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Oxford Radcliffe Hospitals NHS
NHS Trust

The Leeds Teaching Hospitals NHS
NHS Trust

Can rapid integrated Polymerase Chain Reaction (PCR)-based diagnostics for gastrointestinal pathogens and direct sequence typing of *Clostridium difficile* improve routine Hospital Infection Control practice?

Final protocol, version 1.2, 01 October 2010

Authorised by:

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Signature: Date: 01/10/10

GENERAL INFORMATION

This document describes the MassTag PCR diagnostic study. Problems relating to this study should be referred to the Chief Investigator.

- **Compliance**
  The study will be conducted in compliance with the protocol, the Data protection Act (DPA number: Z5886415), and NHS research governance.
- **Sponsor**
  Oxford Radcliffe Hospitals Trust, John Radcliffe Hospital, Headley Way, Oxford, OX9 3DU
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1. SUMMARY

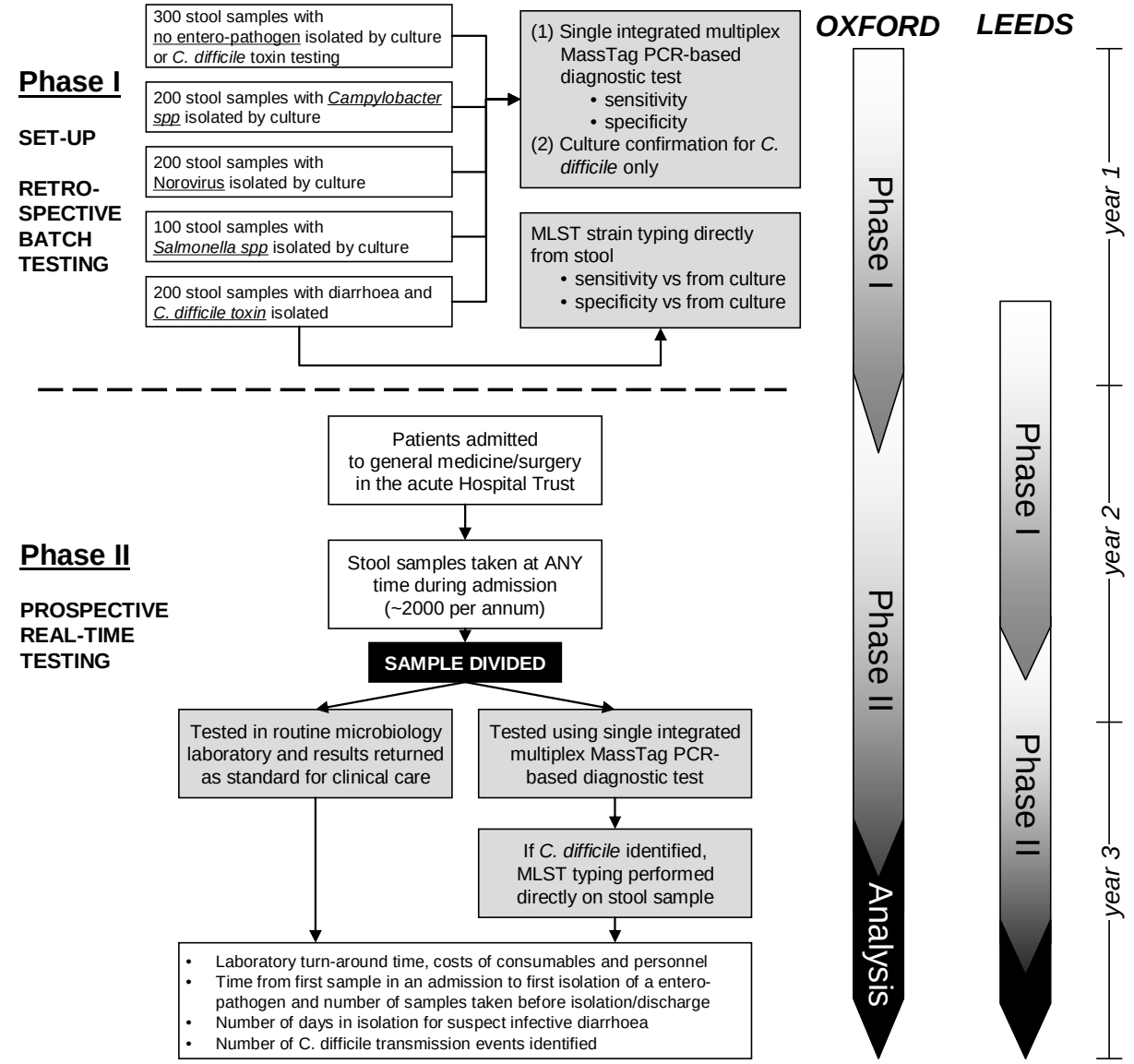
1.1 Abstract and summary of study

The study has 2 research objectives:

- First, to evaluate a new technology - MassTag multiplex PCR - for the simultaneous diagnosis of multiple entero-pathogens directly from stool, in terms of (i) sensitivity, specificity and real-time predictive value to detect a range of pathogens and overall to rule out any infectious causative agent, and (ii) turnaround time (speed of diagnosis), net healthcare costs and utilisation of isolation resources to assess whether it can improve hospital management of patients with suspected infectious diarrhoea, in particular by avoiding/reducing isolation of patients with non-infectious diarrhoea.
- Second, to evaluate rapid multi-locus sequence typing (MLST) of *C. difficile* direct from stool, in terms of (i) turnaround time and net healthcare costs, and (ii) ability to detect outbreaks in clinically useful timeframes and to provide readily available national surveillance data.

These objectives will be addressed by a two-stage diagnostic test study in 2 hospitals (Leeds Teaching and Oxford Radcliffe Hospitals NHS Trusts). Phase I (1 year) is a retrospective batch study based on fixed numbers of samples positive and negative for *C. difficile*, *Campylobacter spp.*, *Salmonella spp.* and Norovirus to estimate sensitivity/specificity; and formal comparison of *C. difficile* MLST typing from cultured isolates versus directly from stool. Phase II (1-1.5 years) is a prospective real-time parallel-group study testing the same stool samples from general medicine and surgery in both 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 healthcare costs and patient-centred outcomes (utilisation of isolation resources, detection of outbreaks). This will supplemented by parallel health economic studies to examine the cost-effectiveness of MassTag multiplex PCR versus current practice, using a semi-Markov model and diagnostic decision-tree, with data on diagnostic procedures and shorter-term consequences derived from Phase II combined with longer-term survival payoffs derived from the study and a structured review of literature. Using the model we will address a range of questions that we expect will inform future clinical decision-making.

