CAT
Critically Appraised Topic

Syndromic testing for gastroenteritis: are conventional diagnostic methods ancient history?

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CLINICAL BOTTOM LINE

Infectious diarrhea is highly prevalent and leads to substantial morbidity and mortality. Guidelines recommend to perform microbiological tests only in case of severe or prolonged illness or in the presence of risk factors for development of severe disease.
Syndromic multiplex PCR testing is an alternative to conventional stool testing based on physician-directed request forms. Molecular multiplex panels provide physicians with a more sensitive test and a more comprehensive assessment of the aetiology of diarrheal disease. But on the other hand, the simultaneous detection of this wide variety of pathogens can pose problems for interpretation and can lead to erroneous conclusions.

In this study, conventional microbiological testing and molecular syndromic testing were performed in parallel during a period of three months to evaluate the contribution of a multiplex panel to the diagnostic process of gastroenteritis (n=1238). The syndromic assay detected **551 additional pathogens** and yielded **244 extra positive samples** compared to the routine conventional laboratory workflow procedure. Only **36.6%** of the request forms were considered '**successful**' (defined as a lab request form requesting the correct causative pathogen). However, 88.2% of these successful requests could be considered as inadequate as far too many tests were randomly requested. The physician directs testing methods **missed the aetiologic diagnosis** (excl. sapovirus and astrovirus) in **32.3%** of the specimens. Astrovirus and/or sapovirus was positive in 31.1% of the samples. In half of the samples (50.9%) testing positive for only adenovirus, norovirus or sapovirus no viral testing was requested. Parasitic infections were suspected in 72.5%. Bacterial infections were theoretically not missed as bacterial stool culture was requested on all stool samples, but some bacteria were not able to be detected by conventional culture (28.6%).
These results show that physician directed test requests for pathogen detection of gastroenteritis is not the best strategy as it seems impossible to predict the pathogen based on clinical presentation alone.

In a second part of this study, we tried to add some clinical useful value to the molecular test reports, especially for results with multiple positive targets. In this CAT, this part of the discussion will be limited to viral pathogens. The PCR data from **4527 positive samples** received over 16 months were retrospectively analyzed to investigate the distribution of the Ct values of each individual pathogen in order to be able to report semi-quantitative results. Semi-quantitative cut-offs were determined by using interquartile ranges for each viral pathogen individually after excluding all samples with a Ct value greater than 35. This approach made it possible to give a more nuanced reporting in case of mixed infections. Using a cut-off value of **35** also led to a **significant reduction of mixed infections reported**.
There is no way to identify the true causative agent in mixed infections, so based on our data we cannot exclude that a pathogen with a high Ct value can be excluded as a causative pathogen. However, linking a lower Ct value of the pathogen to a greater likelihood of being a relevant causative pathogen is common practice. By not reporting very high Ct values and reporting **Ct values semi-quantitatively value was added** to the syndromic reports. This is especially true **in mixed infections**.

CLINICAL/DIAGNOSTIC SCENARIO

With two billion cases of infectious diarrheal disease reported annually worldwide, gastroenteritis is the **second most common infectious illness** around the world leading to substantial morbidity and mortality. Gastroenteritis is defined as a diarrheal disease with increased bowel movement frequency, with or without fever, vomiting and abdominal cramps. More than 3 loose or watery stools in 24 hours can be called diarrhea and is classified as **acute** (< 14 days), **persistent** (> 14 days but < 30 days), **chronic** (> 30 days) or **recurrent** (recurring after 7 days without diarrhea) according to the duration of symptoms (1).
Mainly infants and children under the age of 5 are affected, reaching a yearly number of 1.7 billion cases and to this day, unfortunately approximately 1300 children are dying every day as a result of diarrheal diseases (2). The incidence and risk of mortality is greatest among children living in third world countries (South Asia and sub-Sahara Africa…) facilitated by poor water supplies, sanitation and nutritional deficiencies. In high income countries, few people die from diarrhea, but the illness still results in substantial morbidity and hospitalizations. A widespread variety of agents can cause gastroenteritis, i.e., bacteria, viruses and parasites and distinguishing microbiological aetiology based on clinical presentation alone is often difficult because of similar clinical manifestations. The clinical presentation can vary from mild over severe to even life-threatening. Viral gastroenteritis is most common and although self-limiting, more than 50% of the annual deaths among children are due to diarrhea from viral aetiology (mostly rotavirus and norovirus) due to severe dehydration (1,3). Elderly and immunocompromised patients also have a higher risk of dehydration and hospitalization, leading to increased morbidity and mortality (4,5). Whatever the causative agent, the **treatment** of diarrhea is generally **symptomatic** and directed against the **prevention of dehydration** (1). The use of antibiotics for bacterial gastroenteritis in immunocompetent patients is rarely required.

In most laboratories diagnosis is based on different conventional tests. Pathogens are detected and identified by culture, ELISA, antigen detection by agglutination, lateral flow immunoassays and microscopy. Unfortunately, these methods can only detect a limited number of pathogens implicating that certain pathogens will be missed. Also this approach implies that the physician must suspect the causative agent based on the clinical picture. Today, molecular based methods are increasingly used for the detection of gastrointestinal pathogens. Multiplex panels can simultaneously detect different genome targets and identify a comprehensive variety of enteric pathogens improving diagnostic performance, but also turnaround time.

EPIDEMIOLOGY Bacteria, viruses as well as parasites can be the cause of infectious diarrhea. Aetiology depends on host factors such as age, immune state or co-morbidities and varies among different geographic regions. Viral gastroenteritis is the most common cause of diarrheal disease worldwide and is mainly associated with fecal-oral transmission by ingestion of improperly prepared food (6). Prior to vaccination against rotavirus this virus was the most frequent cause of diarrhea in the pediatric population with almost all children having antibodies by the age of three. Annual numbers of cases for rotavirus decline since many countries are now vaccinating against rotavirus and as a result norovirus became the leading cause of diarrheal outbreaks and hospitalizations (7, 8). The latter is predominantly responsible for 90% of epidemic diarrheal cases. Norovirus is mainly involved in foodborne diarrheal outbreaks and epidemic cases worldwide (9). Adenovirus type 40 and 41, sapovirus and astrovirus are more common in children than in adults and account for approximately 10% of diarrheal cases (10,11). Occasional outbreaks with these viral pathogens have been described (12-15). Norovirus or rotavirus usually cause watery diarrhea, but severe diarrheal illness (fever, blood or mucous in stool, severe abdominal cramps) is mostly caused by *E. coli* (EPEC, ETEC), *Shigella* spp., *Campylobacter* spp. and *Salmonella* spp. (16-18). Parasitic diarrhea is less frequent in developed countries but is believed to be twice as common as bacterial infections. The most frequent protozoan species causing gastroenteritis in industrialized countries is *Giardia lamblia*. Other parasites associated with intestinal illness are *Cryptosporidium* species, *Cyclospora cayetanensis* and *Isospora belli*. The clinical picture of an *Entamoeba histolytica* infection varies from asymptomatic carriage to severe amoebic dysentery. The pathogenicity of *Dientamoeba fragilis* and certainly of *Blastocystis hominis* remains controversial (19). Identifying parasites as the cause of gastroenteritis is not always easy. For example, the pathogenic character of *Giardia lamblia* is disputable as it can be found in many stools of asymptomatic individuals and is even more frequently observed in asymptomatic individuals compared to individuals with acute diarrhea. *Cryptosporidium* is also observed at non negligible rates in asymptomatic children. Therefore a careful interpretation of these protozoa is warranted (16,17,20,21).
Finally, *Clostridioides difficile* is the leading cause of hospital-acquired diarrhea and is generally associated with previous use of antibiotics in hospitalized patients (22). The clinical spectrum ranges from asymptomatic carriage to mild diarrheal illness (with or without fever) to life-threatening pseudomembranous colitis and toxic megacolon. Asymptomatic carriage is very frequently observed in healthy newborns and infants (up to 70%), gradually

decreasing to 5% by the age of 8. In healthy adults the carriage rates range between 4-15%, depending on risk factors such as healthcare work (23,24). Asymptomatic carriers can serve as a reservoir for fecal-oral transmission by spreading *C. difficile* spores to the environment and other patients. Community-acquired *C. difficile* infections is generally less severe than hospital-acquired infections. Community-acquired *C. difficile* infections occur in a significant proportion (41%) of the *C. difficile* infections and mainly affect populations previously thought to be at low risk: young patients and patients with no recent exposure to antibiotics (25).

GUIDELINES CDC and WHO define diarrhea as "three or more episodes of loose stools in 24 hours, or more than normal for the individual". Diarrhea can have an infectious cause, but many non-infectious etiologies such as irritable bowel syndrome, inflammatory bowel disease, food intolerance, malabsorption syndromes and many more can also lead to diarrhea. Culture and culture-independent methods can be used for the detection of infectious agent(s). Most guidelines do not recommend stool testing for community-acquired diarrhea lasting less than a week in patients without risk factors for severe disease. The reason for the restraint to use diagnostic testing is based on the fact that most patients spontaneously recover after a few days without any treatment. If testing is considered the patient's age, severity of disease, duration and geographical location are factors that physicians must examine. Severe, bloody, mucous or persistent diarrhea with or without fever in both healthy and immunocompromised patients is an absolute indication for immediate diagnostic testing for bacteria and *Entamoeba histolytica* regardless of the duration. Broad testing for bacteria, viruses and parasites is advised in persistent community-acquired or travel-related diarrhea. Depending on the consulted guideline persistent diarrhea is defined as >1 week or >2 weeks (26). In case of diarrhea in returning **travelers** and immunocompromised hosts, testing for parasites such as *Cryptosporidium* spp., *Entamoeba histolytica* and *Cyclospora cayetanensis* should be considered (27). As most prevalent **bacterial** pathogens causing inflammatory diarrhea are *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia* all laboratories should be able to detect these enteropathogens by culture or culture-independent methods. *Vibrio* spp. should be considered when the patient mentions eating raw oysters or other raw seafood. Shiga-toxin testing or routinely screening for Shiga toxin-producing *Escherichia coli* O157:H7 and other serotypes is also recommended, especially in children (28). Diarrhea outbreaks in hospitals, daycare, cruises or any long-term (care) institution are a reason to focus on a causative **viral** agent, but bacterial and protozoal causative agents should also be considered (28). Norovirus is the most common cause of community-acquired viral gastroenteritis and needs to be identified as pathogen because contact precautions and isolation measures should be taken. Cytomegalovirus should only be considered in immunocompromised hosts however a negative result does not rule out CMV disease.

A liquid, diarrheal stool sample is the specimen of choice for laboratory diagnosis (28,29). An adequate liquid stool sample is defined as a stool sample taking the shape of the recipient, **corresponding with type 6 and 7 from the Bristol Stool Chart**.
Bacterial pathogens are generally excreted continuously, but a negative stool sample does not completely rule out a bacterial gastroenteritis. In case the first stool sample is negative, an additional stool sample may be submitted for culture when symptoms persist. Rohner et al. claims the detection rate of bacterial pathogens can rise from 87-94% to 98% by analyzing a second stool sample when the initial culture was negative (29). Culture dependent methods are more and more abandoned in favor of culture-independent methods based on nucleic acid amplification tests (NAAT) that can vary in extent from singleplex (one pathogen) to and extended multiplex. This single sample test strategy for bacterial causes is in contrast to parasite examination where multiple samples are necessary to achieve optimal recovery as parasites are typically shed intermittently. As a rule, three consecutive samples collected over a time period of 7-10 days are necessary to obtain the highest positivity rate for microscopic examination.

The diagnosis of **parasitic** gastroenteritis is based on microscopic examination as reference method, but the main disadvantage is the need of an experienced person to recognize the ova and parasites in stool samples and to distinguish similar species. In addition, it is impossible to differentiate between the pathogenic *Entamoeba histolytica* and non-pathogenic *Entamoeba dispar* based on morphology. The use of only one sample is justified for PCR but in case of intermittent shedding with very low DNA extraction, PCR will also remain negative while classical microscopic evaluation of three consecutive samples has been able to detect parasites. Requesting a PCR retest for parasites is therefore indicated if clinical suspicion persists (19). There is no recommendation for routinely performing viral testing because of the possibility of asymptomatic infection or persisting viral shedding after resolution of symptoms (30-34). It is however considered appropriate to test patients for viral gastroenteritis during a known or suspected outbreak, for infection control purposes or in case of diarrhea in immunocompromised patients.

Stools with a positive result for a bacterial pathogen, obtained by multiplex panels, should be cultured on a specific agar in case it concerns notifiable diseases such as shigellosis, enterohemorrhagic *E. coli* or Salmonella *(para)typhi*. Reflex culture is also required when the bacterial strain is needed for epidemiological investigations and for antibiotic susceptibility testing when treatment can affect public health. In general providing empirical treatment for the management of the individual is not recommended (except for immunocompromised patients) (26,28,29,35).

ADVANTAGES AND DISADVANTAGES Conventional laboratory methods are expensive, time-consuming and of limited sensitivity. Syndromic panel-based molecular testing for gastroenteritis provides a **rapid** and **streamlined** alternative to conventional methods for the identification of the microbiological cause of acute or chronic gastroenteritis. These panels target an **extensive range** of different bacterial, viral and parasitic enteropathogens all in one assay and are capable of identifying pathogens that would remain undetected in a physician-directed way of testing (36). A major advantage is the **short turnaround time** (hours), mainly affecting bacterial identification with a higher detection rate compared to stool culture that also generally require up to three or four days for identification and antibiotic sensitivity testing (36). Instead of starting an empiric antibiotic treatment without an identification of the putative pathogen a **more targeted therapy** – if needed – can be initiated less than 24 hours after sampling based on the identified diarrheal aetiology. A rapid pathogen identification can reduce the risk for nosocomial spread as patients can be isolated more quickly. Administration of antibiotics in case of gastroenteritis caused by STEC can lead to a higher risk of hemolytic uremic syndrome and fatal outcomes. Early identification of this pathogen is therefore important. Furthermore, different *E. coli* pathotypes can be differentiated using PCR. Conventional methods mostly only differentiate between O157 or non-O157 Shiga toxin-producing *E. coli* using latex agglutination, but identifying EAEC, EPEC, ETEC and EIEC as such is not possible. Syndromic approach also contributes to a better understanding of the causal spectrum. There is a large amount of new data on pathogens that are not routinely tested by conventional methods. On the other hand this multitude of information raises the question on how to interpret the presence of these organisms.

Conventional stool culture cannot be totally abandoned given the need to perform antibiotic susceptibility tests in case a treatment is indicated and for public health interests in order to determine resistance patterns (37). Reflex culture is also needed to discriminate between EIEC and *Shigella* spp.
Although previously undiagnosed pathogens by routine screening in hospital laboratories can be detected by multiplex panel testing, not all potential aetiologic agents are included such as *Pleisiomonas shigelloides* for which culture remain necessary for detection (38). Despite the fact that not all thinkable enteropathogens are included, syndromic testing is reported to be more accurate, sensitive, specific, and to have much **higher rates of detection** with less false negative results (36). While the sensitivity of conventional stool culture may be affected by the viability of the bacteria and both viral and protozoal detection are limited by their uncultivability, PCR detects the presence of **genomic sequences** from both viable and non-viable organisms. The downside of this advantage is that it is almost impossible to distinguish between a true infection, colonization or the presence of a non-viable organism merely based on a positive PCR signal without clinical judgement. This also implicates that PCR **cannot be used as a test for cure**. The detection and reporting of co-infections certainly increases in PCR driven diagnostics. This could be an advantage, but **the clinical relevance and interpretation of simultaneous detection of multiple positive pathogens** remains a **clinical conundrum** and there is **insufficient data** on how to interpret these findings.

Syndromic panel testing also allows a much higher throughput than conventional testing with less laboratory personnel involved in the workflow and with no need of specific test selection by the physician (39). A disadvantage of molecular syndromic panel testing is the inability to determine the strain's pathogenicity or virulence, for example some STEC strains are highly virulent, others do little harm, some *Yersinia* are non-pathogenic while other biotypes are disease-causing. Additionally, it is impossible to distinguish between EIEC and *Shigella* species without stool culture and selective media.

