Can rapid integrated polymerase chain reaction-based diagnostics for gastrointestinal pathogens improve routine hospital infection control practice? A diagnostic study

Louise Pankhurst,[1,2] Louissa Macfarlane-Smith,[3] James Buchanan,[4] Luke Anson,[1,2] Kerrie Davies,[3,5] Lily O'Connor,[2,6] Helen Ashwin,[5] Graham Pike,[6] Kate E Dingle,[1,2] Timothy EA Peto,[1,2,6] Sarah Wordsworth,[4] A Sarah Walker,[1,2†] Mark H Wilcox[3,5†] and Derrick W Crook[1,2,6*†]

[1]Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK
[2]National Institute for Health Research (NIHR) Oxford Biomedical Research Centre, John Radcliffe Hospital, Oxford, UK
[3]Leeds Teaching Hospitals NHS Trust, Leeds General Infirmary, Leeds, UK
[4]Health Economics Research Centre, Nuffield Department of Population Health, University of Oxford, Oxford, UK
[5]University of Leeds, Microbiology, Leeds General Infirmary Old Medical School, Leeds, UK
[6]Oxford University Hospitals NHS Trust, Oxford, UK

*Corresponding author
†Contributions considered equal

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Scientific summary

Diagnostics for gastrointestinal pathogens

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Scientific summary

Background

A large 1500-bed hospital might expect approximately 9000 cases of potentially infectious diarrhoea necessitating isolation every year. An infecting organism will be identified in as few as 10% of these cases, necessitating substantial isolation capacity primarily for diagnostic reasons. The NHS currently has insufficient single rooms to effectively accommodate all such patients. Single rooms may, therefore, be 'blocked' by patients without infectious diarrhoea as yet unconfirmed, while other patients with infectious diarrhoea are still in open bays because of a lack of free side rooms. A rapid test for identifying cases of infectious diarrhoea could, therefore, provide major benefits to patients.

At present, achieving relative certainty over which diarrhoea cases are infected with enteropathogens can take up to 3 days, and it is this delay in turnaround time from sample collection to test result which has a major impact on bed management and patient pathways. Much current research in microbiological diagnostics is focused on developing simple and rapid molecular tests to identify only the aetiology of infectious conditions such as diarrhoea. However, most molecular tests identify only one organism, and a major hindrance is that many different pathogens can cause infectious syndromes. For example, despite symptoms being similar, infectious diarrhoea may be caused by one of approximately 4–6 common pathogens, or possibly one of a further approximately 20 rarer pathogens, or, extremely rarely, one of hundreds of uncommon organisms. Combining these tests into a multiplex with 10 or more pathogens brings significant challenges in terms of cross-reactivity (primer dimers), random products and inhibition. Thus, multiplexing tests may produce substantial numbers of false positives and false negatives, even compared with the original single polymerase chain reaction (PCR), and may also have decreased sensitivity in terms of the number of copies present in a sample required for pathogen detection. Therefore, the performance of any proposed multiplex assay needs to be rigorously evaluated before it can be considered for NHS use.

Objectives

This diagnostic test study was designed to evaluate a newly developed technology, MassCode multiplex PCR, for the simultaneous diagnosis of multiple enteropathogens directly from stool, in terms of (i) sensitivity/specificity to detect a range of pathogens and overall to rule out any infectious causative agent, and (ii) turnaround time, net health-care costs and utilisation of isolation resources to assess whether or not it could improve hospital management of patients with suspected infectious diarrhoea. The primary focus was on the four most common and important enteropathogens: *Clostridium difficile*, *Campylobacter* spp., *Salmonella* spp. and norovirus.

Methods

These objectives were to be addressed by a two-stage study in two hospitals (Leeds Teaching and Oxford University Hospitals NHS Trusts). Phase 1 was a retrospective study based on fixed numbers of samples positive and negative for *C. difficile* ($n = 200$), *Campylobacter* spp. ($n = 200$), *Salmonella* spp. ($n = 100$) and norovirus ($n = 200$) plus samples negative for all these pathogens ($n = 300$) to estimate sensitivity/specificity of the MassCode assay using standard microbiological methods as the reference. Standardised workflows that could be translated into NHS microbiology laboratories were also to be developed in phase 1. Samples were initially sent to the Oxford University Hospitals NHS Trust service microbiology laboratory for faecal culture and/or *C. difficile* toxin testing by hospital-based doctors or general practitioners as a result

of a suspected enteric infection. Initial diagnosis of the target faecal pathogens was performed according to Public Health England guidelines in the service microbiology laboratory. All enzyme immunoassay (EIA)-positive *C. difficile* samples were cultured in parallel in the research laboratory and only those positive for *C. difficile* on both EIA and culture were included. As norovirus testing was not carried out unless an outbreak was suspected, quantitative PCR (qPCR)-positive norovirus samples were obtained through a separate investigation. The number of *S. enterica*-positive samples was lower than predicted, so additional *S. enterica*-positive samples were sourced from Leeds. Samples were collected by a research assistant not involved in the study, and assigned one of 1000 study numbers at random. All samples were stored at 4 °C prior to processing. Researchers conducting MassCode assays were blinded to results of the reference test and all other clinical information; samples were identified only by their unique study number. MassCode results were used only for the research study and were not returned for patient management. After analysis, any microbiological positives missed by MassCode or unexpected positives identified were retested using single qPCR to confirm or refute the presence of the pathogen in the sample.