1.2 Flow diagram



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2. BACKGROUND

2.1 Introduction

2.1.1 The clinical problem

A large 1,500 bed hospital will have approximately 25,000 cases with **potentially infectious diarrhoea necessitating isolation** under the statutory 2006 Hygiene Code[1] every year. As few as 1 in 10 of these cases will have a infecting organism identified, necessitating substantial isolation capacity primarily for diagnostic reasons. The NHS currently has insufficient single rooms to effectively accommodate all such patients[2]. Single rooms may therefore be "blocked" by patients without infectious diarrhoea as yet unconfirmed, whilst other patients with infectious diarrhoea are still in open bays due to lack of free side rooms. A rapid test for identifying cases of infectious diarrhoea could provide major benefits to the smooth running of hospitals by promoting efficient use of isolation beds.

2.1.2 The microbiological diagnostic problem

Much current research in microbiological diagnostics is focussed on developing simple and rapid molecular tests to identify the aetiology of infectious conditions such as diarrhoea. However, each molecular test focuses on identifying one organism, and a major hindrance is that many different pathogens can cause infectious syndromes. For example, no single test currently exists to identify the cause of a case of infectious diarrhoea, which is likely to be one of ~4-6 common pathogens, or possibly one of a further ~20 rarer pathogens, or, extremely rarely, one of hundreds of uncommon organisms. Despite excellent nucleic acid amplification tests for individual pathogens, no way has previously been found to combine in one reaction an assay to identify an individual pathogen from among many (greater than 10) possibilities.

The development of **MassTag multiplex PCR**, which can detect up to 30 pathogens in a single reaction, potentially offers a cost effective and rapid mechanism of doing exactly this for the first time. Such a test holds out the possibility of radically changing the patient pathway for many infectious syndromes, but in particular, it should alter the urgent need for rapidly increasing the isolation capacity of the NHS to meet the needs of infectious diarrhoea, particularly that caused by *Clostridium difficile*. This would enable the prioritisation of high cost rebuild/refurbishment projects to yield more single rooms to be revisited in many hospitals.

At present, achieving relative certainty over which diarrhoea cases are infected with entero-pathogens 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. Many bacteria e.g. *Campylobacter* spp. and *Salmonella* spp. take a minimum of 48-72 hours to grow and identify (or identify as no growth). In the case of *C. difficile*, the gold standard cytotoxin test takes 1-2 days to provide a result because it relies on cell culture and a negative result is not issued until the test has been re-read at 48 hours, whereas the substantially faster ELISA-based assays have the recognised drawback of lower sensitivity (~75-85%)[3], thus leading to repeat testing over a few days for a substantial minority of cases. As most hospitalised patients with diarrhoea do not have an infectious cause, a better approach would be **same day (<24 hours) differentiation of non-infectious diarrhoea from infectious diarrhoea** caused by the most common entero-pathogens *C. difficile*, *Campylobacter spp.*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, Rotavirus and Norovirus. This would address the current widespread insufficient isolation capacity in the NHS by providing either an almost immediate negative result or a causative pathogen for the vast majority of patients with diarrhoea. In turn, this would allow the instigation of individualised patient treatment and appropriate infection precautions to avoid exposing other patients to the risk of acquiring entero-pathogens and to limit the dissemination of epidemic bacteria (such as *C. difficile*) and viruses (such as Norovirus).

The reduction of *C. difficile* associated infection (CDI) in particular is now a leading government priority, its importance is underlined by a national target of a 30% reduction in cases over 2009-2011. Identifying where there is local or national spread of disease causing strains is an area of active research. One of the limitations is that simple culture and typing methods have not thus far been accessible at the hospital level or in near-to-real time. Therefore, it is widely recognised that a **rapid informative typing scheme for detecting *C. difficile* outbreaks** is urgently needed to assist hospitals identify outbreaks (foci of intense transmission) in real-time. This would allow appropriate interventions to be delivered more rapidly to prevent further onward transmission. Such strain typing for outbreak investigation is well established for other bacteria, e.g. meningococci and *Salmonella spp*. using serological markers. Currently, *C. difficile* typing requires time consuming and resource intensive culture and molecular characterisation, takes at least 1-2 weeks and is only offered on a limited scale; thus yielding data to few hospitals too late to be useful for effective outbreak management. Strain typing data that could be used in near-to-real-time to control transmission during outbreaks in clinically relevant timeframes would be a major advance in current infection control practice. However, such data would also be highly relevant for national surveillance from which early action to control national outbreaks of more virulent strains, such as the ribotype 027, would be possible.

2.2 Rationale

Recent advances in laboratory technologies exploiting genetic information provide potential solutions to both these problems - early differentiation of infectious from non-infectious diarrhoea cases and rapid informative typing for detecting *C. difficile* outbreaks.

2.2.1 MassTag multiplex PCR-based diagnostics

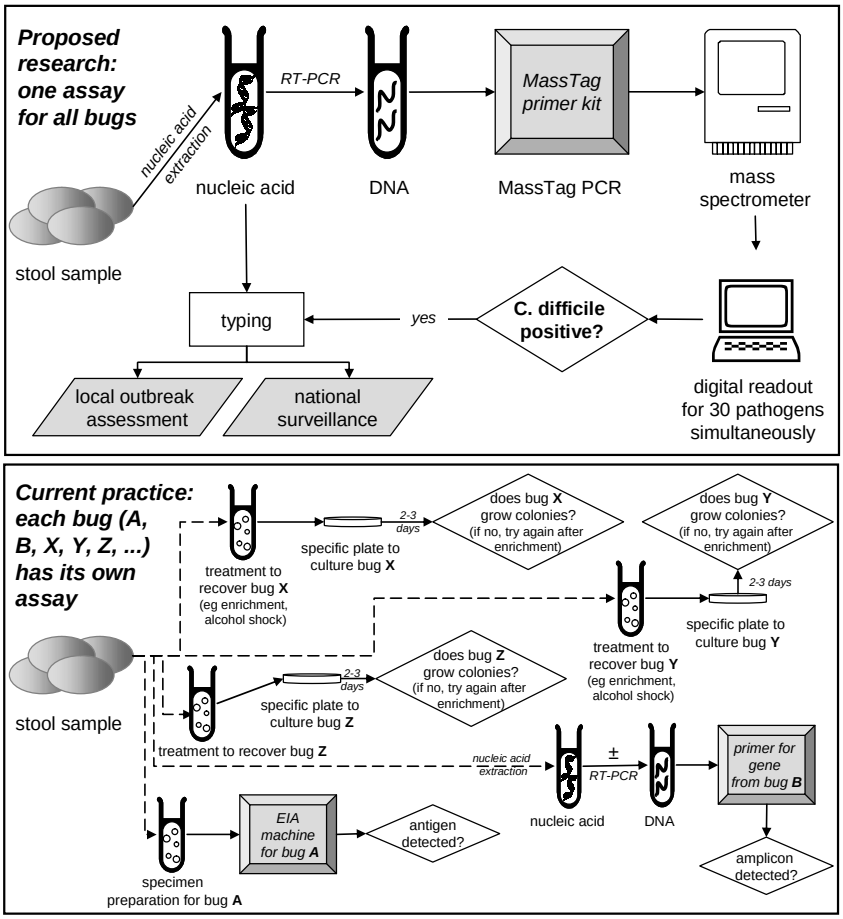
The **first advance is MassTag multiplex PCR-based diagnostics**[4, 5] which provide a rapid (3-4 hours) test for many pathogens (currently up to 30) simultaneously in a single reaction. This offers the opportunity to effectively rule out infectious causes of many syndromes, e.g. infectious diarrhoea, meningitis, infectious arthritis, empyema etc. In contrast, conventional PCR tests are usually limited to detecting one pathogen per test with effectively a maximum of six. Consequently, MassTag multiplex PCR-based diagnostics avoid the need to conduct multiple individual tests (whether antigen testing, culture or conventional PCR) on samples (see diagram on page 10) whilst delivering comparably high sensitivity and specificity to conventional single pathogen PCR tests. This technology is being developed by our collaborators Xynostics Limited to meet a wide range of diagnostic possibilities and in Oxford we are carrying out in house validation procedures.

