CAT
Critically Appraised Topic

Goodbye culture, hello PCR? Implementation of a molecular diagnostic panel for enteropathogens in routine faeces diagnostics

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CLINICAL BOTTOM LINE

Infectious diarrhoea is highly prevalent and causes significant morbidity and healthcare costs. Guidelines recommend to perform microbiological diagnostics only in case of severe or prolonged illness or in the presence of risk factors for development of severe disease, typically using a tiered approach. Detection of a causative agent traditionally required several different techniques, requires a high workload and took a long time to perform. Syndromic molecular panels are changing microbiological diagnostics, with easier test requesting, higher sensitivity, improved workflow and drastically shorter turnaround times. Switching from culture-based methods to PCR also has its disadvantages, however, such as the lack of a cultured isolate for strain typing or antibiotic susceptibility testing. In AZ Sint-Lucas, a molecular diagnostic panel for bacterial enteropathogens (the BD MAX™ Extended Enteric Bacterial Panel) was implemented as routine diagnostic test for all stool samples with a request for culture. In addition, a limited complementary culture is always performed (mostly for detection of non-coli/jejuni *Campylobacter* spp., *Arcobacter* spp. and *Aeromonas* spp.) as well as reflex culture in case of PCR positive results. The implementation of this strategy was retrospectively evaluated over a period of 10 months and compared to the previously used "traditional" culture-based algorithm. A similar (though slightly higher) detection rate of *Campylobacter* and *Salmonella* spp. was noted, with increased detection of STEC, ETEC, *Yersinia* spp. and *Plesiomonas* spp. Median TAT decreased from 2.3 days to 0.9 days. Reagent costs increased total cost (considering labour time) was almost similar. Positive PCR results that could not be confirmed by culture showed on average higher cycle threshold values compared to culture-confirmed results. In conclusion, the combination of a molecular panel and culture harnesses the improved TAT and sensitivity of PCR while overcoming some of its important limitations.

CLINICAL/DIAGNOSTIC SCENARIO

Infectious diarrhea is a global health problem with about two billion cases of infectious diarrheal disease every year. It causes significant morbidity and healthcare costs in developed nations, while in low-and middle-income countries it is a leading cause of death in children under the age of five [1]. Acute diarrhea can be accompanied by vomiting, a syndrome often referred to as acute gastroenteritis (AGE) [2]. There is a wide array of possible causative agents for AGE (including bacteria, viruses and parasites) which are often clinically indistinguishable. In Europe and the United States viruses are the most common of AGE, while in developing nations enteric bacteria and parasites can be more prevalent [1–3]. The most prevalent bacteria causing AGE in industrialized nations are zoonotic (e.g. *Campylobacter* spp., *Salmonella* spp. and shiga-toxin producing *Eschericia coli* or STEC), with seasonal peaks in the summer [4]. In Belgium, the annual number of Campylobacter spp. infections are estimated to range between 150.000 – 450.000, resulting in the loss of 2.500 to 10.000 disability adjusted life years (DALY). Salmonella and STEC infections are about 5 times less prevalent, but cause only around 2 times less DALY loss since the disease is often more severe [5].

The identification of an etiological agent is not always required for patient management, as many acute diarrheal episodes are mild and self-limiting. For the vast majority of AGE episodes, no antibiotic therapy is warranted. Therefore, in immunocompetent patients with mild to moderate watery diarrhea (and/or vomiting), laboratory investigations are generally not recommended [1–3,6]. In several situations, however, a specific diagnosis can be helpful for treatment, local hygienic measures for mitigating outbreaks or public health purposes.

Traditional methods for the diagnosis of gastrointestinal pathogens include bacterial culture, microscopy and antigen testing. Bacterial stool culture typically has a long turn-around time (TAT) (approximately 3-5 days), a relatively low sensitivity and presents a high workload for laboratory personnel [7]. Especially in a low-prevalence setting, with positive stool culture rates typically between 2-4% in Europe and the United states, this translates into a low cost-effectiveness of bacterial stool cultures [8]. Microscopy for parasites is laborious, lacks sensitivity and standardisation and requires high levels of training [9,10]. Rapid antigen tests for viral and parasitic agents often lack sensitivity and specificity [11]. In contrast, molecular diagnostic panels are fast, highly sensitive, highly specific and easy to perform. Molecular diagnostic panels for diagnosis of faecal pathogens typically use multiplex real-time polymerase chain reaction (PCR) to detect nucleic acid sequences of pathogens potentially present in the sample. In the early days of molecular biology, a PCR test required many manual actions and a great amount of technical expertise from the operator. Nowadays there are fully automated, CE-IVD labelled instruments that perform the entire RT-PCR testing process (from extraction to interpretation of the result) with limited user intervention, requiring little knowledge of molecular biology. Generally, these instruments are able to provide standardized results within 1-5 hours, with a hands-on time of only several minutes per sample [7,11–14].

In the AZ Sint-Lucas hospital, a molecular diagnostic panel for gastro-intestinal bacterial pathogens, the "BD MAX™ Extended Enteric Bacterial Panel", was implemented as a routine diagnostic test for stool samples with a request for bacterial culture (See Attachment 1 for more information on the principle of the test). Additionally, a limited number of culture plates are still inoculated to complement the molecular panel, extending the amount of pathogens that can be detected in diagnostic faecal samples. If the molecular panel returns a positive result for certain pathogens, a reflex culture is performed for confirmation, typing and/or antimicrobial susceptibility testing. The goal of this CAT was to evaluate different aspects of this implementation, including compliance with guidelines, evolution of diagnostic yield, cost-effectiveness, and implications for public health. We also provide some insight into the process of determining which molecular panel to implement, considering a specific laboratory setting. Furthermore, we attempted to provide a glimpse into the future of microbial diagnostics of faeces samples, considering new developments and techniques. This appraisal might also serve as a source of information for further discussions regarding adjustments of reimbursement of molecular tests. This CAT will focus on routine faeces diagnostics of bacterial gastro-enteritis (since the molecular panel that was implemented in routine only detects bacterial agents), with limited discussion regarding investigations of viruses and parasites. The topic of *Clostridium difficile* detection will also not be discussed in detail because that topic deserves its own CAT.

QUESTION(S)

1) *What do guidelines recommend regarding the scope of etiologic agents that should be included for diagnosis of acute gastro-enteritis?*
2) *What are the advantages and disadvantages of molecular multiplex diagnostic panels for enteric pathogens (with or without reflex culture), compared to conventional diagnostics?*
3) *How to decide which molecular panel to implement?*
4) *What is the impact after implementing a molecular diagnostic panel with reflex culture compared to the prior period in which conventional diagnostic techniques were used?*

5) *What are possible future directions of diagnostics for enteropathogens?*

SEARCH TERMS

1) *MeSH Database (PubMed)*
    a. *"(("Diarrhea"[Mesh]) OR "Dysentery"[Mesh])) AND (("Real-Time Polymerase Chain Reaction"[Mesh]) OR "Multiplex Polymerase Chain Reaction"[Mesh])) "*
    b. *(((Human[MeSH Terms]) AND ((Diarrheal [MeSH Terms])) OR (Gastroenteritis [MeSH Terms]))) AND (multiplex polymerase chain reaction [MeSH Terms])*
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)*
    a. *"infectious diarrhea AND molecular panel"*
    b. *(("Diarrhea" OR "Vomiting" OR "Gastro-enteritis" OR "diarrhoeae") AND "Multiplex polymerase chain reaction") AND (Diagnosis/Broad[filter])*
3) *SUMSearch (http://sumsearch.uthscsa.edu/)*
    a. *Multiplex polymerase chain reaction AND diarrhea*
4) *The National Institute for Clinical Excellence (http://www.nice.org.uk/)*
    a. *Multiplex polymerase chain reaction*
5) *Cochrane (http://www.update-software.com/cochrane)*
    a. *(Gastroenteritis OR Diarrhea) AND (Multiplex polymerase chain reaction): 0 results*
6) *(Inter)national organizations*
    a. *WGO*
    b. *ACG*
    c. *ASM*
    d. *ESCMID*
7) *UpToDate Online*
    a. Gastro enteritis

APPRAISAL

Question 1) What do guidelines recommend regarding the scope of etiologic agents that should be included for diagnosis of acute gastro-enteritis?

Europe and the United States are regions with a relatively low prevalence of enteropathogenic bacteria and high healthcare resources [1,2]. In this context, detection of a limited set of well-established enteropathogenic bacteria (*Campylobacter, Salmonella, Shigella, Yersinia*) is typically recommended in case of inflammatory diarrhoea (fever, stool mixed with blood or mucus), severe illness or the presence of risk factors for development of severe disease [1,2,15]. Some guidelines suggest stool diagnostic studies should also be performed if symptoms last ≥7-14 days [1,2,6,15]. Diagnosis of viral agents is generally advised in (potential) outbreak settings, such as hospital clusters, food-related outbreaks and specific communal settings [2,3]. Broader diagnostics including bacterial, viral and parasitic agents are advised in case of immunocompromised individuals with chronic diarrhoea (>14 days) and sometimes for returning travellers [2,15]. Note that some episodes of AGE are due to preformed toxins (*S. aureus*, *C. perfringens, B. cereus*) in food which are not routinely looked for by clinical laboratories. Finally, diarrhea can also be non-infectious, e.g. due to irritable bowel syndrome, inflammatory bowel disease or malabsorption of carbohydrates [1,16].

There is some debate whether it is useful to look for community associated enteropathogens in patients hospitalized three days or longer. Some guidelines recommend to reject these samples [3,17]. However in the Belgian setting this could lead to a reduced yield of pathogens of around 10% [18].

The WHO defines diarrhea as "the passage of three or more loose or liquid stools per day (or more frequent passage than is normal for the individual), excluding frequent passing of formed stools and the passing of loose stools by

breastfed babies" [19]. In practice we notice that the presence of diarrhea is often misinterpreted, with up to 20% of stool samples that arrive in the laboratory having a solid consistency [18]. There is some debate regarding the rejection of formed (solid) stool samples for microbiological diagnostics [14] This could, however, lead to an estimated 8.5% of enteropathogens being missed [18]. Therefore, while advocating against the analysis of formed stools, we do not believe these should be rejected entirely.

It is impractical and cost-inefficient to investigate the presence of every possible etiologic agent of AGE in every patient [8]. Most guidelines use a "tiered approach", providing recommendations to investigate the presence of 1) a "minimal set" of bacterial pathogens in patients eligible for laboratory investigation (tier 1) and 2) a more extended array of pathogens of which the composition depends on the type of patient (age, immunocompetence) the clinical presentation (bloody versus watery diarrhea, severity of illness), the exposure (food related, travel related, hospitalized or outpatient, antibiotic use) and the epidemiological setting (outbreak or single case). The amount and types of pathogens investigated also depend on the local epidemiological occurrence of pathogens, the available resources and clinicians' expectations. Because of this, there is no single gold standard strategy for diagnostics of enteric pathogens. The myriad of available textbooks and guidelines present some overlapping advices, while each providing their own accents and recommendations for specific settings. Some examples are given in Table 1 (excluding recommendations regarding detection of parasites, viral agents and C. difficile)

Table 1: examples of guidelines regarding the scope of etiological agents to be investigated in acute gastro-enteritis

	ESCMID 2012 [3]	**IDSA 2017** [2]	**BILULU consensus 2017**[*]
Minimal set (tier 1)	• *Salmonella* • *Shigella* • *Campylobacter*	• *Salmonella* • *Shigella* • *Campylobacter* • *Yersinia* • STEC	• *Salmonella* • *Shigella* • *Campylobacter jejuni/coli* • *Yersinia enterocolitica/pseudotuberculosis*
Extended (tier 2-3)	• *Yersinia* • EHEC/STEC • *Vibrio spp.* • *Aeromonas*	• *Vibrio spp.* • "a broader set of bacterial, viral and parasitic agents should be considered in the context of a possible outbreak"	• EHEC/STEC • *Aeromonas spp.* • *Plesiomonas shigelloides* • *Edwardsiella tarda* • *Campylobacter spp.* (non *jejuni/coli*) • *Arcobacter spp.*
Special situations (tier 4)	• *Listeria* • ETEC, EIEC • Toxins in food (*C. botulinum, B. cereus, S. aureus*)		• *Vibrio spp.* • *Listeria spp.* • *S. aureus* • *Providencia* • *C. botulinum* • *B. cereus*

[*]based on [14,17] EHEC: enterohemorrhagic *Escherichia coli*, STEC: shiga-toxin producing *E. coli*, ETEC: enterotoxin producing *E. coli*, EIEC: entero-invasive *E. coli*.

Ideally, every stool sample should be accompanied with clinical information so the laboratory can adapt its strategy to look for the most likely pathogen(s) responsible in a specific patient, although in practice we notice that clinical information for the laboratory is often limited or lacking entirely. Also, it is not feasible to create a personalized workflow for each patient because of practical reasons. Because of these real-life limitations, laboratories typically

have a “standard workflow” for the detection of the tier 1 (and sometimes some of the tier2-3) pathogens, and a limited collection of “special situations” in which other pathogens are additionally sought after in case of specific clinical or epidemiological arguments (e.g. searching for *Listeria spp*. in case of a neonatal sepsis).

At the time of writing there are many molecular diagnostic panels commercially available, of which there are seven CE-IVD and FDA approved [7,11]. Examples include the BioFire® FilmArray® Gastrointestinal (GI) panel (bioMérieux), the Nanosphere Verigene enteric pathogen panel (Luminex, Austin, TX, USA), The xTAG® gastrointestinal pathogen panel (Luminex, Austin, TX, USA) and the BD MAX™ Extended Enteric Bacterial Panel (BD, Sparks, MD, USA). There are important differences between these different panels such as the spectrum of pathogens assessed (ranging from solely bacteria to panels including both bacteria, viruses and parasites), the price, the throughput capabilities, the hands-on time and the availability of a qualitative or quantitative result (see Attachment 2).

All of the CE-IVD panels (including the BD MAX™ Extended Enteric Bacterial Panel used in AZ Sint-Lucas Gent) detect *Campylobacter* spp., *Salmonella* spp., *Shigella*/EIEC and STEC. The total number of targets included ranges from these four up to 25, with some panels including viral and/or parasitic agents besides bacteria. Some manufacturers (including BD) allow for the user to choose and combine sets of pathogens in a modular fashion. This makes it possible to tailor the molecular panel to individual laboratory needs regarding pathogen scope and cost. Another option with modular panels is to use different modules in a stepwise or tiered way.

Question 2) What are the advantages and disadvantages of molecular diagnostic panels for enteric pathogens (with or without reflex culture), compared to conventional diagnostics?

Advantages of molecular diagnostic panels

A striking difference between molecular diagnostic panels and traditional culture-based techniques is the turn-around time. Molecular panels are able to provide results within hours as compared to days for traditional culture-based techniques. This can lead to a faster initiation of the correct antibiotic treatment, which is especially relevant in severely ill patients. When STEC is detected, antibiotics can be quickly discontinued, as these can increase the risk for haemolytic uremic syndrome when this organism is present [7]. Some studies have shown that the introduction of molecular panels for gastro-intestinal pathogens decreases the amount of antibiotics used, as well as the number technical investigations such endoscopies and radiology [7,20]. Another important advantage of a shorter TAT is the impact on infection control and outbreak management. Patients with a positive PCR can be isolated or cohorted more quickly, which might reduce the risk for nosocomial spread. In contrast, patients placed in precautionary isolation might be released from quarantine faster in case of a negative PCR [13].

It has been consistently shown that using a multiplex PCR approach for gastrointestinal pathogens, a higher sensitivity is reached compared to bacterial culture [21–25]. Especially when antibiotics have already been administered, culture can be false-negative while a PCR test might still be positive because it does not require the organisms to be alive to be detected. Some micro-organisms are difficult or impossible to culture (e.g. parasites), whereas they can be easily detected using PCR. The higher diagnostic yield of PCR panels compared to culture works synergetic with the advantages of the faster TAT to improve treatment and infection control while reducing unnecessary technical interventions [20].

The current CE/IVD labelled molecular panels are highly qualitative, standardized and require little hands-on time to operate [7,11]. In comparison, culture is a slow, labor-intensive, cumbersome and requires a lot of expertise from the technician to distinguish pathogens from commensal flora based on colony morphology and growth on selective agars. Molecular panels therefore allow for an improved workflow compared to traditional culture methods. There is also a faster learning curve for the simple "sample-to result" analysers compared to stool cultures. A final advantage of the use of molecular panels is that it allows for easier test requesting for the clinicians. Gastro-enteritis can be caused by many different, but clinically indistinct, etiologic agents. The use of syndromic panels avoids the need to select between tests (e.g. singleplex PCRs or antigen tests) looking at single organisms based on clinical suspicion, again improving diagnostic yield [7,11].

