PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	A pilot study of rapid benchtop sequencing of Staphylococcus aureus and Clostridium difficile for outbreak detection and surveillance
AUTHORS	David W Eyre, Tanya Golubchik, N Claire Gordon, Rory Bowden, Paolo Piazza, Elizabeth M Batty, Camilla LC Ip, Daniel J Wilson, Xavier Didelot, Lily O'Connor, Rochelle Lay, David Buck, Angela M Kearns, Angela Shaw, John Paul, Mark H Wilcox, Peter J Donnelly, Tim EA Peto, A Sarah Walker and Derrick W Crook

VERSION 1 - REVIEW

REVIEWER	Dr Jonathan Edgeworth Director, Centre for Clinical Infection and Diagnostics Research Department of Infectious Diseases Kings College London St Thomas' Hospital London SE1 7EH UK Competing interests. I am involved in similar studies to determine the clinical utility of NGS for improving infection control practice. No other known conflicts or competing interests
REVIEW RETURNED	28/03/2012

THE STUDY	Most infection specialist recognise that NGS has the potential to improve targeting of appropriate infection control interventions leading to reduced transmission of hospital pathogens; however, the path from sequencing to preventing transmission is a long and complex one. This paper addresses some of the early hurdles namely the ability to 1) sequence clinical isolates and provide meaningful data on relatedness within a few days and 2) use differences in nucleotide variation between isolates to confirm or refute related transmission events for two important hospital pathogens – MRSA and C. difficile. It succeeds on both counts and therefore is a valuable contribution to the literature that deserves publication. It goes on to suggest in many places that from the data presented NGS could "transform" infection control practice. Such comments seem less appropriate here because they have not attempted to address that question and indeed have not presented information on infection control practice in any of the case studies. It does not demonstrate actually or even potentially how the information has a clinical benefit. The comments on transformation of infection control should probably be reserved for publications that have set out to demonstrate NGS data directing the activities of infection control doctors and nurses on the front line in real time. This is an important point because we are in a target-driven environment that has led to an unprecedented focus on implementation of basic and heightened infection control practice.

	One could say that infection control has already been transformed. It is therefore not obvious what additional interventions will be introduced when transmission has been identified by NGS (compared with a cluster of sporadic cases) or what measures can be relaxed in the absence of a confirmed transmission. Therefore my suggestion is that the authors should either provide detailed information on infection control practice and the timing of the provision of sequencing information (where possible) and how it did or might have specifically changed what infection control practice in each case OR tone down their claims for transforming infection control practice based on the data currently as presented.
RESULTS & CONCLUSIONS	It will be helpful to know what more about the background MRSA epidemiology and infection control practice on these units. For the MRSA outbreaks, how may beds in the ICU? were there other MRSA cases on the ICU either at that time or within a period either side? If so could those have been sequenced rather than the MSSAs? How relevant is the sequencing of the MSSAs - this isn't explained; what baseline infection control interventions were in place and what if any additional infection control measures were implemented to control the outbreak? Do they have any explanation for how the outbreak terminated? For the C. difficile outbreaks. What infection control measures were introduced at the time and how might the NGS information have modified practice? Do the authors have any other molecular typing data on the C. difficile isolates (MLST, ribotyping , MLVA) to compare with NGS. Page 9 bottom line "Figure 2B" Page 8 1st paragraph. Although prepared and sequenced together, samples were analysed sequentially to mimic availability of data in a real situation" Not clear what the authors mean Page 13 bottom paragraph. "If this set of cases could have been sequenced in real time….." This is a discussion comment. Page 14 Discussion first line. Too strong a statement to say this paper demonstrates that "bench-top sequencing can transform hospital infection control" particularly since infection control practice is not mentioned throughout and there is no suggestion that NGS was used to introduce or de-introduce an intervention. Page 14 last paragraph. "…in two outbreaks described were used to inform the hospital's response". It is not clear in what way practice was informed. Page 15. Of note the authors state the value of NGS is because " widely used current typing strategies are unable to distinguish isolates belonging to a prevalent strain type". Yet for MRSA they chose to apply NGS to two rare clones, which were identified as an outbreak clinically and using spa typing (confirmed by NGS) and not the current prevalent strain types, ST22 and ST36, for which widely used current typing strategies are ineffective. Page 15 middle top paragraph "strong evidence in favour of a putative transmission event, justifying infection control intervention." again because infection control interventions are not mentioned throughout this statement seems out of context with the data presented. Need to state which intervention. Page 15. last sentence "..bench-top format applied to routine hospital infection control". Surely better to say applied to hospital pathogens. To say routine hospital infection control there has to be some mention of practice and how NGS informed it. Page 16 top. Interesting the authors present PCR, MLST (and presumably spa) as a technique that needs to be sent away because it is not organism specific whereas NGS is something that "individual hospital laboratories" will undertake. I think there is

	currently at least as great a technical and financial hurdle for introducing NGS as there is for other molecular platforms – other potential benefits notwithstanding.