CURRENT WORKFLOW IN AZ DELTA, ROESELARE In the AZ Delta hospital, a molecular diagnostic gastrointestinal panel for bacterial, viral and parasitic pathogens (Seegene Allplex™ Gastrointestinal Panel Assays) was implemented as the routine diagnostic test for stool samples. A reflex culture is performed on selective agars for confirmation, antibiotic sensitivity testing and typing when there is a positive signal for *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Aeromonas* spp. or *Vibrio* spp. When *C. difficile* is detected by PCR a *C. difficile* Quik Chek (Techlab, USA) is used for toxin A/B detection. On top of the molecular panel a request for targeted microscopic parasitic stool test remains possible. If the laboratory receives a second stool sample for

gastrointestinal testing within 96 hours, a Campylosel agar is inoculated for the detection of *Acrobacter* spp. and *Helicobacter pullorum*, extending the number of pathogens that can be detected by the Seegene Allplex Gastrointestinal Panel. On explicit request of the physician a MacConkey agar is inoculated to isolate *Plesiomonas* spp. or *Edwardsiella tarda*.

We started using the molecular diagnostic gastrointestinal panel of Fast Track Diagnostics (Siemens) in 2018. In 2020 the kit was withdrawn from the market. For that reason an alternative was sought and found by Seegene. In the first part of this study we used the Fast Track Diagnostic Gastrointestinal panel. In the second part, the Seegene Allplex Gastrointestinal Panel was used since we are working with this panel to date.

QUESTION(S)

1) *Question 1* How well does physician selection of microbiologic tests based on clinical presentation correlate with the aetiological yield of multiplex PCR?
2) *Question 2* How can Seegene Allplex Gastrointestinal Panel results be reported in a semi-quantitative way?

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: ("Gastroenteritis/diagnosis") AND ("Feces/parasitology" OR "Feces/microbiology" OR "Feces/virology" OR "Diarrhea/diagnosis" OR "Diarrhea/microbiology") AND ("Polymerase Chain Reaction" OR "Multiplex Polymerase Chain Reaction/methods") OR ("Molecular Diagnostic Techniques") AND ("Ct value" OR "Cycle threshold" OR "Clinical correlation to cycle threshold value" OR "Cycle threshold cut-off") OR ("Syndromic Testing Gastroenteritis")*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific: "gastroenteritis + multiplex polymerase chain reaction", "gastroenteritis + Seegene Allplex gastrointestinal panel", "gastroenteritis + clinical significance ct value", "Diarrhea + Seegene Allplex gastrointestinal panel" "gastroenteritis + Seegene Allplex")*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane)*
4) *International organizations: e.g. National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp)*
5) *UpToDate Online: "Approach to the adult with acute diarrhea in resource-rich settings"*

QUESTION 1: HOW WELL DOES PHYSICIAN SELECTION OF MICROBIOLOGIC TESTS BASED ON CLINICAL PRESENTATION CORRELATE WITH THE AETIOLOGICAL YIELD OF MULTIPLEX PCR?

LITERATURE

Usually, the physician decides to order a test to diagnose the aetiology of the infectious diarrhea according to the clinical presentation of the patient. A study comparing the detection rates by physician-selected tests to multiplex PCR assays found that using a multiplex PCR increased the identification rate by 25% when tests other than *C. difficile* were requested (40). Only 8% of *C. difficile* were missed by physician-directed testing, but when only *C. difficile* was sought, additional pathogens were detected in 28% of the cases. Claas et al. (41) discovered that in 65% of all positive stools analyzed by the multiplex panel, the putative pathogen was not requested by the physician (2). Another study from the same research group comparing physician-requested testing and a syndromic multiplex panel (xTAG GPP) demonstrated that in most cases the detected target was not requested by the physician (42). In general, the results obtained by both conventional and molecular methods exhibited a high overall agreement and did have equivalent diagnostic accuracy, although molecular syndromic testing discovered larger numbers of additional enteropathogens and co-infections (43).

Amrud et al. assessed the performance of the **Allplex bacterial and viral gastrointestinal assays to conventional methods** of bacterial culture and stool electron microscopy (44). A more than **two-fold higher detection rate** of diarrhea pathogens (17.8% vs. 44.4%) was observed with the use of the Allplex Gastrointestinal Panel Assay. Bacterial pathogens were detected 2 times more in comparison to conventional cultures (11.9% vs. 25.2%). Samples showing discordant results were retested by monoplex assays and the molecular results could be confirmed in 86% of the cases. But there was also one true positive detection of *Salmonella* spp. by conventional methods that was not detected by PCR. There can be no doubt that higher detection rates are achieved with the use of a syndromic testing panel as many studies are showing improved diagnostic yields (45,46). The use of a bacterial panel also enables the detection of diarrheagenic *E. coli* pathotypes such as enteroaggregative *E. coli*, Enteropathogenic *E. coli* and non-*E. coli* O157 Shiga toxin producing strains.

Vocale et al. did the same experiment with the **xTAG GPP multiplex assay**, **including parasites**, but used enzyme immunoassays as conventional method for viruses (36). The difference in positivity rates was not as high as found by Amrud et al.: 8.3% more positive results were found by the multiplex assay (45.33% vs. 53.61%). Multiple pathogens were found in 35% of the positive specimens (44). Many other studies also confirm that the diagnostic yield is significantly higher when using a multiplex panel instead of conventional testing methods (40,47-50).

Autier et al. evaluated the performance of the Allplex Gastrointestinal **parasite assay** against microscopy. Microscopy showed poor results for detection of *D. fragilis* and *B. hominis,* but the sensitivity of PCR was excellent and superior to microscopic evaluation for all species and only rarely resulted in false negative results for *D. fragilis* and *B. hominis* (51). Likewise, Paulos et al. showed an increased positivity rate for protozoan agents compared to microscopic evaluation (52). Furthermore, the sensitivity of PCR on a single stool sample seems equivalent to the sensitivity of microscopy on multiple stool samples. It has been demonstrated that the parasitic load was significantly lower in microscopy-negative but PCR-positive samples compared to microscopy-positive samples (19).

ANALYSIS OF POSITIVE TEST RESULTS OBTAINED WITH FAST TRACK DIAGNOSTICS AND CONVENTIONAL LABORATORY METHODS DURING A PERIOD OF THREE MONTHS

Conventional microbiological testing and rapid syndromic testing with the Fast Track Diagnostics Gastroenteritis Kit (FTD), detecting 15 gastrointestinal pathogens (see table I), were performed in parallel during a period of three months (01/11/2018 – 31/01/2019) to evaluate the contribution and the added value of a multiplex panel to the aetiological diagnosis of acute or chronic diarrhea. All stool samples were obtained from patients with a clinical picture consistent with gastrointestinal illness and for all these samples the results of physician-directed testing by conventional methods were compared to those derived from multiplex PCR testing.

A total of **1238 stool requests** were analyzed by conventional microbiologic tests during this period according to the selection of tests requested by the physician. A gastrointestinal multiplex was performed at the same time. Stools were submitted for conventional lab testing with a median of 2 (with a range from 1 to 6) requested microbiological tests. The physicians were able to request the following tests: stool culture, *C. difficile* rapid immunoassay with simultaneous detection of GDH and toxin A and B, lateral flow immunochromatographic tests for adenovirus, norovirus and rotavirus, lateral flow immunochromatographic test for *Cryptosporidium* spp. and *Giardia lamblia* and microscopic examination for the detection of parasites. In 91 samples only one single microbiological test was selected by the physician, mainly being bacterial culture (87/91) despite the fact that the most important cause of diarrhea is mostly a virus.

The syndromic approach could find one or more pathogens in **511 stool samples** (**41.3%**) from **470 different patients** with an identification of **832 pathogens** in total. Only **267 (21.6%)** stool samples tested positive by the standard laboratory methods identifying 281 pathogens. **No pathogens were isolated or detected by conventional laboratory methods in samples that were labeled negative by PCR**. Amrud et al. detected no viral discrepant results. Only one discrepant *Salmonella* spp. was observed (PCR negative and culture positive) (44). No additional parasitic pathogens were found with conventional methods compared to PCR (51).
An overview of the requested tests for conventional laboratory testing and the positivity rates compared to those of the multiplex panel is shown in table 1. The most detected causative agents by both conventional methods and multiplex PCR were **viruses**, with **adenovirus** being most prevalent.
Toxigenic *Clostridioides difficile* was the second most detected pathogen by multiplex PCR, but the detection rate was much lower using conventional methods based on the physician's request. The high number of children in the studied population explains the difference in positivity rates between both methods as physicians do usually not request *C. difficile* in young children. The positivity rate of **parasitic agents** was higher by the use of multiplex PCR compared to conventional testing as physicians do only request parasitic examination when a parasitic infection is clinically suspected. A slightly higher positivity rate for bacteria was obtained by syndromic testing.

In 293 stool samples (**57.3%**) only one pathogen was detected by the FTD assay. The most common pathogens detected in **single infections** were *C. difficile*, norovirus (genotype II) and adenovirus. **Two or more pathogens** were detected in a high number (**42.7%**, n=218) of positive samples when analyzed by FTD. **Co-detection** of 2, 3, 4 and even 5 pathogens was observed in 132 (25.8%), 70 (13.7%), 14 (2.8%) and 2 (0.4%) samples, respectively. Only performing the requested conventional tests would have only detected **28.9%** of all mixed infections. In general, **adenovirus** (n=143) and **sapovirus** (n=84) were involved in most **mixed infections**.

Table 1 An overview of the requested tests (conventional methods) compared to the FTD Gastrointestinal panel and both its positivity rates

Pathogen	**Conventional methods**		**FTD Gastrointestinal Panel**	
	No. samples	No. positive	No. samples	No. positive
Bacteria	**1222**	**50 (4.1%)**	**1238**	**70 (5.6%)**
Campylobacter		29		38
Campylobacter spp.		1		-
Campylobacter coli		2		-
Campylobacter jejuni		26		-
Salmonella spp.		9		10
Shigella spp.		0		0
STEC		7		11
Yersinia spp.		5		11
Viruses	**1009**	**197 (19.5%)**	**1238**	**563 (45.5%)**
Adenovirus	334	107		187
Astrovirus	42*	-		58
Norovirus	378	75		161
Norovirus GI				34
Norovirus GII				127
Rotavirus	297	15		41
Sapovirus	96*	-		116
Parasites	**434****	**36 (8.3%)**	**1238**	**51 (4.1%)**
Cryptosporidium spp.		24		36
Entamoeba histolytica		0		0
Giardia lamblia		12		15
Clostridioides difficile	**451**	**35 (7.8%)**	**1238**	**148 (11.9%)**

* Number of times viral testing was requested

**404 requests for Combined Rapid Antigen Test (Cryptosporidium and Giardia lamblia) and 28 for direct microscopic examination

A lab order was considered '**successful**' if the physician requested the correct causative pathogen on the lab request form. These so-called successful orders can be divided into two groups based on how many identification tests were ordered by the physician, i.e. "overshooting" and "adequate". An **adequate** order is defined as an order requesting correct the conventional test for the pathogen(s) detected by PCR. Overshooting or inadequate request forms are defined as request forms containing tests requests for more pathogens than detected by PCR.

Out of the 1238 stool samples for analysis 511 (41%) tested positive by PCR. Only **187** (36.6%) of 511 samples could be marked as **successful** requests and **only 22** of these were classified as an **adequate targeted order**. Astrovirus and sapovirus were excluded from this analysis because the physician was unable to request a rapid antigen test for these pathogens.

All **22 adequate requests** were only requesting for culture. In nine out of these 22 PCR positive samples (44 %) no intestinal pathogens were isolated by culture. In five out of these 22 samples an additional test (3 for *C. difficile* and 2 for viruses; one for norovirus and one for adenovirus) was requested and found positive by conventional testing and PCR.
Concerning the samples labeled as '**overshooting**' **(165 samples)**, only culture and *C. difficile* were requested in **23** requests, where either culture (15/23) or *C. difficile* (8/23) was the inappropriate test request. In all other **142** samples, more than two analyses (e.g. culture, adenovirus and norovirus or parasites, culture, norovirus and rotavirus) were requested. Although at least one of these requests is an appropriate request, these requests can be regarded as **inadequate** as too many pointless tests were requested (figure1). Culture was requested in all inadequate requests, but only 34 turned out positive (20.6%) and not even half of the viral tests requested turned positive by PCR (41.7%). More than three-quarters of the requests for parasite examination was inadequate.

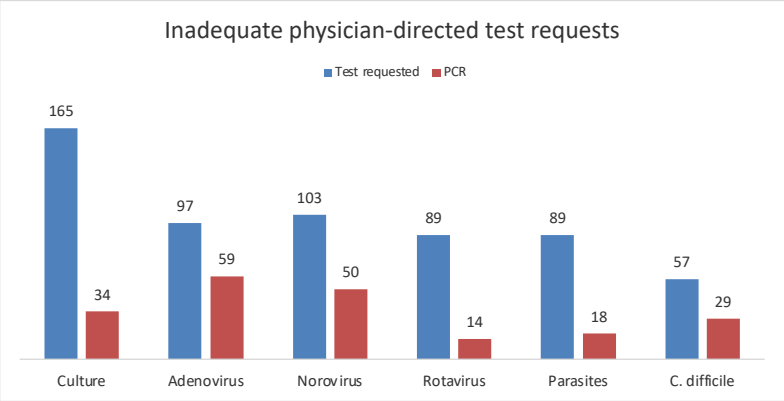


Figure 1 Inadequate physician-directed test orders. Blue = test requested, red = PCR positive for the requested pathogen

Astrovirus and sapovirus could not be requested for routine testing and is is considered as a separate group. **159** samples were positive for either astrovirus and/or sapovirus on samples with a request to test for viral causes. 101 samples tested positive for sapovirus, 43 for astrovirus and 15 for both sapovirus and astrovirus. In addition, astrovirus and sapovirus were detected 58 and 116 times respectively by PCR, but a viral aetiology of the diarrhea was clinically not suspected in 36 (20.7%) samples (table 2). In approximately 57% of the cases the requesting physician asked for all three viruses.

Table 2 Viral ordering profile in case of positive astrovirus or sapovirus by FTD multiplex

	Astrovirus	**Sapovirus**
All three pathogens	32/58 (**55%**)	68/116 (**59%**)
Two pathogens		
Adenovirus and norovirus	2/58 (3.4%)	8/116 (6.9%)
Adenovirus and rotavirus	3/58 (5.2%)	14/116 (12.1%)
Norovirus and rotavirus	1/58 (1.7%)	0
One pathogen		
Adenovirus	3 (5.2%)	2 (1.7%)
Norovirus	1 (1.7%)	4 (3.4%)
Rotavirus	0	0
No viral pathogen request	16/58 (27.6%)	20/116 (17.2%)

The remaining '**unsuccessful**' **165** samples had a request that did not, or only partially, match the PCR result. All of samples detected a causative pathogen that was not considered by the physician based. After **exclusion of 20 samples** with only *C. difficile* being positive in children under the age of two, **145** samples left. 106 had a request profile that did not match the enteric pathogens found using syndromic testing. In the other **39 samples**, the request form matched at least one, but not all pathogens found by PCR. Testing for the identified pathogen by PCR was **not requested** by the clinician in **42.5%** of the specimens. This demonstrates the difficulty in predicting the aetiology of diarrhea and preselecting appropriate assays for pathogen detection based on the clinical presentation.

All samples yielding a positive signal for any **bacterial species** mentioned in table 3 requested a stool culture. In case no isolate could be recovered for *Campylobacter* spp., *Salmonella* spp., *Yesinia* spp. and STEC, the cycle threshold value was greater than 30. However, there were two exceptions to this: one *Yersinia* spp. and one STEC (Ct values between 20 and 30). No bacterial enteropathogens were detected by stool culture in PCR negative samples, so **no enteropathogens were missed by syndromic testing**, indicating its high sensitivity. These findings underline the limitations of stool culture which still is considered as gold standard for detection of bacteria in stool. A few reports showed a higher bacterial yield using PCR compared to bacterial culture, which also implies molecular methods are more sensitive than culture.