Disadvantages/limitations of molecular diagnostic panels

While there are obvious advantages to molecular panels as mentioned above, they also harbour some intrinsic limitations. The fact that multiplex PCR tests detect nucleic acids does not require the organism to be alive, however it therefore also not distinguish whether the organism is alive. Because the viability/infectivity of an organism is not determined, and very low quantities of nucleic acids can be detected, the problem of interpreting (weakly) positive results (high Ct values) arises. Especially in children and in regions with lower sanitation, high rates of asymptomatic carriage of enteropathogens are detected [7]. A study in Belgian children detected a pathogen in 56.2% of gastroenteritis episodes using the Luminex GI Pathogen panel, however the panel was also positive in 24.2% of asymptomatic controls [23]. Also, prolonged shedding of (dead) organisms after an infection is possible, resulting in prolonged PCR positivity after the disease has been resolved. For the interpretation of a positive PCR result, the clinical context has to be taken into careful consideration, maybe even more so than with a positive culture. The high sensitivity also results in a higher frequency of mixed infections that can be detected, which also poses interpretation difficulties, since it is difficult to assess how much each pathogen contributes to the clinical picture. Some studies have suggested that the Ct value might help in assessing whether the detected organism(s) are of clinical importance or represent a "carrier state" or old infection [26–28] Most current CE-IVD molecular panels for faeces diagnostics, however, do not claim to provide a quantitative assessment of the included pathogens [7,11]. Another intrinsic limitation of PCR is the lack of antibiotic susceptibility testing, since no organism is isolated. The impact for the individual patient of this limitation is small, since there is a low failure rate of empiric treatment of gastro-enteritis. On a larger scale, however, this could have implications for public health, as trends in resistance patterns and mechanisms can no longer be monitored if culture would be no longer performed [12,13,28]. Another limitation relating to the lack of organism isolation is that it cannot be typed to determine pathogenicity or virulence. For example, some *Yersinia* biotypes are disease-causing agents while others are considered non-pathogenic, some STEC strains are highly virulent whilst others cause little harm to the patient. A typical molecular diagnostic panel cannot distinguish between the different types/strains and can therefore not distinguish low-virulent from highly virulent strains. From a public health or epidemiological standpoint, advanced characterization and typing of organisms is relied on for outbreak investigation and monitoring of epidemiological spread [12,13,28]. Lastly, while the clustering of several pathogens related to a clinical syndrome in a single molecular test has diagnostic advantages, it inevitably excludes some potential etiologic agents. More than 40 bacterial agents, as well as a multitude of viruses and parasites have been identified to cause gastroenteritis, therefore it is currently impossible to include all these in a single molecular panel [8]. Panels do not include atypical pathogens that can cause disease in immunocompromised

patients, such as CMV, HSV, mycobacteria and *Treponema pallidum*. Also, certain parasites that can be found in travellers' diarrhea are often omitted [7]. A negative molecular panel therefore does not exclude the presence of any causal agent altogether. Furthermore, molecular panels might limit our ability to detect new causes of diarrheal disease [6]. Some emerging pathogens such as *Arcobacter spp*. and *Escherichia albertii* were discovered using culture-based techniques and are not included in current molecular panels . Culture-based techniques theoretically do not have a "hard limit" on the number of different species that can be detected, although in practice selective media and laboratory practices selecting certain colony morphologies do impose limits on the diversity of species that can be detected. PCR multiplex panels, in contrast, have a limitative, defined list of targets that are examined. In other words, a pathogen will not be detected if it is not included in the panel. Finally, the cost of reagents for PCR-based techniques is currently still higher than the cost of culture-based techniques, while reimbursement of faecal diagnostic testing in Belgium does not differentiate between these techniques. The higher cost for the laboratory might be saved by lower healthcare costs altogether (cf. supra), however for the laboratory the current reimbursement strategy does not incentivize a switch to molecular panels (although there might be non-financial advantages for the laboratory to switch to a molecular panel, see below).

Note that while theoretical TATs of molecular panels are in the order of magnitude of hours, the TAT in practice is always somewhat longer. Because of technical and cost constraints, batching of samples (1 or 2 batches per day in AZ Sint-Lucas Gent) is performed, resulting in some loss of speed [23]. Furthermore, a technical oversight of results is often required to verify a that the RT-PCR result is reliable, e.g. through visual check of the amplification curve. The latter might also delay reporting of results, especially if the measurement has to be repeated due to technical issues such as inhibition.

A combination of culture and molecular panels, the best of both worlds?

Many of the above-mentioned limitations of molecular panels can be obviated by combining culture methods and PCR-based techniques. There are two main options: 1) performing of a "reflex" culture when the PCR panels is positive for one or more pathogens and 2) performing of a "complementary" culture to increase diagnostic yield beyond the hard limits of the molecular diagnostic panel. The 2016 ACG clinical guideline alludes to reflex culture with regard to public health purposes in the following statement: *"The future may hold a combination approach where specimens that have yielded a positive result by culture-independent testing are then submitted to public health laboratories for subtyping and sensitivity analysis"* [6]. Another advantage of performing reflex culture is confirmation of viability of the organism, which might have clinical implications. Culture can also be used to "fill the gaps" of molecular panels, e.g. for rare or emerging pathogens that are not included in molecular panels. Although the latter strategy requires routine inoculation of culture plates which increases workload for laboratory technicians. In the AZ Sint-Lucas Ghent, a combination of reflex culture and a limited complementary culture were implemented together with the PCR panel (See detailed description below).

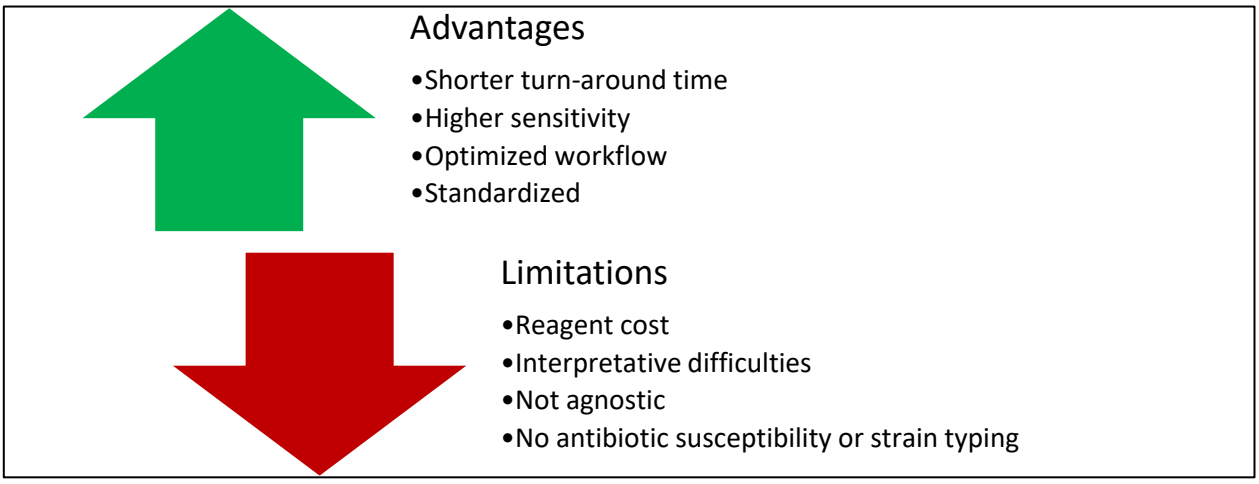

Figure 1: summary of advantages and limitations of current molecular diagnostic panels

Question 3) How to decide which molecular panel to implement?

The choice of molecular panel depends on several factors, such as: 1) what molecular equipment is already available in the laboratory? 2) how much experience and expertise with molecular techniques does the laboratory have 3) how many samples need to be analysed per day (i.e. what is the sample turnover)? 4) what is the desired turnaround time (including on weekends and holidays)? 5) is there a need/demand for other molecular panels to be implemented, and can these be combined in a single (new) analyser? 6) how many pathogens does the lab want (or do the clinicians expect the lab) to detect? 7) is it desirable to be able to use a stepwise or modular approach? 8) technical performance of the molecular test and finally 9) cost [13].

In the AZ Sint-Lucas Ghent, a technical validation of two panels was performed, the BioFire®FilmArray® Gastrointestinal (GI) Panel (Biomérieux) and the BD MAX™ Extended Enteric Bacterial Panel (Becton Dickinson). The results of the panels were compared with conventional culture methods (data not shown in this CAT). Both techniques showed high sensitivity (higher than culture), high specificity and good concordance with culture, confirming literature reports [21,24,25,29]. While the FilmArray® panel provides detection of a more extensive set of pathogens (See Attachment 3), it also has some important disadvantages compared to the BD MAX™. First, it does not provide a quantitative measurement such as a Ct value since it uses nested PCR with a qualitative result reporting. Second, per FilmArray® machine only 1 sample can be analysed simultaneously (although this can be extended by purchase of additional modules) which has implications for batching and throughput. Finally, the reagent cost per sample is substantially higher compared to the BD MAX™. An analysis with FilmArray® costs almost 10-fold the amount of a BD MAX™ Extended Enteric Bacterial Panel. Note that the BD MAX™ can also accommodate a viral and parasite panel, at an additional cost. This modular setup allows for tailoring of diagnostics depending on the clinical need. For example, the viral panel is applied in the AZ Sint-Lucas Ghent for children below 2 years old with diarrhea, but not for adults (except in the case of suspected in-hospital outbreak). The parasite panel is not routinely used in AZ Sint-Lucas (yet), however perhaps in the future a “stepwise” work-up of difficult diarrhea cases could be done using a combination of the different BD MAX™ enteric panels.

The BD MAX™ analyser allows for batches of 24 samples to be analysed simultaneously per analyser, with minimal hands-on time per sample. The molecular biologists and laboratory technicians were already trained and accustomed to the BD MAX™ analyser as two of these were already present in the laboratory, which is an important advantage against any new analyser. Furthermore, the BD MAX™ is a relatively small benchtop analyse that performs all analysis

steps (from extraction to PCR cycling and detection) in a closed system. This allowed for the analyser to be placed in the routine "core" microbiology lab, in contrast to panels requiring separate extraction and PCR steps (e.g. the Seegene Allplex™ kits for diarrhea). In combination with the ease of use and short hands-on time, this allows for the BD MAX™ Extended Enteric Bacterial Panel to be performed on moments of limited staff availability such as weekends and holidays, improving TATs overall.

After consideration of the advantages and limitations of both culture and PCR techniques (as mentioned in question 2), the number of samples arriving in the laboratory each day and the fact that there were already two BD MAX™ analysers present in the laboratory, it was decided that the BD MAX™ Extended Enteric Bacterial Panel would be implemented for routine stool diagnostics. The BD MAX™ molecular panel is complemented with a limited "complementary" culture for the detection of pathogens not included in the panel (mostly for non-*jejuni/coli Campylobacter spp*., *Arcobacter spp* and *Aeromonas spp.*). The complementary culture (using CAT-broth, AY-agar and blood agar with filter method) was thoroughly validated for its ability to grow these pathogens using spiked samples. Different media were tested (raw stool sample, Cary-Blair, with or without CAT-broth) and found to be suitable. If the BD MAX™ panel returns a positive result, reflex culture is also performed in order to obtain a cultured isolate for antibiotic susceptibility testing, strain typing and/or referral to a reference centre. This reflex culture is performed starting from the Cary-Blair medium, which has been validated to yield similar results as culture directly from the stool sample. Exceptions for reflex cultures is a PCR positive result for a pathogenic *E. coli* (STEC or ETEC), as these are difficult to distinguish from non-pathogenic *E. coli* which are abundant in faeces. One to two PCR batches are performed each day to minimize "waiting time" for stool samples (and thus TAT). Figure 2 shows the differences between the "old" traditional culture algorithm and the "new" algorithm using BD MAX™ . PCR results are technically and clinically validated every day of the week (in weekends the microbiologist on weekend duty), after which they become visible for the requesting clinicians. If the Ct value is high (>30), a comment is added to the result "Low bacterial load, clinical relevance?" If the Ct value is low, the result is communicated by telephone. If the Ct value is high and the reflex culture could not confirm the PCR result, the following comment is added to the report: "The positive PCR result could not be confirmed in culture".

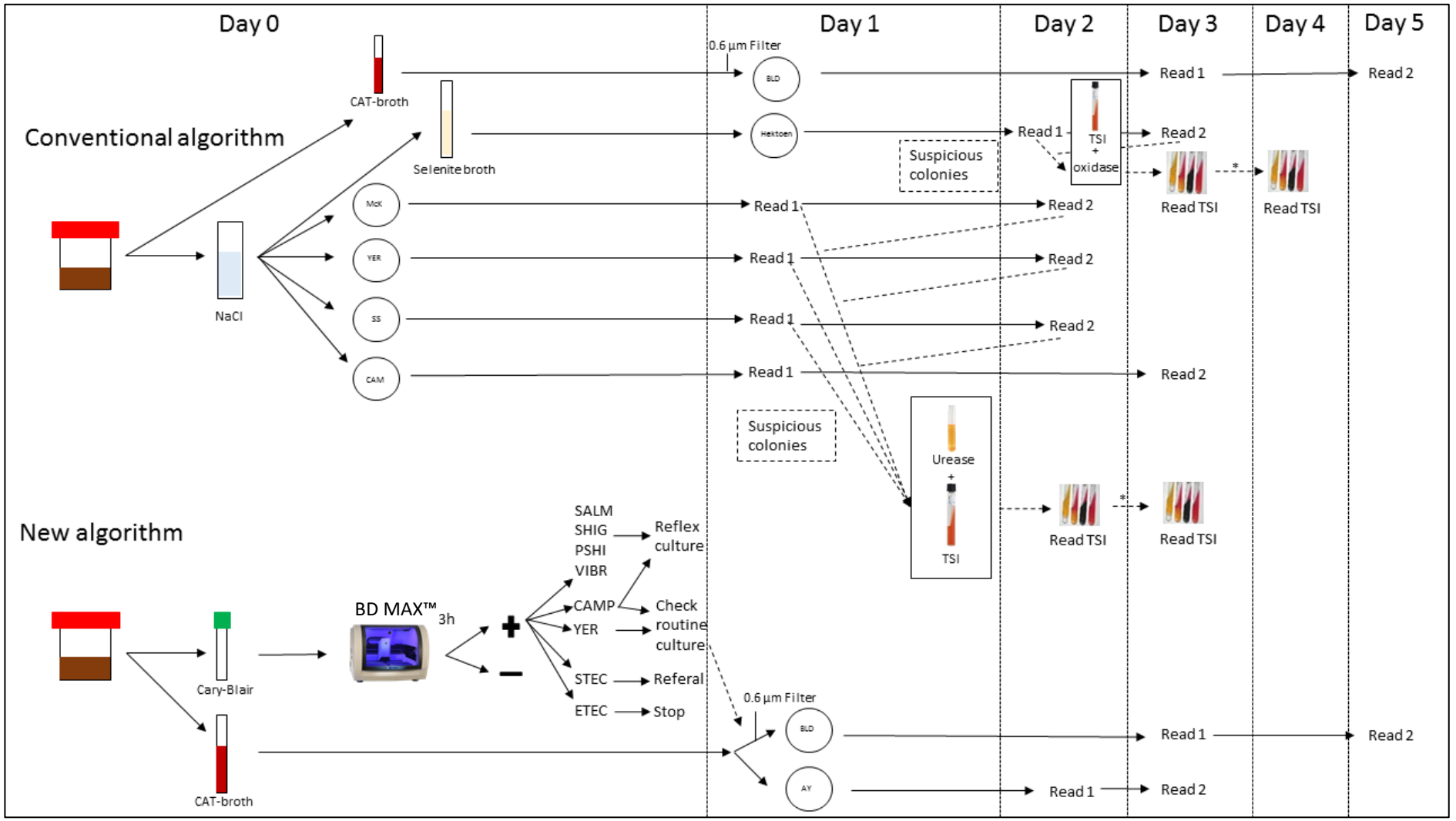

Figure 2: depiction of the workflow of faeces diagnostics, before and after implementation of routine multiplex PCR. **if suspicious colonies are identified on Read 2. McK: McConkey agar, YER: Yersinia agar, SS: Salmonella-Shigella agar, CAM: Campylosel agar, BLD: blood agar, Hektoen: Salmonella agar, TSI: triple sugar iron agar, CAT: cefoperazone, Amphotericin B, Teicoplanin , AY: aeromonas-yersinia agar. Note: urease could also be performed on Hektoen plates, and not all suspicious colonies were inoculated on TSI (depending on the urease result)*

As internal quality control a commercial material containing stabilized non-infectious bacteria (NATtrol GI control, ZeptoMetrix) is analyzed whenever there is a switch to a new lot of reagent. A "general bacteriology" EQC (UK NEQAS) is used for external validation whenever it contains a GI pathogen detected by culture.

Question 4) What is the impact after implementing a molecular diagnostic panel with reflex culture in AZ Sint-Lucas Ghent compared to the prior period in which conventional diagnostic techniques were used?