For suspected infectious diarrhoea, the technology tests a stool sample against a panel of pre-specified gastrointestinal pathogens. The panel consists of a pair of primers (short runs of nucleotides unique to each pathogen) for each of the multiple pathogens. Each primer is coded up with a unique tag of different mass; thus, there are up to 60 tagged primers in each reaction. The panel is combined with the patient sample and rapid endpoint PCR then selectively amplifies any pathogen DNA in the original patient sample. The amplified DNA is identified by reading the corresponding mass tags (which remain attached to the primers incorporated in the amplified DNA) in a mass spectrometer. These increasingly simple instruments are very sensitive and can easily detect and differentiate between the specially designed mass tags, in a matter of seconds. Simple software analyses the instrument's output and reveals the presence of any pathogens in the original sample. The low concentrations that can be detected in this system means that fewer rounds of PCR amplification are needed, which substantially reduces the reaction time and leads to a very rapid turnaround. Whilst setting up real-time PCR for the detection of *C. difficile* is a current research interest for several groups[3], we are not aware of any competing approaches to better diagnosing *C. difficile* with a rapid test other than PCR.

The current gastrointestinal/food poisoning panel (ready for marketing) includes 23 primer

pairs targeted at *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium difficile*, *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella spp.*, *Salmonella typhi/paratyphi*, *Vibrio spp.*, *Vibrio vulnificus*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Escherichia coli*, Escherichia EPEC/EHEC, Escherichia EPEC, Escherichia EHEC, Escherichia EIEC, Escherichia EAEC, Escherichia O157:H7, Rotavirus A/B/C, Norovirus, Sapovirus, Human astrovirus 1-8, and Calicivirus. This panel is modifiable and can be easily updated to include new pathogen strains as they are discovered. The current panel already includes most of the key high burden or high impact pathogens targeted for surveillance by the HPA (Adenovirus, Astrovirus, Botulism, Calicivirus, Campylobacter, Cryptosporidium, *E. coli O157*, *Giardia lamblia*, Norovirus, *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella typhi*, *paratyphi A* and *paratyphi*[1]). Current research interest (outside the scope of this study) is in including gastrointestinal parasites such as *Giardia lamblia,* Cryptosporidium and *Entamoeba histolytica*.

Currently, the product for MassTag PCR will be a kit containing the primer mix for incorporation in the PCR reaction. DNA extraction, and preparation of the sample for amplification including, where necessary, reverse transcription for RNA viruses will need to be undertaken as preliminary steps (see diagram). The stages for successful assay will need to be organised as modules, as is currently the case for many PCR diagnostic tests undertaken in routine diagnostic laboratories. This technology is highly suitable for ultimate automation in a single integrated platform which incorporates all the steps in a single machine such as modern ELISA machines. The digital outputs from the mass spectrometer will facilitate integration with laboratory computer systems.



This diagnostic approach could have a major impact on patients in NHS hospitals by achieving the early recognition of infectious diarrhoea. This would substantially improve the care of patients by ensuring only appropriate cases are kept in isolation, and as a consequence, could not only reduce the transmission of entero-pathogens but also greatly improve the use of single rooms by reducing their unnecessary use for non-infectious diarrhoea cases awaiting results of tests to rule out infectious diarrhoea. We are not aware of any other rapid sensitive and specific technology that can test for multiple (up to 30) entero-pathogens in a single sample and is ready for evaluation of costs and benefits in clinical practice. This technology will be the first diagnostic approach that can integrate so many pathogens in a single test and is ground breaking. However, despite its potential advantages, **whether such a test will actually deliver cost-effective improvements in patient management and**

[1] http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1195733811919?p=1191942150126

outcomes, and whether it can be generalised across the NHS is unknown. In particular, the key risk is that the PCR test identifies a high proportion of patients with colonisation rather than infection (true "colonisation positive", false "infection positive") which could lead to unnecessarily increased anxiety for patients, considerable additional unnecessary treatment costs, and also increase (rather than decrease) pressure on side rooms. Rolling out such new technology across the NHS requires an evidence base covering both costs to microbiology service and benefits to patients, which will be provided by this study. An added benefit from simultaneously testing for these multiple pathogens would be increased understanding of the epidemiology of carriage and disease caused by a variety of entero-pathogens in the UK.

2.2.2 Multi-locus sequence typing (MLST) for *C. difficile*

The **second advance is real-time *C. difficile* strain typing** using multi-locus sequence typing (MLST) approaches directly on stool samples without first culturing them to obtain samples of the infecting organism itself. Currently, typing is not routinely used in *C. difficile* diarrhoea outbreak management for two reasons: first, the long time lag in obtaining information, because an organism needs to be grown before being processed through a typing scheme which is labour intensive and time-consuming; and, second, the fact that the typing schemes proposed for *C. difficile* thus far have recognised key limitations. For example, the MLST scheme of Lemee et al[6] is critically limited in typing resolution by its choice of relatively non-variable and difficult-to-sequence target genes, in common with many others.

MLST is a proven technology for understanding the molecular epidemiology and population biology of many bacterial species. It has already been applied to various collections of *C. difficile* isolates[6-8]. Our pilot work (funded through the NIHR Oxford Biomedical Research Centre) has adapted this scheme to include longer allele sequences, thus improving discrimination, and we have also substituted a null allele for one which is present in all strains. This more robust version of the MLST scheme has been applied to a diverse set of *C. difficile* strains supplied by the principal investigator Professor Mark Wilcox and achieves equivalent discrimination as ribotyping, which is the scheme used nationally. The enhanced MLST has been successfully used to type 300 cultured *C. difficile* isolates obtained from patients in the John Radcliffe Hospital and the surrounding community. An internet accessible web site and database have been set up through PubMLST (http://pubmlst.org/).