As mentioned earlier, a disadvantage of molecular methods is the inability to distinguish between viable and death bacteria. So, the fact that a bacterium is found by PCR but is not confirmed by culture poses a problem. Because all samples were from patients with a clinical picture of gastroenteritis it was assumed that the molecular diagnosis was correct in these cases. Reporting false positive signals cannot be totally excluded. Therefore, it remains important that test results be interpreted against the background of the patient's symptoms, risk profile and history.

Table 3 Stool culture vs. PCR (bacteria)

	FTD positive	Stool culture positive	Stool culture negative
***Campylobacter* spp.**	38	29 (76.0%)	9 (24.0%)
***Salmonella* spp.**	10	9 (90.0%)	1 (10.0%)
***Yersinia* spp.**	11	5 (45.5%)	6 (54.5%)
STEC	11	7*(63.6%)	4 (36.4%)
Total	70	50 (**71.4%**)	20 (28.6%)

*Seven *E. coli* isolates were obtained by culture. Only 3 of these were confirmed as being a *Shiga toxin-producing E. coli* strain. Two samples were negative for Shiga toxins and for two samples no confirmation was received.

Single detection of *C. difficile* was present in 57 stool samples. 17 patients were younger than two years old, 4 were between the age of 3 and 10, and the other 36 patients were older than 35 years. Only three pediatric stool samples were requested for *C. difficile* detection. The clinical relevance of *C. difficile* in young children is unclear and most of these positive pediatric samples can therefore be considered as not clinically relevant. **Mixed infections with *C. difficile*** appeared in 91 stool samples with 81 pediatric patients younger than 3 years and only 10 samples originating from adults. In only 15 samples *C. difficile* was requested, including all 10 adult samples. The most detected concomitant enteropathogens were **adenovirus**, followed by **sapovirus** and **norovirus** genotype II. These data support the fact that *C. difficile* will rarely be the putative causative pathogen in children with diarrhea and that in most cases another co-detected pathogen will be responsible for the symptoms. It is useful to mention that it was decided to not report *C. difficile* in pediatric samples (< 2 years), unless the request form explicitly mentions that *C. difficile* needs to be ruled out.

When delving into the positive viral hits revealed by the FTD multiplex panel, a **single virus** was found in **178** samples with **norovirus** genotype I and II (n=60) being the predominantly identified causative organism, followed by adenovirus (n=44) and sapovirus (n=33), whereas a **mixed infection** of multiple viruses was observed in **143** samples (**65.6%** of all mixed infections).
Astrovirus and sapovirus were identified as single causative enteropathogens in only 22 and 32 cases respectively. In more than half of the samples positive for only adenovirus, norovirus or rotavirus a **viral aetiology was not suspected** by the treating physician (table 4). In respectively 4, 5 and 1 samples the physician did request a test for a viral pathogen but not the right one.

Table 4 Viral aetiology suspected based on clinical presentation when a single viral pathogen was identified by FTD

	Adenovirus (n=44)	Norovirus (n=60)	Rotavirus (n=12)
Clinical suspicion of specific viral aetiology	19/44 (43.2%)	25/60 (41.7%)	5/12 (41.8%)
No viral suspicion	23/44 (52.3%)	30/60 (50.0%)	6/12 (50.0%)

No false positive results were obtained by rapid viral testing. The **sensitivity** of the rapid antigen tests for viral enteropathogens is good, respectively being 86.2%, 97.4 % and 88.2% for adenovirus, norovirus and rotavirus (table 5). Sixty-three rapid antigen tests for rotavirus were not performed as the patient was older than 2 years. Only five of these (7.9%) were positive with Ct values ranging from <20 to >30.

Table 5 Comparison between results acquired by physician-requested testing and FTD (viral)

	FTD positive	Rapid antigen test positive	Rapid antigen test negative	Rapid antigen test not requested
Adenovirus	**187**	107 (**56.1%**)	17 (9.1%)	63 (34.8%)
Norovirus GI	34	11 (32.4%)	1 (2.9%)	22 (**64.7%**)
Norovirus GII	127	64 (**50.4%**)	1 (0.8%)	62 (48.8%)
Norovirus I & II	**161**	75 (46.6%)	2 (1.2%)	84 (**52.2%**)
Rotavirus	**41**	15 (36.6%)	7 (17.1%)*	19 (**46.3%**)
Total	389	197 (**50.6%**)	26 (6.7%)	166 (42.7%)

*Rotavirus rapid antigen test was not performed in 5 cases as the patient was older than 2 years, however 2 of them had a Ct value <20 and 3 had a Ct value >30 (FTD).

All requested combined rapid antigen test for *Cryptosporidium* spp. and *Giardia lamblia* turning positive for the parasite gave a positive signal by PCR, indicating a very high sensitivity of the rapid antigen test (table 6). Only four samples positive for *Cryptosporidium* spp. or *Giardia lamblia* were requested for parasite microscopic evaluation. Microscopic evaluation missed *Cryptosporidium* spp. in two samples (one had a Ct value <20, the other with Ct value between 20-30).

We can conclude that the global agreement of the syndromic panel with immunochromatographic lateral flow tests for adenovirus, norovirus and protozoa was greater than stool culture and the rapid antigen test for rotavirus.

Table 6 Comparison of results acquired by physician-requested testing and FTD (protozoa)

	FTD positive	**Rapid antigen test positive**	**Rapid antigen test negative**	**Rapid antigen test not requested**
***Cryptosporidium* spp.**	36	24* (**66.7%**)	0	11 (30.6%)
Giardia lamblia	15	12 (80.0%)	0	3 (20.0%)
Total	51	36 (**70.6%**)		14 (27.5%)

*One sample was a follow-up sample after therapy initiation. The rapid antigen test was not executed.

When the **conventional microbiological test** procedure was followed **281 pathogens** were detected in **267 positive samples out** of all 1238 samples included. All 50 **bacterial isolates** recovered from stool culture were detected by the syndromic assay. **20 additional positive bacterial specimens** were discovered with the use of the multiplex, although frequently being detected at Ct values >30, except for one *Yesinia* spp. isolate and one STEC were detected at a Ct value between 20 and 30. In **50.8%** of diarrhea due to **a single viral pathogen**, a viral cause was not considered based on clinical presentation. Routine diagnostics for viral enteropathogens was requested in only 57.3% of the cases and yielded a negative result in 11.7% of all requested rapid antigen tests. The performance of the combined *Giardia* and *Cryptosporidium* rapid antigen test seems to have a slightly better performance compared to viral rapid tests**. Importantly, not one samples with a negative syndromic test result yield any additional pathogen using physician-directed testing.**

We conclude that **physician-directed testing** is frequently little more than a guess game. It is an **inadequate approach** to obtain correct diagnosis in patients with gastroenteritis. Not only because of the hurdle to suspect the aetiology correctly based on the clinical presentation, but also as commercial tests are not available for all gastrointestinal pathogens which in turn leads to underdiagnosis. Sapovirus and astrovirus were missed in 22.7% and 10.5% of positive samples, respectively, and detection of especially sapovirus should be considered.
The results acquired by directed testing methods **missed the aetiologic diagnosis** (excl. sapovirus and astrovirus) in **32.3%** of the samples. By using syndromic testing almost **three times as many pathogens** were detected and a twofold more positive samples were obtained compared to request form directed testing. An adequate test profile is rarely requested. In most over-requested samples at least one gastrointestinal pathogen could be determined, but this way of testing is very time consuming, work-intensive and usually redundant. In particular, performing bacterial culture on all feces samples despite the very low positivity rate (4.1%). Although syndromic multiplex PCR testing can sometimes be challenging in terms of interpretation can lead to **erroneous conclusions**, we decided to implement a molecular syndromic test panel as the rapid turnaround time and completeness of the panel is considered more important by the physicians than the possible interpretation difficulties.

QUESTION 2: HOW CAN SEEGENE ALLPLEX GASTROINTESTINAL PANEL RESULTS BE REPORTED IN A SEMI-QUANTITATIVE WAY?

LITERATURE

The utility of multiplex PCR assays, along with the importance of cycle threshold values and the latter's interpretation are indispensable ever since the beginning of the Sars-COV-2 pandemic. A cycle threshold value refers to the number of amplification cycles required to exceed the detection threshold for a positive signal and is inversely proportional to the quantity of copies of the target gene in the sample. This logically means that a higher pathogen load correlates with a lower Ct value. Only reporting whether a person is positive or not is not enough since the magnitude of positivity as viral load correlates with cycle threshold values and the likelihood for experiencing symptoms, but also the contagiousness of the patient so Ct values do have potential utility in providing information regarding genomic load. This information may guide clinicians to distinguish between colonization and infection and the related infection-control decisions (53,54,55).

As far as known, cut-offs have been determined by the National Reference Centrum for the semi-quantitative reporting of Sars-COV-2 ranging from 'very strongly positive' to 'weakly positive' based on the viral load, $\geq 10^7$ copies/mL to $< 10^3$ copies/mL respectively, and isolation measures are based on this reporting (56). Cycle threshold values are not absolute, they can slightly differ among different testing machines or assays and even between different samples from the same patient but are mostly in the same order of magnitude. However, reporting Ct values is controversial in that regard, but an important advantage of reporting Ct values is the rapid traceability of epidemiologic outbreaks. When many low or high Ct values are reported, an outbreak is likely expanding or waning, respectively. Furthermore, information about the Ct value can be informative to flag patients at risk for developing severe disease as large studies found that patients with Ct values of Sars-COV-2 equal to or below 25, were more prone to severe disease and even death (57,58).

Multiplex PCR assays allow a rapid detection of a broad range of pathogens with high sensitivity and specificity. Syndromic panels can be helpful since there is a significant overlap in clinical sings between pathogens causing gastrointestinal disease and not all pathogens were routinely searched for with conventional methods. The main flip side of broad testing is the questionable clinical significance of several targets such as *Clostridioides difficile*, enteropathogenic and enteroaggregative *E. coli* and the interpretation and management of multiple positive targets. In theory, Ct values could also be suitable to differentiate between an asymptomatic carriage and causative pathogen in cases where multiple pathogens are detected, implicating testing for gastrointestinal pathogens is only appropriate when clinical context is suggestive. Broad testing can lead to incidental detection that makes it difficult to predict the clinical relevance of these pathogens found. In order to differentiate between carriage and infection or causative pathogen based on Ct values in case multiple pathogens are detected, it would be helpful to have information about Ct values for different pathogens compared between a symptomatic and an asymptomatic group. If significantly different pathogenic load between both groups is present, this information can be used for interpretating mixed infection. When a low load (high Ct values) of a specific pathogen is observed in the control group, it is possible to consider this pathogen irrelevant (carriage). When detecting a pathogen with a high Ct value (that is known to be present at low loads in the control group) in combination with another pathogen with a low Ct value, the high Ct pathogen can be considered as carriage. It is known that *C. difficile* can be carried asymptomatically in the gastrointestinal tract in 1-5% of adults and 40% of children under the age of one (22,24,59). Also postdiarrheal enteric pathogen carriage can result in a positive result (53,60). The cycle threshold values are correlated to the concentration of pathogenic load (cfr. own validation, question 2). Barely any studies suggesting cycle threshold cut-offs for a semi-quantitative categorical classification based on Ct values were found for the whole spectrum of causative pathogens of gastroenteritis.
The correlation between the **cycle threshold value** and **clinical presentation** and outcomes in pediatric as well as an adult population with gastrointestinal infections was reviewed for the most frequently observed gastrointestinal pathogens. *Clostridioides difficile* was the most common studied pathogen, followed by norovirus and rotavirus. In following section, associations between Ct values and clinical presentation, i.e., symptomatic or asymptomatic and patients with or without diarrhea, will be discussed for viral, bacterial and parasitic pathogens that may play a causative role in diarrhea.

Concerning **viral pathogens**, most studies investigated the difference in Ct values between asymptomatic and symptomatic patients (case-control studies). Significant differences in viral load, and thus cycle threshold values, between asymptomatic and symptomatic patients for **norovirus genotype II and rotavirus**, have been previously reported (30,31,61-64). A difference of approximately 1 log or more, which is equal to 3.32 cycle

thresholds, is observed between the median Ct value of asymptomatic and symptomatic people in lots of studies (table 7). A pathogen-quantity dependent association with diarrhea was present in most case-control studies. According to **Liu et al**. (64) a value of **28.8** was used as a discriminating cut-off for diarrhea caused by **norovirus** in children younger than five years old. For rotavirus a threshold of **26.9** was used. An optimal **cycle threshold cut-off value** for **norovirus** was set on **30 and 33** by **Phillips** et al. (31), for children younger and adults, respectively. According to the same research group, PCR detects additional infections in gastrointestinal diseases at levels correlated with sub-clinical infections and defined **24-27** as an optimal cut-off for **rotavirus** (65). The latter studies are the only one examining an adult population. Another study assessed by Trang et al. determined a **norovirus threshold** value of **21.36** below which diarrhea is most likely caused by norovirus in children < 5 years (66). They observed a bimodal distribution of norovirus Ct values with significantly higher rotavirus co-infection rates at low norovirus genotype II viral loads. No such associations were found for norovirus genotype I (61,62). No significant difference in Ct value was found regarding infectiousness for norovirus and duration of symptoms for norovirus and rotavirus (67). Severe symptoms as severe diarrhea, vomiting and dehydration caused by **rotavirus** were significantly associated with a lower median Ct value (62,68), whereas this association is less pronounced for norovirus genotype II and even less for genotype I. Only two studies found no significant difference in Ct value between both groups (62,69). Norovirus, astrovirus and rotavirus were rarely found in asymptomatic children and the viral load was significantly lower than in samples from children with diarrhea so values below 45 were associated with disease. These viruses can be found in stool of asymptomatic persons. **Lower quantities of viral load** are found **in asymptomatic carriers compared to patients** with diarrhea due to norovirus, astrovirus or rotavirus (30,32,70) and may reflect shedding after a previous (a)symptomatic infection. Two studies did not observe a significant difference in Ct values of astrovirus, adenovirus and sapovirus between patients and controls in contrast with the findings of Liu et al., suggesting a stronger ct-dependent association with diarrhea for **adenovirus and astrovirus rather than sapovirus** in children < 5 years old according to this study, with a maximal discriminating cut-off of **30.2**, **28.1 and 34.1**, respectively (64). **Sapovirus** was sparsely encountered in cases but highly associated with shedding (34). **In contrast to** the statements above, viral load did not seem to discriminate between carriage or disease for none of the viruses in PCR positive subject according to a large case-control study in The Netherlands (71).
A large study in Italy observed the epidemiological pattern of enteric viruses in children hospitalized with acute diarrhea over a period of 11 years. In approximately 50% of all samples (n=4161) at least one viral pathogen was detected, and 167 samples contained 2 or more viruses (8). Rotavirus was often involved in co-infections, primarily with norovirus genotype II. A remarkable correlation was noticed between the viral load of rotavirus and astrovirus in co-infections. **Rotavirus** and **astrovirus** were detected at **lower cycle thresholds**, up to a difference of 9 Ct values, **than the concomitant virus** (primarily norovirus genotype II) in mixed infections. In other words, their viral load is generally higher than the co-infecting norovirus, perhaps mirroring asymptomatic carriage in a pediatric population.