Data collection

A query in the laboratory information system was performed to obtain data for stool cultures performed between 01/01/2015 and 18/04/2022. The following information was obtained: age, sex, turn-around time, culture result, PCR result and time hospitalised at the moment of sampling. Quality control and study samples were excluded from further analysis. The period of 01/01/2015-27/06/2021 was defined as the "traditional culture" period, while the period 28/06/2021 – 18/04/2022 is the "PCR with limited culture and reflex culture" period (Figure 3). Between 16/09/2020 and 27/06/2021, validation took place. PCR results from this period were excluded. Before the introduction of PCR, if the laboratory received two samples from the same patient within 24 hours, the second sample was rejected. After the introduction of PCR, this period was extended to 7 days. The rationale for this decision was threefold: 1) PCR has a higher sensitivity 2) PCR remains positive for longer and 3) PCR has a higher reagent cost. Data were analysed using Microsoft Excel and R Studio (version 4.02).

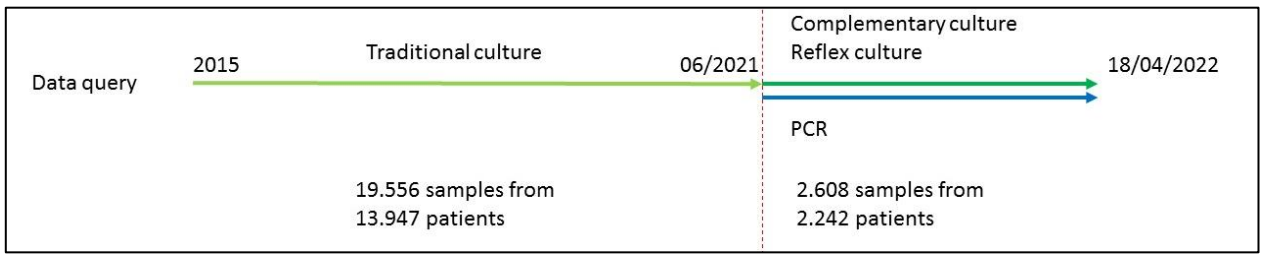

Figure 3. Timeline of the study period

General information

Between 2015 and 2021, around 3000 faeces samples with a request for bacterial pathogen detection were received each year. This translates into an average number of samples per month and per day of around 250 and 8.3, respectively (Figure 4). There was a slight decrease in sample numbers in 2020, although a recovery has been seen in 2021 and 2022.

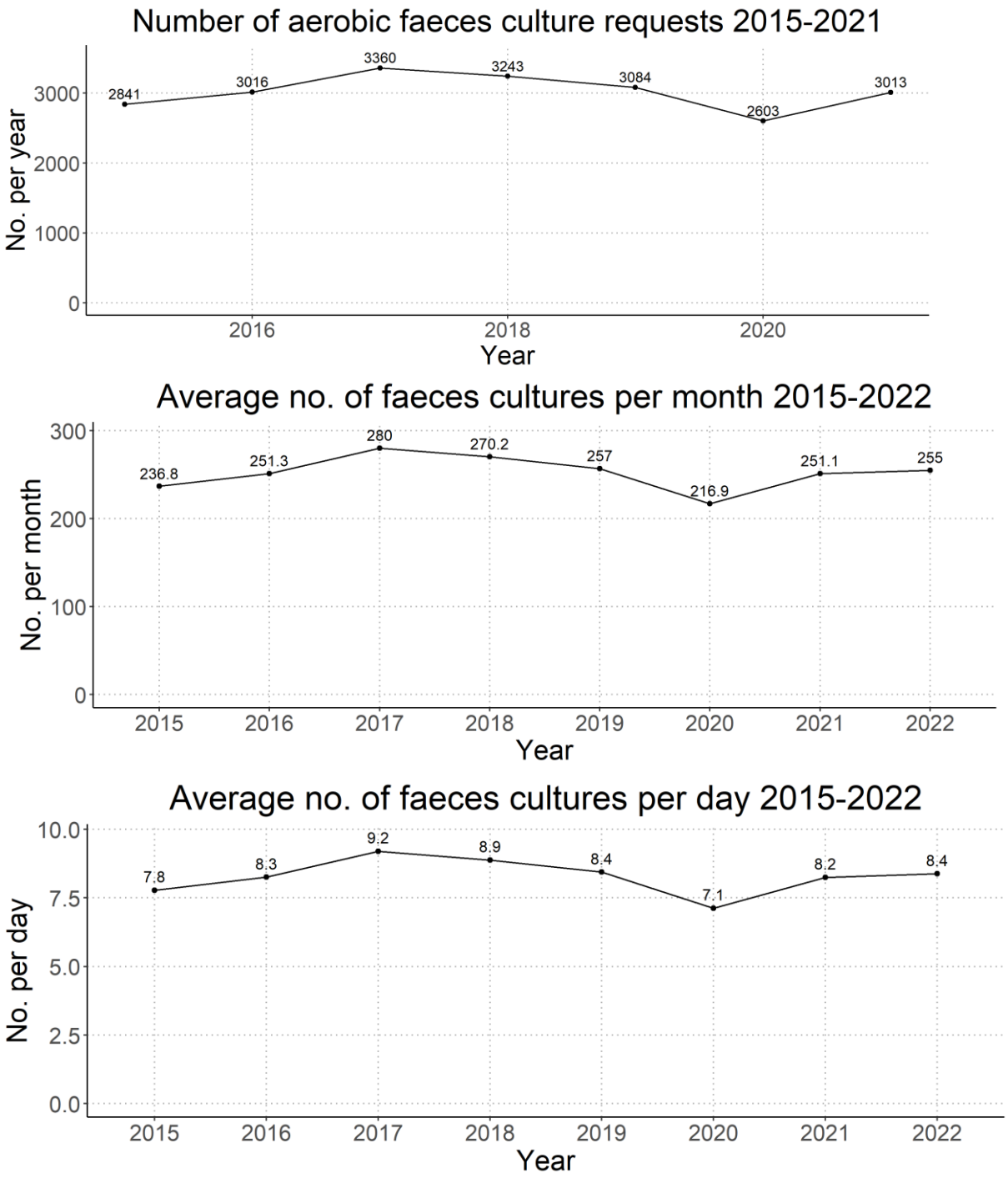

Figure 4. Number of sample requests for bacterial pathogen detection in faecal samples

The age distribution of the investigated patients was bimodal, with a peak <=2 years and between the ages of 60 and 90. There is also a small bump around 20-40 years. There were slightly more requests for women with an average female/male ratio of 56/44%. More than a quarter of samples were from outpatients, another quarter from internal medicine patients. Pediatrics and geriatrics are the third and fourth most requesting wards for stool cultures, respectively (Figure 5).

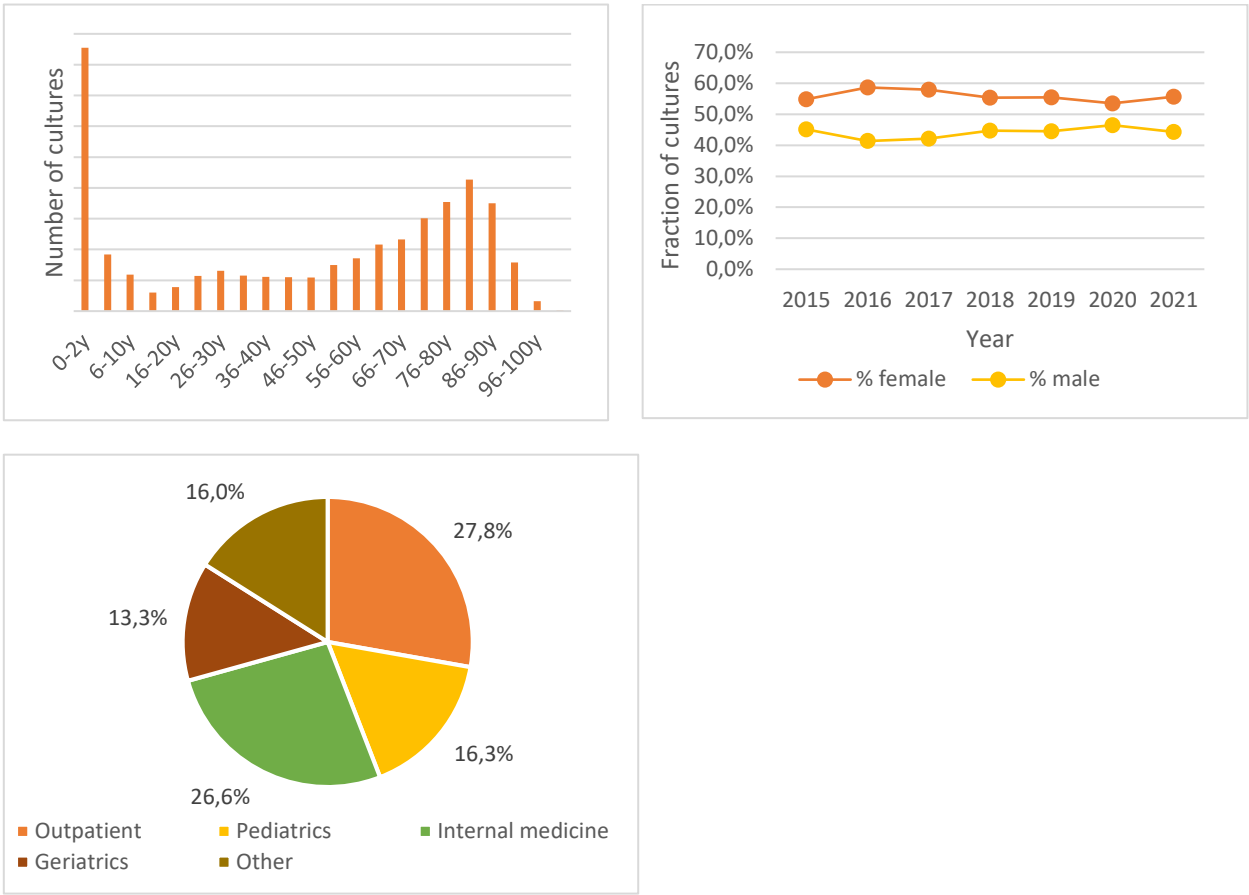

Figure 5. General information stool culture samples collected between 2015 and 2022 A) age distribution of stool culture requests B) sex distribution of culture requests C) distribution of requesting wards

Diagnostic yield analysis

Confirming reports in literature, the rate of negative cultures is high with on average only 5.0% of traditional cultures (975 of 19556 samples) positive for bacterial enteropathogens (excluding toxigenic *Clostridium difficile*, which is detected in around 4% of stool culture samples in AZ Sint-Lucas Gent [30]). For PCR, this number was 5.6% (146 of 2608 samples). If the results of PCR and the limited/reflex culture are combined, considering a sample positive if either the PCR or culture was positive, a positivity rate of 6.8% is reached (177 of 2624 samples). The evolution of the positivity rate over the last 7 years is shown in Figure 6.

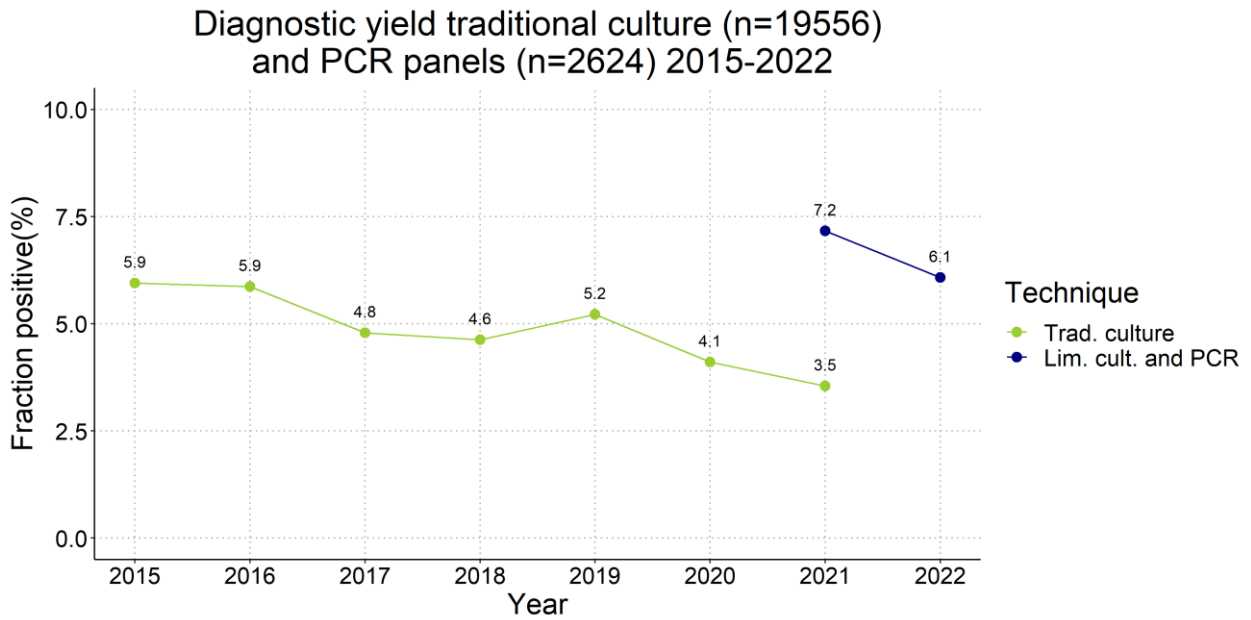

Figure 6. Positivity rate of the traditional culture and the new algorithm (limited culture and PCR). For the new algorithm, if *either* the limited culture *or* the PCR was positive, the sample was considered positive. If both the culture and the PCR were positive for the *same organism*, the result was only counted once.

The most commonly detected organisms using traditional culture were 1) *Campylobacter coli/jejuni* (2.98%), *Salmonella spp*. (0.7%), *Arcobacter spp.* (0.7%) and *Aeromonas spp.* (0.2%). Multiple pathogenic organisms were detected in 0.13% of samples (n=25). Using the BD Max extended enteric panel, the most commonly detected organisms were *Campylobacter coli/jejuni* (2.68%), STEC (1.0%), *Salmonella spp. (*0.88%) and *Yersinia enterocolitica* (0.58%). There was one PCR panel (0.04%)with a mixed result (*Campylobacter coli/jejuni* + *Salmonella spp.*)

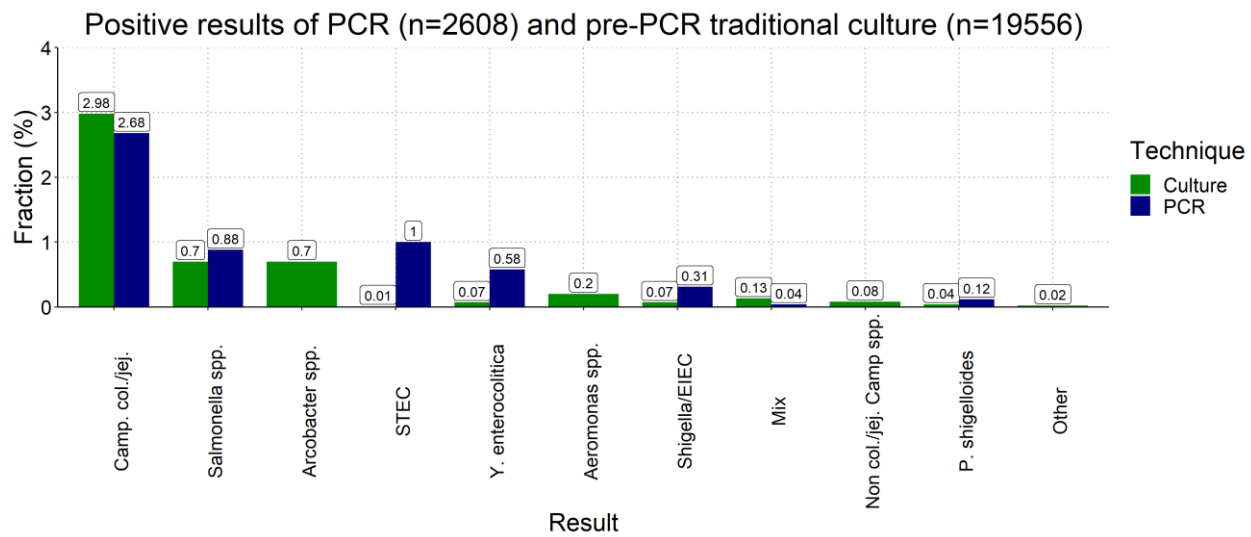

Figure 7. Detected micro-organisms over the entire study period, comparing traditional culture with PCR. *This Figure excludes the results obtained with the limited culture in the new algorithm.*

A detailed overview of all detected pathogens per technique and per year is presented in Table 2.

Table 2: Diagnostic yield of different pathogens per year per technique. The "Other" category contains 3 *Vibrio spp.* (same patient), 1 *Salmonella typhi* and *1 Yersinia pseudotuberculosis*. ETEC is not shown in this table as it is not routinely reported to the clinicians.

