptomatic stages may cause contamination. By comparison, healthcare outbreaks are often linked to person-to-person contact through feces, vomitus, surfaces, aerosolization and droplets (1,7,9).

Multiple factors may contribute to high infectivity. For example, the infectious dose with 18 to 1000 viral particles is small. Conversely, the excretion level is high with 10^8 to 10^{10} virions per gram of feces and may continue for more than two weeks after the symptomatic stadium (8,9).

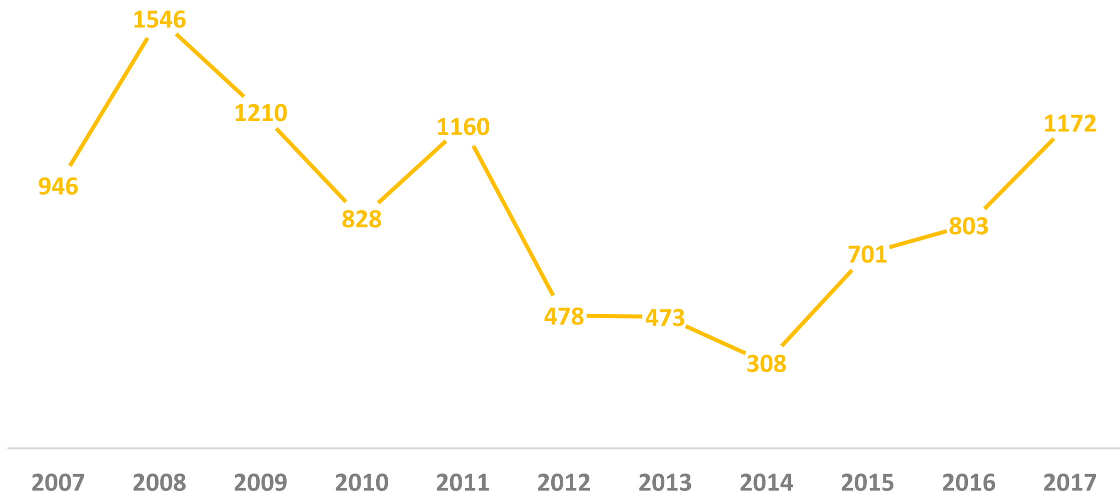

Figure 1: Number of registered norovirus infections in Austria from 2007 - 2017. Credit: I. Lederer and S. Köberl-Jelovcan, “Nationale Referenzzentrale für Noroviren - Jahresbericht 2017,” pp. 1–16, 2017.

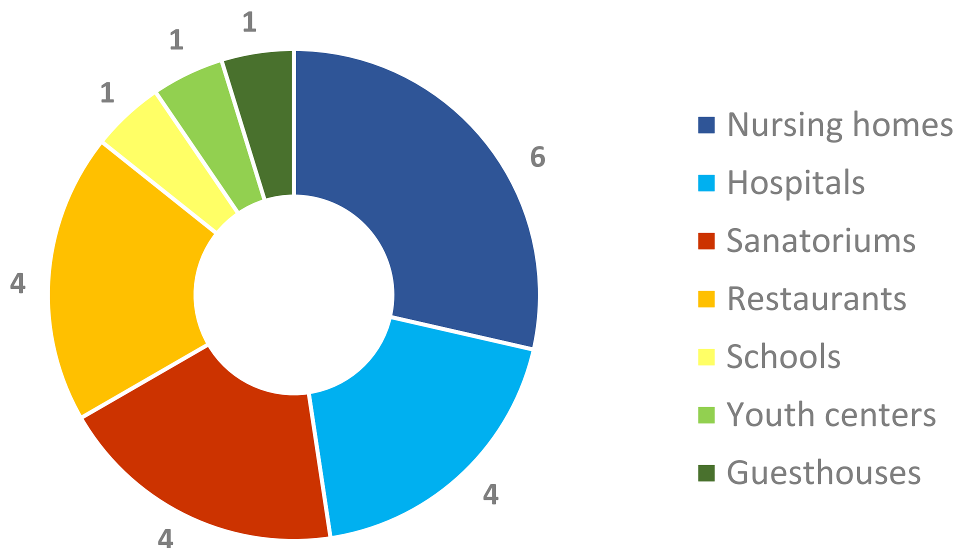

Figure 2: Norovirus outbreak settings in Austria in 2017. Credit: I. Lederer and S. Köberl-Jelovcan, “Nationale Referenzzentrale für Noroviren - Jahresbericht 2017,” pp. 1–16, 2017.

1.2 Genome

Norovirus belongs to the non-enveloped, positive-sensed *Caliciviridae* family. The genome RNA is linear, single-stranded and around 7.7 kb in size. Serving as an mRNA, it is a template for a complementary negative strand being transcribed into genome RNA through the viral polymerase. As soon as they enter the target-cell and bound to ribosomes, protein translation starts (9,10).

The norovirus genome is subdivided into three open reading frames (ORFs). ORF1 at the 5' region encodes a polyprotein, which is transformed into six non-structural proteins, including the norovirus protease and RNA-dependent RNA polymerase (2). The second ORF encodes the major capsid protein, which stabilizes the shape of the virions (10). ORF3 at the 3' end is known as the most variable region and encodes the minor capsid proteins. At the 5' end, the genome is linked to the viral protein genome (VPg). It has an essential role in virus infectivity and in the initial translation. The 3' end presents a poli-A tail that helps with translation. In Fig. 3, the norovirus genomic organization, as it was used for the design of the primer pool used in RT-PCR, is shown (9).