Phase 1 was supplemented by a parallel questionnaire survey examining current practice and costs of managing infectious diarrhoea, to inform subsequent health economic analysis. Three questionnaires were developed, for completion by infection control teams, microbiologists and microbiology laboratory managers.

If MassCode met pre-specified criteria indicating it had the potential for successful NHS translation, phase 2 was intended to be a prospective real-time parallel-group study testing the same stool samples from general medicine and surgery both in the routine microbiology laboratory and by the new technology to estimate positive/negative predictive value in a real-world setting and to directly compare turnaround time, net health-care costs and patient-centred outcomes.

Results

Developing standardised workflows for MassCode

The first major challenge was to identify an extraction method that successfully isolates deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) from a wide range of physiologically diverse organisms, since the organism present in the sample is unknown when DNA/RNA is extracted for molecular testing. Experiments designed to optimise nucleic acid extraction and purification performed using *C. difficile* and *S. enterica* identified stool transport and recovery (STAR) buffer with bead beating as an initial lysis step followed by purification with QIAsymphony as the best procedure. However, nucleic acid yields were still relatively low for *S. enterica*. Further exploration suggested that a biochemical or biological mechanism may result in poor DNA yield from the wider Enterobacteriaceae family (to which *S. enterica* belongs).

MassCode phase 1

A total of 948 clinical samples were collected and extracted using the optimised protocol; 200 were positive for *Campylobacter* spp., 199 for *C. difficile*, 60 for *S. enterica* and 199 for norovirus and 295 samples were negative (some samples had more than one pathogen). Sensitivities for each organism compared with the microbiological reference ranged from 43% to 94% and specificities from 95% to 98%. Including qPCR results in the reference standard increased sensitivity and specificity modestly, to 60–95% and 97–100%, respectively. The best-performing organism was *C. difficile,* although *Campylobacter* spp. and norovirus also had sensitivities and specificities well above the 75% threshold required to proceed to phase 2, with 95% confidence interval (CI) lower limits exceeding 83% (sensitivity) and 92% (specificity). However, the sensitivity of *S. enterica* remained well below this threshold; even including qPCR results the upper 95% CI limit around the estimated 60% sensitivity was just below 75%. Of equal concern was the large number of unexpected positives not confirmed with qPCR, particularly for *S. enterica*, *Giardia lamblia* and *Cryptosporidium* spp. Additional testing suggested that *Cryptosporidium* primers were cross-reacting with *Candida* spp. However, a few unexpected positives were confirmed by qPCR, highlighting potential benefits from routine testing for rarer organisms.

An independent oversight committee reviewed these results; as the MassCode assay had clearly failed the pre-specified threshold sensitivity to proceed to phase 2, further investigation was abandoned. However, as findings indicated that detecting *S. enterica* might provide generic challenges to other gastrointestinal multiplex assays, and given the lack of a large independent validation of a different assay (Luminex), the funders agreed that the Luminex assay should be run in Leeds on the same set of samples.

Luminex assay

Researchers conducting Luminex assays were blinded to results of the MassCode and reference microbiology tests; samples were identified only by their unique study number. Of the original 948 DNA/RNA extracts used for the MassCode assay, 937 had sufficient material remaining to be tested using the Luminex assay in a direct comparison and 839 had sufficient material remaining for fresh re-extractions and testing.

For *Campylobacter* spp., *C. difficile* and norovirus, high sensitivities (> 92%, most > 97%) and specificities (> 96%) were observed, regardless of extraction method and regardless of whether comparisons were made with microbiology alone (primary comparison for MassCode) or microbiology plus qPCR results. Interestingly, lower sensitivities were observed using freshly extracted material (*Campylobacter* spp., 98%; *C. difficile*, 96%; norovirus, 92%). However, major differences in results with the same Luminex assay were found for *S. enterica*. On the original MassCode/Oxford extracts, sensitivity against microbiological testing was 84% (95% CI 73% to 93%). Although this clearly would have some limitations in clinical practice, it was a substantial improvement over MassCode. However, sensitivity when assayed using a fresh/Leeds extract dropped to 46%, very similar to MassCode, with a corresponding increase in specificity from 92% to 99%. Including qPCR results in the reference standard gave similar results, with sensitivity for detecting *S. enterica* from freshly extracted material only 60% with an upper 95% CI limit just below 79%. Again, there were a number of unexpected positives for both the target four and additional pathogens included in the assay, more so with the original MassCode/Oxford than the fresh/Leeds extracts.