01/01/2015-18/04/2022 (n=24.788)	Period	Negative	*Campylobacter col./jej.*	*Salmonella spp.*	*Arcobacter spp.*	*Aeromonas spp.*	*Yersinia enterocolitica*	STEC	*Shigella spp.*/EIEC	*Non Camp col./jej. spp.*	*P. shigelloides*	Mixed	Other	Grand Total
Trad. culture	2015	2672 (94,1%)	101 (3,6%)	25 (0,9%)	30 (1,1%)	3 (0,1%)	1 (0%)		3 (0,1%)			6 (0,2%)		2841 (100%)
	2016	2839 (94,1%)	107 (3,5%)	21 (0,7%)	34 (1,1%)	5 (0,2%)	2 (0,1%)	1 (0%)	2 (0,1%)			5 (0,2%)		3016 (100%)
	2017	3199 (95,2%)	93 (2,8%)	20 (0,6%)	27 (0,8%)	8 (0,2%)			4 (0,1%)		2 (0,1%)	7 (0,2%)		3360 (100%)
	2018	3093 (95,4%)	96 (3%)	17 (0,5%)	16 (0,5%)	6 (0,2%)	3 (0,1%)		1 (0%)	3 (0,1%)	1 (0%)	4 (0,1%)	3 (0,1%)	3243 (100%)
	2019	2923 (94,8%)	102 (3,3%)	27 (0,9%)	15 (0,5%)	7 (0,2%)	4 (0,1%)		2 (0,1%)	1 (0%)	1 (0%)	1 (0%)	1 (0%)	3084 (100%)
	2020	2496 (95,9%)	56 (2,2%)	22 (0,8%)	8 (0,3%)	7 (0,3%)	2 (0,1%)		2 (0,1%)	7 (0,3%)	2 (0,1%)	1 (0%)		2603 (100%)
	2021 (jan-june)	1359 (96,5%)	28 (2%)	4 (0,3%)	6 (0,4%)	4 (0,3%)	1 (0,1%)			4 (0,3%)	2 (0,1%)	1 (0,1%)		1409 (100%)
Lim. and refl. culture	2021 (july-dec)	1527 (95,2%)	36 (2,2%)	14 (0,9%)	6 (0,4%)	5 (0,3%)	6 (0,4%)			5 (0,3%)	1 (0,1%)	4 (0,2%)		1604 (100%)
PCR	2021(july-dec)	1483 (93,9%)	46 (2,9%)	15 (0,9%)			11 (0,7%)	18 (1,1%)	2 (0,1%)		3 (0,2%)	1 (0,1%)		1579 (100%)
Lim. and refl. culture	2022 (jan-apr)	979 (96%)	15 (1,5%)	7 (0,7%)	3 (0,3%)	4 (0,4%)	5 (0,5%)		2 (0,2%)	3 (0,3%)		1 (0,1%)	1 (0,1%)	1020 (100%)
PCR	2022 (jan-apr)	979 (95,1%)	24 (2,3%)	8 (0,8%)			4 (0,4%)	8 (0,8%)	6 (0,6%)					1029 (100%)
Grand Total		23549 (95%)	704 (2,8%)	180 (0,7%)	145 (0,6%)	49 (0,2%)	39 (0,2%)	27 (0,1%)	24 (0,1%)	23 (0,1%)	12 (0%)	31 (0,1%)	5 (0%)	24788 (100%)

Figure 8 is a graphical depiction of Table 2, showing diagnostic yield per year per technique per organism.

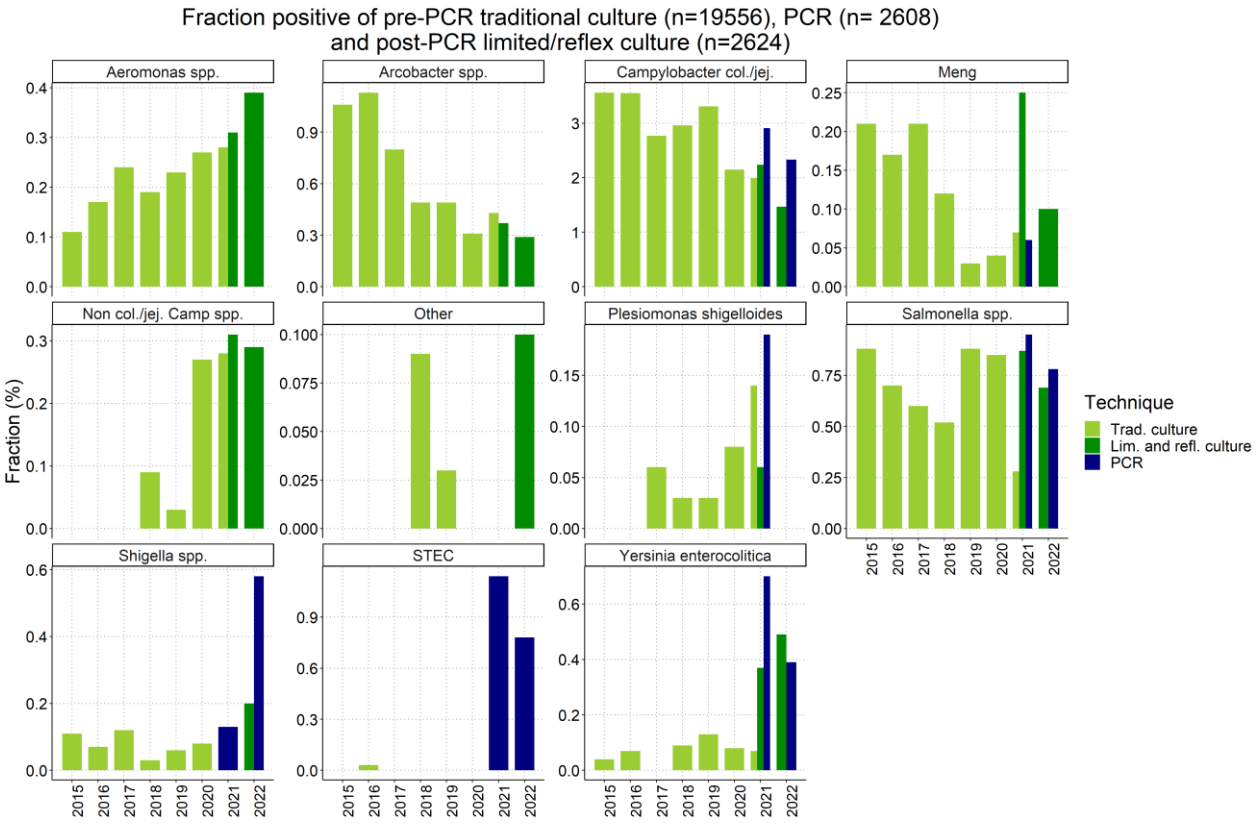

Figure 8. Diagnostic yield per year per organism, coloured by technique used.

For some organisms, there seems a clear increase in detection rate, such as for STEC, *Y. enterocolitica* and *Shigella/EIEC*. For *Campylobacter* and *Salmonella spp.*, positivity rates are in line with previous years.

Concordance between culture and PCR analysis

For the period 28/06/2021 – 18/04/2022 (post-PCR implementation), both PCR and a limited culture were performed (a complementary culture as well as reflex culture when the PCR was positive) on 2598 samples (Figure 3). Note that some pathogens are detectable by culture but are not included in the PCR panel. This concerned 33 pathogens in 28 samples (1.08%): *Aeromonas spp.* (n=12), Arcobacter spp. (n=12) and *non-jejuni/coli Campylobacter spp.* (*C. fetus*, n=1 and *C. upsaliensis*, n=8). Conversely, some pathogens are picked up by PCR panel while not (routinely) looked for using culture. This occurred in 36 samples (1.38%) in the same period with detection of STEC in 26 cases and ETEC in 10 cases, although ETEC is not routinely reported to clinicians. For the pathogens that can be detected by both techniques there were 89 pathogens (in 86 samples) where both PCR and culture were positive ("concordant"), 33 samples in which the culture was negative but PCR was positive, and 2 samples in which culture was positive but PCR was negative (Table 3).

Table 3: concordance analysis between PCR and limited (complementary) + reflex culture. Green shade indicates concordant results for organisms that both techniques can detect. Red indicates discordant results. Yellow shade indicates organisms that can be detected with one technique but not with the other. For this table, cultures/PCRs in which multiple were species detected were split up by organism.

Post-PCR period (28/06/2021-18/04/2022, n= 2598)	**Limited + reflex culture result**										% "missed" by culture
PCR result	**Negative**	***C. col./jej.***	***Salmonella spp.***	***Y. enterocolitica***	***Shigella spp.***	***P. shigelloides***	***Y. pseudo-tuberculosis***	***Aeromonas spp.***	***Arcobacter spp.***	***Non col./jej. Camp spp.***	
Negative	2421	1		1			1	12	12	9	
C. col./jej.	18	54									**25,0%**
Salmonella spp.	3		21								**12,5%**
Yersinia enterocolitica	4			11							**26,7%**
Shigella/EIEC	6				2						**75,0%**
Plesiomonas shigelloides	2					1					**66,7%**
STEC	26										
ETEC	10										
% "missed" by PCR		**1,9%**		**8,3%**							

Note that *Shigella* might also be detected by culture while EIEC cannot (as it is not easily distinguishable from non-pathogenic *E. coli* species). For the 6 *Shigella*/EIEC organisms that were "missed" by the culture it is thus unknown how many concerned *Shigella spp.* and how many concerned EIEC. This might give an underestimation of culture capabilities for this organism. On the other hand, when the PCR panel was positive for a given pathogen, the laboratory technicians sometimes tried long and hard to isolate the organism in culture (occasionally even performing multiple re-inoculations on different sample media). This might overestimate the capabilities of "routine" culture slightly, as less effort might have been done if the technician would have been blinded from the PCR result.

In the samples with a positive PCR result but negative culture, there is a difference in the average Ct values of "discordant" and "concordant" culture-PCR results, with discordant samples showing higher Ct values (Figure 9). This is consistent with a study by Gueudet et al. That showed a significant difference in Ct values of Campylobacter between samples which with "PCR positive/culture negative" result (Ct value <30 in 18.9% of cases) and "PCR positive/culture positive" result (Ct value <30 in 96.2% of cases) [31]. In conclusion, despite the overlap in Ct values between culture positive and culture negative results, the Ct value of the PCR can help to interpret the clinical significance of the result.

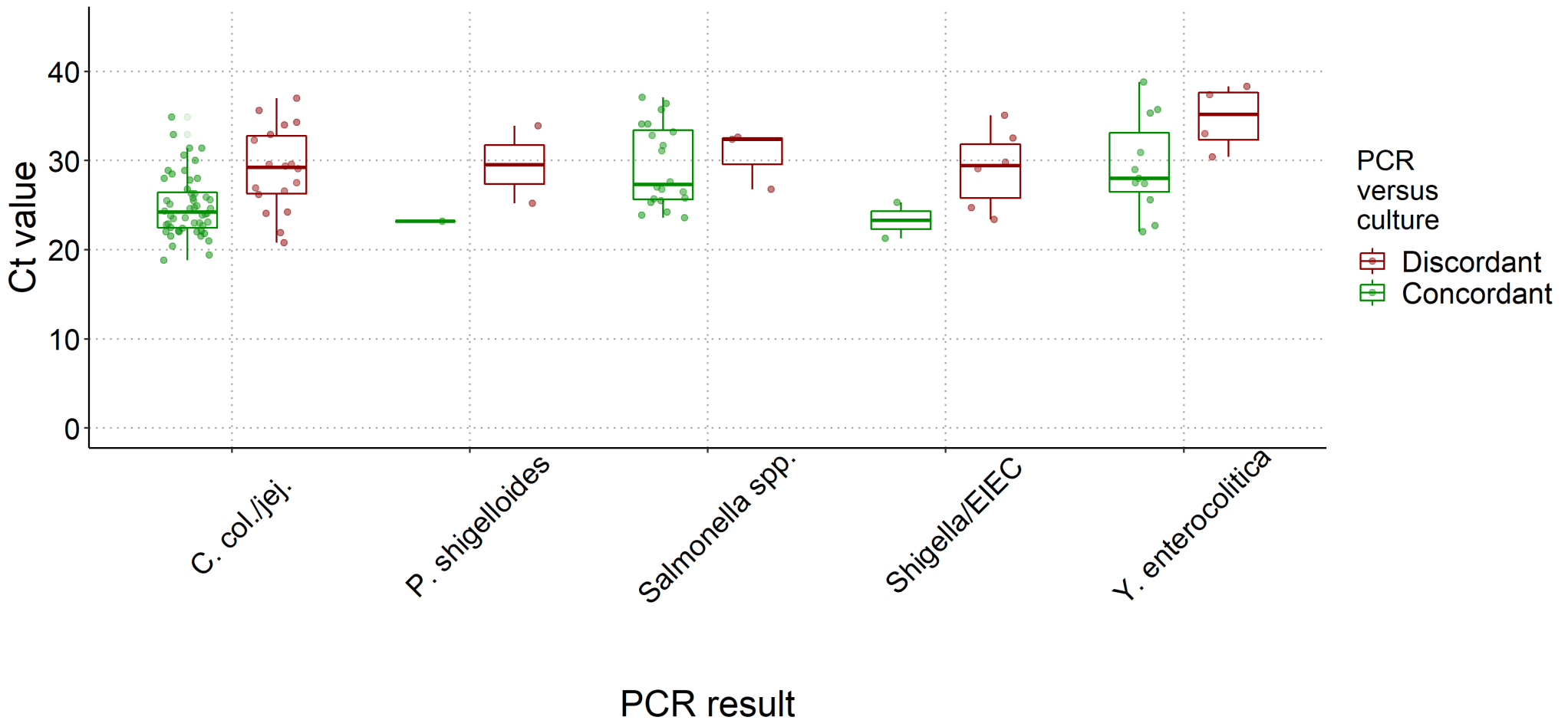

Figure 9. Relationship between Ct value and culturability. Concordant (n=86) and discordant (n=33) PCR results (organisms detectable using both culture and PCR). Samples from 112 different patients. One patient provided 4 samples (all *Shigella/EIEC*), in which 2 were concordant and 2 were discordant.

False positives

The amplification curves of the RT-PCR reaction need to be manually checked to verify whether the software of the analyser correctly identified positive and negative samples. In some instances, the analyser generated a positive result for a certain micro-organism (e.g. *Vibro spp*.) however the amplification curve was not sigmoidal and the sample was subsequently made negative by the micriobiologist, considering the result as "false-positive". In these cases, the analyser had called "background noise" positive because it reached a certain fluorescence threshold (See Attachment 6 for an example). The ratio of false-positive to true-positive results varied per organism, with *Vibrio* showing only false-positives and no true-positives (100% FP), while *Campylobacter* and *Salmonella* only showing true-positives and no false-positives (so 0% FP) (Figure 10). This is probably reflective of assay design and optimization to detect some organisms more correctly than others. In total there were 37 false-positives that occurred in 14 separate runs. Indeed, some runs contained many false-positives (up to 6 in a single run), while other runs contained only one. One of the BD Max analysers was accountable for 12 of these 14 runs that contained false-positives.

Another problem that occurred during the study period was contamination. There were 3 weakly positive *Shigella*/EIEC results following a strongly positive *Shigella*/EIEC sample (one in the same run and two in two consecutive runs). On repeat testing, these samples returned a negative result. This was suggestive for contamination, within or outside of the analyser.

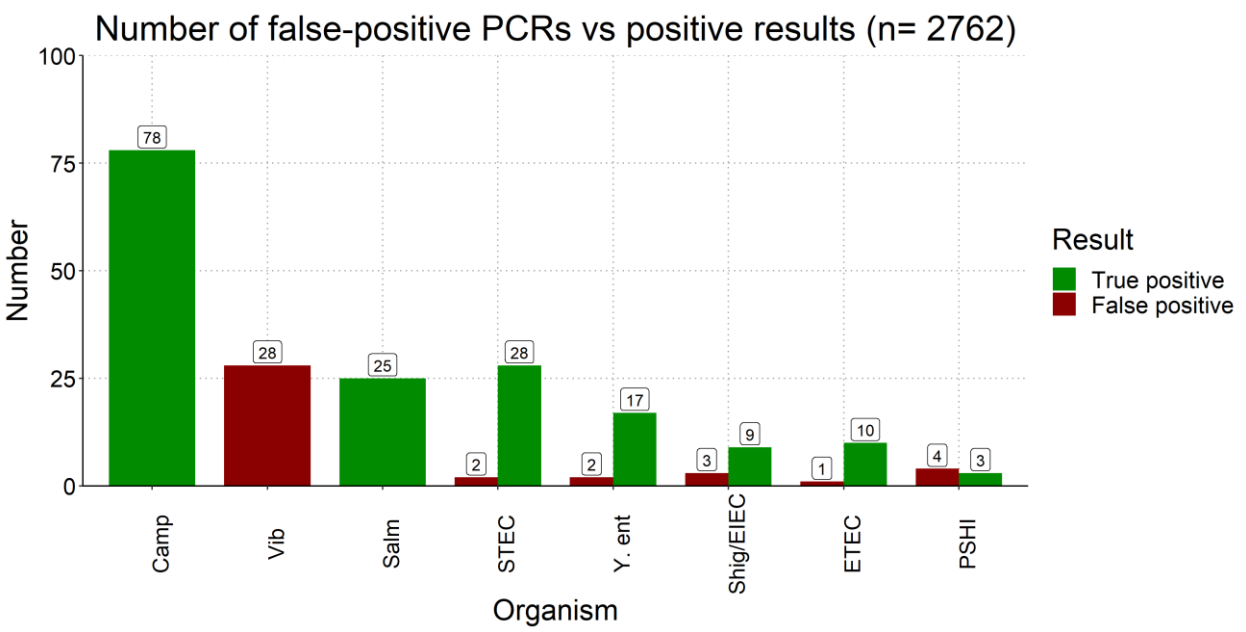

Figure 10. False-positive compared to "true" positive results. Note that the 3 false-positive *Shigella*/EIEC samples were considered contamination. The other false-positives concerned amplification curves flagged positive by the analyser software that were considered negative by the microbiologist.