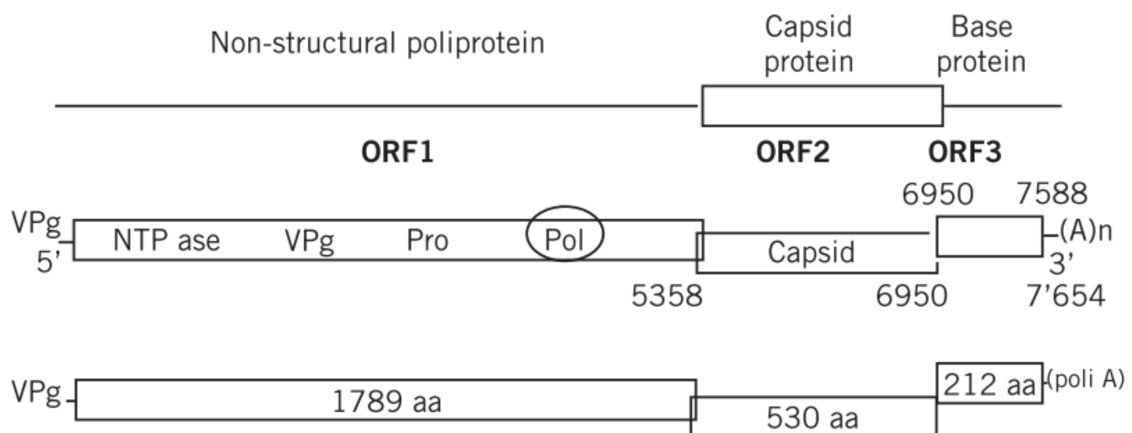

Figure 3: The norovirus genome. Image Credit: S. G. Morillo and M. do C. S. T. T. Timenetsky, "Norovirus: An overview," Rev Assoc Med Bras, vol. 57, no. 4, pp. 453–458, 2011.

1.3 Genogroups and genotypes

The norovirus genus is subdivided into six genogroups (GI and GVI) and more than 40 genotypes based on amino acid diversity in ORF1. Human norovirus infections are mostly caused by genogroup I, II and IV. About 70% of norovirus outbreaks arise from a specific strain from genogroup II, the norovirus GII.4 (2,10). Infections caused by GII.4 strains tend to be more severe and are strongly associated with person-to-person contact, compared to GI viruses, which are more often associated with foodborne diseases (11).

1.4 Infection

1.4.1 Symptomatic infection

A symptomatic norovirus infection is usually a mild, self-limited illness of short duration (8). The median incubation time for norovirus infections is 1.2 days. Then symptoms as vomiting, abdominal cramps, fever, watery diarrhea and systemic symptoms as headaches, chills and myalgias arise. The most dominant symptoms are vomiting and diarrhea, which usually do not last longer than one to three days. Some patients even suffer from leukocytosis, thrombocytopenia, neck stiffness, photophobia, and dizziness. (2,9). The highest peak of viral shedding in feces is about three days after the onset of symptoms, which often overlaps with the last symptomatic or the first asymptomatic day. Viral shedding may also occur three to 14 hours before symptom onset and up to four weeks after symptomatic episodes (1).

1.4.2 Asymptomatic infection

Patients with an asymptomatic infection have no signs of symptoms, but still transmit the virus. Reasons for such an infection may be long-term shedding from a previous symptomatic episode or the lack of susceptible factors to symptomatic infections. Asymptomatic infections sum up to 30% of all norovirus infections, whereas among children, percentages tend to be higher (2,10,12-14).

1.4.3 Sequelae

Vulnerable populations, such as young children, elderly, immunocompromised and patients who have received organ transplants, are at higher risk of a severe disease progression and complications (8). For example, elderly patients are more likely to have a fatal outcome (7). Among children, the duration of illness is often twice as long and the severity and number of its symptoms greater (2).

Transplant recipients and patients with primary immune deficiency are exposed to the risk of long-lasting viral shedding and prolonged symptomatic illness. The most common sequel is chronic diarrhea. As stated in a study, the duration of chronic diarrhea among immunocompromised varied between four weeks to nine years, of which a third was accompanied by concomitants like wasting and weight loss. In some rare cases, it even resulted in failure to thrive, renal failure and pneumatosis intestinalis (14).

Symptoms seen in term and preterm neonates may vary widely. One very serious symptom is necrotizing enterocolitis, the most frequent and lethal disease of the gastrointestinal tract of preterm infants (Fig. 4). Cases of benign convulsions have been reported in infants and children as well. The rates ranged from 9% to 30%, a frequency about 6 times higher than for infections with rotavirus (2,14,15).

Secondary complications of norovirus infection are diverse. They vary from extraintestinal manifestations (renal and liver failure, encephalopathy, convulsions) to inflammatory effects (necrotizing enterocolitis) and even mechanical defects (intussusception) (14).