MLST of pure *C. difficile* DNA extracted from cultured isolates is straightforward. However, we have also extended the technology to type *C. difficile* directly from total DNA extracted from stool specimens, a complex mixture of human, bacterial and undigested food (plant and animal) DNAs. We have developed an approach which has proven successful in pilot work, and will also be a key step in implementing the MassTag PCR diagnostics (see diagram above). It overcomes three main obstacles to the use of total stool DNA extracts for PCR:

(i) Lysis of both *C. difficile* cells (Gram positive) and spores in faeces, thus maximising the proportion of *C. difficile* DNA contained in the total DNA extract.
(ii) Removal of PCR inhibitors from the extracted DNA.
(iii) Specific PCR amplification of *C. difficile* sequences from a complex mixture of DNAs (human, bacterial and food) containing variable amounts of *C. difficile* DNA.

To address (i) we have used the "FastPrep" homogeniser (MP Biomedicals) to lyse both *C. difficile* cells and spores contained in stool samples[9, 10]. This has the dual advantages of efficiency and reproducibility. Up to 48 samples can be lysed in 40 seconds by homogenisation in the presence of a "lysing matrix" comprising ceramic spheres, silica spheres and a glass bead. Cross contamination is eliminated by the use of closed lysing matrix tubes. The integrity of the DNA is retained, with high yields of high molecular weight material and no sheared low molecular weight DNA visible on agarose gel electrophoresis. The extraction of DNA lacking inhibitors from the homogenisated stool ((ii) above) has been accomplished using a "Fastprep" kit (MP Biomedicals), which binds DNA using a silica matrix which is then washed

thoroughly prior to elution of clean DNA. The specific amplification of *C. difficile* sequences by PCR for MLST ((iii) above) was achieved by careful design of PCR primers and the use of a hotstart taq DNA polymerase.

This approach is conservatively yielding high quality *C. difficile* sequence for >90% of stools with a positive toxin assay based on ELISA. This approach obviates the need to culture the stool, can be done on stored stools and in optimal circumstances will yield typing in 5-7 working days. Using robotically operated facilities would reduce time to completion in the majority of cases to 3 days, well inside the time needed for clinical decision making. Such near-to-real time discriminatory typing of this pathogen directly on stool samples could not only improve control of outbreaks in hospitals, but would also be highly valuable to national surveillance which would benefit future patients by providing early warning of new, potentially more dangerous, transmissible strains.

This study provides a unique opportunity to evaluate the utility of this system in a real-world setting as a second step following the rapid MassTag PCR-based test described above to allow strain typing to be readily scaled up cost efficiently in a clinically realistic timeframe. This will allow genuine outbreaks to be identified in near-to-real time - in contrast to the current Department of Health outbreak definition of two cases occurring on a ward within 7 days when these cases may or may not be related. Whilst such a scheme would have relatively little benefit for the individual infected patient, identifying such outbreaks will delineate more clearly the key mechanisms of spread in NHS Trusts. If a substantial proportion of cases can be linked into outbreak chains in near-to-real time, widespread implementation of such a system would provide unprecedented opportunities to change infection control practice to interrupt transmission as it actually occurs within outbreaks - rather than as it is believed to occur. Reducing cases would directly benefit uninfected patients. Further, current surveillance data provide only sporadic information. Thus, even if highly discriminatory fingerprinting methods are used, it is difficult to be certain how much observed shift is 'natural' genomic drift. Systematic data on all cases, as would be provided by this study, would provide a much more thorough understanding of genetic changes in circulating strains which would provide a more secure foundation for a national surveillance system. The *Clostridium difficile* Ribotyping Network for England (CDRNE) is well placed to rollout any such new methods should they be of proven benefit (led on behalf of the Health Protection Agency by Prof Wilcox, principal investigator).

2.3 Objectives

This diagnostic test study therefore has two research objectives:

(1) to evaluate a newly developed technology - MassTag multiplex PCR - for the simultaneous diagnosis of multiple entero-pathogens directly from stool, in terms of core metrics of performance:
   (i) sensitivity/specificity and real-time predictive values to detect a range of pathogens and overall to rule out any infectious causative agent.
   (ii) turnaround time (speed of diagnosis), net healthcare costs and utilisation of isolation resources to assess whether it can improve hospital management of patients with suspected infectious diarrhoea, in particular by avoiding/reducing isolation of patients with non-infectious diarrhoea.

(2) to evaluate rapid multi-locus sequence typing (MLST) of *C. difficile* direct from stool, in terms of
   (i) turnaround time and net healthcare costs
   (ii) ability to detect outbreaks in clinically useful timeframes and to provide readily available national surveillance data

Parallel health economic evaluation will combine data on diagnostic procedures and short-term consequences collected in this study with longer-term survival payoffs modelled from the

study and a structured literature review. Using these models we will address a range of questions to inform future clinical decision-making, in particular

Clinical

a) Will earlier diagnostic information improve bed stock management by reducing the number of suspected infective cases being kept in isolation?
b) Will earlier detection of food-borne infections allow outbreaks to be identified and contained more efficiently?
c) Will earlier recognition of specific conditions such as *C. difficile* or bacterial dysentery allow more rapid initiation of appropriate therapy with consequently better outcomes?

Laboratory

d) Will a move from culture methodology to MassTag multiplex PCR be less labour intensive, permit higher throughput in laboratories, and facilitate better patient management through integration of digital output into the NHS IT system.

Our goal is for the modelling to put plausible bounds on the costs, effects and cost-effectiveness of these impacts, and to also help identify ways in which patient management might have to be modified to obtain maximum benefit from them.

3. STUDY CENTRES

Both objectives will be addressed in parallel studies in two of the largest Trusts in the UK (see Flowsheet, section 1.2, p6):

- The Oxford Radcliffe Hospitals NHS Trust (Chief Investigator: Prof Derrick Crook)
- The Leeds Teaching Hospitals NHS Trust (Principal Investigator: Prof Mark Wilcox)

Inclusion of two centres allows us to quantify the impact of the new technology on the background of different local practices and current systems, and to directly address variability and robustness across the two centres as part of implementation. In particular, the faster but potentially sub-optimally sensitive ELISA technology is used to test for *C. difficile* toxin in Oxford whereas the gold standard cytotoxin method that takes up to 2 days to complete is used in Leeds – these are the two major methodologies used across the UK. The two phases will be staggered in Oxford and Leeds hospitals (see Flowsheet and below) to support optimal translation. The two new technologies will initially be implemented and evaluated in Phase I in the Oxford microbiology laboratory (months 1-12). From month 10, the technologies will be rolled out to the Leeds microbiology laboratory, including on-the-job training with the system in Oxford, after which Phase I evaluation in Leeds will then proceed (Phase I months 10-21). The Phase II real-time parallel group study will be conducted over 18 months in Oxford and 12 months in Leeds (months 13-30 and 22-33 of the project respectively), leaving the final months of the three year study for data analysis and report/manuscript preparation. It is essential to run Phase II over at least 1 year in each hospital as the burden of different entero-pathogens varies strongly with season.