TAT analysis

TAT was defined as the time between sample receipt in the laboratory and isolate confirmation, meaning it became visible for clinicians on the laboratory report. For PCR, this included the time it took for technical and clinical validation by the microbiologist. Table 4 shows the median TAT and percentiles 25, 75 and 90 for the traditional culture, PCR and limited/reflex culture. Figure 11 is a graphical representation of this table, providing more in depth information on the shape of the distribution of TAT of results.

Table 4: Turnaround time (TAT) of PCR and culture techniques (days)

	P25	Median (P50)	P75	P90
Trad. Culture (n=19556)	1.92 days	2.32 days	3.04 days	3.97 days
PCR (n=2608)	0.36 days	0.90 days	1.11 days	1.35 days
Limited/reflex culture(n=2608)	2.83 days	3.0 days	3.8 days	4.78 days

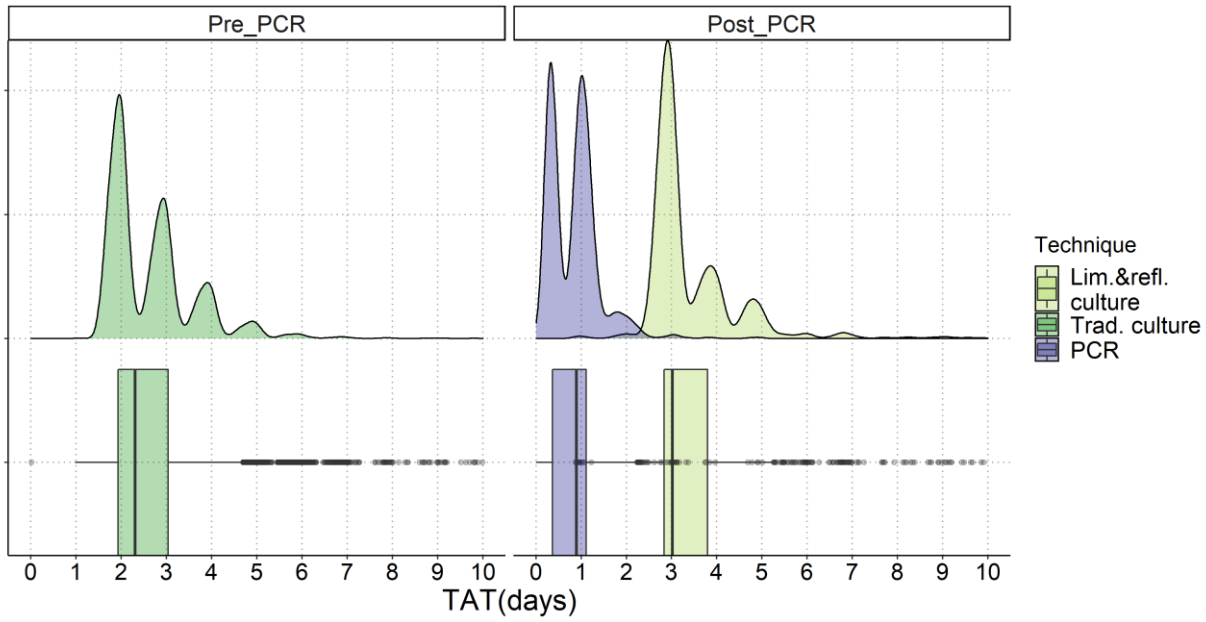

Figure 11. TAT density and boxplots for traditional culture, PCR and limited/reflex culture.

The median TAT for an aerobic culture isolate in the pre-PCR era was 2.3 days, with 90% of results reported within 4.0 days. For PCR, these numbers are 0.9 days and 1.4 days respectively, implicating a 61% faster median result and an 66% faster reporting of nine out of 10 results. The median TAT of the complementary culture is slightly longer compared to the median TAT of the traditional culture.

Note the bimodal distribution of TATs for PCR results, indicative of samples that are analysed same-day or next-day, depending on whether they arrived before or after the PCR batch (which is usually performed once or twice a day). For culture, multiple “humps” are seen as well, indicating different days of plate reading and confirming of results (Figure 11).

Stool shape

As some guidelines suggest the rejection of solid stool samples, we investigated the diagnostic yield of stools of different macroscopy, as well as the fraction of positive results that are obtained from different stool shapes.

Solid stools have the lowest positivity rate (around 3%) compared to semi-solid, slimy, fluidy, bloody and watery stools. Watery stools showed the highest positivity rate for cultures (9.7%) and bloody stools the highest positivity rate for PCR (8.6%). A rejection of solid stool samples would lead to 19.1% less samples to be analysed, however solid stool samples still account for around 12% of positive results, despite the low positivity rate, simply because of the large number of solid stool samples that are received (Figure 12).

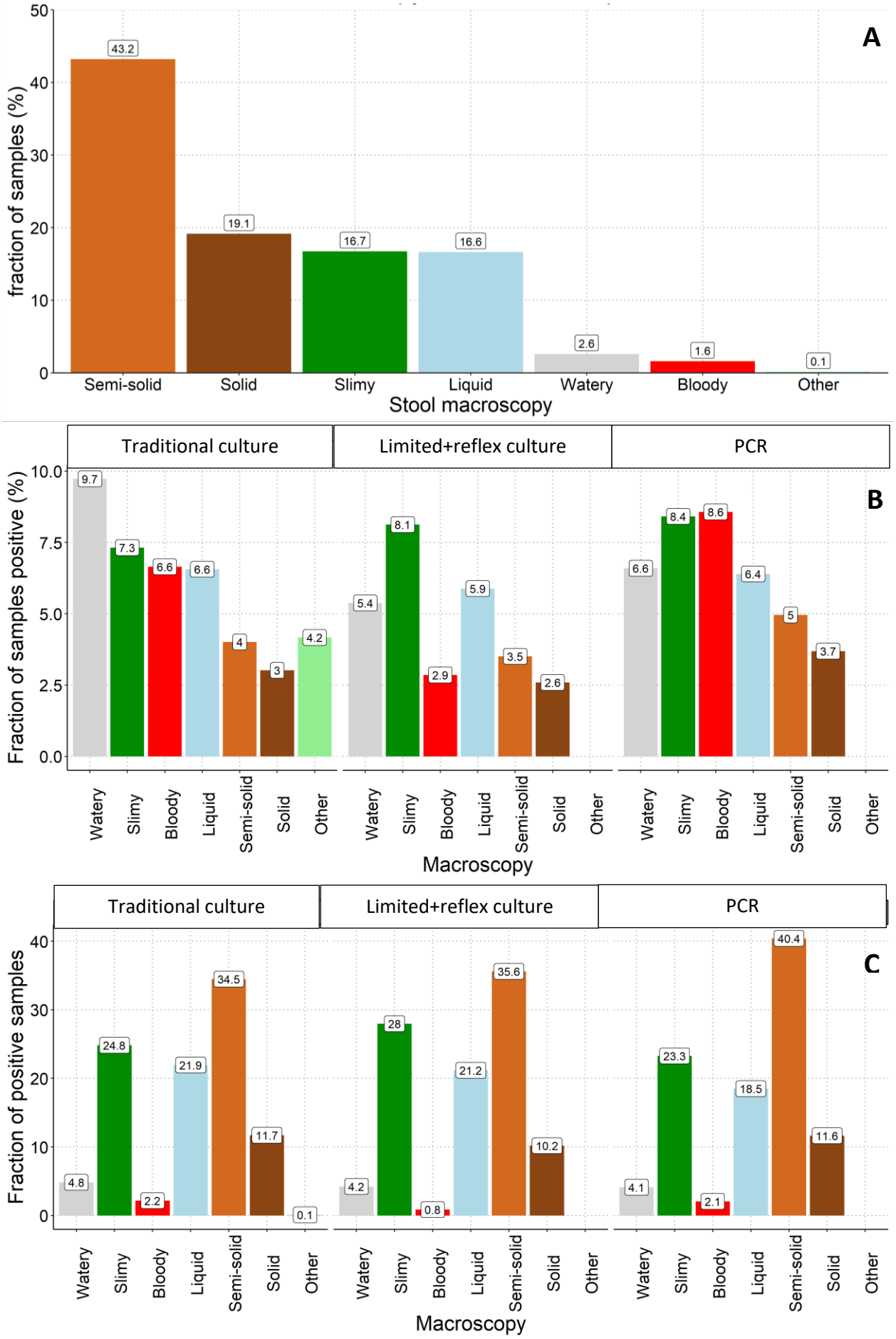

Figure 12. A) macroscopy of received stool samples **B)** positivity rate of stool samples of different shape (of all stool samples of shape x or y, how many were positive?) **C)** fraction of all positive results obtained from stools of different shapes (of all positive stool samples, how many had shape x or y?)

We also investigated whether another spectrum of pathogens is detected depending on the stool shape. Looking at Figure 13, there are no clear trends between the different stool shapes regarding which pathogens are mostly detected (except maybe a lower fraction of *Campylobacter* spp. detected solid stool samples) (Figure 13).

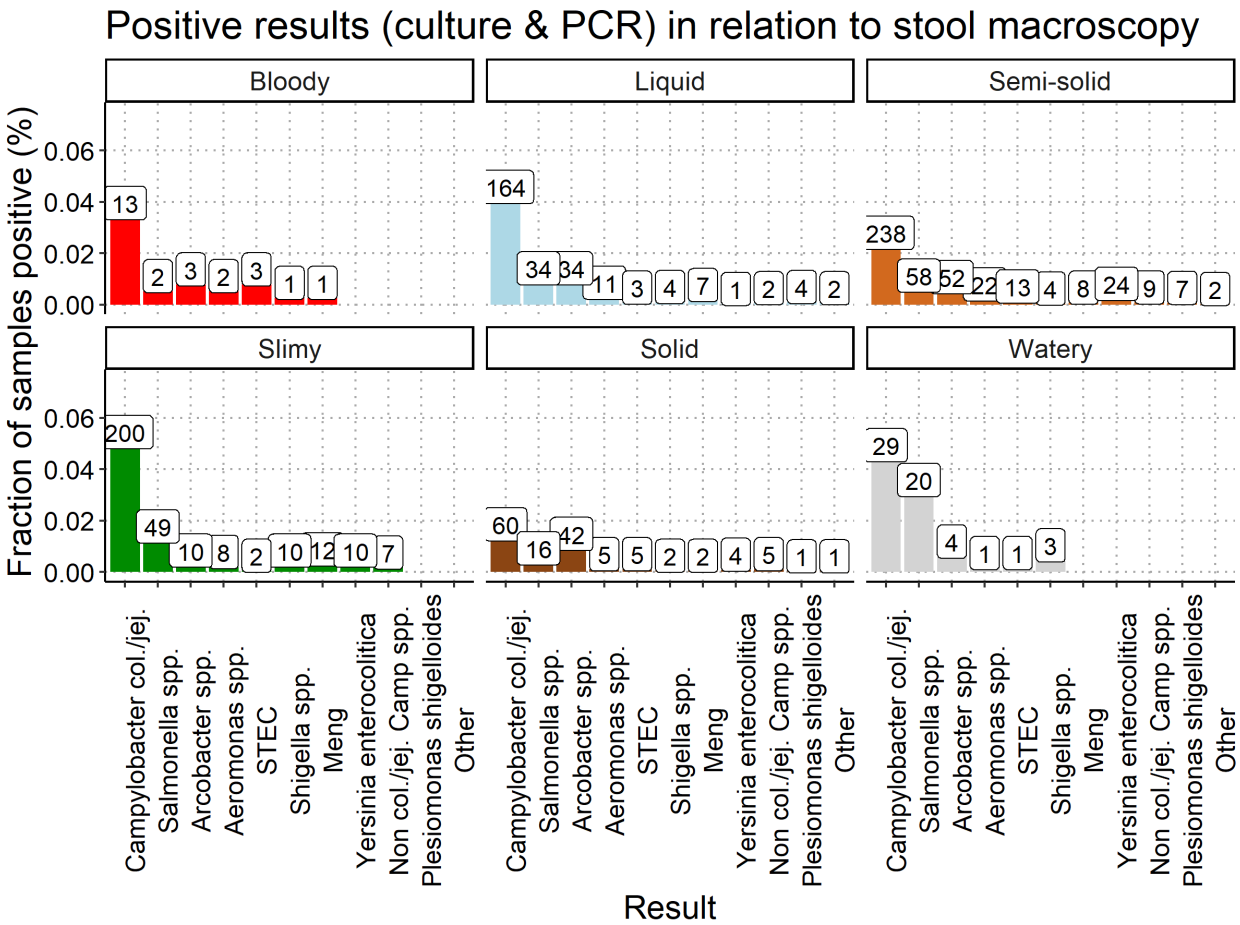

Figure 13. Which organisms are detected in stools of different macroscopy? In the y-axis, the fraction of samples in which a certain organism is detected is shown. Each column also has a label depicting the absolute number. This graph contains results from both culture and PCR.

Time after admission and diagnostic yield

Of all faeces samples, around 30% were taken from patients who were hospitalized for 3 days or longer. The positivity rate of samples from these patients (2.0% for culture, 1.8% for PCR) was lower compared to patients that were hospitalized for fewer than 3 days or who were not hospitalized at all (6.2% for culture, 6.8% for PCR). Despite the lower positivity rate, 12.6% of positive culture results are obtained from patients admitted to the hospital for 3 days or longer. In this group, relatively fewer *Campylobacter* infections are detected and relatively more *Arcobacter* infections. For the other pathogens, the distribution looks similar.

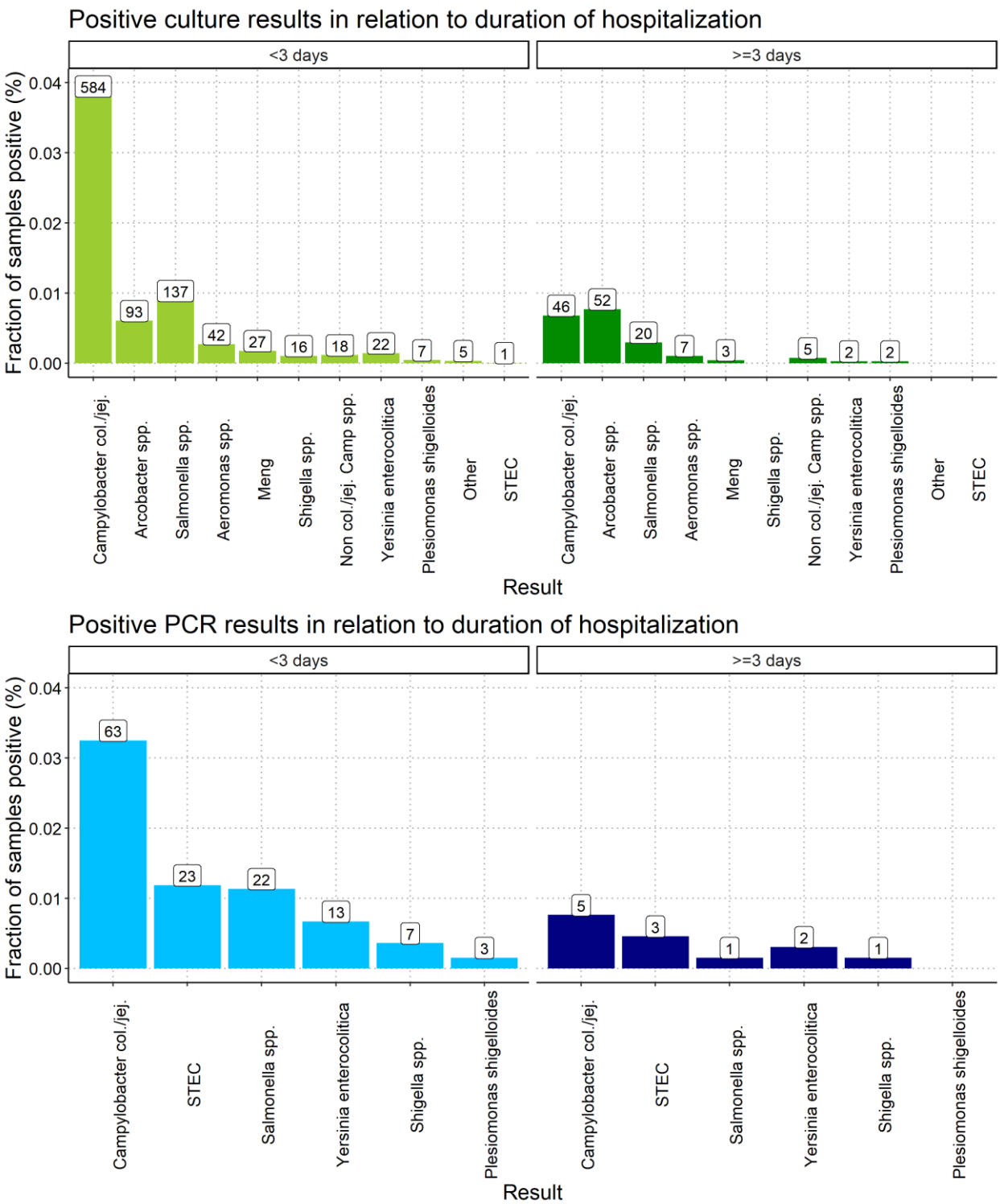

Figure 14. Relation between time hospitalized and pathogens detected. Fraction of positive samples of patients that are admitted to the hospital for 3 days or longer, compared to outpatients and hospitalized patients admitted for less than 3 days. In the y-axis, the fraction of samples in which a certain organism is detected is shown. Each column also has a label depicting the absolute number. This graph contains results from both culture and PCR.