Combined analysis

As several unexpected positives were confirmed by qPCR results (implying that the target pathogen was genuinely present in the sample but missed in the original microbiology work-up), in a combined analysis we used the overall best information as to what organisms were present from both microbiology and qPCR. The pathogens identified in each sample, i.e. what result would be returned to the doctor for patient management, varied across the different assays. Even restricting analysis to the four main pathogens, overall agreement with combined microbiology plus PCR as the reference standard was 85.6% ($\kappa = 0.81$), 87.0% ($\kappa = 0.84$) and 89.8% ($\kappa = 0.87$) for the MassCode assay, Luminex assay/MassCode extract and Luminex assay/fresh extract, respectively. Although the Luminex assay on freshly extracted samples was most accurate overall (89.8%), it was still incorrect for 1 in 10 samples and would have concluded that 5.2% of samples did not come from patients with infectious diarrhoea caused by the main four pathogens, even though these pathogens were in fact present in the sample. Including additional pathogens covered by both assays, overall agreement with the combined result from microbiology and PCR as the standard reference was poorer at 64.7% ($\kappa = 0.58$), 82.9% ($\kappa = 0.79$) and 86.8% ($\kappa = 0.83$) for the MassCode assay, Luminex assay/MassCode extract and Luminex assay/fresh extract, respectively.

Survey of current NHS practice regarding management of potentially infectious diarrhoea

Three surveys were sent to a representative sample of 54 NHS trusts; 41% of trusts contacted responded to at least one survey. On average, infection control teams currently spend 21% of their time on the routine management of diarrhoea. Infection control teams spend around 1 hour 40 minutes per day tracking patients with suspected or confirmed diarrhoea of infectious origin, using a mixture of manual paper-based systems and computer systems. The mean percentage of patients with suspected infectious diarrhoea who are isolated in a side room is 95%, with 44% of all trusts isolating all such patients in a side room. When insufficient side rooms are available to manage multiple patients with suspected infectious diarrhoea, most trusts prioritise patients by isolating those with particular pathogens/strains or the most severely ill. The average length of time it takes to isolate a patient is 2 hours 40 minutes, with

patients making two bed moves on average during their inpatient stay. The mean number of wards closed annually per trust as a consequence of outbreaks of infectious diarrhoea was approximately 12.

As expected, the average length of time between taking a stool sample and receiving test results was around 1 day for *C. difficile* and norovirus, but around 2 days for *Cryptosporidium* spp. and 2.5 days for *Shigella* spp., *Escherichia coli*, *Campylobacter* spp. and *Salmonella* spp.

Infection control, microbiology and laboratory staff were also asked to consider the impact of a new diagnostic test which could detect 30 pathogens in a single reaction. Most trusts were positive about the consequences of this scenario. Several potential benefits were identified, including more informed and faster decisions regarding the need for isolation and de-isolation; more effective use of limited side room space and reduced bed-blocking; improved patient treatment outcomes; and earlier identification of outbreaks and implementation of cohorting. Concerns were, however, raised about the need for such tests to be accurate and the requirement for samples to be taken as simply as possible.

Conclusions

The comments from infection control, microbiology and laboratory staff in response to the potential future scenario (and the free-text comments throughout the rest of the surveys) indicate a clear need for the type of interventions that have been considered in this study. Respondents identified several difficulties currently faced in this clinical context, including the lack of side room capacity and the existence of bed-blocking. Respondents also revealed a clear appetite for molecular testing to assist with the management of patients with suspected infectious diarrhoea, highlighting a variety of potential benefits.

Overall, the Luminex xTag® assay showed similar or superior sensitivity and specificity to the MassCode assay. In particular, using fresh extracts, the number of unexpected positives using the Luminex assay was relatively small across all organisms. However, these high specificities came at the cost of low sensitivity to detect a key enteric pathogen, *S. enterica*; such test sensitivity is too low for this assay to be a realistic option for most microbiology laboratories and would necessitate continued investment into other mechanisms for identifying this pathogen. Interestingly, this low sensitivity was very similar to that observed for the MassCode assay, suggesting that extraction efficiency is genuinely a major obstacle for nucleic acid-based tests for this organism, and possibly the whole family of Enterobacteriaceae, regardless of platform. However, whereas for the MassCode assay this low sensitivity was also paralleled by relatively low specificity and a substantial number of false positives, for the Luminex assay using fresh extracts the number of false positives was also fairly low. Nevertheless, overall, even on fresh samples the Luminex assay would have made an incorrect decision on the pathogens present in 1 in 10 samples, and would have missed an important pathogen in 1 in 20. It should be highlighted that traditional microbiological processes also missed a number of pathogens, although whether some of these were co-colonisers rather than true co-infections is unclear.

Overall, this large and comprehensive assessment of two multiplex assays (MassCode and Luminex) for gastrointestinal pathogens has demonstrated that neither is currently ready for deployment in the NHS. The MassCode assay, in particular, will no longer be developed. To improve workflows in service microbiology laboratories, to reduce workload for infection control practitioners, and to improve outcomes for NHS patients both with potentially infectious diarrhoea and without diarrhoea but at risk of transmission, further research on multiplex gastrointestinal diagnostics is urgently needed.

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