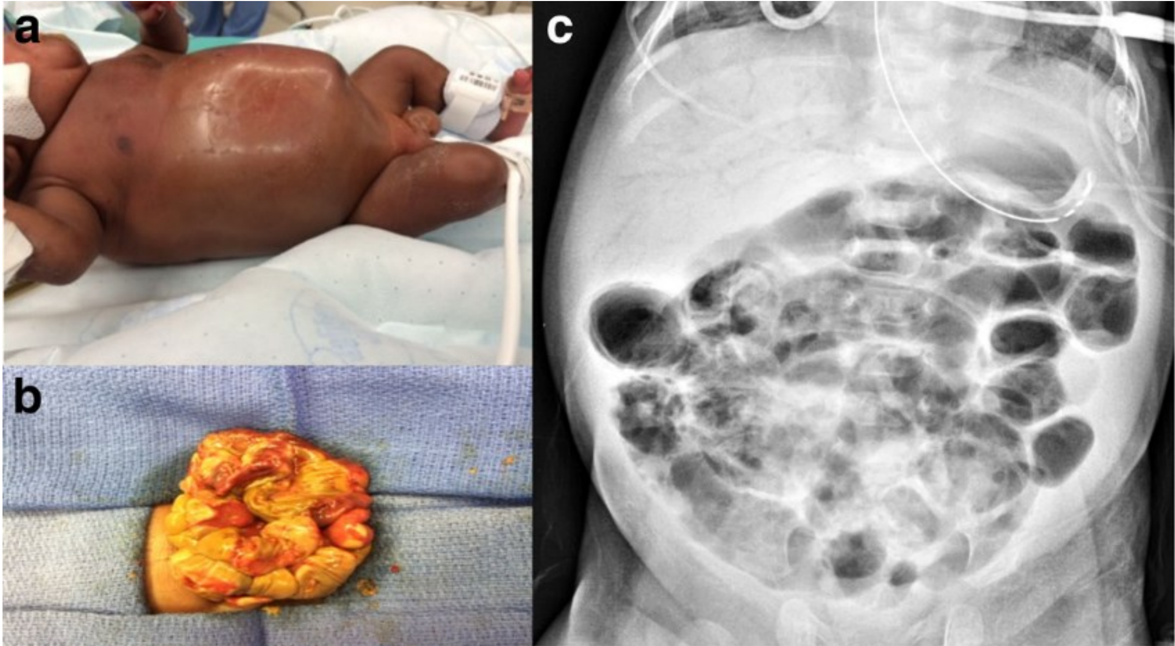

Figure 4: Clinical findings of necrotizing enterocolitis. Image credit: N. Denning and J. M. Prince, “Neonatal intestinal dysbiosis in necrotizing enterocolitis,” Mol. Med., vol. 24, no. 4, pp. 1–10, 2018.

1.5 Diagnostic approaches

The main challenge in controlling norovirus outbreaks is an early identification of norovirus as the cause of a gastroenteritis outbreak. To distinguish foodborne outbreaks of gastroenteritis caused by noroviruses from those caused by bacteria, the clinical and epidemiologic profile “Kaplan’s criteria” is being used. For an exact diagnosis, laboratory testing is needed (1,17).

The Kaplan’s criteria include:

1) Vomiting in more than half of affected persons
2) Average incubation period of 24 to 48 hours
3) Average illness duration of 12 to 60 hours
4) Absence of bacterial pathogen in stool culture

The classic diagnosis method is electron microscopy (EM), which presents the 27 nm viral particles as shown in Fig.5. Since it requires a highly qualified microscopist and expensive equipment, the EM got replaced by more efficient diagnosis methods in the early 1990s (8).

The enzyme-linked immunosorbent assay (ELISA) uses antibodies to detect specific antigens expressed on noroviruses. It is a simple, rapid and cheap method to detect the virus directly from feces. Due to their high specificity but low sensitivity, these antigen tests are more useful in detecting norovirus in an outbreak with multiple stool samples tested, rather than in a sporadic case (1,9).

The RT-PCR, which is seen as the method of choice for norovirus identification, detects genome sequences of norovirus RNA in stool filtrates. But since RT-PCR does not differentiate between infectious and inactivated virus particles, it does not always imply infectivity (1).

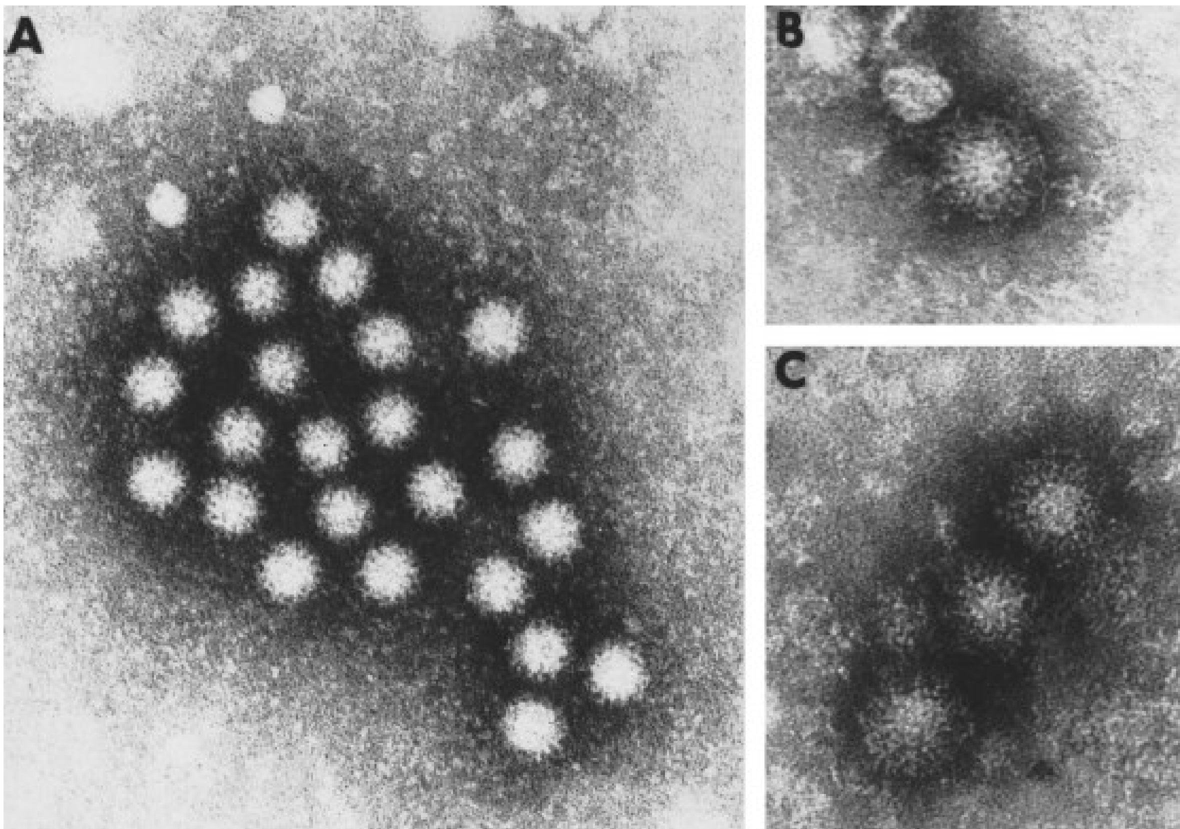

Figure 5: Norwalk virus in a stool filtrate. Image credit: A. Z. Kapikian, “The Discovery of the 27-nm Norwalk Virus: An Historic Perspective,” J Infect Dis, vol. 181, no. 2, pp. 295–302, 2000.