Costs analysis

Since the BD MAX™ analysers were already in place for other molecular tests, the investment cost of purchasing this equipment is not included in the cost analysis described here. However, for laboratories considering to purchase a new analyser this investment should also be taken into account. Several other costs (the cost of reflex culture, performed in around 5% of cases, the maintenance cost for the BD MAX™ analysers and VAT) were omitted from this cost analysis.

Reagent cost (see Attachment 4) for the traditional culture workflow was estimated to be around €9.4 while the current workflow (excluding reflex cultures) amounted to a reagent cost of €18.3 (€4.2 for the culture and €14.1 for the PCR panel), all figures excluding VAT. However, since the number of plates that needs to be inoculated and read is reduced, this decreases the personnel time spent per faeces sample from around 15 minutes to around 7 minutes. If a gross cost of €0.672 per minute of hands-on time is taken into account, the total cost of the previous algorithm sums up to about €19.5, while the current algorithm is around €23.0 (€7.5 for the culture and €15.5 for the PCR). This small difference in cost was considered an acceptable investment to improve workflow, standardisation, TATs and sensitivity.

According to the RIZIV/INAMI, routine stool culture should look for *Salmonella spp., Shigella spp., Yersinia spp*. and *Campylobacter spp*. There is some additional nomenclature for parasites and rota/adenovirus (in children <2 years old), but not for additional (emerging) pathogens such as *Arcobacter, Plesiomonas* or *Aeromonas* species (see attachment 5) The RIZIV/INAMI does not have a specific nomenclature number for molecular panels, which is not incentivizing for laboratories to make investments in this promising technology with potential benefits for patient care, unless it leads to cost and/or time savings for the laboratory itself. Clinical laboratories are making this switch nonetheless in order to provide a desired service for clinicians and patients, and to gain experience with these assays. Another advantage for the laboratory is increased quality and standardisation through reduction of interpersonal variability in the assessment of bacterial cultures. Indeed, varying technical competency and experience in the laboratory team leads to divergent interpretations of bacterial cultures. This is much less the case for easy-to operate molecular panels that provide objective test results [32].

Molecular techniques are here to stay and their use is steadily increasing, leading to lowered costs and improved technical performance (a trend which COVID-19 greatly accelerated in the past two years). There should be some thought on how the benefits of this technology of the future might be more acknowledged, since studies have shown that they might lead to cost savings for healthcare overall by reducing hospital stay and other technical investigations Now the cost is carried by the laboratory but the potential savings might be gained elsewhere in the healthcare system [33].

Question 5) What are potential future directions faeces diagnostics?

Ct values as cut-offs?

The Ct value, or the number of amplification cycles required for a positive PCR result, is inversely related to the pathogen quantity in the tested sample. It is therefore often used as a proxy of viral or bacterial load. A recent systematic review summarized the available evidence on the association between Ct values and patient presentation (asymptomatic vs symptomatic) and clinical outcomes (good versus poor outcome). The most commonly studied pathogens in this regard were *C. difficile* as well as norovirus and rotavirus. There were also a limited number of studies on *Shigella* spp., pathogenic *E. Coli*, *Campylobacter* spp., *Salmonella* spp. *Vibrio cholera*e and *Aeromonas* species. In general, lower Ct were reported in cases versus controls, with an increased chance for the presence of clinical symptoms such as vomiting, dehydration and diarrhea if Ct values were lower. Additionally, some studies (especially for noro and rotavirus, as well as *Shigella/*EIEC) suggest associations between lower Ct values and severity of symptoms. Regarding duration of clinical symptoms, no difference in bacterial or viral load was observed for any pathogen [27]. The study concludes that Ct values may have utility (next to the clinical characteristics) in defining symptomatic causality, aiding to distinguish between infection and colonization. These conclusions are backed with the most evidence for *C. difficile* and noro/rotaviruses, with only limited (and sometimes inconclusive) data for non-*C. difficile* bacteria and parasites, indicating that Ct values should be used with caution for these agents [27].

For some pathogens such as EPEC, EAEC and *Aeromonas* bacterial loads are typically less clearly discernible between cases and controls because of more asymptomatic carriers of these bacteria [34].

Note that Ct values are not interchangeable between laboratories as this is not a universally standardized parameter. Its value depends on multiple variables such as the timing between onset of symptoms and sampling, specimen source, transport media, the amount of sample used, the extraction procedure, master mix components, PCR reaction efficiency and many more. This implicates that Ct values cannot be directly compared between the different commercially available CE-IVD multiplex kits, and therefore a “universal” Ct cut-off discerning cases from controls or mild from severe diseases is not possible at this moment. Furthermore, the BD MAX™ Extended Enteric Bacterial Panel, although providing a Ct value, only claims to detect nucleic acids qualitatively. In other words, the assay is not intended to be used in a quantitative manner. In fact, this is the case for many (if not all) of the molecular panels mentioned in Attachment 3, with some panels not providing a Ct value at all (such as the FilmArray® GI panel).

Ideally, until further standardization occurs, each laboratory would identify the optimal Ct cut-off for discerning pathogens as “disease-causing” or “innocent bystander” for the molecular panel used in that laboratory and the patient population served. As there is overlap in the Ct value distributions between these two groups, this cut-off would be associated with a certain sensitivity and specificity. It should also be stressed that the Ct value alone is insufficient to make this distinction, and clinical information should always be considered in parallel [27].

Future developments in the use of PCR panels

Reimbursement

At the time of writing, there is a discrepancy between the reimbursement criteria (See attachment 5) which still reflect the traditional microbiological methods (culture, antigen testing and microscopy) and the clinical reality of rapid implementation of molecular panels. A pragmatic approach could be to replace the words “culture” with “detection” in these criteria, to delete the word “enrichment” for parasites (as this is not necessary for molecular methods) and perhaps to incorporate restriction periods (e.g. once every 1 to 7 days) to avoid excessive testing.

Another potential approach would be to create several clinical gastro-intestinal "syndromes", which could then be coupled to the reimbursement of detection of specific sets of pathogens (combining bacteria with viruses and/or parasites). For example, for a community acquired acute gastro-enteritis in a normally healthy individual a less broad spectrum of pathogens needs to be searched after compared to a gastro-enteritis in an outbreak setting or immunocompromised individual.

Quality control

Reflex culture has several advantages including the possibility for antimicrobial susceptibility testing, but it can also be used as a quality control measure for the used culture-based methods. If the PCR returns a positive result reflex culture should be able to grow the respective organism, provided the bacterial load is sufficient and the bacteria are still viable. Vice versa, reflex culture confirms the specificity of the molecular panel by ascertaining the presence of a certain bacteria using another identification method (typically MALDI-TOF MS).

For internal quality control of the GI PCR panel, commercially available materials containing target sequences can be used. The external quality control options are limited, however (to our knowledge) at least a single provider exists (QCMD). As a surrogate for an external quality control program, diagnostic yield can be compared to national surveillance data. In Belgium this is accessible through the Epistat website of Sciensano [35]. The proportions and trends of detected pathogens should parallel provincial (or even national) proportions and trends of reported pathogens. A limitation of this strategy is that not all pathogens are reported to Sciensano.

Susceptibility testing and strain typing

Some concerns regarding widespread implementation of molecular panels include the lack of antibiotic susceptibility testing in current molecular panels, the lack of a viable strain for typing, and the loss of culture capabilities and expertise which might limit our ability to detect new or aberrant causes of gastro-enteritis. A potential solution for these limitations of PCR would be for moderate and large size laboratories to retain culture capabilities to perform reflex cultures in case of a positive PCR result, while smaller laboratories send these samples to a reference laboratory [32]. Regarding strain typing and outbreak investigation, new technologies such as metagenomics could provide potential solutions (See below).

An alternative to phenotypic methods of antibiotic resistance testing is the detection of resistance genes which can be also performed using PCR. This genotypic approach is becoming more widespread and is already routinely used for the detection of methicilline resistant *Staphylococcus aureus* (mecA gene), vancomycine resistant *enterococci* (vanA and vanB genes) and carbapenemase producing enterobacterales (e.g. OXA48) [36]. Besides some small studies showing the proof-of-concept of this approach to demonstrate antimicrobial resistance in stool samples, this technique is not yet commercially available at this moment [37,38]. Note that genotypic approaches for resistance testing have their own set of challenges because of the genetic complexity of antibiotic resistance. The detection of a resistance gene may not necessarily imply phenotypic resistance, for example if the gene is expressed at low levels or is not functional [36,39]. More research and development is needed in this area before this technique can compete with phenotypic methods for detection of antimicrobial resistance.

Emerging pathogens

Multiplex molecular GI panels have increased the understanding of the pathogenic role of several species, as better detection led to a better estimate of the true incidence. For example, culture methods cannot accurately detect and distinguish the different types of pathogenic *E. coli* which are implicated in GI disease. Systematic detection of diarrheagenic pathotypes of *E. coli* by several molecular panels point to a clear pathogenic role of some types (STEC, ETEC, EIEC), while it seems that EAEC is less clearly associated with disease. Similarly, the pathogenic potential *Aeromonas* and *Plesiomonas* became more clear thanks to their inclusion in several molecular panels [34].

While molecular panels might increase understanding of pathogens included in their scope, all other potentially present micro-organisms are invariably missed. For example, some *Arcobacter* spp. have been implicated in GI disease, however none of the commercial panels include target sequences for *Arcobacter* [40]. Other examples include *Eschericia albertii*, *Providencia alcalifaciens* and *Klebsiella oxytoca* as potential emerging pathogens in AGE [8]. Also, the lack of thorough characterisation of pathogens included in molecular panels might mean that changes in pathogenicity will go undetected. Examples include hypervirulent or aberrant strains, such as the outbreaks of *C. difficile* NAP1/027 in Canada or specific strains of STEC with more pathogenic potential such as the O157:O7 or the O104:H4 outbreaks [32]. Finally, targeted multiplex PCR panels do not allow for the discovery of new emerging pathogens. The latter requires an unbiased, hypothesis-free approach, such as culturomics or metagenomics [41].

Metagenomics

PCR techniques determine the presence or absence of a specific target gene (or set of genes in multiplex PCR) using sequence-specific primers and probes. Next-generation sequencing (NGS) techniques, in contrast, can potentially provide information on the entire genome of all microorganisms in clinical samples, including the presence of virulence genes or antibiotic resistance genes. Microbial NGS approaches directly on uncultured samples are sometimes referred to as “metagenomics” and include several different techniques. One approach is the targeted amplicon NGS, in which PCR is first used to enrich microbial DNA by amplyfing the 16S rRNA (for bacteria) or the 18S rRNA (for fungi) before sequencing. An advantage of this technique is that less sequencing depth is required for accurate readings, as regions of interest are artificially multiplied while the background DNA is not. A disadvantage is that it is not completely hypothesis free, as it required a choice in what DNA fragments should be amplified. Another limitation is that only the 16S rRNA or 18S rRNA is sequenced, and not the entire genome of the potential pathogens [42].

A truly hypothesis free technique that provides information on the entire genome is “shotgun metagenomics”, in which all nucleic acids in a sample extracted, randomly broken up into short fragments and subsequently massively sequenced in parallel. This technique has been put forward as a potential “universal diagnostic test”, able to detect (as well as determine the relative abundance) of both bacteria, fungi, viruses, archaea and parasites.

Although there have been some proof-of concept studies showing clinical applications of shotgun metagenomics, this technique is currently almost exclusively being used in research settings because of cumbersome work-up, high cost and long turnaround time compared to syndromic PCR panels. Although as the cost of sequencing decreases, these techniques might gradually find their way into the clinical laboratory. A potential place to start are those settings in which there is a high suspicion of infectious etiology of a clinical syndrome, but conventional and PCR techniques fail to demonstrate a putative organism. In these cases the culprit is usually a more rare organism which is not easy to cultivate, typically in the setting of immunocompromised patients [42]. Amplicon NGS (in the form of 16S rRNA or 18S rRNA PCR and sequencing) is already used routinely in some centres for work-up of culture-negative

prosthetic joint infection and endocarditis. Sequencing data can also be used to demonstrate the clonal nature of pathogens involved in outbreak settings, as well as to construct phylogenetic trees to investigate transmission routes.

One Health

Many of the causative agents of AGE are of zoonotic in origin and individuals with AGE are often infected through the ingestion of contaminated foods. Therefore, diagnostics of faeces is close to the monitoring of microbes in food industry, and a lot of research into virulence factors and antimicrobial resistance has been performed by research groups in the latter field. It has been widely recognized that there is a relationship between the virulence and antimicrobial resistance of pathogens among animals and in the environment and those implicated in human health and disease. The One Health approach recognizes this connection between the health of humans and that of animals and the environment, and promotes collaboration across disciplines in these different fields with a goal of optimizing control and prevention of infectious diseases [43]. Put differently, lessons learned in veterinary medicine and food industry and techniques applied in those fields can be used to drive forward clinical diagnostics in human medicine. For example, an approach that is currently used in food industry is the detect virulence genes such as invA (Salmonella), NHE (B. cereus), hly and ActA (Listeria). The presence of virulence genes ascertains that the detected bacterial species has pathogenic potential, and is less likely to be an innocent bystander [44].

COMMENTS

/

TO DO/ACTIONS

1) The information obtained in this CAT will be added to the validation report of the BD MAX enteric panel (continuous validation).
2) The findings will be summarized in a poster format.
3) The findings in this CAT will be used to inform decisions on whether or not the complementary culture will be kept as a parallel track next to the PCR test with reflex culture.

ATTACHMENTS

Attachment 1: BD MAX™ Extended Enteric Bacterial Panel technical principle

To perform a PCR, an extraction step is almost always necessary to purify the nucleic acids present in the sample. This is typically performed using lysing reagents that break up cells and nanoparticle magnetic beads that are able to selectively bind nucleic acids (Figure S3). After extraction, a PCR reaction is performed in a thermocycler using a mastermix that contains primers specific for a nucleic acid sequence of the investigated pathogen. In multiplex PCR, multiple primers are used in the same reaction vessel, allowing for the simultaneous amplification of nucleic acids from different pathogens (See Attachment 2 for the BD MAX™ targets). The detection of amplification product is done using fluorescent sequence-specific hybridization probes, directed at specific sequences for the different pathogens in the panel. Each probe has a different excitation and emission wavelength, allowing for the simultaneous detection of multiple amplification products. "Real-time" PCR indicates that a signal (e.g. fluorescence) (which corresponds to the amount of amplified target sequence) is measured every amplification cycle, resulting in a "fluorescence" or "amplification" curve when plotted (Figure S1). When the fluorescence exceeds a predefined threshold, the sample is considered "positive" for the pathogen from which the genetic material was amplified. The number of PCR amplification cycles required to reach this threshold is called the "Cycle threshold" or "Ct value". This technique allows for a (semi) quantitative determination of the amount of starting material in the sample, giving an estimate of the bacterial or viral load. This is in contrast to end-point PCR in which the signal is only measured at the end of the PCR (typically after 40-45 cycles), which can only give a qualitative result (positive or negative). Note that some real-time PCR panels do not provide a fluorescence curve or Ct value to the user, but only provide a qualitative result (e.g. the FilmArray® GI panel).