Most studies found significant quantity-dependent associations with diarrhea when investigating the Ct value of **bacterial non-*C. difficile* pathogens** in cases and controls and concluded there is a direct proportional relationship between the bacterial load of **EPEC**, **ETEC-*estA*, EIEC/*Shigella* spp**. **and *Campylobacter* spp.** and severity of symptoms (62,64,70,72). **Vomiting** was mainly associated with *Campylobacter* spp. and ETEC-*estA* and **dehydration** with *Shigella spp.* (62). Results from case-control studies (63,69) indicate a strong **bacterial load dependent** association with diarrhea for **EIEC/*Shigella*** spp. and **ETEC** producing heat-stable toxin (*estA*). A moderate association was found for *Campylobacter* spp. and EPEC by Liu et al. however significantly higher loads were found by Bruijnestein et al. (71) and Kabayiza et al. (62). EPEC load in stool was considered significantly higher associated among children with a single pathogen infection or in children younger than 12 months and the odds of diarrhea increase by each Ct value (= log 10) that decreases but was only moderately associated with diarrhea in another study (64,72,73). Unlike with viral pathogens where there seems to be a correlation between higher viral loads and acute diarrhea, no consistent conclusions can be made concerning the "diarrhea-associated" character of different bacterial pathogens, as contradictory associations were observed across studies for multiple bacterial pathogens, unless for **ETEC-*estA*** and probably ***Shigella* spp**. although the suggested cut-off values range from 22.8 to 31 and 26.1 to 33 for ETEC-*estA* and *Shigella* spp. respectively (table 8) (64,70).

The results of most studies (62,64,70,72) on ***Cryptosporidium*** spp. seem to prove that the **protozoal load** is **significantly higher in cases than controls**. According to a study of Liu et al. (64), this correlation would be even stronger in **children younger than 5 years old**. The same associations are described for *Cyclospora cayentanensis* and *Entamoeba histolytica* and all were more prevalent in symptomatic patients than in healthy controls with a suggested cut-off of 35 (64,70,72), while *Dientamoeba fragilis* and *Blastocystis* species were detected to a

lesser extent in cases compared to controls according to two large studies in The Netherlands, which questions the clinical relevance (72,75). For ***D. fragilis* and *B. hominis*** no correlation between parasitic load and symptoms was found by Haque et al. (case vs. control) (74). There are conflicting results concerning ***Giardia lamblia*** parasitic load and its association with diarrhea (72,74,75). Mixed protozoal infections were associated with a high (>35) Ct value (52).

For all gastroenteric viruses, except for sapovirus, lower Ct values seem to be helpful for defining symptomatic causality and correlates to a great extent to severity of symptoms however some studies publish conflicting results. Clinical utility of Ct values in non-*Clostridioides* bacterial and parasitic gastrointestinal pathogens was less evident, unless for *Shigella* spp. and ETEC-e*stA*. Preliminary data indicate such an association for *Cryptosporidium* spp., but evidence is too scarce to make conclusions for other parasitic gastrointestinal pathogens.

Caution must be taken **when interpretating results of these different studies**. Despite similar trends across different studies, results were inconsistent. Most studies were performed in pediatric populations and in a wide diversity of settings and mostly in non-industrialized countries. Therefore, the clinical impact of Ct values remains to be determined in adult populations and in industrialized countries, but it is highly probable that the same trends and correlations will be observed. The use of pathogenic load measured by PCR can possibly be used to discriminate between carriage and disease, although Ct values cannot be used as an independent marker and subsequently must be interpreted in the clinical context (8,48).

Table 7 Median Ct values of norovirus and adenovirus in symptomatic patients and asymptomatic carriers

	Symptomatic patients	**Asymptomatic patients**
Norovirus (31,61,62-64)	27 (n=104)	34.6
	25.8 (n=51)	29.5
	34 (n=589)	37
	26.4 (n=467)	30.1
	6.85 log copies/mL (n=138)	5.07 log copies/mL
Adenovirus (30,32,62,65,69,70)	18 (n=153)	>37
	10.6 log copies/mL (n=113)	>8.33 log copies/mL
	24.4 (n=19)	>26
	21.16 (n=238)	>23.29
	26.26 (n=103)	>27.34
	17.21 (n=15)	>30.98

DNA/RNA extraction procedures, primers and probes are varying between different PCR assays and so do the Ct values and detection rates between different laboratories. A validated semi quantitative reporting for every single agent in every assay or for each individual laboratory seems a way towards uniformization of results, although some variable factors will remain (e.g. sample manipulation, transport…) (19,70). **Diagnostic stewardship** is recommended in patients with non-specific presentation and high Ct values for pathogens with a low pre-test probability (patients did not appear to have gastroenteritis) in low-prevalence settings as non-reproducible results are likely to occur in this group of patients (76). As the development and validation of multiplex panels may occur in different geographical areas than where the multiplexes are used, DNA variations in local subtypes can cause false negative results, mostly occurring in protozoan species (77). Eibach et al. suggested that a **diagnostic algorithm** for pathogens with a high attributable fraction such as norovirus, rotavirus and *Shigella* spp. based on quantitative PCR could provide more relevant results as co-infections are frequently seen in cases and in control groups and might potentially result in unnecessary therapies (78). Case-control studies must be performed within the population to which the assay will be deployed in order to correctly interpret positive findings (71).

Table 8 Median Ct values of bacterial species in symptomatic patients and asymptomatic carriers

	Median ct value patients	Median ct value controls	p-value
***Campylobacter* spp.**			
Kabayiza et al. (62)	29.75	33.02	**0.007**
Elfving et al. (70)	31.8	33.3	0.12
Bruijnestein et al. (72)	25	35	**< 0.005**
EIEC/*Shigella* spp.			
Kabayiza et al.	30.35	33.99	0.1
Elfving et al.	29.2	34.5	**< 0.0001**
Bruijnestein et al.	24	-	*
EPEC			
Barletta et al.	299 bacteria/mg	29 bacteria/mg	**0.016**
Kabayiza et al.	34.84	35.95	0.05
Bruijnestein et al.	24	32	**< 0.005**
ETEC-*estA*			
Kabayiza et al.	24.75	34.37	**0.04**
Elfving et al.	32.6	37.3	**0.0001**
Bruijnestein et al.	25	34	**< 0.005**
***Salmonella* spp.**			
Kabayiza et al.	41.41	40.70	0.23
Elfving et al.	42.2	40.6	0.22
Bruijnestein et al.	27.5	37	**< 0.005**

**Shigella spp.* was not observed in the control population

DETERMINATION OF VIRAL CYCLE THRESHOLD CUT-OFFS AND ITS IMPACT ON REPORTING RESULTS TO CLINICIANS (AZ DELTA)

2.1 Materials and method

This retrospective study was performed at AZ Delta, Roeselare, Belgium, a 1369-bedsize hospital and Sint-Andries Hospital, Tielt counting 288 beds. Between November 2020 and February 2022, a total of 8442 stool samples from symptomatic patients were sent to the laboratory and were analyzed by the Allplex Gastrointestinal full panel (Seegene, Seoul, South Korea), hereafter Allplex GIP. All samples positive for bacterial pathogens but *E. coli* pathotypes were brought into culture for antibiotic susceptibility testing. We assessed the performance of the Allplex GIP, with particular attention to the technical performance of the viral panel.

The Allplex GIP is a CE-IVD multiplex one-step realtime (RT)-PCR assay that allows simultaneous detection of 13 bacteria, 6 viruses and 6 parasites in four PCR assays (one viral, one parasitic and two bacterial). This panel includes following gastrointestinal pathogens: *Aeromonas* spp. (Aer), *Campylobacter* spp. (Cam), *Clostridioides difficile* toxin B (CdB), *Salmonella* spp. (Sal), *Shigella* spp./EIEC (Sh/EI), *Vibrio* spp. (Vib), *Yersinia enterocolitica* (Yer), EAEC (aggR), EPEC (eaeA), *Escherichia coli* O157 (E. coli O157), ETEC (lt/st), hypervirulent *Clostridioides difficile* (CD hyper), STEC (stx1/2), *Blastocystis hominis* (BH), *Cryptosporidium* spp. (CR), *Cyclospora cayetanensis* (CC), *Dientamoeba fragilis* (DF), *Entamoeba histolytica* (EH), *Giardia lamblia* (GL), adenovirus (AdV), astrovirus (AstV), norovirus GI (NoV-GI), norovirus GII (NoV-GII), rotavirus (RotV) and sapovirus (SV) and provide a quantitative indication by cycle threshold value of the detected enteropathogens.

2.2 Global analysis of our dataset

The data of 8442 stool samples during a period of one year and three months was studied in a retrospective way. **4527** (**53.6%**) samples were found **positive** by Allplex GIP with a total of **7676** pathogens. Figure 2 represents positivity rate for all pathogens. The median age of patients with a positive stool sample was 5.8 years (IQR 1.4-66.5 years), the average age was 29.8 years (figure 3).

A **single pathogen** was detected in **2484** samples (**54.9%**), whereas **co-infections** were detected in **2043** (**45.1%**) samples. A co-infection with 2 and 3 pathogens was found in respectively 1267 (62.0%) and 526 (25.7%) samples, 4 or more pathogens in were observed 249 (12.2%) samples. These numbers are similar to previously published data (48). Out of all samples with a single infection due to *C. difficile*, eighty-nine (39.2%) samples belonged to children under the age of 2 years. The number of each pathogen detected by Seegene Allplex GIP categorized by age is presented in table 9. The **most prevalent species** were *Dientamoeba fragilis* (n=907) and *Blastocystis hominis* (n=893), both with a positivity ratio of 11%, *enteropathogenic E. coli* (n=878, 10%) followed by norovirus genotype II (n=793, 9%) and rotavirus (n=763, 8%). No positive samples for *Entamoeba histolytica* and *Cyclospora cayetanensis* were acquired.

An equal distribution was observed in the aetiology of diarrhea, with a slight lean towards bacterial pathogens due to the inclusion of *C. difficile*. *Blastocystis hominis* (n=448), *Dientamoeba fragilis* (n=302) and rotavirus (n=261) were the pathogens mostly found in **mono-infections**. Most common **co-infecting pathogens** were enteropathogenic *E. coli*, norovirus genotype II and *Dientamoeba fragilis* (table 9). In **441 samples viral co-infections** were observed, 290 of these included norovirus genotype II and 272 rotavirus. The diarrheagenic *E. coli* subtypes (EAEC and EPEC) are detected together in 106 samples (27.0% of EAEC samples, 16.6% of EPEC samples). *Giardia lamblia* was the parasite most frequently associated with co-infections relative to its involvement in mono-infections.

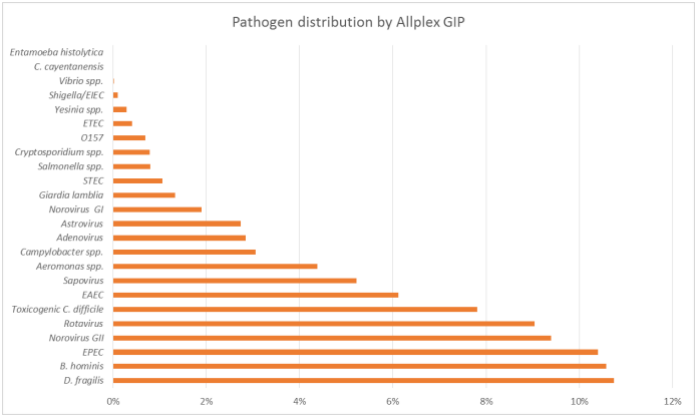


Figure 2 Pathogen distribution by Allplex GIP

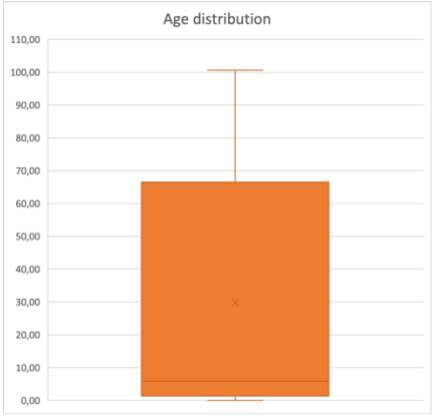


Figure 3 Age distribution of positive samples

		Total no./no. associated with co-infections						
Pathogen	No (pos. ratio)	No. associated with co-infections (%)	< 1 year (n=748)	1-5 years (n=1529)	6-12 years (n=274)	13-20 years (n=109)	21-60 years (n=577)	> 60 years (n=1290)
Bacteria	**3067 (36.6%)**							
Aeromonas spp.	470 (5.6%)	331 (**70.4%**)	136/109	126/109	15/13	8/4	50/29	135/67
Campylobacter spp.	258 (3.1%)	152 (65.5%)	19/16	54/47	13/9	19/10	65/27	88/46
Clostridioides difficile	658* (7.8%)	431 (65.5%)	270/206	184/161	0/0	2/1	45/19	156/42
E. coli								
EAEC	517 (6.1%)	393 (**76.0%**)	111/96	280/241	7/5	5/3	38/24	76/27
EPEC	878 (10.4%)	638 (**72.7%**)	159/137	342/295	33/24	10/8	115/73	219/111
ETEC	35 (0.4%)	21 (60.0%)	3/3	8/8	2/2	0/0	8/5	14/4
STEC	90 (1.1%)	70 (77.8%)	8/8	16/14	5/5	5/3	16/12	40/31
O157**	59 (0.7%)	52 (88.1%)	10/8	12/12	2/2	3/3	9/7	24/22
EIEC/*Shigella* spp.	9 (0.1%)	5 (55.6%)	0/0	1/0	0/0	2/1	6/4	0/0
Salmonella spp.	68 (0.8%)	31 (45.6%)	11/5	19/10	12/5	5/2	6/5	15/4
Yersinia spp.	25 (0.3%)	14 (56.0%)	1/1	7/4	0/0	0/0	3/2	14/8
Vibrio spp.	2 (0.0%)	2 (100%)	0/0	1/1	0/0	0/0	1/1	0/0
Viruses	**2628 (31.1%)**							
Adenovirus	240 (2.8%)	194 (**80.8%**)	86/68	129/107	4/4	3/3	8/7	10/7
Astrovirus	231 (2.7%)	172 (74.5%)	65/50	137/110	4/0	1/1	13/9	11/5
Norovirus genotype I	160 (1.9%)	127 (**79.4%**)	26/25	86/77	5/5	3/2	9/7	31/11
Norovirus genotype II	793 (9.4%)	571 (**72.0%**)	220/173	403/306	20/15	10/6	69/36	71/35
Rotavirus	763 (9.0%)	497 (65.1%)	161/105	335/284	2/2	25/23	55/31	165/57
Sapovirus	441 (5.2%)	292 (66.2%)	140/100	268/179	9/6	1/0	15/5	8/3
Parasites	**1979 (23.4%)**							
Blastocystis hominis	893 (10.6%)	438 (49.0%)	4/4	101/92	89/68	48/31	185/96	466/154
Cyclospora cayentanensis	0 (0.0%)							
Cryptosporidium spp.	67 (0.8%)	44 (65.7%)	3/2	38/28	8/5	3/1	13/7	2/1
Dientamoeba fragilis	907 (10.7%)	594 (65.5%)	18/10	449/331	176/107	53/34	101/57	110/66
Entamoeba histolytica	0 (0.0%)							
Giardia lamblia	112 (1.3%)	77 (68.8%)	3/3	48/43	9/7	2/1	24/11	26/12

*In 2 cases of CdB detection, also CD 027 was detected

**18/59 positive O157 samples were also positive for STEC with approximately the same Ct value. 15/59 had a Ct value between 30-40 and 10/59 had a Ct value more than 40

Norovirus has the highest positivity rate among viruses, counting for 11.3% including 793 cases of norovirus genotype II and 160 cases of norovirus genotype I, followed by rotavirus (5.9%) and sapovirus (5.2%). Most of all **viruses** (78.2%) were detected in children of **5 years** and younger. The positivity ratio of sapovirus, adenovirus and astrovirus was significantly higher in children ≤5 years (resp. 17.9%, 9.4% and 8.8%) compared to patients older than 21 years (resp. 0.1%, 0.96% and 1.2%).
Concerning bacterial pathogens, more enteroaggregative and enteropathogenic *E. coli* were found in samples of children ≤5 years old (resp. 17.2% vs. 6.1% and 22.0% vs. 17.9%). ***Campylobacter*** spp. and ***Yersinia*** spp. were detected mostly samples from patients older than **21 years**, counting for respectively 8.2% and 0.9% (compared to 3.2% and 0.3% in children ≤5 years). *C. difficile* was detected in 19.9% of the children and in 10.7% of the adult population. **Diarrheagenic *E. coli* pathotypes** – EAEC, ETEC and EPEC – were frequently detected as an aggregated group in patients **younger than 5 years** old (63.1% of the diarrheagenic *E. coli pathotypes*).
Two-fold more samples containing *Dientamoeba fragilis* were from children ≤5 years old (20.5% vs. 11.3%), whereas *Giardia lamblia* was roughly equally present in populations ≤5 years and >21 years old. ***Blastocystis hominis*** was most frequent in patients **older than 21 years** (34.9% vs. 4.6%).