1.6 Prevention and Therapy

For lack of vaccine and anti-viral treatment, the main approach to preventing norovirus infections is proper hygiene (1,11). In case of an outbreak, personnel, patients and visitors should pay very particular attention to washing their hands, at best with soap and running water for at least 20 seconds. Despite the lack of evidence for effectiveness, alcohol-based hand sanitizers are recommended in addition (7,11). Furthermore, frequently touched surfaces as toilets, beds, sinks, telephones and door handles need to be cleaned with a bleach solution. (2,11). For personnel, also protective equipment as gloves and masks should be available (11).

2. Objectives

(1) To compare results obtained by the Anchor Norovirus PCR Kit (Anchor Diagnostics, Hamburg, Germany) after RNA extraction with the EMAG® nucleic acid extraction platform (bioMerieux, Marcy I´Etoile, France) using the specific B protocol vs. the RIDA®GENE Norovirus assay (R-Biopharm, Darmstadt, Germany) in combination with the EMAG® nucleic acid extraction platform (reference test system).

(2) To compare results obtained by the Anchor Norovirus PCR Kit after RNA extraction with the MagNA Pure 24 (Roche Diagnostics, Penzberg, Germany) platform using the Fast Pathogen 200 protocol vs. RIDA®GENE Norovirus assay in combination with the EMAG® nucleic acid extraction platform (reference test system).

(3) To evaluate the accuracy of the Anchor Norovirus PCR Kit in combination with the two different extraction platforms using reference material including samples containing different norovirus isolates.

(4) To compare results achieved from clinical samples obtained by the two different test systems with those obtained by the reference test system.

(5) To compare turn-around time and hands-on time of the 3 different analytical workflows.

3. Materials and Methods

3.1 Study design

3.1.1 Analytical performance

The accuracy of the Anchor Norovirus PCR Kit assay after nucleic acid extraction with the EMAG® platform and that of the Anchor Norovirus PCR Kit after nucleic acid extraction with the MagNA Pure 24 platform were determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2017 Viral Gastroenteritis EQA Pilot Study and the QCMD 2018 Viral Gastroenteritis EQA Pilot Study (www.qcmd.org). The matrix for all members of the panels (9 members in 2017, 10 members in 2018) was stool. The composition of the panels is shown in Table 1.

Table 1: Composition of the QCMD panels used in this study.

Vial no.	Panel 2017	Panel 2018
1	**Norovirus (GII.4-GII.p16)**	**Norovirus (GII)**
2	Rotavirus	Adenovirus (Type 41)
3	Astrovirus	Rotavirus
4	Rotavirus	**Norovirus (GI)**
5	Adenovirus (Type 41)	**Norovirus (GII)**
6	**Norovirus (GI)**	Rotavirus
7	**Norovirus (GII)**	Negative
8	**Norovirus (GII)**	**Norovirus (GII.4-GII.p16)**
9	Negative	Astrovirus
10	-	Sapovirus

3.1.2 Clinical performance

One hundred and nine anonymized left-over stool samples that had been obtained from female and male patients with clinical presentation compatible to viral gastroenteritis were studied. All samples had been obtained with FecalSwab™ (Copan Diagnostics, Murietta, CA, USA) stool collection vials (Fig. 6). Patients had been admitted to outpatient clinics of the Department of Internal Medicine or the Department of Pediatrics and Adolescent Medicine of the Medical University of Graz, Austria. The mean age of patients was 13 years (age range, 0-93 years). The female-to-male ratio was 45:55%.

Figure 6: FecalSwab™ stool collection vials (picture taken by Alexandra Radler on June, 12, 2019).

3.1.3 Lab flow analysis

For the lab flow analysis, the turn-around time including hands-on time were estimated. Times required for the Anchor Norovirus PCR Kit assay after nucleic acid extraction with the EMAG® platform, that of the Anchor Norovirus PCR Kit after nucleic acid extraction with the MagNA Pure 24 platform, and that for the RIDA®GENE Norovirus assay in combination with the EMAG® nucleic acid extraction platform were compared.

3.2 Methods

3.2.1 Extraction of nucleic acids

Nucleic acids extraction was performed on the EMAG® and on the MagNA Pure 24 platforms. The Anchor Norovirus PCR Kit was used in combination with each extraction platform.

On the EMAG® platform (Fig. 7), all samples were extracted with the EMAG® accessory products using the specific B protocol. The input volume was 500 µl. After lysis, 5 µl of Internal Control RNA (IC) provided with the RIDA®GENE Norovirus assay or Anchor Norovirus PCR Kit was added. The elution volume was 50 µl.

On the MagNA Pure 24 platform (Fig. 8), extraction was performed using the Fast Pathogen 200 protocol. The input volume was 200 µl, the elution volume was 50 µl. After lysis, 5 µl of Internal Control RNA was added.

Figure 7: EMAG® nucleic acid extraction platform (picture taken by Alexandra Radler on April 5, 2019).