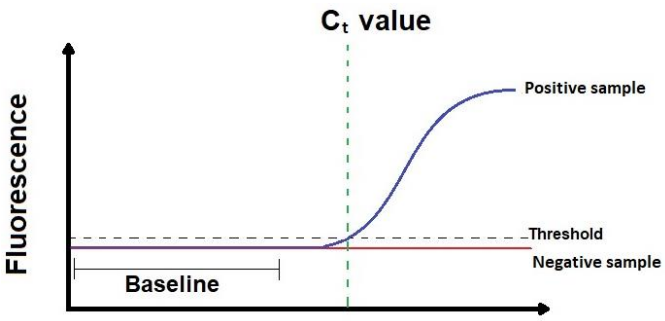

Figure S1. Fluorescence or amplification curve of a real-time PCR assay. Ct: cycle treshold. Adapted from [31]

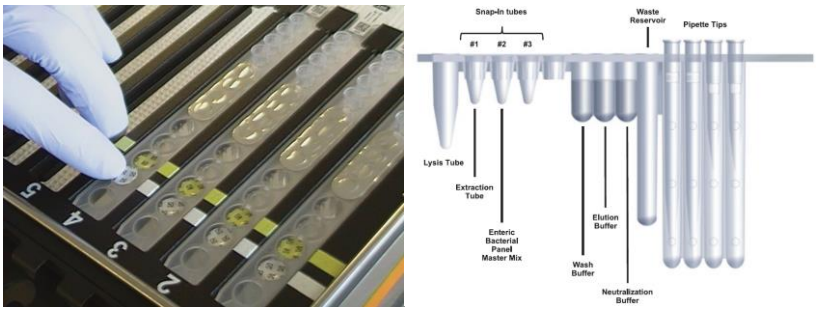

Figure S2: BD MAX™ reagent strips [46]

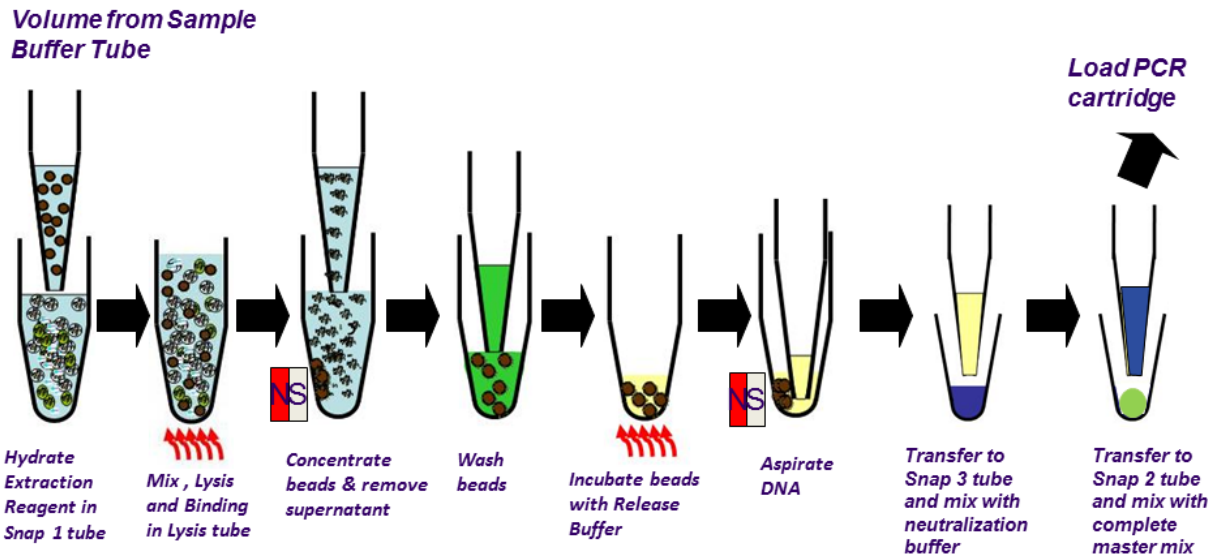

Figure S3: BD MAX™ sample preparation principle [46]

Attachment 2: wavelengths and gene targets used in the BD Max extended enteric bacterial panel [46]

Per sample, the BD max performs 2 PCR reactions.

Wavelenght (nm)	Excitation (nm)	Emission (nm)	Color	Target (gene)
475/520	464-490	510-530	Green	*Campylobacter* spp (tuf)., *Yersinia* spp. (invA)
530/565	520-540	559-571	Yellow	*Salmonella* spp (SpaO). , *ETEC* (eltA, sta1, sta2)
585/630	555-598	618-638	Orange	*Shigella/EIEC* spp. (ipaH), *Vibrio* spp.(atpA)
630/665	622-636	657-670	Red	*STEC/S. dysenteriae* (stx1a, stx2a), *Plesiomoas spp.* (not-specified)
680/715	673-683	710-790	Paars	SPC

Attachment 3

Scope of pathogens detected and list-prices of several CE-IVD molecular syndromic panels for GI pathogens (based on [7,11] and others)

		BD MAX™ Extended Enteric Bacterial Panel (BD)	**CLART Enterobac (Genomica, Spain)**	**Verigene II Gastrointestinal Flex assay (Luminex)**	**QIAstat-DX GI panel (Qiagen)**	**NxTAG GI pathogen panel (Luminex)**	**BioFire® FilmArray® GI panel (Biomérieux)**	**Allplex GI panels (Seegene, Accuramed)**	**Gastrofinder 2SMART (PathoFinder)**	**EntericBio panels (Serosep, Mediphos)**	**ampliCube (Mikrogen)**	**RIDAGENE (R-Biopharm)**
Bacteria	Campylobacter	X	X	X	X	X	X	X (bac I)	X	X (Dx)	X (bact)	X (bac I)
	Salmonella	X	X	X	X	X	X	X (bac I)	X	X (Dx)	X (bact)	X (bac I)
	Shigella/EIEC	X	X	X	X	X	X	X (bac I)	X	X (Dx)	X (bact)	X (bac I)
	STEC	X	X	X	X	X	X	X (bac II)	X	X (Dx)	X(bact)	X (bac I)
	Yersinia enterocolitica	X (extended)	X	X	X	X	X	X (bac I)	X	X (Dx)	X (bact)	
	Vibrio spp	X (extended)		X	X	X	X	X (bac I)		X (Dx)		
	Plesiomonas Shigelloides	X (extended)		X	X		X					
	ETEC	X (extended)	X	X	X	X	X	X (bac II)	X		X (bact)	
	EAEC				X		X	X (bac II)				
	EPEC		X		X		X	X (bac II)	X			
	C. difficile (toxigenic)		X	X	X	X	X	X (bac I & II)	X			
	Aeromonas spp.		X					X (bac I)				
Viruses	Adenovirus	X (vir)		X	X	X	X	X (vir)	X	X (vir)	X (vir)	X (Vir III)
	Norovirus	X (vir)		X	X	X	X	X (vir)	X	X (vir)	X (vir)	X (Vir III)
	Rotavirus	X (vir)		X	X	X	X	X (vir)	X	X (vir)	X (vir)	X (Vir III)

	Sapovirus	X (vir)		X	X	X	X	X (vir)	X	X (vir)	X (vir)	
	Astrovirus	X (vir)		X	X	X	X	X (vir)	X	X (vir)	X (vir)	
Parasites	Cryptosporidium spp.	X (par)		X	X	X	X	X (par)	X	X (Dx)		X (Par)
	Cyclospora cayetanensis			X	X		X	X (par)				
	Entamoeba histolytica	X (par)		X	X	X	X	X (par)	X	X (Dx)		X (Par)
	Giardia duodenalis	X (par)		X	X	X	X	X (par)	X	X (Dx)		X (Par)
	Blastocystis hominis			X				X (par)				
	Dientamoeba fragilis			X								X (Par)
	Microsporidia			X								
	Strongyloides stercoralis			X								
Price per sample (catalog, excl. VAT)		**Bac + ext**: €22.5 **Viral**: €18 **Par**:€18	Unknown	Unknown	€120	€22 (excl. extraction)	€136.6 (2016 price)	**Bac I:** €12.1 **Bac II:** €12.1 **Vir:** €8.8 **Par:** €8.3 Sum: €41.3	€40	**Dx:** €24.3 **Viral:** €20.5 Sum: €44.8	**Bacterial:** €22.9 **Viral**: 25.6€ Sum: €48.5	Unknown

Attachment 4: price of reagents and time cost: old algorithm versus new algorithm (prices excluding VAT)

Classic culture (old algorithm)	Manufacturer	Price/box	#/box	Price/sample
Saline	Biotrading	144.00 €	360	0.40 €
SS agar	BioMérieux	8.80 €	20	0.44 €
McK agar	BioMérieux	6.28 €	20	0.31 €
CAM agar	BioMérieux	106.61 €	20	5.33 €
YER agar	BioMérieux	11.31 €	20	0.57 €
Selenite Broth	BD	45.50 €	100	0.46 €
HEKT agar	BioMérieux	8.80 €	20	0.44 €
Urease (40% of samples)	BioMérieux	26.91 €	200	0.13 €
Oxidase (15% of samples)	Oxoid	121.79 €	200	0.61 €
TSI (40% of samples)	BD	63.69 €	100	0.64 €
CAT broth	Biotrading	1.10 €	1	1.10 €
Personnel hands-on time (estimated) – 0.672 euro per minute	15 minutes			10.08 €
Sum traditional culture				**19.52** € (9.44 € reagents)
Molecular test with limited culture (new algorithm)*	**Manufacturer**	**Price/box**	**#/box**	**Price/sample**
PCR cartridge	BD	2.91 €	24	0.12 €
Personnel hands-on time for PCR panel– 0.672 euro per minute	2 minutes			1.34 €
GI (extended) bacterial panel**	BD	14.00 €	1	14.00 €
Sum (PCR)				15.47€ (14.12 € reagents)
CB medium	Copan	50.56 €	50	1.01 €
AY agar	BD	14.36 €	20	0.72 €
COH agar	BioMérieux	9.40 €	20	0.47 €
0.6 µm filters	Novolab	89.03 €	100	0.89 €
CAT broth	Biotrading	1.10 €	1	1.10 €

Personel hands-on time (estimated) for complementary culture– euro per minute	5 minutes			3.36 €
Sum (limited culture)				7.55 € (4.19€ reagents)
Sum (PCR + culture)				**23.01**€ (18.31 € reagents)

*excluding the cost of reflex culture **this excludes a yearly maintenance fee for the BD MAX™ analysers

Attachment 5: RIZIV nomenclature applicable to diagnosis of enteropathogens (in Dutch to exactly represent the text as stated in the law)

Nomenclatuurnummer en beschrijving (ambulant - gehospitaliseerd)	**B-waarde (B = €0,032268 (1/01/2021))**	**€**	**Diagnoseregel**
549813 549824 Opzoeken van parasieten, na verrijking, in faeces	400	12.9 €	
549872 549883 Opzoeken van Cryptosporidium, na verrijking, in faeces	400	12.9 €	
549894 549905 Opzoeken van Microsporidia	1000	32.3 €	78*
549835 549846 Kweek die tenminste het opzoeken van Salmonella, Shigella, Yersinia en Campylobacter omvat, met identificatie van de kiemen in faeces	600	19.4 €	
549850 549861 Kweek van Clostridium difficile en opsporen van toxines A of B van Clostridium difficile in faeces	800	25.8 €	37**
552311 552322 Opzoeken van rotavirus bij een kind, jonger dan twee jaar	200	6.5 €	
552333 552344 Opzoeken van adenovirus bij een kind, jonger dan twee jaar	200	6.5 €	
552016 552020 Opzoeken van infectieuze agentia met een immunologische techniek (max 3 per afname)	250	8.1 €	

** De verstrekkingen 550970 - 550981 en 549894 - 549905 mogen enkel worden aangerekend bij transplantpatiënten, infectie met het HIV of bij behandeling met immunosuppressiva.*
*** De verstrekking 549850 - 549861 mag enkel aangerekend worden aan de ZIV bij personen boven 2 jaar, tenzij na transplantatie*

Attachment 6: false-positives and contamination

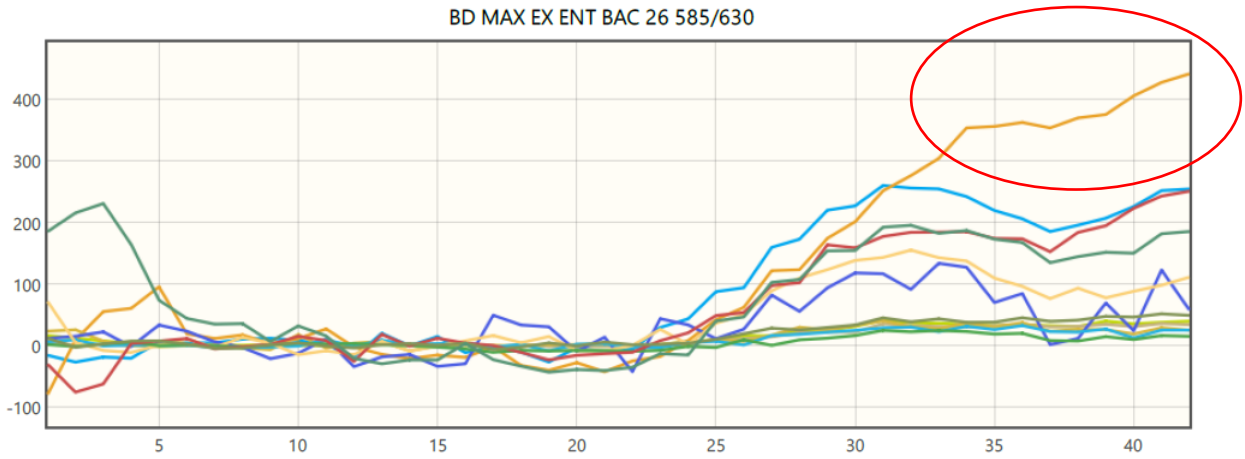

Figure S4: example of a false positive result for *Vibrio spp*. The lightbrown sample was flagged as positive since it exceeds a certain fluorescence threshold, however there is no sigmoidal shape of the amplification curve (red circle).

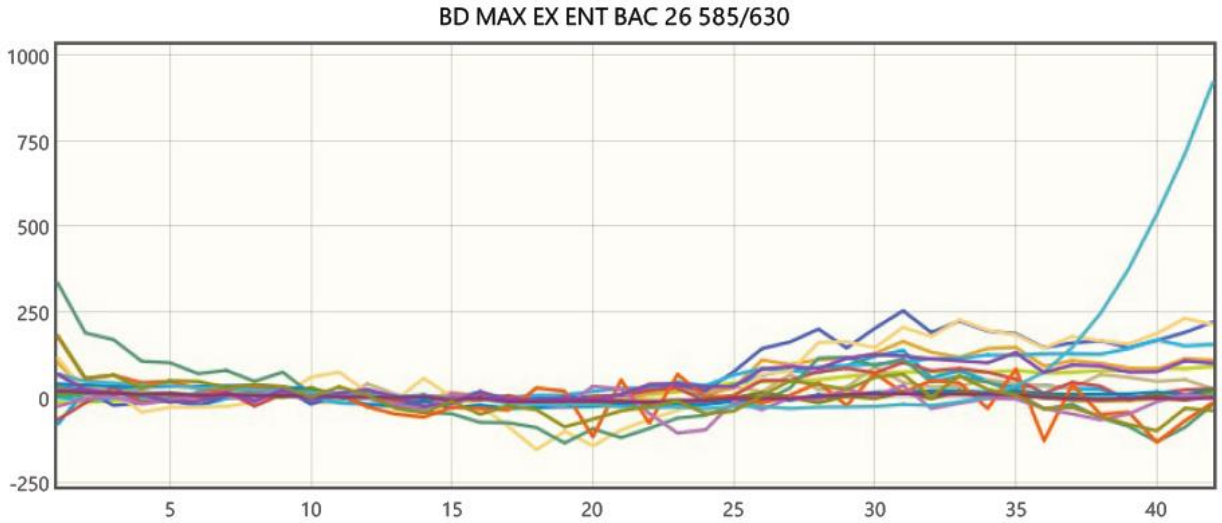

Figure S5 weak positive result for *Shigella/EIEC*. When this sample was repeated, the result was negative. Since there had been a strong positive sample in the previous run, this was considered to be contamination.