4. SELECTION OF SAMPLES

4.1 Inclusion criteria

In **Phase I (retrospective diagnostic study)**, 200 stool samples with *C. difficile* toxin, norovirus (by RT-PCR) and *Campylobacter spp.* (by culture); 100 stool samples with *Salmonella spp.* (by culture); and 300 stool samples negative for all pathogens (total 1000 samples to be tested in each hospital) will be extracted completely at random from stores within each microbiology laboratory (samples are stored for approximately the last 12 months) without reference to age/location or any other factor. These samples will be presented for testing in a randomised order.

In **Phase II (prospective diagnostic study)**, parallel testing will be performed on all stool samples taken

- during admission to general medical and surgical wards in the John Radcliffe Hospital, Oxford, and one of the two main Leeds hospitals
- arriving at microbiology laboratories at any time of day during Monday through Thursday

These specialities are chosen because they account for half to two-thirds of the *C. difficile* cases (~300 and ~1000 in Oxford and Leeds respectively in 2007/8) and nearly all other entero-pathogens, whilst also experiencing significant pressure on side rooms due to other factors such as MRSA colonisation. For example, in a study in Leeds General Infirmary, one in three requests for isolation for infection control reasons in these specialities could not be met[2]. The Leeds Teaching Hospitals NHS Trust is comprised of two large hospitals (Leeds General Infirmary and St James's Hospital), each with general medical and surgical wards – one of these two hospitals will be used for Phase II evaluation to provide a similar number of samples as from Oxford. The choice of hospital will be made at the time Phase II evaluation is ready to start, depending on the ward case-mix at the time (this is currently under review) to provide a comparative range of specialities to general medicine/surgery at the John Radcliffe Hospital Oxford. Restricting data collection on isolation for diarrhoea of unknown origin to defined locations with high burden is a more efficient strategy to test proof-of-principle for translation than including multiple areas of very large acute hospital Trusts.

Restriction of samples to those arriving at microbiology laboratories on Monday through Thursday is a pragmatic choice given personnel costs for the study. Study staff will only be employed for standard working hours, and therefore analyses will be conducted on all eligible samples and also restricting to those arriving in microbiology laboratories during standard working hours.

All stool samples will be tested using the MassTag system for the multiple entero-pathogens. Each sample will be noted as diarrhoeal (adopting the shape of the container) or not, in order for results for *C. difficile* to be compared with toxin testing where national guidance is to test only diarrhoeal stool samples.

As individual patients will continue to be managed according to routine microbiology service (see section 5.1.2 below), there are neither risks nor benefits for patients whose samples are used within this study. However, we recognise that these new technologies, although having risks as detailed above, may also have substantial benefits. Therefore we plan a single formal interim analysis of Phase II when this study could be terminated early if substantial and statistically significant improvements had already been identified without evidence of concomitant risks (see section 6.3, p19 for details).

5. STUDY DESIGN

This study is a two-stage diagnostic test study carried out in two acute NHS Trusts.

5.1 Planned interventions

(1) A single integrated multiplex MassTag PCR-based diagnostic test to directly test stool samples for a panel of gastrointestinal pathogens including (but not restricted) to *C. difficile*, Norovirus, *Campylobacter spp.* and *Salmonella spp.* versus reference-standard culture-based methods and (*C. difficile* only) fast and slow toxin detection based methods (ELISA and cytotoxin respectively).
(2) A follow-on *C. difficile* strain typing scheme based on MLST performed directly on stool samples.

5.1.1 Phase I

Phase I (1 year) is a retrospective batch study in two parts

- estimation of sensitivity/specificity of the new MassTag multiplex PCR-based diagnostics versus reference standard molecular RT-PCR for Norovirus; toxin detection methods, ELISA (Oxford) and cytotoxin (Leeds) for *C. difficile*; or culture-based methods (other pathogens) using fixed numbers of samples positive for *C. difficile* toxin (200), *Campylobacter spp.* (200), *Salmonella spp.* (100) or Norovirus (200), or negative for all entero-pathogens (300) (total 1000 samples to be tested).
- comparison of ***C. difficile* MLST typing directly from stool** versus cultured isolates.

Current reference standard for diagnosis of *C. difficile* infection is a positive toxin test (by ELISA or cytotoxin) from a diarrhoea sample[3, 11]. During Phase I (and II, see below), any stool sample positive for *C. difficile* toxin or by PCR will also be cultured, to provide additional comparisons for sensitivity/specificity (false positive identification in particular) and isolation rates.

5.1.2 Phase II

Phase II (1-1.5 years) is a prospective real-time parallel-group study testing the same stool samples from hospitalised patients in general medical and surgical specialities in both the routine microbiology laboratory (using standard ELISA (Oxford) or cytotoxin (Leeds) and culture-based methods) and by the new MassTag multiplex PCR-based technology with follow-on *C. difficile* strain typing from stool samples. This study will enable

- estimation of positive/negative predictive value for various pathogens in a real-world setting
- direct comparison of turnaround time, net healthcare costs and patient-centred outcomes (utilisation of isolation resources, detection of outbreaks), as well as implementation robustness (see section 6.1, p18 below).

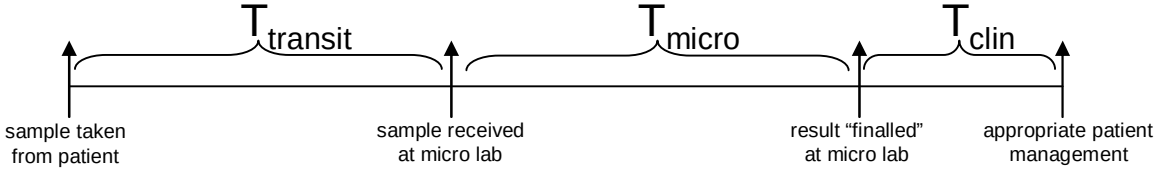
During Phase II, the new MassTag system would be run **in parallel** with the service microbiology laboratory, the usual way in which new tests are evaluated and incorporated into routine service laboratory practice. Whilst all results would be entered onto electronic databases, **all patients will be managed only according to the results from the routine microbiology service**.

There are several major scientific and pragmatic reasons supporting this choice over a randomisation of either patients or wards to standard versus new test:

- Scientifically, a major question remains as to whether PCR-based tests are over-sensitive for detecting infection – that is, that many PCR positives are solely due to colonisation and are not relevant or useful clinically. Given that the new test has the potential to provide multiple positive hits from a single sample, we do not consider that the risks of moving directly from sensitivity/specificity testing on selected samples to clinical practice justify

the risks of inappropriate management and treatment of colonisation. We also judge that ethics committees will have similar concerns.