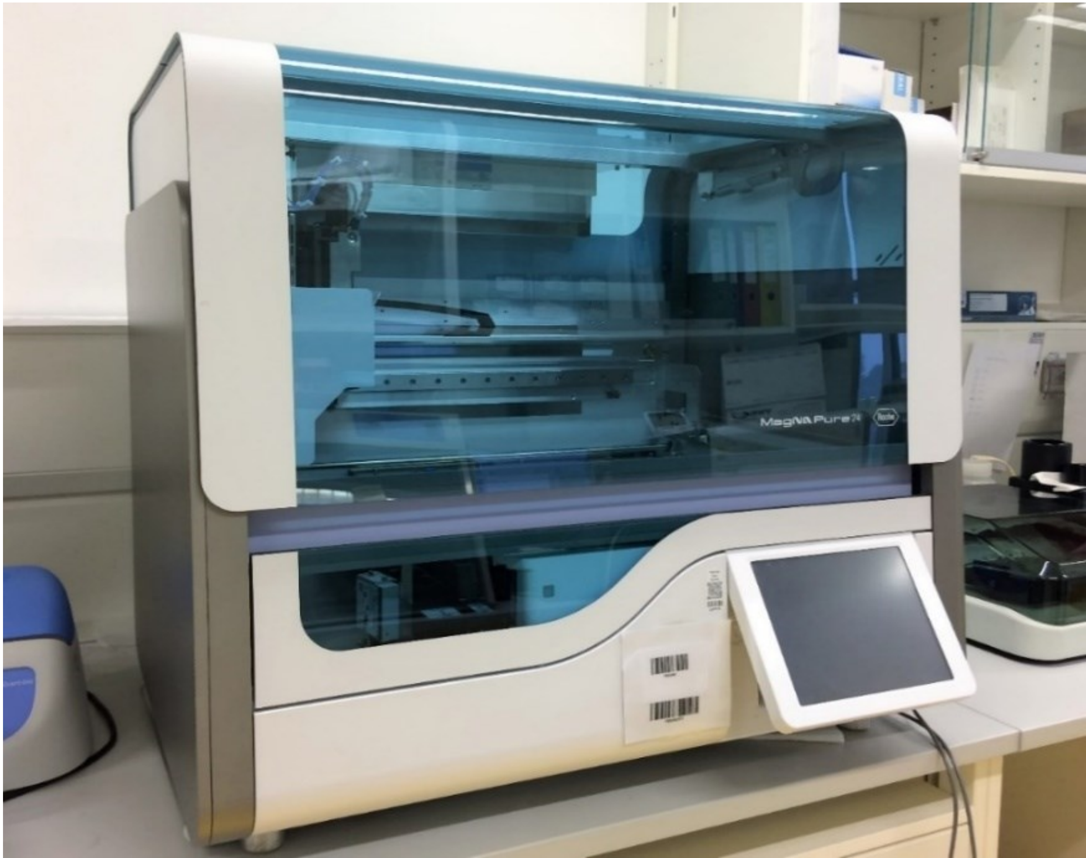

Figure 8: MagNA Pure 24 nucleic extraction platform (picture taken by Alexandra Radler on April 5, 2019).

3.2.2 Amplification and detection

Nucleic acid extracts were amplified and detected with the Anchor Norovirus PCR Kit and the RIDA®GENE Norovirus assay on the Light Cycler® 480 II instrument (Fig. 9). The Anchor Norovirus PCR Kit is a ready-to-use system for reverse transcription, amplification, and detection of norovirus nucleic acids. The kit is able to differentiate between norovirus genogroups GI and GII. It includes a Master A and a Master B reagent, containing all necessary components (PCR buffer, polymerase and reverse transcriptase, magnesium ions, dNTPs, primers and probes) to allow PCR. For one reaction, 5 µl of Master A and 5 µl of Master B are used for the PCR Mix. In addition, the performance of the PCR is monitored with a combined Norovirus GI and GII Positive Control and a Norovirus GI and GII Negative Control. 10 µl of PCR Mix plus 15 µl of the extracted sample or the Positive Control or the Negative Control are pipetted into a well of the PCR plate.

In this study, the reference test system used the RIDA®GENE Norovirus assay on the Light Cycler® 480 II instrument for amplification and detection. The RIDA®GENE Norovirus assay includes a Reaction Mix, an Enzyme Mix, an Internal Control, PCR Water (serving as Negative Control), and a Positive Control. For the PCR Mix, 19.3 µl of Reaction Mix, and 0.7 µl of Enzyme Mix are pipetted. Five µl of eluate, Positive Control, or Negative Control are added to the PCR mix and pipetted into a well of the PCR plate. After sealing the plate, amplification and detection are performed on the Light Cycler® 480 II.