The bacterial pathogens identified by PCR were isolated in only 49.8% of all **reflex bacterial cultures**. For *Aeromonas* spp. a yield of 30.2% was reached by stool culture, for *Yersinia* spp., *Salmonella* spp. and *Campylobacter* spp. respectively 56.0%, 91.2% and 73.6%. The proportion of non-cultivable ***Aeromonas*** spp. (328/470) and ***Salmonella*** spp. (5/68) increased with the Ct value, starting at a value of 31 which equals a very low number of bacteria. It is remarkable that ***Salmonella* spp**. are most frequently **observed in mono-infections** and that they are cultivable in 92.6% of the cases indicating its pathogenicity independent of the Ct value. All positive ***Yersinia*** spp. with a Ct value <35 were cultivable (5/5), whereas all 11 negative cultures had a Ct value greater than 35 (11/20). However 9 samples were cultivable even with a Ct value of >40. ***Campylobacter*** spp. was not cultivable in 26.4% of the cases, but the remarkable thing is that not all negative cultures were associated with a higher Ct value; 37 out of all 68 culture-negative samples had a Ct value lower than 35.

In this appraisal we will zoom in further on the <u>viral enteropathogens</u>.

<u>2.3 Determination of viral cut-offs</u>

In order to determine cut-offs for viral enteropathogens, median Ct values of mono- and co-infections and median Ct values of two different populations, ≤5 years old and >21 years old were compared. If these median Ct values and distributions are comparable, it is justified to consider all obtained results as one group, independent of age and number of positive targets. In **attachment 1** box plots are illustrated for each virus and for the age groups ≤5 years and >21 years.
Table 10 shows the **median Ct values** of the different viruses in mono-infections and mixed infections and in both patient populations. Patients older than 6 years and younger than 21 were considered irrelevant as diarrhea occurs only in a very small proportion of this population. A **first relevant observation** from table 10 is the clearly lower median Ct value for norovirus genotype I in single infections compared to co-infections. A **second observation** is the difference in median Ct value for adenovirus between both age groups. In patients older than 21 years the Ct values for adenovirus seem to be significantly higher than in patients with the age of 5 years or younger. When digging deeper into detail, 14/18 samples from patients >21 years old were co-infected samples. 11/14 were co-infected with a pathogen with a Ct value <30 (ranging from 9.4 to 29.24), most of these being another virus. 4/18 patients had a mono-infection with adenovirus, but only one with a Ct value <35 (13.68). This may indicate that adenovirus is less pathogenic in adults than in children.

The histograms presented in **attachment 2** do not take the expected shape with a decrease in number of hits at high Ct values. This observation was prominently present for adenovirus, norovirus GI and GII and rotavirus. An upward shape is observed starting at a Ct value of 34 with appearing new higher peaks which is not an expected pattern for symptomatic patients. Another remarkable thing is the **difference in the range of Ct values** at which viruses are present (figure 4). For example, the lowest observed Ct value for adenovirus is 7.03, while for norovirus GII 12.04 is measured as lowest Ct value and 15.34 for sapovirus. **Adenovirus, astrovirus and rotavirus** are detected at **remarkably lower Ct values** than norovirus GI and GII and sapovirus.

Table 10 Median Ct values of different viruses in both single and co-infections and in different patient populations

	Median Ct value single infection	Median Ct value co-infection	Median Ct value ≤ 5 years	Median Ct value >21 years
Adenovirus	16.89	19.71	**16.45**	**34.58**
Astrovirus	18.47	18.62	17.63	21.21
Norovirus				
Genotype I	**25.45**	**36.67**	36.04	29.47
Genotype II	**21.96**	**26.00**	24.35	28.21
Rotavirus	16.43	15.18	15.33	16.75
Sapovirus	24.46	25.63	24.90	28.01

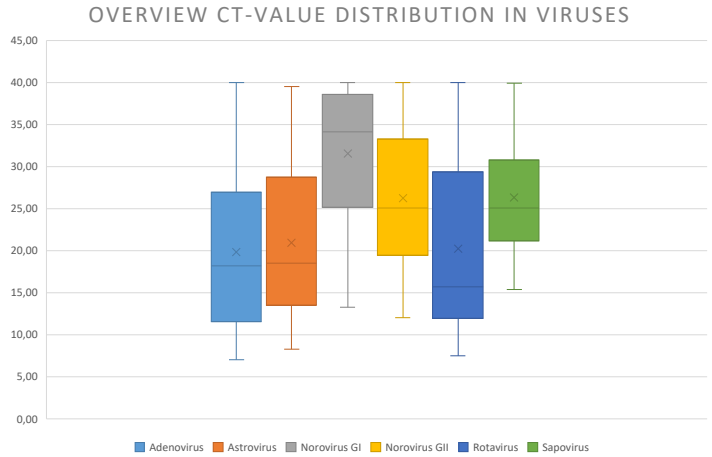


Figure 4 An overview of the distribution of Ct values for different viral pathogens

As demonstrated in '*Question 1'* multiplex panels yield more pathogens in a very sensitive assay compared to physician-based requesting and conventional laboratory testing but they pose a problem as most panels available report qualitative data, e.g., positive or negative. Seegene is capable of reporting quantitative results of each pathogen by Ct values. Either quantitative or qualitative reporting can cause troubles concerning interpretation and numbers of **intra assay** (extraction and amplification efficiency) and **extra assay** (nucleotide degeneration in the stool sample) factors can affect the Ct value. Reporting a qualitative result on the lab report can lead to medication overuse or unnecessary isolation measurements and that's not an improvement compared to physician-based requesting. Other options could be reporting concentrations as 'copies/mL' or Ct values however this approach poses the same issues regarding interpretation: *what does this concentration/Ct value mean? Is it high? Can this load correlate to the clinical presentation?* etc. Clinicians do not know how to interpret qualitative results and frequently ask *how* positive a result is and whether it is clinically relevant or not, so **we considered a first step to harmonization of gastrointestinal viral pathogen reporting in a semi-quantitative way by means of a relative reporting to a large symptomatic population** (n=8442).

Looking at samples positive for at least one viral target (2060), regardless of the presence or absence of a bacterial/protozoal agent, **norovirus genotype II and rotavirus are the most frequently detected viral pathogens**, subsequently followed by sapovirus, adenovirus, astrovirus and norovirus genotype I.
All samples were sorted by virus and divided into groups according to the Ct value: <10, 10-20, 20-30, 30-35 and >35. In table 11 and figure 5 an overview can be found. We observed a lot of samples with viral co-infections at high Ct value ranges. A virus with a high Ct value (>35) was frequently accompanied by another pathogen (mostly a virus) with a lower Ct value (<35).

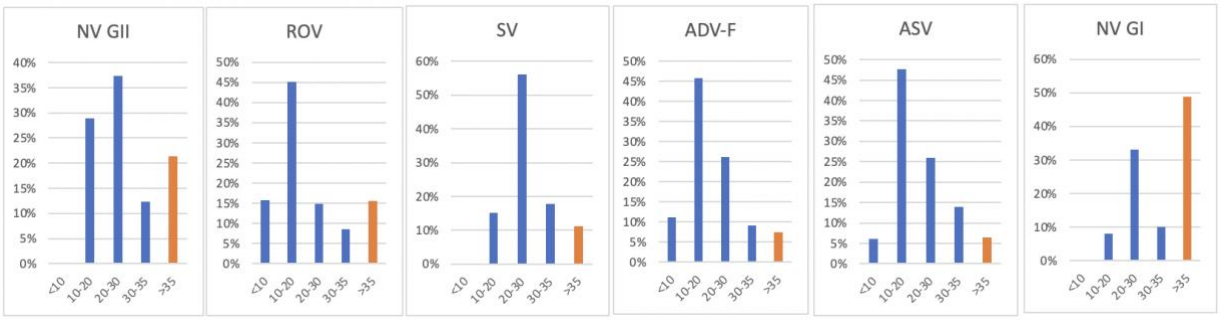


Figure 5 Grouped distributions according to Ct values

Table 11 Grouped distributions according to Ct values (numeric)

	NV GII		ROV		SV		ADV-F		ASV		NV GI	
<10	0	0.0%	120	15.7%	0	0,0%	27	11.3%	14	6.1%	0	0.0%
10-20	229	28.9%	345	45.2%	67	15.2%	110	45.8%	110	47.6%	13	8.1%
20-30	296	37.3%	113	14.8%	247	56.0%	63	26.3%	60	25.9%	53	33.1%
30-35	98	12.4%	66	8.7%	78	17.7%	22	9.2%	32	13.8%	16	10.0%
>35	170	21.4%	119	15.6%	49	11.2%	18	7.5%	15	6.5%	78	48.8%
No other pathogen	29	**3.7%**	36	**4.72%**	12	**2.7%**	5	**2.1%**	1	**0.4%**	2	**1.3%**

It is clear that very large numbers with a Ct value >35 were detected, which does not correspond to clinically relevant Ct values according to literature. All results with a value greater than 35 were analyzed individually. The results for which another virus was detected with a Ct value <35 (ranging from Ct 8 to Ct 34.9) were erased so that only the results positive for that particular virus with a high Ct value were left (presumably the very weak positives). Those remaining numbers are shown in orange in the lower graphs (figure 6). This trend in numbers of strong signals compared to the number of weak signals, much more following a gaussian distribution, correlates better to clinical importance. A negligible percentage of samples with a single positive viral pathogen remained of uncertain importance. All viral pathogens previously observed at Ct values are now considered as falsely positive. False positive results are described in other studies when multiple viral pathogens are detected but no cause was found (8,79).

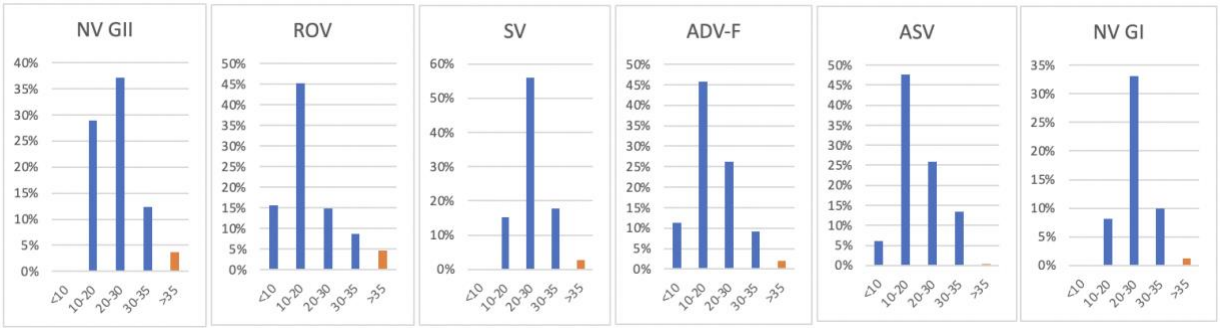


Figure 6 Grouped distributions according to Ct values but after exclusion of viral pathogens with Ct value >35 associated with another pathogen with lower Ct values

We determined Ct 35 as out cut-off and decided to report all Ct values greater than 35 for viral pathogens as negative. The cut-off of 35 and semi-quantitative reporting leads to a better and more interpretable lab report with less positive targets to be assessed by the clinician. **The <u>first reason</u> for this cut-off value** is, as above mentioned, that Ct values >35 are very frequently detected in the presence of another pathogen (mostly a virus) with a significant lower Ct value. This results, for the example in NV G1 and NV GII, that approximately 48.8% and 21.4% of all NV G1 and NV GII results will no longer be reported (table 11). The flipside of this cut-off is that a few samples will be missed and will be reported falsely negative, however the clinical significance of such low viral loads are questionable. As Ct values >35 are considered to be of questionable clinical relevance, *Boers* et al. showed the overall agreement of two multiplex panels increased from 91% up to 95% by excluding discordant results with a Ct value >35 (80). **The <u>second reason</u> is the observation and confirmation of false positive results frequently occurring in Ct values >35** (see infra). **The <u>last reason</u> is that detecting Ct values higher**

than 35 is technically less likely to be correct (and it's clear that Seegene is trying to avoid a certain cut-off by adjusting the detection threshold line).

Cut-offs were determined for each viral agent in order to report results in semi-quantitative way. We used the dataset of all viruses with a Ct value <35. Subsequently interquartile ranges were determined for each agent and those were used as values to distinguish between 'weak positive' (>p75), 'positive' (p50-p75), 'strong positive' (p24-p50) and 'very strong positive' (p<25) (table 12 and 13).

Table 12 Interquartile ranges

	25th percentile	50th percentile	75th percentile
Norovirus GII	18.31	22.14	27.52
Norovirus GI	21.03	25.50	29.24
Adenovirus	11.12	16.34	23.84
Astrovirus	13.39	17.12	27.71
Rotavirus	10.94	14.36	21.26
Sapovirus	20.91	24.25	23.84

Table 13 Cut-offs for semi-quantitative reporting base on interquartile ranges mentioned in table 12

	Very strong positive	Strong positive	Positive	Weak positive	Negative
Norovirus GII	<18	≥18 en <22	≥22 en <28	≥28 en < 35	≥35
Norovirus GI	<21	≥21 en <26	≥26 en <29	≥29 en <35	≥35
Adenovirus	<11	≥11 en <16	≥16 en <29	≥29 en <35	≥35
Astrovirus	<14	≥14 en <17	≥17 en <28	≥28 en <35	≥35
Rotavirus	<11	≥11 en <14	≥14 en <21	≥21 en <35	≥35
Sapovirus	<21	≥21 en <24	≥24 en <29	≥29 en <35	≥35

2.4 Performance of the Seegene Allplex Gastrointestinal Panel

Analyzing the raw data of a few random samples revealed the same phenomenon as in previous section, namely that a lot of mixed infections are viral co-infections. In this section 5 samples that demonstrates us something will be discussed (figure 7). The first sample is positive for adenovirus (Ct 39.97), norovirus genotype I (Ct 39.89) and **rotavirus** (Ct 8.09). The second sample is positive for norovirus genotype I and II (Ct 39.51 and 39.52), sapovirus (Ct 30.98) and **rotavirus** (Ct 9.51). The third sample is positive for **sapovirus** (Ct 20.99), norovirus genotype I (Ct 37.75) and *Clostridioides difficile* (Ct 30.58). The fourth sample is positive for norovirus genotype II (Ct 35.43), **rotavirus** (ct 14.22) and *Clostridioides difficile* (Ct 40.90). The fifth sample is positive for **norovirus genotype I** and II (Ct 17.02 and 37.77) and rotavirus (Ct 38.97). These five co-infected samples with a suspicion of false positivity (marked in red) due to the presence of another parameter that is strongly positive were sent to AZ Sint-Jan Brugge for comparative research with a sensitive single-plex PCR (GI-TAC assay) (figure 7). **In several cases, it is confirmed that false positive results are generated in the presence of another strongly positive target.** In the first sample for example ROV was detected but ADV-F and NVGI were not by the GI-TAC assay. The same observations were found for sample 3, 4 and 5. To further investigate this problem dilution series of (1) cultivable bacteria, (2) strongly positive samples and (3) bought standardized viral DNA were made and analyzed by Seegene's Allplex panel.