RELEVANT EVIDENCE/REFERENCES

1) *Guidelines and Recommendations*
   a. BILULU consensus document 2017
   b. A Guide to Utilization of the Microbiology Laboratory for Diagnosis of Infectious Diseases: 2018 Update by the Infectious Diseases Society of America and the American Society for Microbiology – Miller et al. CID 2018
   c. 2017 Infectious Diseases Society of America Clinical Practice Guidelines for the Diagnosis and Management of Infectious Diarrhea – Shane et al. CID 2017
   d. American College of Gastroenterology (ACG) Clinical Guideline: Diagnosis, Treatment, and Prevention of Acute Diarrheal Infections in Adults – Riddle et al. 2016
   e. World gastroenterology Organisation (WGO) Acute Diarrhea in Adults and Children: A Global Perspective - 2012
   f. American Society for Microbiology (ASM) Practical Guidance for Clinical Microbiology laboratories: Diagnosis of Bacterial Gastroenteritis – Humphries et al 2020

   g. *The National Institute for Clinical Excellence (http://www.nice.org.uk/)*
      i. *"BD MAX Enteric Bacterial Panel for identifying pathogens in contagious gastroenteritis" (link)*
      ii. *"Integrated multiplex PCR tests for identifying gastrointestinal pathogens in people with suspected gastroenteritis (xTAG Gastrointestinal Pathogen Panel, FilmArray GI Panel and Faecal Pathogens B assay)" (link)*
      iii. "Diarrhoea and vomiting caused by gastroenteritis in under 5s: diagnosis and management" (link)

h. Uptodate
   i. Approach to diarrhea in children in resource-rich countries
   ii. Approach to the adult with acute diarrhea in resource-rich settings

2) *Systematic Reviews and Meta-analyses*
   a. Systemaic Review on the Correlation of Quantiative PCR Cycle Treshold Values of gastrointestinal pathogens with patient clinical presentation and outcomes – Bonacorsi et al 2021.

3) *Reviews*

   a. An Overview of the Molecular Methods in the Diagnosis of Gastrointestinal Infectious Diseases – M. Amjad 2020
   b. Advanced PCR-based molecular diagnosis of gastrointestinal infections: challenges and opportunities – Zboromyrska et al 2016
   c. Humphries RM, Linscott AJ. Laboratory diagnosis of bacterial gastroenteritis. Clin Microbiol Rev. 2015 Jan;28(1):3-31.
   d. …

4) *Original Articles*
   a. Comparison of the BD MAX® Enteric Bacterial Panel assay with conventional diagnostic procedures in diarrheal stool samples - https://pubmed.ncbi.nlm.nih.gov/26563899/
   b. Multicenter Clinical Validation of the Molecular BD Max Enteric Viral Panel for Detection of Enteric Pathogens - https://pubmed.ncbi.nlm.nih.gov/31270179/
   c. Multisite Evaluation of the BD Max Extended Enteric Bacterial Panel for Detection of Yersinia enterocolitica, Enterotoxigenic Escherichia coli, Vibrio, and Plesiomonas shigelloides from Stool Specimens - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5654910/
   d. Evaluation of Copan FecalSwab™ preserved stool specimens with the BD MAX™ Enteric Bacterial Panel and the BD MAX™ Extended Enteric Bacterial Panel - https://www.sciencedirect.com/science/article/pii/S0732889320301012?via%3Dihub
   e. Evaluation of the FecalSwab for Stool Specimen Storage and Molecular Detection of Enteropathogens on the BD Max System - https://pubmed.ncbi.nlm.nih.gov/32461284/
   f. How to Interpret a Positive Campylobacter PCR result using the BD MAX system in the absence of positive culture? – Gueudet et al 2019
   g. (op het einde aan te vullen met referentielijst)

5) Reference Works, Handbooks and Databases

   a. European Manual of Clinical Microbiology, 1st edition 2012 (ESCMID)
   b. Manual of Clinical Microbiology, 12th edition
   c. Mandell, Douglas and Bennet's Principles and Practice of Infectious diseases, 8th edition

6) *Posters, "grey literature", presentations*

   a. BD MAX™ Extended Enteric Bacterial Panel product insert
   b. Efficiënte, kwalitatieve en snelle faecesdiagnostiek: zijn moleculaire panels de oplossing? – M. Cauchie 2014
   c. CAT HOET
   d. ESCMID poster: Cattoir L, Vandecandelaere P, Cartuyvels R, et al. Implementation of rejection criteria for stool culture: a ballad of loss and gains?
   e. ESCMID poster: Van Acker J,assessing the value of systematic culture in diagnostic algorithms for detection of toxigenic Clostridium difficile. 2015

NUMBERED REFERENCE LIST

1. Farthing M, Salam MA, Lindberg G, et al. Acute diarrhea in adults and children: A global perspective. J Clin Gastroenterol **2013**; 47:12–20. Available at: https://journals.lww.com/jcge/Fulltext/2013/01000/Acute_Diarrhea_in_Adults_and_Children__A_Global

.7.aspx. Accessed 28 December 2021.

2. Shane AL, Mody RK, Crump JA, et al. 2017 Infectious Diseases Society of America Clinical Practice Guidelines for the Diagnosis and Management of Infectious Diarrhea. Clin Infect Dis **2017**; 65:e45–e80. Available at: https://pubmed.ncbi.nlm.nih.gov/29053792/. Accessed 28 December 2021.

3. ESCMID. European Manual of Clinical Microbiology. 2012.

4. Lal A, Hales S, French N, Baker MG. Seasonality in Human Zoonotic Enteric Diseases: A Systematic Review. PLoS One **2012**; 7. Available at: /pmc/articles/PMC3317665/. Accessed 23 January 2022.

5. Wetenschappelijk Instituut Volksgezondheid. Voedsel- en watergerelateerde infectieziekten - Epidemiologische surveillance in België. 2017. Available at: https://epidemio.wiv-isp.be/ID/reports/Voedsel- en watergerelateerde infectieziekten - Epidemiologie - Jaarrapport 2015-2016.pdf.

6. Riddle MS, Dupont HL, Connor BA. ACG clinical guideline: Diagnosis, treatment, and prevention of acute diarrheal infections in adults. Am J Gastroenterol **2016**; 111:602–622. Available at: https://journals.lww.com/ajg/Fulltext/2016/05000/ACG_Clinical_Guideline__Diagnosis,_Treatment,_and .14.aspx. Accessed 28 December 2021.

7. Teh R, Tee W De, Tan E, et al. Review of the role of gastrointestinal multiplex polymerase chain reaction in the management of diarrheal illness. J Gastroenterol Hepatol **2021**; 36:3286–3297. Available at: https://pubmed.ncbi.nlm.nih.gov/34129249/. Accessed 29 December 2021.

8. Janda JM, Abbott SL. Revisiting Bacterial Gastroenteritis, Part I: Issues, Possible Approaches, and an Ever-Expanding List of Etiologic Agents. Clin Microbiol Newsl **2011**; 33:71–76.

9. Platts-Mills JA, Liu J, Houpt ER. New concepts in diagnostics for infectious diarrhea. Mucosal Immunol 2013 65 **2013**; 6:876–885. Available at: https://www.nature.com/articles/mi201350. Accessed 29 December 2021.

10. Zboromyrska Y, Vila J. Advanced PCR-based molecular diagnosis of gastrointestinal infections: challenges and opportunities. http://dx.doi.org/101586/1473715920161167599 **2016**; 16:631–640. Available at: https://www.tandfonline.com/doi/abs/10.1586/14737159.2016.1167599. Accessed 29 December 2021.

11. Amjad M. An Overview of the Molecular Methods in the Diagnosis of Gastrointestinal Infectious Diseases. Int J Microbiol **2020**; 2020.

12. Ramanan P, Bryson AL, Binnicker MJ, Pritt BS, Patel R. Syndromic Panel-Based Testing in Clinical Microbiology. Clin Microbiol Rev **2017**; 31. Available at: https://pubmed.ncbi.nlm.nih.gov/29142077/. Accessed 29 December 2021.

13. Bloomfield MG, Balm MND, Blackmore TK. Molecular testing for viral and bacterial enteric pathogens: gold standard for viruses, but don't let culture go just yet? Pathology **2015**; 47:227–233. Available at: https://pubmed.ncbi.nlm.nih.gov/25719855/. Accessed 29 December 2021.

14. Humphries RM, Linscott AJ. Practical Guidance for Clinical Microbiology Laboratories: Diagnosis of Bacterial Gastroenteritis. Clin Microbiol Rev **2015**; 28:3. Available at: /pmc/articles/PMC4284301/. Accessed 29 December 2021.

15. National Institute for Health and Care Excellence (NICE). BD MAX Enteric Bacterial Panel for identifying pathogens in contagious gastroenteritis | Advice | NICE. 2015. Available at: https://www.nice.org.uk/advice/mib32. Accessed 29 December 2021.

16. Approach to the adult with acute diarrhea in resource-rich settings - UpToDate. Available at: https://www.uptodate.com/contents/approach-to-the-adult-with-acute-diarrhea-in-resource-rich-settings?topicRef=16294&source=see_link. Accessed 29 December 2021.

17. Leber AL. Clinical Microbiology Procedures Handbook. 2016.

18. Cattoir L, Vandecandelaere P, Cartuyvels R, et al. Implementation of rejection criteria for stool culture: a ballad of loss and gains? 2017. Available at: http://www.bilulu.be/cms01/index.php/activities/science/posters.

19. WHO. Diarrhoeal disease. 2017. Available at: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease. Accessed 29 December 2021.

20. O'Neal M, Murray H, Dash S, Al-Hasan MN, Justo JA, Bookstaver PB. Evaluating appropriateness and

diagnostic stewardship opportunities of multiplex polymerase chain reaction gastrointestinal testing within a hospital system. Ther Adv Infect Dis **2020**; 7. Available at: https://pubmed.ncbi.nlm.nih.gov/33014363/. Accessed 29 December 2021.

21. Simner PJ, Oethinger M, Stellrecht KA, et al. Multisite Evaluation of the BD Max Extended Enteric Bacterial Panel for Detection of Yersinia enterocolitica, Enterotoxigenic Escherichia coli, Vibrio, and Plesiomonas shigelloides from Stool Specimens. J Clin Microbiol **2017**; 55:3258–3266. Available at: https://pubmed.ncbi.nlm.nih.gov/28878009/. Accessed 29 December 2021.

22. Anderson NW, Buchan BW, Ledeboer NA. Comparison of the BD MAX enteric bacterial panel to routine culture methods for detection of Campylobacter, enterohemorrhagic Escherichia coli (O157), Salmonella, and Shigella isolates in preserved stool specimens. J Clin Microbiol **2014**; 52:1222–1224. Available at: https://pubmed.ncbi.nlm.nih.gov/24430460/. Accessed 29 December 2021.

23. Tilmanne A, Martiny D, Quach C, et al. Enteropathogens in paediatric gastroenteritis: comparison of routine diagnostic and molecular methods. Clin Microbiol Infect **2019**; 25:1519–1524. Available at: https://pubmed.ncbi.nlm.nih.gov/31374260/. Accessed 29 December 2021.

24. Knabl L, Grutsch I, Orth-Höller D. Comparison of the BD MAX® Enteric Bacterial Panel assay with conventional diagnostic procedures in diarrheal stool samples. Eur J Clin Microbiol Infect Dis **2016**; 35:131–136. Available at: https://pubmed.ncbi.nlm.nih.gov/26563899/. Accessed 29 December 2021.

25. Spina A, Kerr KG, Cormican M, et al. Spectrum of enteropathogens detected by the FilmArray GI Panel in a multicentre study of community-acquired gastroenteritis. Clin Microbiol Infect **2015**; 21:719–728. Available at: https://pubmed.ncbi.nlm.nih.gov/25908431/. Accessed 29 December 2021.

26. Schwenk HT, Bio LL, Kruger JF, Banaei N. Clinical Impact of Clostridium difficile PCR Cycle Threshold-Predicted Toxin Reporting in Pediatric Patients. J Pediatric Infect Dis Soc **2020**; 9:44–50. Available at: https://pubmed.ncbi.nlm.nih.gov/30476169/. Accessed 29 December 2021.

27. Bonacorsi S, Visseaux B, Bouzid D, et al. Systematic Review on the Correlation of Quantitative PCR Cycle Threshold Values of Gastrointestinal Pathogens With Patient Clinical Presentation and Outcomes. Front Med **2021**; 8. Available at: https://pubmed.ncbi.nlm.nih.gov/34631732/. Accessed 29 December 2021.

28. Yalamanchili H, Dandachi D, Okhuysen PC. Use and Interpretation of Enteropathogen Multiplex Nucleic Acid Amplification Tests in Patients With Suspected Infectious Diarrhea. Gastroenterol Hepatol (N Y) **2018**; 14:646. Available at: /pmc/articles/PMC6284344/. Accessed 29 December 2021.

29. Harrington SM, Buchan BW, Doern C, et al. Multicenter evaluation of the BD max enteric bacterial panel PCR assay for rapid detection of Salmonella spp., Shigella spp., Campylobacter spp. (C. jejuni and C. coli), and Shiga toxin 1 and 2 genes. J Clin Microbiol **2015**; 53:1639–1647. Available at: https://pubmed.ncbi.nlm.nih.gov/25740779/. Accessed 29 December 2021.

30. Van Acker J, Cox I, Verfaillie C, Van den Abeeele A-M. Assessing the value of systematic culture in diagnostic algorithms for detection of toxigenic Clostridium difficile. 2015. Available at: https://www.escmid.org/escmid_publications/escmid_elibrary/material/?mid=26280.

31. Gueudet T, Paolini MC, Buissonnière A, et al. How to Interpret a Positive Campylobacter PCR Result Using the BD MAX TM System in the Absence of Positive Culture? J Clin Med **2019**; 8. Available at: https://pubmed.ncbi.nlm.nih.gov/31817056/. Accessed 29 December 2021.

32. Janda JM, Abbott SA. Culture-independent diagnostic testing: have we opened Pandora's box for good? Diagn Microbiol Infect Dis **2014**; 80:171–176. Available at: https://pubmed.ncbi.nlm.nih.gov/25200256/. Accessed 23 January 2022.

33. Goldenberg SD, Bacelar M, Brazier P, Bisnauthsing K, Edgeworth JD. A cost benefit analysis of the Luminex xTAG Gastrointestinal Pathogen Panel for detection of infectious gastroenteritis in hospitalised patients. J Infect **2015**; 70:504–511. Available at: https://pubmed.ncbi.nlm.nih.gov/25449904/. Accessed 29 December 2021.

34. Schuetz AN. Emerging agents of gastroenteritis: Aeromonas, Plesiomonas, and the diarrheagenic pathotypes of Escherichia coli. Semin Diagn Pathol **2019**; 36:187–192. Available at: https://pubmed.ncbi.nlm.nih.gov/31036328/. Accessed 23 January 2022.

35. Sciensano. Epistat – Belgian Infectious Diseases. Available at: https://epistat.wiv-isp.be/dashboard/. Accessed 23 January 2022.

36. Kaprou GD, Bergšpica I, Alexa EA, Alvarez-Ordóñez A, Prieto M. Rapid Methods for Antimicrobial

Resistance Diagnostics. Antibiot (Basel, Switzerland) **2021**; 10:1–30. Available at: https://pubmed.ncbi.nlm.nih.gov/33672677/. Accessed 21 May 2022.

37. Omolajaiye SA, Afolabi KO, Iweriebor BC. Pathotyping and antibiotic resistance profiling of Escherichia coli isolates from children with acute diarrhea in amatole district municipality of Eastern Cape, South Africa. Biomed Res Int **2020**; 2020.

38. Ohashi Y, Fujisawa T. Detection of antibiotic resistance genes in the feces of young adult Japanese. Biosci microbiota, food Heal **2017**; 36:151–154. Available at: https://pubmed.ncbi.nlm.nih.gov/29038771/. Accessed 21 May 2022.

39. Rifai N, Horvath AR, Wittwer C. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 6th ed. Elsevier, 2018.

40. Ramees TP, Dhama K, Karthik K, et al. Arcobacter: an emerging food-borne zoonotic pathogen, its public health concerns and advances in diagnosis and control - a comprehensive review. Vet Q **2017**; 37:136–161. Available at: https://pubmed.ncbi.nlm.nih.gov/28438095/. Accessed 23 January 2022.

41. Fournier PE, Drancourt M, Raoult D. New Laboratory Tools for Emerging Bacterial Challenges. Clin Infect Dis **2017**; 65:S39–S49. Available at: https://pubmed.ncbi.nlm.nih.gov/28859351/. Accessed 23 January 2022.

42. Chiang AD, Dekker JP. From the Pipeline to the Bedside: Advances and Challenges in Clinical Metagenomics. J Infect Dis **2020**; 221:S331–S340. Available at: https://pubmed.ncbi.nlm.nih.gov/31538184/. Accessed 23 January 2022.

43. Guardabassi L, Butaye P, Dockrell DH, Fitzgerald JR, Kuijper EJ. One Health: a multifaceted concept combining diverse approaches to prevent and control antimicrobial resistance. Clin Microbiol Infect **2020**; 26:1604–1605. Available at: https://pubmed.ncbi.nlm.nih.gov/32702500/. Accessed 23 January 2022.

44. Yuliangsih S, Waturangi DE, Yogiara. Microbial analysis and virulence genes detection of milk preserved using heat-assisted pulsed electric field. BMC Res Notes **2021**; 14:1–7. Available at: https://bmcresnotes.biomedcentral.com/articles/10.1186/s13104-021-05805-3. Accessed 23 January 2022.

45. Ct Values Simplified: A Practical Guide for Researchers. Available at: https://bitesizebio.com/24581/what-is-a-ct-value/. Accessed 30 December 2021.

46. BD. BD MAX™ Enteric Bacterial Panel. 2019; Available at: https://www.bd.com/resource.aspx?IDX=28623. Accessed 23 January 2022.