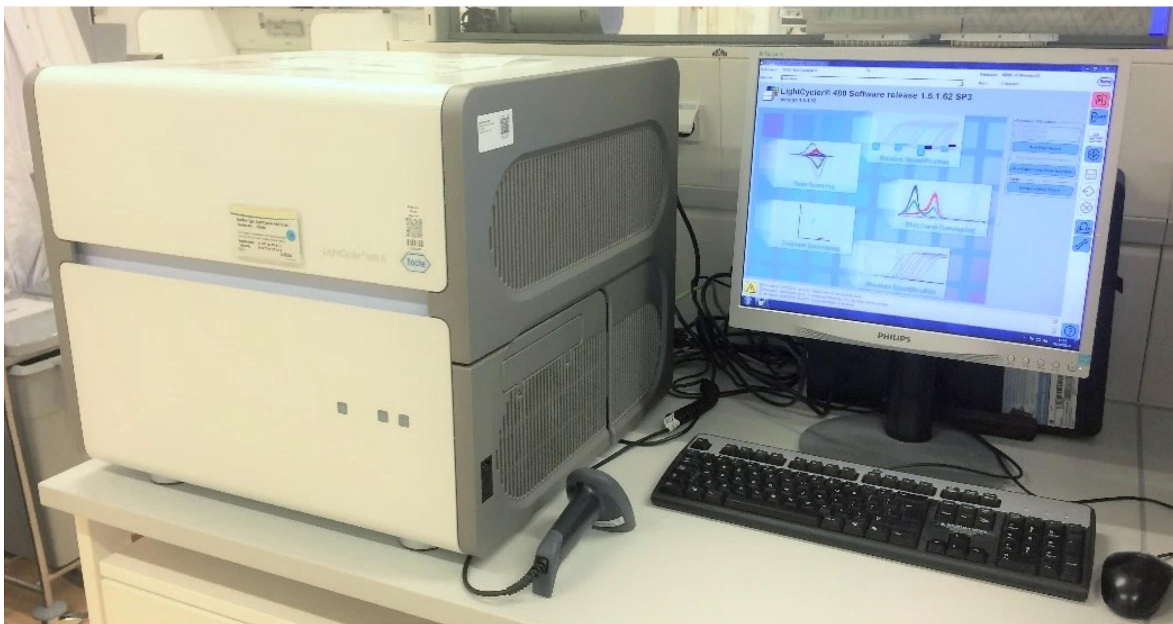

Figure 9: Light Cycler® 480 II (picture taken by Alexandra Radler on April 5, 2019).

4. Results

4.1 Analytical performance

When accuracy was determined, all 8 of 8 panel members of both panels containing norovirus were identified as positive by the EMAG® extraction platform using the specific B protocol or the MagNA Pure 24 extraction platform using the Fast Pathogen 200 protocol (Table 2, Table 3). Furthermore, viruses other than norovirus were not detected. The negative panel members (vial no. 9 in 2017 and vial no. 7 in 2018) and the Sapovirus (vial no. 10 in 2018) were not tested as with the Fast Pathogen 200 protocol, only eight samples can be processed in a single run. Ct values of panel members containing norovirus are shown in Table 4. When comparing the Ct values obtained from the EMAG® platform and the MagNA Pure 24 platform, the EMAG® platform showed lower values for all members, with differences exceeding three cycles (corresponding to approximately 1.0 $\log_{10}$ in case of a quantitative assay).

Table 2: Results of accuracy testing utilizing the QCMD 2017 Viral Gastroenteritis EQA Programme when tested using the EMAG® and the MagNA Pure 24 platforms.

Vial no.	Sample content	Result obtained with EMAG®	Result obtained with MagNA Pure 24
1	**Norovirus (GII.4-GII.p16)**	**Positive**	**Positive**
2	Rotavirus	Negative	Negative
3	Astrovirus	Negative	Negative
4	Rotavirus	Negative	Negative
5	Adenovirus (Type 41)	Negative	Negative
6	**Norovirus (GI)**	**Positive**	**Positive**
7	**Norovirus (GII)**	**Positive**	**Positive**
8	**Norovirus (GII)**	**Positive**	**Positive**
9	Negative	NT[a]	NT

[a]NT, not tested

Table 3: Results of accuracy testing utilizing the QCMD 2018 Viral Gastroenteritis EQA Programme panel when tested using the EMAG® and the MagNA Pure 24 platforms.

Vial no.	Sample content	Result obtained with EMAG®	Result obtained with MagNA Pure 24
1	**Norovirus (GII)**	**Positive**	**Positive**
2	Adenovirus (Type 41)	Negative	Negative
3	Rotavirus	Negative	Negative
4	**Norovirus (GI)**	**Positive**	**Positive**
5	**Norovirus (GII)**	**Positive**	**Positive**
6	Rotavirus	Negative	Negative
7	Negative	NT	NT
8	**Norovirus (GII.4-GII.p16)**	**Positive**	**Positive**
9	Astrovirus	Negative	Negative
10	Sapovirus	NT	NT

Table 4: Ct values of positive panel members of the QCMD 2017 and 2018 Viral Gastroenteritis EQA Programme panel when tested using the EMAG® and the MagNA Pure 24 platforms.

Vial no.	Sample content	Ct values obtained with EMAG®	Ct values obtained with MagNA Pure 24
17/1	**Norovirus (GII.4-GII.p16)**	**23.89**	**28.60**
17/6	**Norovirus (GI)**	**25.22**	**30.33**
17/7	**Norovirus (GII)**	**25.70**	**32.33**
17/8	**Norovirus (GII)**	**26.47**	**31.92**
18/1	**Norovirus (GII)**	**26.67**	**31.91**
18/4	**Norovirus (GI)**	**24.92**	**30.41**
18/5	**Norovirus (GII)**	**26.36**	**31.28**
18/8	**Norovirus (GII)**	**20.70**	**25.50**

4.2 Clinical performance

Three liquefied stool samples clotted during extraction (one with the EMAG® protocol and two with the Fast Pathogen 200 protocol) and were thus excluded from further analysis.

Of 109 samples, 69 were found to contain norovirus RNA with the reference test system (RIDA®GENE Norovirus assay in combination with the EMAG® nucleic acid extraction platform). When employing the Anchor Norovirus PCR assay in combination with nucleic acid extraction on the EMAG® platform, all 69 samples tested positive. In combination with nucleic acid extraction on the MagNA Pure 24, all 69 samples tested positive, too. With both test systems, all samples except of one were found to contain norovirus genogroup II. The remaining sample was found to contain norovirus genogroup I.

Norovirus RNA was not detected in 40 samples with the reference test system. With the Anchor Norovirus PCR Kit in combination with the EMAG® specific B protocol, 36 of 40 (90%) samples gave a negative result. Four samples were found to be inhibited. With the Anchor Norovirus PCR Kit in combination with the MagNA Pure 24 Fast Pathogen 200 protocol, 4 of 40 (10%) samples gave a negative result, 36 samples were found to be inhibited.

When results obtained either by the Anchor Norovirus PCR Kit in combination with the EMAG® platform or by the Anchor Norovirus PCR Kit in combination with the MagNA Pure 24 platform were compared to those obtained with the reference test system, no discordant results were observed (Table 5, Table 6).