– Scientifically, if the PCR-based test is not over-sensitive, parallel testing will actually provide a more powerful assessment of the role of this new technology in clinical practice than randomisation. This is because the pathway from sample collection to appropriate clinical action is composed of three major parts – a time in transit from patient to microbiology laboratory ($T_{transit}$); a time being tested in the microbiology laboratory (T_{micro}), the end result of which is a result being declared "final"; and a time for this finalled result to lead to appropriate patient management (T_{clin}):



Whilst randomising patients/wards to new versus standard test methodologies would enable comparisons of all three components of this pathway, these comparisons would be on **different** samples. As there is no reason to expect the new technology to provide improvements in $T_{transit}$ or T_{clin}, we would essentially be comparing T_{micro} across these different samples and trusting the randomisation to ensure that the samples were comparable in their other characteristics ($T_{transit}$ and T_{clin} in particular). However running the MassTag PCR test **in parallel** with the standard microbiology service will enable us to directly compare T_{micro} on the **same** sample run on each of the tests (analogous to a cross-over trial where the same patient receives both treatments). Thus we will obtain a direct estimate of the effect of the new test on the sample pathway, making the reasonable assumption that $T_{transit}$ and T_{clin} are unchanged.

Further, whilst the total time from sample collection to appropriate patient management could be compared between new versus standard test methodologies with patient/ward level randomisation, in reality this would be an extremely insensitive comparison because the contribution of $T_{transit}$ and T_{clin} to the total time would be independent of the test methodologies and thus essentially random with respect to them. This would dilute any real differences in T_{micro} between the test methodologies.

Current reference standard for diagnosis of *C. difficile* infection is a positive toxin test (by ELISA or cytotoxin) from a diarrhoea sample[3, 11]. During Phase II (and I, see above), any stool sample positive for *C. difficile* toxin or by PCR will also be cultured, to provide additional comparisons for sensitivity/specificity (false positive identification in particular) and isolation rates.

5.2 Methods for reducing bias

We plan to reduce the potential for bias to influence study results in the following ways:

– in Phase I, different laboratory staff will perform sample selection and sample testing. The order of the 1000 samples will be randomised at each hospital (ie samples positive for different pathogens will not all be grouped together). The researchers conducting the MassTag assays will therefore be blind (masked) to the results of the standard (reference) assays in Phase I.
– in Phase II, researchers conducting the MassTag assays will complete these tests considerably before the culture based methods used in the standard service microbiology laboratories. Further the research laboratories are physically separate from the service laboratories. Therefore the researchers conducting the MassTag assays will therefore also be blind (masked) to the results of the standard (reference) assays in Phase II.
– in Phase II, whilst all samples will be tested and data recorded electronically in real-time,

comparison with the standard microbiology service will only be performed at one interim analysis during the study (see section 6.3, p19 for details) and at the end of the study. These results will not be released outside of the steering group. This will prevent knowledge of any current differences between methods influencing laboratory staff personnel behaviour.

5.3 Economic evaluation

The economic analyses will estimate the likely costs and effects of using MassTag multiplex PCR for the simultaneous diagnosis of multiple entero-pathogens, and will consist of several linked components. In the first instance the economic analysis will estimate the net cost to the NHS of the new technology. However, attention will also be paid to estimating generalisability of results to the NHS by combining data on diagnostic procedures and shorter-term consequences derived from our study with longer-term survival payoffs modelled from the specific studies below and a structured literature review.

5.3.1 Survey of current procedures for and costs of diagnosing gastrointestinal pathogens

In year 1, a survey will be undertaken across a sample of general medical and surgical specialities in Acute NHS trusts across the UK, to obtain information on the current procedures for diagnosing gastrointestinal pathogens and managing patients with suspected infectious diarrhoea, including current laboratory and isolation procedures, turnaround times, and other information. A detailed questionnaire will be developed in collaboration with clinicians and infection control staff from Leeds and Oxford, piloted in a small number of centres (including validation checks), and then sent to a larger sample of approximately 50 hospitals in the second half of year 1 of the study. This will provide data on how typical the two study centres are, and thereby make it possible to generalise the results by modelling likely impact of these technologies across the wider NHS.

The costs of using MassTag multiplex PCR in comparison with current diagnostic methods will be assessed using a combination of observational work, logs, diaries, and interviewing to assess laboratory practices and staffing. A survey of laboratory practices will be conducted among the 50 hospitals chosen above. In particular, we will estimate the institutional costs of running a "faeces bench" in the microbiology laboratory versus implementing and automating the MassTag PCR technology (see diagram p9). This would be done primarily in year 2.

5.4 Data collection

For each stool sample included in Phase II (see section 4.1, p13) four types of data will be recorded (none being personal identifiers):

(i) basic demographic data from the NHS patient administration system
- sex of patient
- year of birth of patient (this is not considered identifiable in HES extracts)
- specific ward where sample taken
- date of admission/discharge to hospital and this ward

(ii) basic information about the stool sample from the NHS microbiology system
- date and time of collection of the stool sample
- whether or not the stool was diarrhoeal (adopting the shape of the container)
- date and time of receipt of the stool sample at the standard service microbiology laboratory
- date and time the standard service microbiology laboratory result was declared final

(iii) information about the MassTag system from the research microbiology laboratory
- date and time of receipt of the stool sample at the research microbiology laboratory
- breakdown of times of each of the steps described on p9
- date and time the MassTag microbiology laboratory result was declared final

(iv) information about days of isolation for suspected infectious diarrhoea

- for each stool sample included in the study, the infection control research nurse will record whether and how long each patient had suspected and/or confirmed infective diarrhoea for, the location of the patient during this period (side-room (reason), cohorted (reason), other), whether and how long each patient should have been isolated for (reason). In Oxford this information will be routinely recorded as part of the Infection Control service database (this information is currently collected periodically for specific audits). In Leeds, this type of information is also collected for periodic audits: for this study it will be entered onto a specific database using only a pseudo-anonymised identifier (see below).

Patients will not be identified by names or NHS/hospital numbers: rather a (pseudo-anonymised) independent identifier will be allocated when data are extracted (in order to link repeated samples from the same person and to allow identification of possible *C. difficile* outbreaks). The link between personal pseudo-anonymised identifiers will be held in a physical patient register in Leeds and, in Oxford, as part of the Infections in Oxfordshire Research Database (approved by Oxfordshire Research Ethics Committee C).