Well	Name	Type	FAM	C(t)	FAM	C(t)	HEX	C(t)	Cal Red 610	C(t)	Cal Red 610	C(t)	Quasar 670	C(t)	Quasar 670	C(t)	HEX	C(t)	Auto Interpretation
A01	2051611801	SAMPLE	ASV	C(t)	NVG2	C(t)	ADV-F	C(t)	SV	C(t)	NVG1	C(t)	ROV	C(t)		C(t)	IC	C(t)	"ADV-F,NVG1,ROV"
			-	N/A	-	N/A	+	"39,97"	-	N/A	+	"39,89"	+	"8,09"			+	"26,89"	
A06	2051611801		Sh/EI	C(t)	Cam	C(t)	Yer	C(t)	Vib	C(t)	CdB	C(t)	Aer	C(t)	Sal	C(t)	IC	C(t)	
			-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	"31,16"	
B02	2051981301	SAMPLE	ASV	C(t)	NVG2	C(t)	ADV-F	C(t)	SV	C(t)	NVG1	C(t)	ROV	C(t)		C(t)	IC	C(t)	"NVG2,SV,NVG1,ROV"
			-	N/A	+	"39,52"	-	N/A	+	"30,98"	+	"39,51"	+	"9,51"			+	"26,75"	
B07	2051981301		Sh/EI	C(t)	Cam	C(t)	Yer	C(t)	Vib	C(t)	CdB	C(t)	Aer	C(t)	Sal	C(t)	IC	C(t)	
			-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	"31,47"	
C01	2051794701	SAMPLE	ASV	C(t)	NVG2	C(t)	ADV-F	C(t)	SV	C(t)	NVG1	C(t)	ROV	C(t)		C(t)	IC	C(t)	"SV,NVG1,CdB"
			-	N/A	-	N/A	-	N/A	+	"20,99"	+	"37,75"	-	N/A			+	"28,27"	
C06	2051794701		Sh/EI	C(t)	Cam	C(t)	Yer	C(t)	Vib	C(t)	CdB	C(t)	Aer	C(t)	Sal	C(t)	IC	C(t)	
			-	N/A	-	N/A	-	N/A	-	N/A	+	"30,58"	-	N/A	-	N/A	+	"30,59"	
D02	2052037301	SAMPLE	ASV	C(t)	NVG2	C(t)	ADV-F	C(t)	SV	C(t)	NVG1	C(t)	ROV	C(t)		C(t)	IC	C(t)	"NVG2,ROV,CdB"
			-	N/A	+	"35,43"	-	N/A	-	N/A	-	N/A	+	"14,22"			+	"26,71"	
D07	2052037301		Sh/EI	C(t)	Cam	C(t)	Yer	C(t)	Vib	C(t)	CdB	C(t)	Aer	C(t)	Sal	C(t)	IC	C(t)	
			-	N/A	-	N/A	-	N/A	-	N/A	+	"40,90"	-	N/A	-	N/A	+	"30,86"	
G03	2052129501	SAMPLE	ASV	C(t)	NVG2	C(t)	ADV-F	C(t)	SV	C(t)	NVG1	C(t)	ROV	C(t)		C(t)	IC	C(t)	"NVG2,NVG1,ROV"
			-	N/A	+	"37,77"	-	N/A	-	N/A	+	"17,02"	+	"38,97"			+	"27,30"	
G08	2052129501		Sh/EI	C(t)	Cam	C(t)	Yer	C(t)	Vib	C(t)	CdB	C(t)	Aer	C(t)	Sal	C(t)	IC	C(t)	
			-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	"32,06"	

Figure 7 Raw data of six samples. Five samples are co-infections with multiple viruses with a suspicion of false positive results (marked in red in the column 'auto-interpretation'.

(1) Cultivable bacteria from pure cultures were brought into cultures. A 0.5 McF (1.5 x 10^8 CFU/mL) suspension in physiologic sterile water (Mini Plasco 0.9% NaCl) was made for each bacterium and this suspension serves as the basis for further dilutions. These data are not shown in this CAT. All following targets of the Allplex GI-Bacteria (I) and (II) assay are functional: stx1/2, eaeA, O157, Sh/EI, Cam, Yer, Vib, CdB, Aer and Sal. No cross-reactivity was observed with parasitic pathogens (Allplex GI-Parasite assay) and a one-time false positive viral (NVGII) was received (Ct 39.05) (Allplex GI-Virus assay). Following targets were not tested as no germs were available: lt/st, aggR and CDhyp. Ct values vary from 17.25 to 30.93 and these fluctuations can be relied on the efficiency of the PCR, cycle threshold settings and multi-copy genes (table 14).

Table 14 Ct values of the original 0.5 McF dilutions

	Ct value
C. difficile	25,49
Campylobacter coli	24,37
Campylobacter jejuni	17,25
VTEC	23,40
Vibrio cholerae	21,7
Aeromonas hydrophila	26,52
***Aeromonas* species**	30,93
Salmonella newport	29,56
Salmonella enteritidis	26,28
Salmonella typhimurium	26,11
***Shigella* species**	29,26
Yersinia enterica	25,09

To evaluate the attribution of high concentration sodium chloride in Mini Plasco 0.9%, two different dilution series of 1/10 (1 log) were made of the above-mentioned pathogens; one in 0.9% NaCl, another in e-NAT medium. The dilution series were brought into culture and the colony forming units were counted (see attachment 3). The detection rate is higher in more diluted samples when diluted in **e-NAT** rather than in 0.9% NaCl, so our assumption of NaCl inhibiting the extraction can be stated as correct. The limit of detection for PCR is mainly 10 to 1000x higher compared to culture (suspension in physiological water) except for *Salmonella* spp. diagnostics where an enrichment medium is used. When counting back to how many CFU/mL should be present in the 0.5 McF suspension for each pathogen, most pathogens fulfil the criterium of a maximum deviation of 1.5x10^8 +/- 1 log (attachment 3), except for *C. difficile*, *Aeromonas* spp and *Yersinia* spp. *Vibrio* spp. was uncultivable.

The **efficiency of the PCR** based on Ct values given by either Seegene viewer or the CFX was evaluated. The efficiency of the PCR determined based on the Ct values generated by the Seegene viewer is significantly lower than that determined using the auto baseline method in the **CFX**. A reduction in 1 log should be equal to a difference in 3.32 Ct values (= the slope) in case of a 100% efficient PCR and this premise is nearly reached with the CFX. Slopes ranged from 2.52 to 4.10 for Ct values interpreted by Seegene viewer and from 2.97 to 3.71 for CFX, this corresponds to an efficiency (%) ranging from **-149.09** to **-75.32** and **-117.29** to **-86.17**, respectively. **This means that the Seegene viewer artificially raises the cycle threshold cut-off for every pathogen individually compared to the auto baseline method in the CFX. The PCR assay as such is not that bad but the interpretation of these values is manipulated by Seegene viewer.** This sometimes results in very high Ct values for the bacterial parameters without the suspicion of false positivity. The regression analysis of both Seegene viewer and CFX are shown in figure 8 and 9.

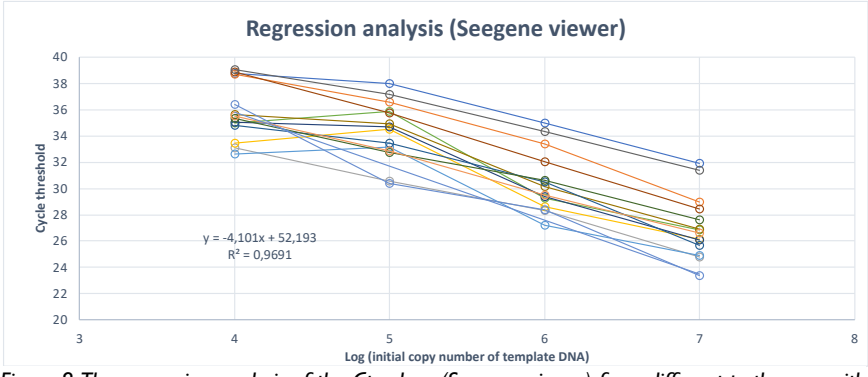


Figure 8 The regression analysis of the Ct values (Seegene viewer) from different pathogens with the log 10 dilutions of the DNA template showing the linearity and the efficiency of the PCR assay.

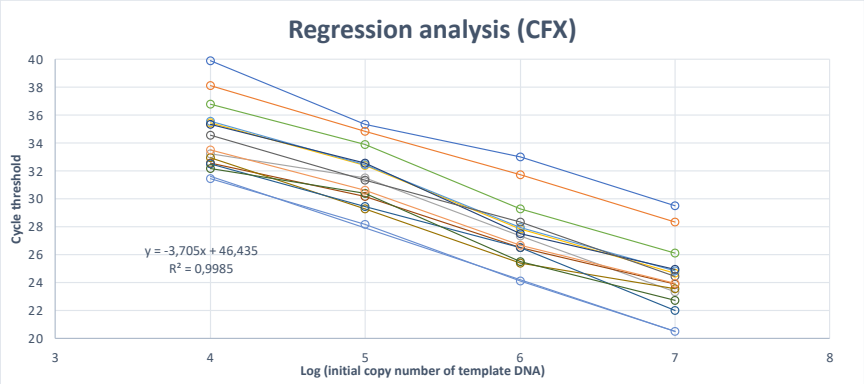


Figure 9 The regression analysis of the Ct values (CFX) from different pathogens with the log 10 dilutions of the DNA template showing the linearity and the efficiency of the PCR assay.

(2) Eight samples were selected and hypothetically Ct values were calculated reliant on the start Ct value; an increase of 1.66 Ct is presumed per 0.5 log dilution (as 1 log = 3.32). Every single virus was present at low Ct values (as a mono-infection) in one of the samples, but by exception three samples were selected for rotavirus. All eight samples and their dilution series were once again analyzed by Seegene Allplex Gastrointestinal Panel. The efficiency for Ct values reported by the Seegene viewer ranged from **87.19%** to **121.62%** for viral pathogens. An **inverse linear correlation between the viral load and Ct value** could be demonstrated (figure 10).
We observed **additional positive results** that were not observed in the initial run of the samples. The sample containing astrovirus (Ct 9.74) gave a positive result for **norovirus GII** (Ct 39.65) and **rotavirus** (Ct 38.81) but only in the first dilution (-0.5 log), similarly **sapovirus** (Ct 37.91) was detected in a sample containing adenovirus (Ct 10.68). **Norovirus GII** (Ct 38.38) was detected in a sample with rotavirus (Ct 13.29) ONLY in the fifth dilution, although the other blue (NVGII) and red curves (NVGI) were not detected as 'positive pathogen' by Seegene viewer (figure 11). This observation advocates false positive results as no norovirus GII was observed in previous dilutions. These results are presumably attributable to **non-specific amplification**, but all those false positive results were reported as 'positive' by Seegene viewer.

Another dilution series of a sample containing astrovirus in high viral loads showed following results: the first three dilutions showed the presence of norovirus GII and GI, sapovirus and rotavirus next to astrovirus in Ct values ranging from 38.99 to 44.62 but **only** one additionally found **rotavirus** (ct 39.58) was **reported** by Seegene viewer according to the cut-offs that were set by Seegene viewer itself. Figures 12 shows the 'co-existence' of all those viruses. It is suspicious and inexplicable that only one out of 12 additionally 'found' targets were reported by Seegene. **This confirms the above-mentioned observation that Seegene itself choses a random cut-off for each pathogen individually** as ROV was reported at Ct 39.58, but NVGI and SV were not at Ct 39.06 and Ct 39.12 respectively. **Throughout the validation course it is remarkable that especially norovirus genotype II emerges in high ct-ranges (>35) whenever especially another pathogen is present at high viral loads.** ASV and NVGII are detected in FAM, ADV in Hex 5, SV and NVGI in calred 610 and ROV in quasar67, so the co-existent false positive results are not only related to the channel in which they are detected but can also be the result of overlapping melting curves.

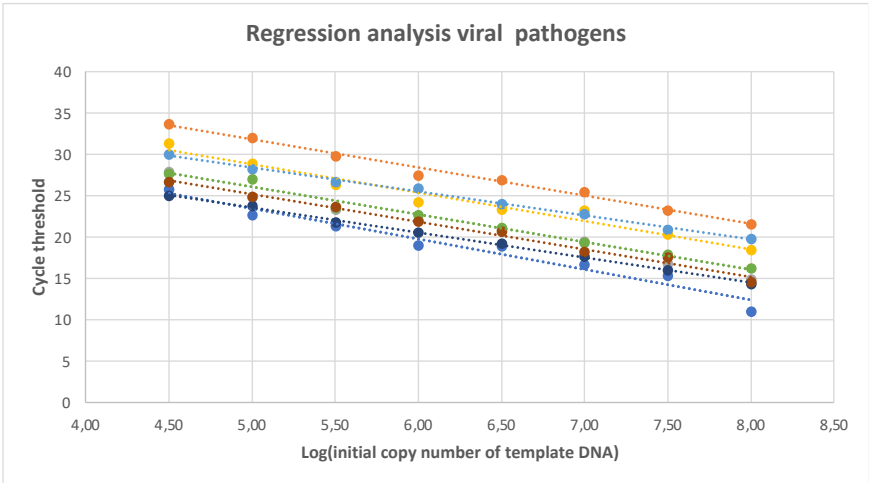


Figure 10 The regression analysis of the Ct values (Seegene vieuwer) from different viral pathogens with the 0,5 log dilutions of the DNA template showing the linearity and the efficiency of the PCR assay.

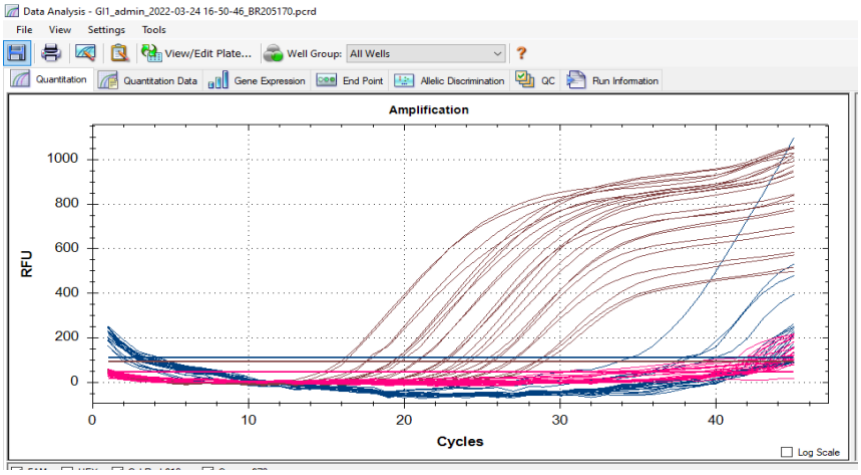


Figure 11 Curves obtained by Seegene viewer for a sample only containing rotavirus. Blue lines are norovirus genotype II, red ones are norovirus genotype I

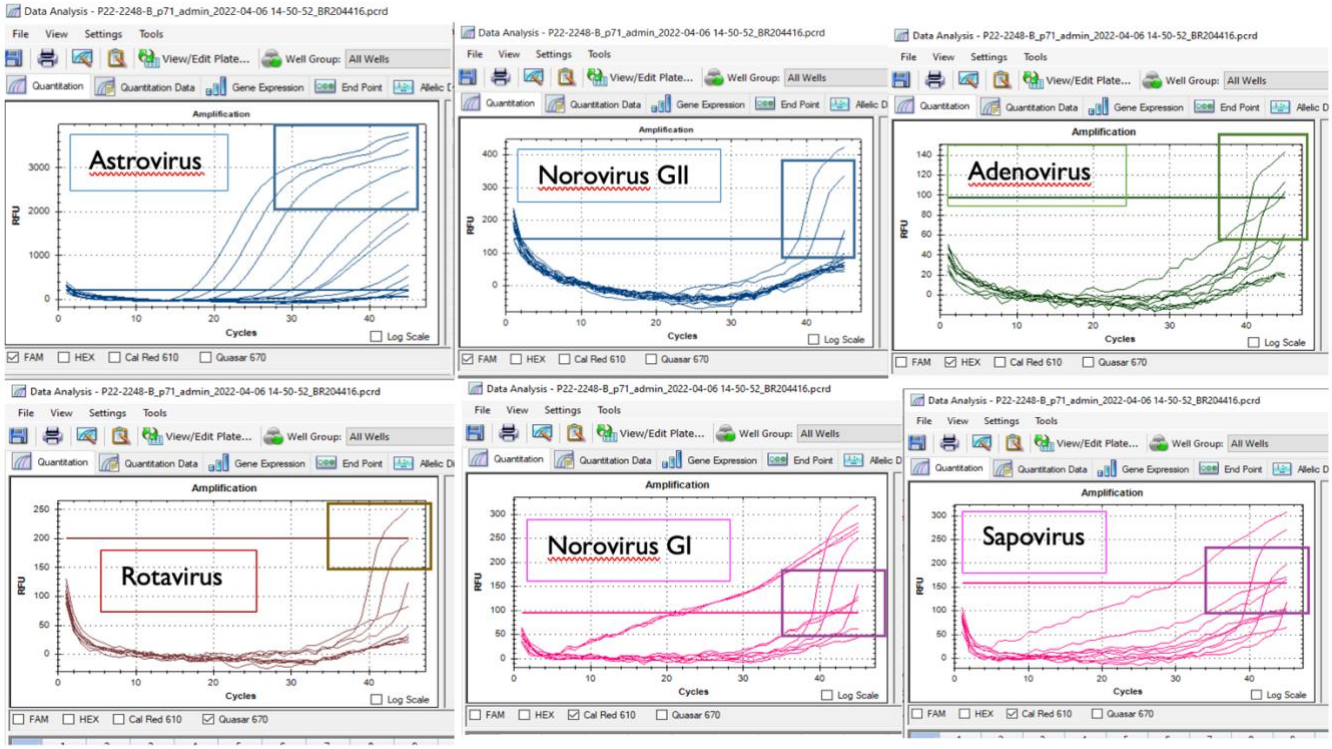


Figure 12 Co-detection of different viruses in a dilution series of a sample containing high viral loads of astrovirus. The detection of norovirus GII, adenovirus, rotavirus, norovirus GI and sapovirus are considered to be false positives.