Table 5: Results obtained with the Anchor Norovirus PCR Kit in combination with the EMAG® platform compared to those obtained with the reference test system (RIDA®GENE Norovirus assay in combination with the EMAG® platform).

		Anchor Norovirus PCR Kit + EMAG®		
		Positive	**Negative**	**Inhibited**
RIDA® GENE + EMAG®	**Positive**	**69**	**-**	**-**
	Negative	**-**	**36**	**4**
	Inhibited	**-**	**-**	**-**

Table 6: Results obtained with the Anchor Norovirus PCR Kit combined with the MagNA Pure 24 platform compared to those obtained with the reference test system (RIDA®GENE Norovirus assay in combination with the EMAG® platform).

		Anchor Norovirus PCR Kit + MagNA Pure 24		
		Positive	**Negative**	**Inhibited**
RIDA® GENE + EMAG®	**Positive**	**69**	**-**	**-**
	Negative	**-**	**4**	**36**
	Inhibited	**-**	**-**	**-**

4.3 Lab flow analysis

For the lab flow analysis, the turn-around time including hands-on time for the three different test systems were estimated and compared. When 8 samples were extracted on each platform, the overall time required for the nucleic acid extraction was 80 minutes on the EMAG® platform and 40 minutes on the MagNA Pure 24 platform. The hands-on time was 20 minutes and 15 minutes, respectively.

5. Discussion

Molecular assays for detection of norovirus RNA have been significant tools for the diagnosis of viral gastroenteritis. Rapid and accurate detection of norovirus RNA is of paramount importance for a reliable diagnosis allowing rapid clinical decisions including prevention of gastroenteritis outbreaks.

The Anchor Norovirus PCR Kit is based on real-time PCR and has been designed for detection of clinically relevant norovirus RNA. In this study, the new assay was evaluated in combination with two different extraction platforms (EMAG®, MagNA Pure 24) and results were compared to those obtained with the reference test system (RIDA®GENE Norovirus assay in combination with the EMAG® platform). For detection and quantitation of norovirus RNA, liquefied stool was used.

The accuracy of the Anchor Norovirus PCR Kit was tested by using the 2017 and 2018 Quality Control for Molecular Diagnostics (QCMD) Viral Gastroenteritis EQA Pilot Study panels including norovirus, adenovirus, astrovirus, rotavirus, sapovirus, and negative members. As the kit did not detect pathogens other than norovirus, all results correspond to the expected results. When comparing the Ct values obtained from the EMAG® platform and the MagNA Pure 24 platform, the EMAG® platform showed lower values for all members, with differences exceeding three cycles (corresponding to approximately 1.0 $\log_{10}$ in case of a quantitative assay). Recent studies have shown differences in the extraction efficiency when comparing the EMAG® platform to other extraction platforms (17,18).

The clinical performance was evaluated by using 109 anonymized left-over stool samples (three samples had clotted during extraction and thus were excluded from further analysis), of which 69 contained norovirus when tested with the reference test system. Of 40 samples that tested negative with the reference test system, 36 showed inhibition, all of them when employing the Anchor Norovirus PCR Kit in combination with the MagNA Pure 24 platform and four of them with the EMAG® platform. In PCR based assays, inhibition may always occur. Inhibitors may prevent amplification through co-purification and subsequent inhibition of the enzymatic

reaction. Blood, sputum, urine, tissues, and feces are common specimens that may contain inhibitors. Additional sources may be reagents and materials which are exposed to the samples during nucleic acid extraction. There are various ways to prevent adverse effects from inhibitors not eliminated during nucleic acid extraction, such as choice and amount of DNA polymerase, and quantity of the DNA or RNA template. It is obligatory to add an Internal Control in every molecular test to detect interference from inhibitors during amplification and thus exclude false-negative results. In case of a positive target result and a negative IC, the result is considered as valid because the IC result is not required in this combination. In case of a negative target result and a negative IC, the result is considered as invalid (19). Thus, 10% of results obtained from negative specimens extracted with the EMAG® platform and 90% of results obtained from negative specimens extracted with the MagNA Pure 24 platform were found to be invalid. This very high percentage of inhibitions makes the MagNA Pure 24 unreliable for extraction of norovirus RNA from stool samples if the Fast Pathogen 200 protocol is used.

Lab flow analysis showed that the EMAG® platform required both longer overall and longer hands-on time in comparison to the MagNA Pure 24 platform. However, the EMAG® platform is able to test up to 24 samples in one run, while the MagNA Pure 24 platform extracts only 8 samples in parallel when using the Fast Pathogen 200 protocol. Furthermore, on the EMAG® platform the IC must be added manually to the clinical sample, which also extends hands-on time.

Limitations of this study include the low number of samples and that the samples were stored up to 3 months at minus 20 C. However, the study design was chosen according to real-world conditions in a high-throughput routine diagnostic laboratory. Larger studies including more samples are suggested.

In conclusion, rapid detection of norovirus RNA in stool samples is advisable; however, the Anchor Norovirus PCR Kit in combination with the MagNA Pure 24 platform when using the Fast Pathogen 200 showed minor performance due to the significantly increased number of inhibitions. In combination with the EMAG® platform, significantly less inhibitions were observed. The Anchor Norovirus PCR Kit

in combination with the MagNA Pure 24 platform showed a significantly shorter turn-around time; however, only 8 samples can be run in parallel.

6. References

1. Ong CW. Norovirus: A challenging pathogen. Healthc Infect [Internet]. 2013;18(4):133–42. Available from: http://dx.doi.org/10.1071/HI13016

2. Robilotti E, Deresinski S, Pinsky BA. Norovirus. Clin Microbiol Rev. 2015;28(1):134–64.

3. Kapikian AZ. The Discovery of the 27-nm Norwalk Virus: An Historic Perspective. J Infect Dis. 2000;181(2):295–302.

4. Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinjé J, Parashar UD. Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis. 2008;14(8):1224–31.