5.5 Quality control

Quality control has an important role in molecular diagnostic testing for the diagnosis of infectious disease. Appropriate positive and negative controls will be included, and the results of positive controls will be monitored over time to ensure consistency and reproducibility. For the MassTag PCR in Phase II we will also assay the samples forming the external Quality Assurance Scheme for the relevant gastro-intestinal pathogens. All reasonable precautions will be taken to avoid the introduction of PCR contamination (introduction of post amplification products into the PCR set up areas), which can lead to false positive results. For example the extraction of total stool DNA from clinical samples will be performed in a separate laboratory to the *C. difficile* MLST PCR and post PCR anaylsis.

6. STATISTICAL CONSIDERATIONS

6.1 Outcome Measures

Outcomes measured will be

(i) Sensitivity and specificity (**primary outcomes for Phase I**), and positive and negative predictive value of the new MassTag PCR test compared with currently used methods based on toxin detection assays for *C. difficile* (ELISA and cytotoxin), culture for *Campylobacter spp.* and *Salmonella spp.*, and RT-PCR for Norovirus.

(ii) Resource utilisation in terms of test turnaround time from sample receipt at the service microbiology laboratory to test result being declared final in each of the service microbiology laboratory and research laboratory (**primary outcome for Phase II**), and net healthcare costs (including consumable and personnel costs). We make the generalisability assumption that the time from the result from the service microbiology laboratory being finalled to appropriate clinical management would be the same as the hypothetical (non-observed) time from new MassTag PCR result being finalled to appropriate clinical management (see p15 above). Thus a comparison of test turnaround time (time from sample receipt to final result) is directly analogous to the patient-centred comparison of time from sample being taken to appropriate clinical management.

(iii) Time from first stool sample being taken in an admission to first isolation of a bacterial entero-pathogen, number of samples taken between first stool sample and first isolation of a bacterial entero-pathogen or discharge, and number of pathogens identified for each patient during an admission.

- A cost-effectiveness analysis based on the incremental cost (ii) for reductions in turnaround time for positive isolations will also be conducted (see above for more details of economic analyses).

(iv) Number of days in isolation for suspected infective diarrhoea. This will provide a direct measure of the contribution of suspected infective diarrhoea to isolation pressure in general medical/surgical specialities in the two Trusts – side-room suspected infectious diarrhoea isolation days and cohorted suspected infectious diarrhoea isolation days at different times of year, which can be compared with test turnaround time (ii) in order to estimate likely isolation-room savings. It will also delineate the contribution of isolation of patients with suspected infectious diarrhoea to failure to isolate other patients.

(v) Number of cases defined as an outbreak according to DH definitions (two cases on a ward within 7 days) infected with the same strain of C. difficile according to MLST.

6.2 Sample Size

In Phase I, testing 200 positive detections of *C. difficile*, *Norovirus*, *Campylobacter spp.* with reference standard culture/toxin detection and new PCR-based tests would produce a 95% confidence interval around sensitivities of 90% and 96% of ±4.2% and ±2.7% respectively. Testing 100 positive isolations of *Salmonella spp.* would produce a 95% confidence interval around a sensitivity of 90% of approximately ±5.9%. Samples positive for each pathogen will act as negative controls for other pathogens together with 300 samples negative for all pathogens (total 1000 samples to be tested in each hospital). All *C. difficile* toxin positive samples will also be cultured, and sensitivity/specificity of toxin - (ELISA/cytotoxin) versus PCR-based tests compared with culture will be assessed using the methods of Newcombe[12]. Phase I will take 1 year in each Trust (including time to set up and quality control the MassTag assay).

General medicine/surgery account for around half the *C. difficile* cases (~300 and ~500 in Oxford and one of the two Leeds hospitals respectively in 2007/8) and most other entero-pathogens. Based on the length of Phase II (18 vs 12 months respectively) and other eligibility criteria (specimens arriving at microbiology on Mon-Thurs from general medical/surgical wards in Oxford and one of the two Leeds hospitals), during Phase II we

expect (per hospital) 250-350 samples positive for *C. difficile*, *campylobacter spp*. and norovirus; and ~100 positive for *salmonella spp*.. 250-350 (100) samples will provide at least 90% power to detect increases in the percentage returned within various time thresholds of 10-15% (16-24%). In total, we estimate 1800 and 1200 stool samples will be tested from Oxford and one Leeds hospitals respectively in Phase II.

6.3 Interim Monitoring and Analyses

Analysis of sensitivity/specificity of MassTag PCR versus standard reference methods and MLST directly from stool versus culture will be conducted at the end of Phase I in each Trust. Results in the two centres will be combined in the absence of qualitative heterogeneity - otherwise reasons for any such heterogeneity will be sought.

A small steering group will be set up to review results of Phase I (in each hospital and overall) in order to determine whether or not Phase II should proceed. Criteria for not moving to Phase II would be based on extremely poor sensitivity for detecting key organisms (under 75%) such that the test would not be likely to be adopted in routine NHS practice. The choice of a threshold of 75% is based on estimated sensitivity of the currently widely used ELISA tests for detecting *C. difficile*. The steering group will comprise investigators (Crook, Peto, Wilcox, Walker) plus two independent researchers - **Professor Ajit Lalvani** (Imperial College, expertise in development, assessment and implementation of interferon gamma release assays: approached and agreed in principle), and (as Chair) **Dr Christine McCartney** (Director of the HPA Regional Microbiology Network: approached and agreed in principle) and one independent member from outside academia, **Ms Katherine Innes Ker**, who has extensive commercial experience and will also represent the patient community (approached and agreed in principle). Inclusion of investigators and independent researchers in one oversight committee is appropriate because there is no patient management which could be influenced by knowledge of results to date, as no results are being returned for clinical care.

Phase II analyses of outcome measures described in 6.4 below will be carried out twice: once in a formal interim analysis at the start of the third year of the study (after 12 and 3 months Phase II data from Oxford and Leeds respectively) and once at the end of phase II in each site. Results from each site will be analysed separately and combined into an overall estimate for each outcome using meta-analysis techniques. The interim analysis would be reviewed by the steering group who will be asked to give advice on whether the accumulated data from the study, justify termination of the study, in terms of either the strength of the evidence being such that the MassTag assay would likely be adopted NHS-wise or never adopted at all.

6.4 Statistical analysis

We will use time-to-event methods (life-tables, Kaplan Meier estimates, logrank test) to compare our primary outcome, test turnaround time, between the groups in Phase II because we expect that its distribution will be strongly non-normal with a "fat tail" - a few results with long turnaround times. We will also use these methods to compare time from first sample being taken to discharge/first isolation of pathogen. In order to test whether MassTag PCR is over-sensitive, we will classify each patient admission according to pathogens isolated before discharge, and compare these patterns between MassTag and standard tests. If MassTag PCR is over-sensitive we would expect it to identify significantly more pathogens not identified using standard methods. If, as hoped, it has improved (but not over) sensitivity, we would expect the same pathogens to be identified on a per admission basis, but significantly shorter times to first pathogen isolation/discharge on the time-to-event analysis. Consumable and personnel costs will be compared using means, standard deviations and t-tests as these take into account overall population-level costs. An institutional perspective will be considered for the cost-effectiveness analysis.