(3) Vircell rotavirus was bought (purified RNA of rotavirus) and a 10 log dilution series was made. A false positive result for *Aeromonas* spp. (Ct value 41.23) emerged only at dilution 8. False positive results of bacterial pathogens are further not discussed as reflex culture is done, which is helpful in further interpretation.

Physician-based directed testing shows to be **inappropriate** for diagnosing gastroenteritis most of the time. Many pathogens are missed because they are not suspected as possible causative agents based on clinical presentation. There is a high workload and the turnaround time is usually high, certainly in case bacterial culture is requested. Syndromic multiplex PCR testing is a highly sensitive, fast and cost-efficient alternative method. Syndromic testing is a broad way of testing and a lot more pathogens can be found compared to conventional testing, leading to **higher positivity rates**. PCR has been considered error-free so far, but we show that Seegene can **occasionally generate false positive results for viral parameters**. False positive results of bacteria and parasites are not discussed in this CAT. Multiplex assays pose a problem concerning the interpretation of positive pathogens if quantitative results are reported. This quantitative reporting complicates the interpretation when multiple positive pathogens are found. We aimed for a more interpretable lab report by reducing the number of positive targets in one sample by setting a cut-off and reporting the positive pathogens semi-quantitatively instead of qualitative.

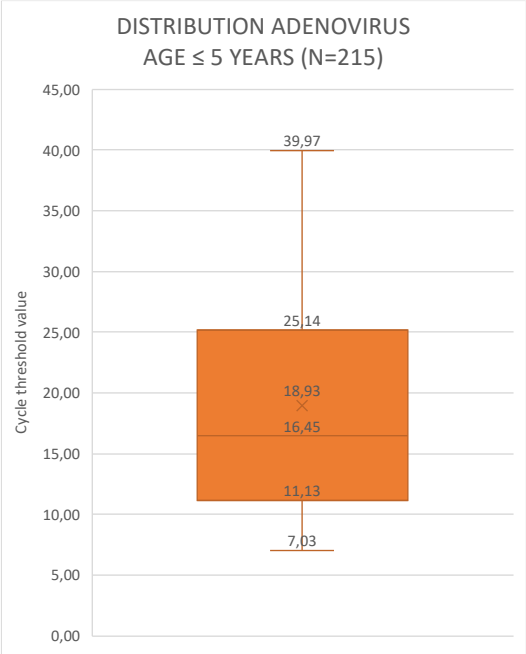
In the absence of an asymptomatic control population, we used a very large population of patients with gastroenteritis. So relative reporting to this large population allowed us to determine what is 'weak positive' and what is 'strong positive' in patients with gastroenteritis. A Ct value of 35 was used as cut-off as viral pathogens with a Ct value higher than 35 were mostly co-detected with another viral pathogen with a low Ct value.
We **cannot prove a causal relationship** since we only have Ct values in a population with gastroenteritis, but Ct values can be used as a proxy of probability. We cannot say that a certain Ct value stands for causality of symptoms but we can say that a certain Ct value is high or low in a population with gastroenteritis, so that the pathogen is likely or less likely to be the causative pathogen. **This is especially an interesting concept in co-infections**. We demonstrated that a high Ct value excludes a pathogen as causative pathogen with a high probability when in presence of another pathogen with a low Ct value. The ideal scenario would be to have an asymptomatic population to compare Ct values to explore causative relationships and to set a cut-off for all pathogens individually in order to discriminate between infection and colonization.

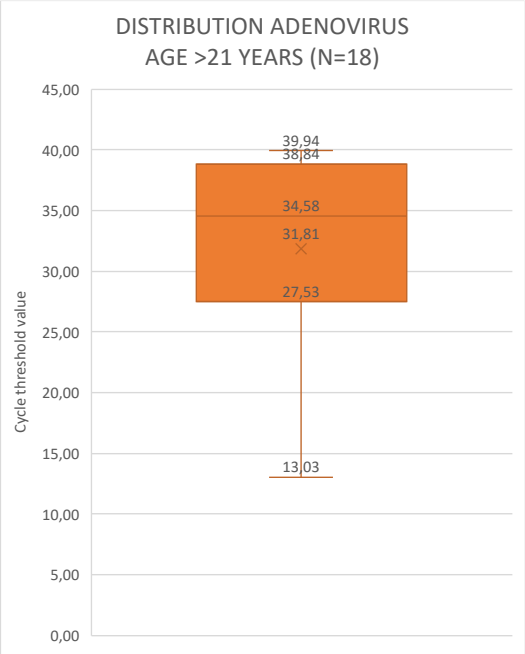
TO DO/ACTIONS

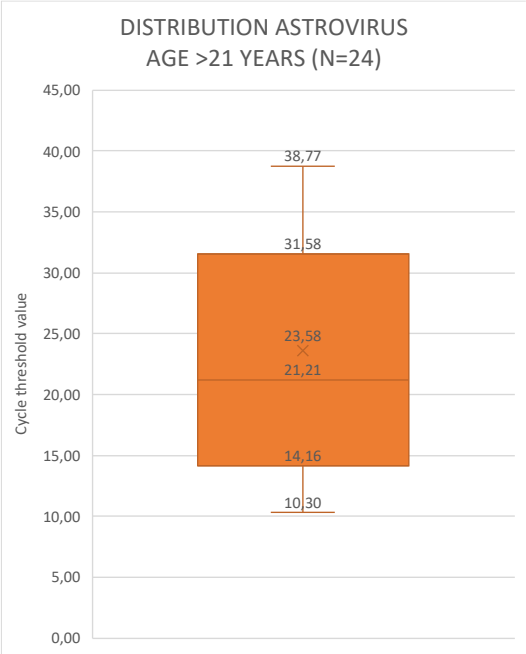
1) Determine semi-quantitative cut-off values for parasites and bacteria.
2) Performing the same analysis on a large group of asymptomatic individuals and compare the positivity rates and Ct values (significantly different or not?).
3) Performing the same analysis for other multiplex panels in order to determine semi-quantitative cut-off values

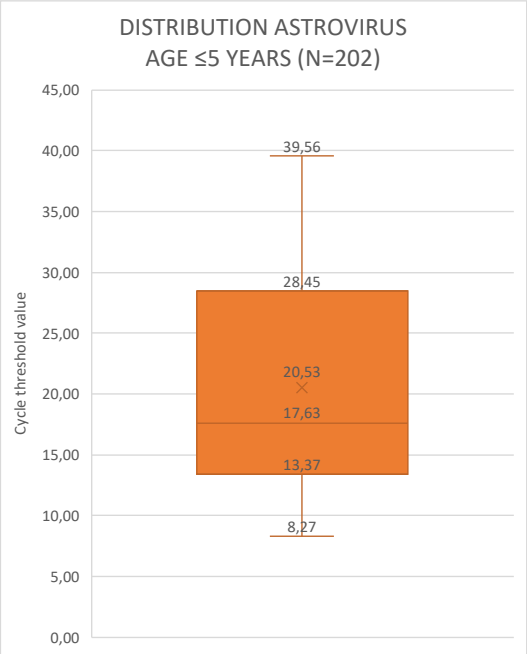
ATTACHMENTS

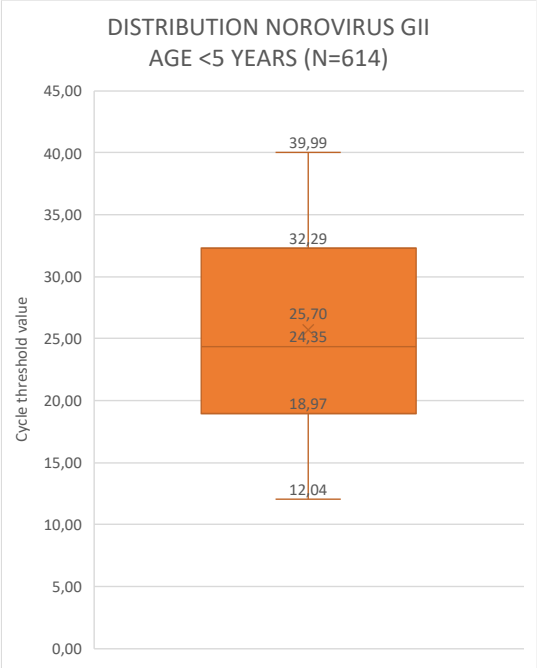
Attachment 1

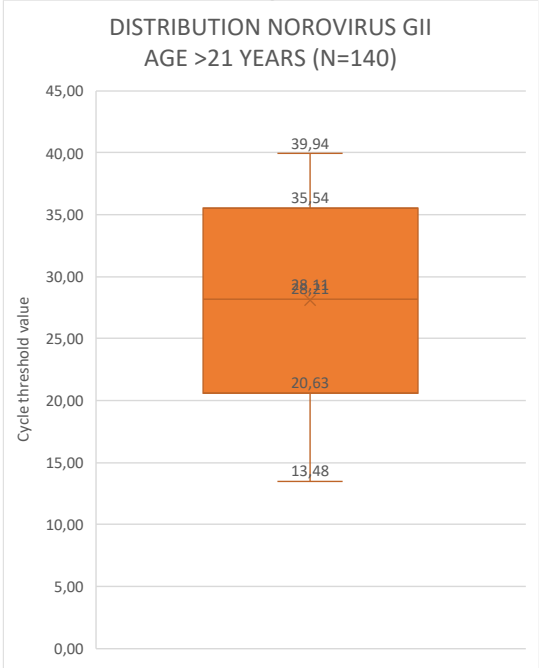


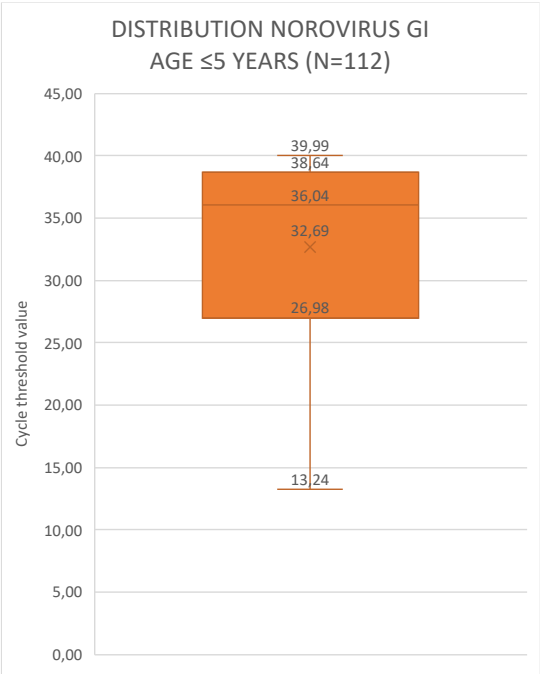


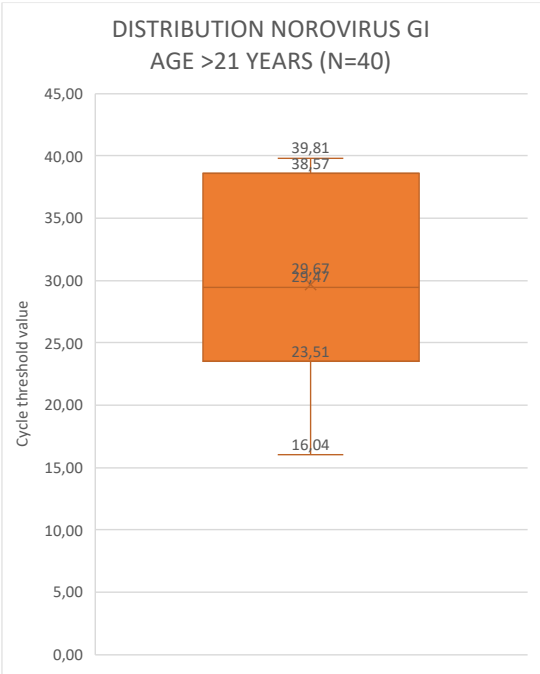


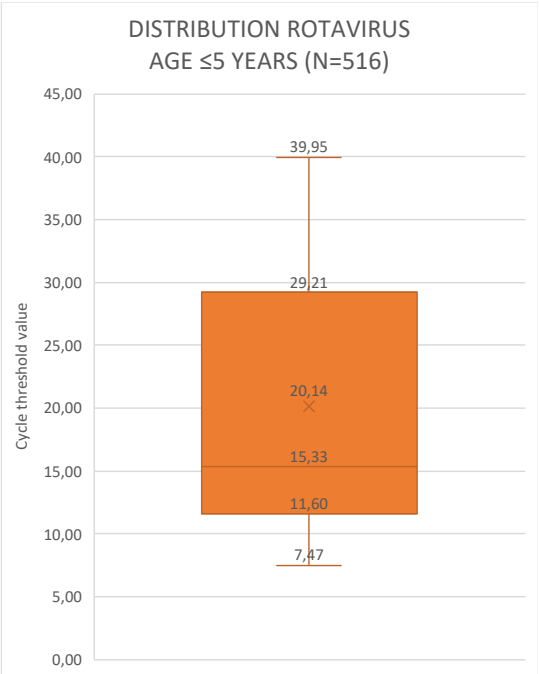



DISTRIBUTION NOROVIRUS GII
AGE <5 YEARS (N=614)
Cycle threshold value
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
39,99
32,29
25,70
24,35
18,97
12,04


DISTRIBUTION NOROVIRUS GII
AGE >21 YEARS (N=140)
Cycle threshold value
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
39,94
35,54
20,63
13,48


DISTRIBUTION NOROVIRUS GI
AGE ≤5 YEARS (N=112)
Cycle threshold value
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
39,99
38,64
36,04
32,69
26,98
13,24


DISTRIBUTION NOROVIRUS GI
AGE >21 YEARS (N=40)
Cycle threshold value
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
39,81
38,57
29,67
23,51
16,04