5. Lederer I, Köberl-Jelovcan S. Nationale Referenzzentrale für Noroviren - Jahresbericht 2017. 2017;1–16.

6. Karst SM. Pathogenesis of noroviruses, emerging RNA viruses. Vol. 2, Viruses. 2010. 748-781 p.

7. Capece G, Gignac E. Norovirus. StatPearls. 2018.

8. Glass RI, Parashar UD, Estes MK. Norovirus Gastroenteritis. N Engl J Med. 2009;361(18):1776–85.

9. Morillo SG, Timenetsky M do CSTT. Norovirus: An overview. Rev Assoc Med Bras. 2011;57(4):453–8.

10. Bartnicki E, Cunha JB, Kolawole AO, Wobus CE. Recent advances in understanding noroviruses. F1000Res [Internet]. 2017;6:79. Available from: https://f1000research.com/articles/6-79/v1

11. Barclay L, Park GW, Vega E, Hall A, Parashar U, Lopman B. Infection control for norovirus. Clin Microbiol Infect. 2014;20(8):731–40.

12. Qi R, Huang Y, Liu J, Sun Y, Sun X, Han H-J, et al. Global Prevalence of

Asymptomatic Norovirus Infection: A Meta-analysis. EClinicalMedicine [Internet]. 2018;2–3:50–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2589537018300269

13. Newman KL, Moe CL, Kirby AE, Flanders WD, Parkos CA, Leon JS. Norovirus in symptomatic and asymptomatic individuals: Cytokines and viral shedding. Clin Exp Immunol. 2016;184(3):347–57.

14. Petrignani M, Verhoef L, de Graaf M, Richardus JH, Koopmans M. Chronic sequelae and severe complications of norovirus infection: A systematic review of literature. J Clin Virol [Internet]. 2018;105(May):1–10. Available from: https://doi.org/10.1016/j.jcv.2018.05.004

15. Nino DF, Sodhi CP, Hackam DJ. Necrotizing enterocolitis: new insights into pathogenesis and mechanisms. Nat Rev Gastroenterol Hepatol. 2016;13(10):590–600.

16. Schmid D, Lederer I, Jelovcan S, Kasper S. Vorgehen bei gastroenteritis-ausbrüchen durch norovirus …. AGES. 2011;

17. Harbusch NS, Bozic M, Konrad PM, Winkler M, Kessler HH. Evaluation of a new extraction platform in combination with molecular assays useful for monitoring immunosuppressed patients. J Clin Virol [Internet]. 2018;108(July):59–63. Available from: https://doi.org/10.1016/j.jcv.2018.09.006

18. Garcia M, Chessa C, Bourgoin A, Giraudeau G, Plouzeau C, Agius G, et al. Comparison of eMAG TM versus NucliSENS EasyMAG performance on clinical specimens. J Clin Virol [Internet]. 2017;88:52–7. Available from: http://dx.doi.org/10.1016/j.jcv.2017.01.004

19. Kessler HH. Molecular diagnostics of infectious diseases. 3rd ed. Kessler HH, editor. Graz, Austria: Walter de Gruyter; 2014.

7. List of figures

Figure 1: Number of registered norovirus infections in Austria from 2007 - 2017. Credit: I. Lederer and S. Köberl-Jelovcan, "Nationale Referenzzentrale für Noroviren - Jahresbericht 2017," pp. 1–16, 2017.

Figure 2: Norovirus outbreak settings in Austria in 2017. Credit: I. Lederer and S. Köberl-Jelovcan, "Nationale Referenzzentrale für Noroviren - Jahresbericht 2017," pp. 1–16, 2017.

Figure 3: The norovirus genome. Image Credit: S. G. Morillo and M. do C. S. T. T. Timenetsky, "Norovirus: An overview," Rev Assoc Med Bras, vol. 57, no. 4, pp. 453–458, 2011.

Figure 4: Clinical findings of necrotizing enterocolitis. Image credit: N. Denning and J. M. Prince, "Neonatal intestinal dysbiosis in necrotizing enterocolitis," Mol. Med., vol. 24, no. 4, pp. 1–10, 2018.

Figure 5: Norwalk virus in a stool filtrate. Image credit: A. Z. Kapikian, "The Discovery of the 27-nm Norwalk Virus: An Historic Perspective," J Infect Dis, vol. 181, no. 2, pp. 295–302, 2000.

Figure 6: FecalSwab™ stool collection vials (picture taken by Alexandra Radler on June, 12, 2019).

Figure 7: EMAG® nucleic acid extraction platform (picture taken by Alexandra Radler
on April 5, 2019).

Figure 8: MagNA Pure 24 nucleic extraction platform (picture taken by Alexandra Radler on April 5, 2019).

Figure 9: Light Cycler® 480 II (picture taken by Alexandra Radler on April 5, 2019).

8. List of tables

Table 1: Composition of the QCMD panels used in this study.

Table 2: Results of accuracy testing utilizing the QCMD 2017 Viral Gastroenteritis EQA Programme when tested using the EMAG® and the MagNA Pure 24 platforms.

Table 3: Results of accuracy testing utilizing the QCMD 2018 Viral Gastroenteritis EQA Programme panel when tested using the EMAG® and the MagNA Pure 24 platforms.

Table 4: Ct values of positive panel members of the QCMD 2017 and 2018 Viral Gastroenteritis EQA Programme panel when tested using the EMAG® and the MagNA Pure 24 platforms.

Table 5: Results obtained with the Anchor Norovirus PCR Kit in combination with the EMAG® platform compared to those obtained with the reference test system (RIDA®GENE Norovirus assay in combination with the EMAG® platform).

Table 6: Results obtained with the Anchor Norovirus PCR Kit combined with the MagNA Pure 24 platform compared to those obtained with the reference test system (RIDA®GENE Norovirus assay in combination with the EMAG® platform).