The number of days in isolation with suspected and/or confirmed infective diarrhoea, the

number of days in isolation for other reasons, and the number of days not in isolation with suspected and/or confirmed infective diarrhoea will be estimated from twice-monthly isolation audits in eligible wards for both side-rooms and cohorted bays. These estimates will be a direct measure of the contribution of suspected infective diarrhoea to isolation pressure in general medical/surgical specialities in the two Trusts, and will identify the current "gap" in isolation capacity together with reasons for this gap. We will combine these estimates with reductions in turnaround time and test sensitivity/specificity in order to estimate likely isolation-room savings.

Analyses will be conducted on all eligible samples, and restricting to samples arriving in microbiology laboratories during standard working hours; the latter comparison being the fairest test as staff will be active in both service and research laboratories during this period. However, analysing all samples arriving at microbiology laboratories Monday-Thursday will also provide an indirect assessment as to whether improved service could also be provided with reduced staffing cover using the new technology. Analysis for *C. difficile* will also be conducted restricting to diarrhoeal samples only in order to follow national guidance for *C. difficile* testing.

Phase II analyses of the MLST typing scheme will include estimation of the distribution of turnaround time (from sample receipt to typing classification) using Kaplan-Meier. Turnaround time of the current national standard, ribotyping, is a minimum of 8 days, and therefore we will estimate the proportion of results that can be identified within 8 days, and also within 3 days which is clinically judged to be the maximum effective timescale for intervention. The expected 400-500 C. difficile cases within Phase II within each Trust would provide at least 80% power for the width of the 95% confidence interval around these proportions to be ±10-15% (depending on the estimated proportion itself). We will compare the Department of Health definition of an outbreak (two cases on a ward within 7 days) with strain relatedness according to MLST typing.

Additional epidemiological analyses of data collected within this study will be essential to quantifying the role of such new PCR-based tests in clinical practice. In particular, the key risk is that the PCR test identifies a high proportion of patients with colonisation rather than infection (true "colonisation positive", false "infection positive") which could lead to unnecessarily increased anxiety for patients and considerable additional unnecessary treatment costs. This risk is magnified by the fact that the MassTag technology simultaneously tests for multiple organisms – any of which could be either infecting or colonising. In contrast, the expected benefits are that the PCR test identifies a pathogen earlier in time. For *C. difficile* in particular, this would be from a sample toxin negative on ELISA/cytotoxin tests, with only subsequent samples becoming positive both for toxin on ELISA/cytotoxin and by PCR (see diagram). Further, it is possible that toxin can still be detected after all pathogens have been eliminated, in which case toxin tests would suggest that a patient is still infected, where PCR tests would indicate that no living pathogen remains – and expensive antibiotics with side-effects could be safely

Hypothesis: PCR is over-sensitive to colonisation

Mass Tag- PCR	-	-	+	+	+
(Toxin)	-	-	-	-	-
Culture	-	-	-	-	-

Hypothesis: PCR allows earlier diagnosis

Mass Tag- PCR	+	+	+	+	-
(Toxin)	-	-	+	+	-
Culture	-	+	+	+	-

Hypothesis: PCR allows earlier detection of elimination

Mass Tag- PCR	+	+	+	-	-
(Toxin)	-	-	+	+	+
Culture	-	+	+	+	-

time →

discontinued more quickly. We will investigate the pattern of positivity/negativity of samples for *C. difficile* on toxin and PCR tests over time within individual patient admissions, and compare with results from culture which will be performed on all Phase II samples positive for toxin or PCR to investigate these hypotheses.

For the health economic analyses, data will be analysed on the actual management of patients in the prospective real-time parallel-group Phase II, to estimate resource use from hospital stay and isolation using routine diagnostic methods, and to then model the likely effect of the PCR-based technology on management and therefore costs. The modelling would include estimates of infection transmission and outbreaks, informed by the results of the MLST typing scheme, the wider survey of hospital practice (section 5.3.1, p16) and wider literature. The model would take the form of a semi-Markov model, with a decision-tree component to replicate the initial diagnostic procedures and associated sensitivity and specificity, coupled to a 1-day cycle length Markov model to replicate the in-patient stay with probabilities of isolation, infection and discharge. Uncertainty would be handled within a probabilistic sensitivity analysis framework, using empirical data from the study on rates, probabilities and proportions, and variance estimates for other parameters from literature. The model construction and use will take place in years 2 and 3.

7. ETHICAL CONSIDERATIONS AND APPROVAL

7.1 Ethical considerations

As individual patients will continue to be managed according to routine microbiology service, there are in general neither risks nor benefits for patients whose samples are used within this study. As there is no alteration to patient management, and patients are not being randomised either individually or as groups (clusters), informed consent is not required. All data will be pseudo-anonymised; names will not be held.

However, we recognise that these new technologies, although having risks as detailed in section 5.1.2 (p14 above), may also have substantial benefits. Therefore we plan a single formal interim analysis of Phase II when this study could be terminated early if substantial and statistically significant improvements had already been identified without evidence of concomitant risks (see section 6.3, p19 for details).

Of the organisms that may be identified from this new technology (see section 2.2.1, p8), one, *E. coli O157,* is a pathogen which can have life-threatening consequences but is not routinely identified in the service laboratories. In order to ensure that any potentially life-saving results from this study are used to inform patient care, if an *E. coli O157* is identified by the MassTag system, the researchers will immediately inform the service laboratory using the laboratory sample identifier (names will still not be used). The service laboratory can then immediately initiate their standard tests for *E. coli O157,* and it would be the results of these standard tests which would be used to inform clinical management, ensuring that any false positives from the new (untested) system are not used inappropriately. All other pathogens identified by the new system are either already routinely assayed for by the service laboratories, or are not immediately life-threatening.

7.2 Ethical approval

The study protocol version 1.0 was approved by Oxfordshire Research Ethics Committee A on 9 December 2009, subject to the minor amendment to notify the service laboratory if *E. Coli O157* is detected included in version 1.1 section 7.1 above.

7.3 Ethical approval

The study protocol version 1.0 was approved by Oxfordshire Research Ethics Committee A on 9 December 2009, subject to the minor amendment to notify the service laboratory if *E. Coli O157* is detected included in version 1.1 section 7.1 above.

8. PUBLICATION

The results from the two centres will be analysed together and published as soon as possible for each Phase. Main publications will follow the STARD guidelines for reporting diagnostic studies (www.stard-statement.org. No verbal or written report may be made without the approval of the steering group to ensure that the overall conduct of the study is not prejudiced by early release of data. The members of the steering group will be listed with their affiliations in the Acknowledgements of the main publications of each Phase.

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