DISTRIBUTION ROTAVIRUS
AGE ≤5 YEARS (N=516)
Cycle threshold value
45,00
40,00
35,00
30,00
25,00
20,00
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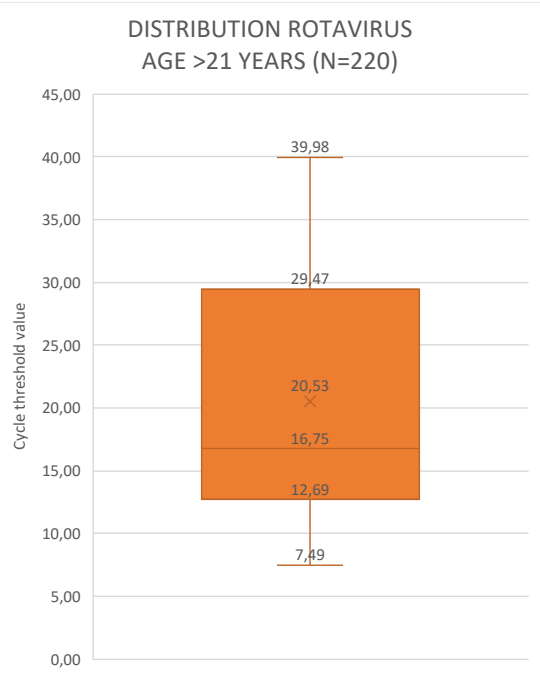
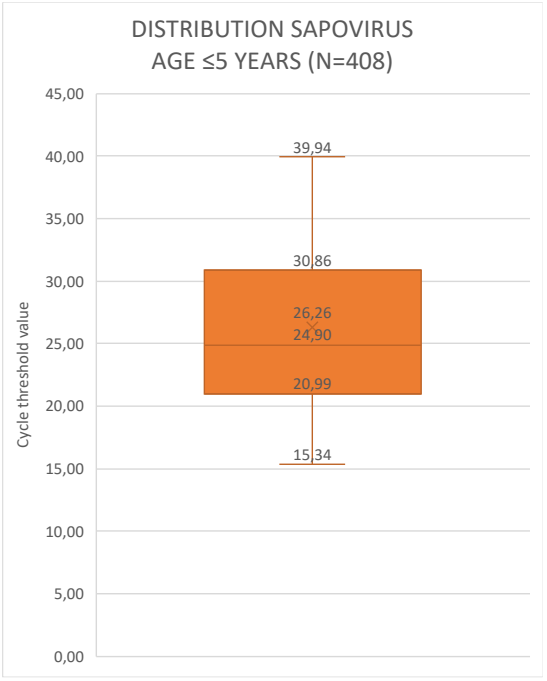
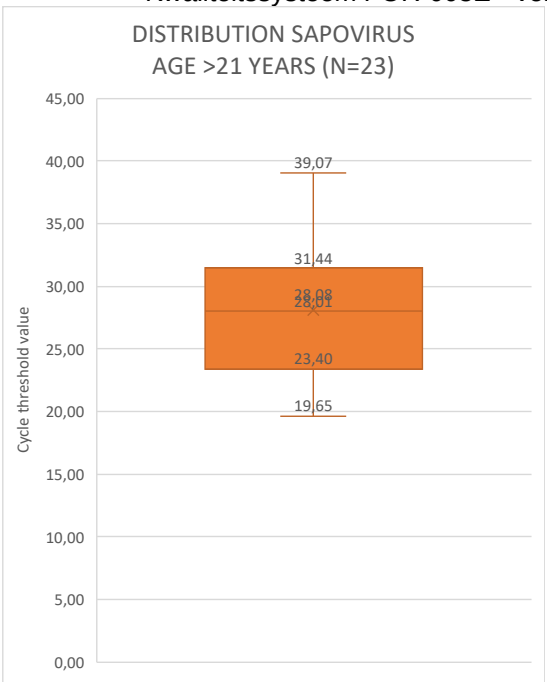
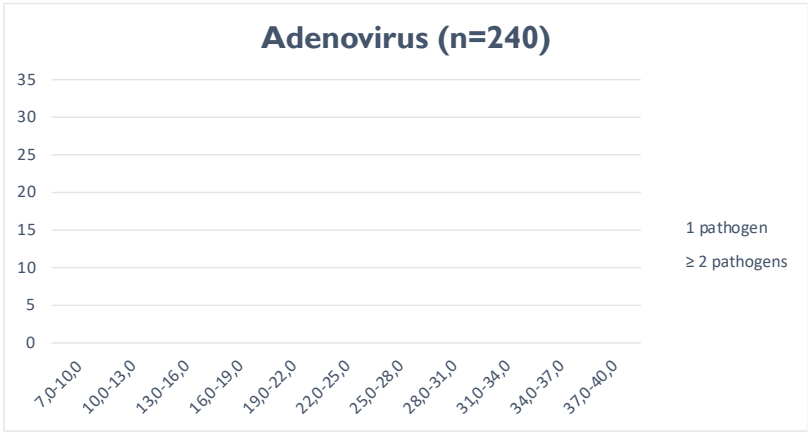
10,00
5,00
0,00
39,95
29,21
20,14
15,33
11,60
7,47

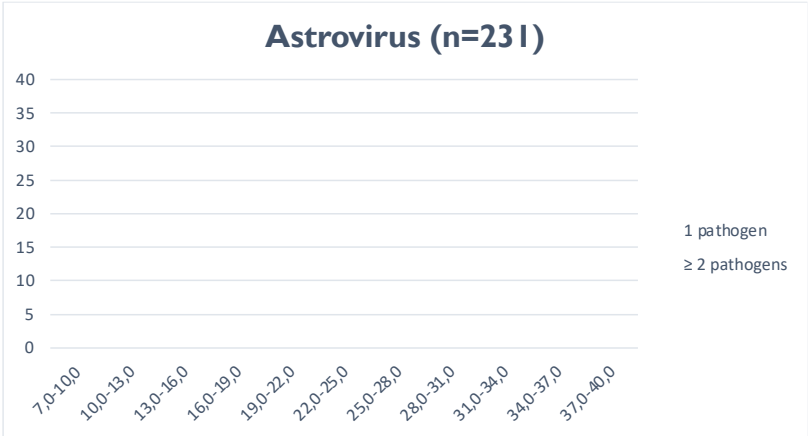

DISTRIBUTION ROTAVIRUS
AGE >21 YEARS (N=220)
Cycle threshold value
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
39,98
29,47
20,53
16,75
12,69
7,49

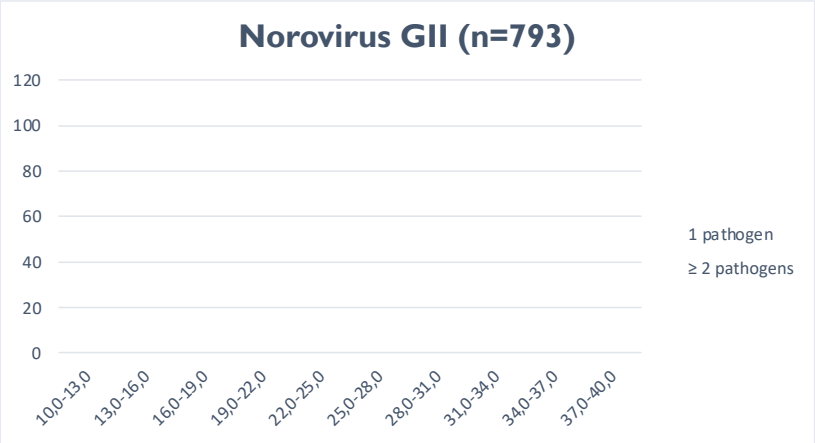


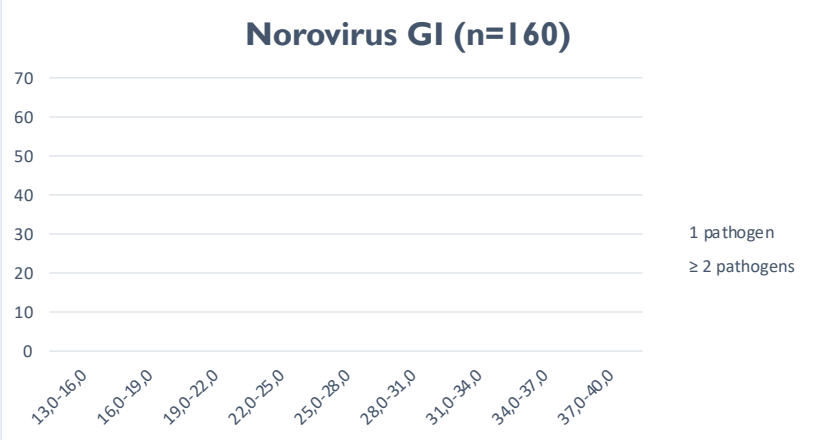


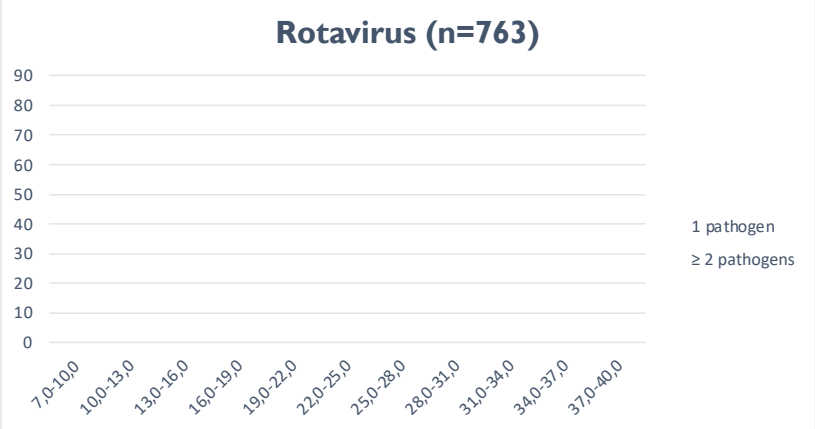
Attachment 2

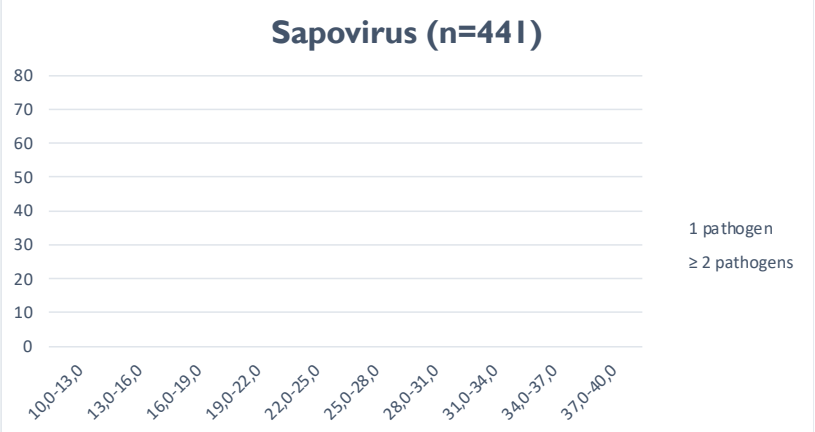





Norovirus GII (n=793)
120
100
80
60
40
20
0
10,0-13,0
13,0-16,0
16,0-19,0
19,0-22,0
22,0-25,0
25,0-28,0
28,0-31,0
31,0-34,0
34,0-37,0
37,0-40,0
1 pathogen
≥ 2 pathogens


Norovirus GI (n=160)
70
60
50
40
30
20
10
0
13,0-16,0
16,0-19,0
19,0-22,0
22,0-25,0
25,0-28,0
28,0-31,0
31,0-34,0
34,0-37,0
37,0-40,0
1 pathogen
≥ 2 pathogens


Rotavirus (n=763)
90
80
70
60
50
40
30
20
10
0
7,0-10,0
10,0-13,0
13,0-16,0
16,0-19,0
19,0-22,0
22,0-25,0
25,0-28,0
28,0-31,0
31,0-34,0
34,0-37,0
37,0-40,0
1 pathogen
≥ 2 pathogens


Sapovirus (n=441)
80
70
60
50
40
30
20
10
0
10,0-13,0
13,0-16,0
16,0-19,0
19,0-22,0
22,0-25,0
25,0-28,0
28,0-31,0
31,0-34,0
34,0-37,0
37,0-40,0
1 pathogen
≥ 2 pathogens

Attachment 3

		Dilution in physiologic sterile water											
		Culture											
		Dilution in eNAT											
					dilution	1/10	1/100	1/1000	1/10^4	1/10^5	1/10^6	1/10^7
					estimated cfu/ml	1,5x10^7	1,5x10^6	1,5x10^5	1,5x10^4	1,5x10^3	150	15
	Stam	Nr. plaat bacterio	Ordernummer			Ct. 1	Ct. 2	Ct. 3	Ct.4	Ct.5	Ct.6	Ct.7
1	C. *diffile*	1C4-5	extern		0.9% NaCl			36,05	40,48	0		
					culture			4	1	0		
					eNAT	31,93	34,99	38,01	38,77	0	0	0
5	*Campylobacter coli*	1C4-10	extern		0.9% NaCl			38,14	38,61	0		
					culture			>150	82	15		
					eNAT	28,95	33,42	36,57	38,72	43,24	42,44	0
6	*Campylobacter jejuni*	1D1-28	06391-1406M		NaCl			33,01	36,69	36,15		
					culture			>150	>100	27		
					eNAT	24,79	28,33	30,54	33,11	36,16	36,8	39,89
13	VTEC	1D1-71	26155-1406M	stx1/2	0.9% NaCl			33,36	38,73	0		
				eaeA	0.9% NaCl			32,6	39,79	0		
				O157	0.9% NaCl			35,18	39,52	0		
					culture			>150	46	4		
				stx1/2	eNAT	26,15	28,61	34,53	33,47	39,11	41,58	0,00
				eaeA	eNAT	24,92	27,22	33,14	32,65	37,5	39,71	0
				O157	eNAT	26,84	29,27	35,89	34,97	41,75	0	0
21	Vibrio cholerae	1B1-2	QC		0.9% NaCl			33,04	35,92	0		
					culture			0	0	0		
					eNAT	26,11	29,39	34,68	35,03	37,5	40,00	0
22	Aeromonas hydrophila	1A1-3	QC		0.9% NaCl			39,20	40,17	43,48		
					culture			106	15	3		
					eNAT	28,46	32,01	35,76	38,87	41,09	40,53	0
23	Aeromonas species		00816-2210R		NaCl			40,38	43,11	0		
					culture			69	5	0		
					eNAT	31,36	34,35	37,14	39,04	41,01	43,98	0
25	Salmonella newport		20553-2202L		0.9% NaCl			36,45	42,60	0		
					culture			>150	92	9	massief	0
					eNAT	26,94	30,14	34,9	35,66	37,55	42,48	42,06
26	Salmonella enteritidis				0.9% NaCl			36,39	40,69	0		
					culture			>150	101	14	massief	massief
					eNAT	25,66	30,48	33,46	34,79	38,18	38,42	0
27	Salmonella typhimurium				0.9% NaCl			38,44	40,78	0		
					culture			>150	69	3	massief	>150
					eNAT	27,62	30,60	32,73	35,32	38,50	42,23	0
28	Shigella spp				0.9% NaCl			35,03	38,83	40,85		
					culture			>150	25	8		
					eNAT	23,39	28,38	30,39	36,39	37,49	39,08	44,99
29	Yersinia enterica				0.9% NaCl			36,31	41,78	0		
					culture			76	15	0		
					eNAT	26,64	29,48	32,85	35,52	39,40	39,52	43,23

	1,5x10^7	1,5x10^6	1,5x10^5	1,5x10^4	1,5x10^3	150	15
Stam	Ct. 1	Ct. 2	Ct. 3	Ct.4	Ct.5	Ct.6	Ct.7
C. diff			4,00E+05	1,00E+06	0		
Campylobacter Coli			massief	8,20E+07	1,50E+08		
Campylobacter Jejuni			massief	massief	2,70E+08		
VTEC			massief	4,60E+07	4,00E+07		
Vibrio cholerae			0	0	0		
Aeromonas hydrophila			1,E+07	2,E+07	3,E+07		
Aeromonas species			6,90E+06	5,00E+06	0		
Salmonella newport		massief	massief	9,20E+07	9,00E+07	massief	0
Salmonella enteritidis			massief	1,01E+08	1,40E+08	massief	massief
Salmonella typhimurium		massief	massief	massief	3,00E+07	massief	>150
Shigella spp			>150	2,50E+07	8,00E+07		
Yersinia enterica			7,60E+06	1,50E+07	0		

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