REVIEWER	Michael Metzker, Ph.D. Associate Professor Baylor College of Medicine USA
REVIEW RETURNED	11/04/2012

THE STUDY	Question #M2: Control samples/sequences in conducting phylogenetic studies are absence from the study. Moreover, no statistical framework is provided to assess the strenght of the differences between samples. Question #M5: Regions of genomic alignment are missing from the method section. Selection of appropriate (i.e., unrelated) controls is missing as well. The authors state using a JC method in modeling genetic changes, but why this model? Many studies use multiple models to remove model bias from the analysis. No description is provided regarding a statistical method, such as the widely used Bayesian posterior probability method. Question #M6: Without appropriate controls, drawing conclusions such as "NGS confirms transmission..." (title on page 10) and "NGS refutes transmission..." (title on page 11) are baseless. Moreover, even with appropriate controls, a statistical framework is necessary to determine the significance of sequence clusters. Question #M8: No statistical methods are provided. Question #M9: See #M8 Question #M11: No supplemental material was provided.
RESULTS & CONCLUSIONS	Question #R1: The results are incomplete due to the lack of appropriate controls and statistical framework. The study could benefit from analyzing more complex evolutionary models. Question #R2: The problems associated with the previous question makes the intrepretation of the results no credible. Question #R3: Figures 2, 3, and 4 could be better illustrated to show the genomic regions that resulted in the phylogenetic trees. Question #R4: Interpretation is limited by the problems identified above in my answer to question #R1
GENERAL COMMENTS	The authors should provide the reader with guidance as to why they chose the MiSeq system over the Ion Torrent PGM (another benchtop sequencer). Where does the PacBio system fit in rapid analysis. The authors note that they provide the "...first demonstration of rapid sequencing in a benchtop format...", but reference 8 (Holger et al.) makes the same claim. Please reconcile this statement. Can the authors also provide more description as to why with 77.6x and 50.4x coverage (page 9), only 80% of the reads mapped to their respective genomes?

VERSION 1 – AUTHOR RESPONSE

Reviewer: Dr Jonathan Edgeworth

Most infection specialist recognise that NGS has the potential to improve targeting of appropriate infection control interventions leading to reduced transmission of hospital pathogens; however, the path from sequencing to preventing transmission is a long and complex one. This paper addresses some of the early hurdles namely the ability to 1) sequence clinical isolates and provide meaningful data on relatedness within a few days and 2) use differences in nucleotide variation between isolates to confirm or refute related transmission events for two important hospital pathogens - MRSA and C. difficile. It succeeds on both counts and therefore is a valuable contribution to the literature that deserves publication.

It goes on to suggest in many places that from the data presented NGS could "transform" infection control practice. Such comments seem less appropriate here because they have not attempted to address that question and indeed have not presented information on infection control practice in any of the case studies. It does not demonstrate actually or even potentially how the information has a clinical benefit. The comments on transformation of infection control should probably be reserved for publications that have set out to demonstrate NGS data directing the activities of infection control doctors and nurses on the front line in real time.

We agree, and modified our wording to avoid any suggestion this paper is sufficient to transform infection control. Instead we now suggest NGS may “support” or “enhance” infection control. Significant further evaluation of the impact of this technology is required, a point made in our final paragraph of the discussion, and which has also been added to a new paragraph discussing the infection control interventions driven by the NGS data.

This is an important point because we are in a target-driven environment that has led to an unprecedented focus on implementation of basic and heightened infection control practice. One could say that infection control has already been transformed. It is therefore not obvious what additional interventions will be introduced when transmission has been identified by NGS (compared with a cluster of sporadic cases) or what measures can be relaxed in the absence of a confirmed transmission.

This is an excellent point, many hospitals already implement heightened infection control. However this technology provides a rapid means of identifying when these measures might not have been fully implemented or when transmission is occurring in spite of them. We have added details of the infection control interventions driven by the sequencing data to our results. We have also expanded our discussion of the impact of sequence data in our case studies to show some of the possible outcomes from availability of this data, but accept that formal trials of its use will be required in future.

Therefore my suggestion is that the authors should either provide detailed information on infection control practice and the timing of the provision of sequencing information (where possible) and how it did or might have specifically changed what infection control practice in each case OR tone down their claims for transforming infection control practice based on the data currently as presented.

Addressing the comments above we have both toned-down the “transformational” assertion, and also provided more detailed information on the infection control impact of the sequencing data.

It will be helpful to know what more about the background MRSA epidemiology and infection control practice on these units. For the MRSA outbreaks, how may beds in the ICU? were there other MRSA cases on the ICU either at that time or within a period either side? If so could those have been sequenced rather than the MSSAs? How relevant is the sequencing of the MSSAs - this isn't explained; what baseline infection control interventions were in place and what if any additional infection control measures were implemented to control the outbreak? Do they have any explanation for how the outbreak terminated?

More information has been provided in the methods section about the units involved and the baseline infection control interventions. Further details are provided in the results covering the extra interventions introduced. As detailed in the responses to reviewer 2, the additional *S. aureus* samples sequenced in the month following the ICU outbreak provide important control data for comparison with the outbreak data, providing data on the background level of diversity present. We have clarified the methods to state these cases were sequenced irrespective of their antibiotic susceptibility.

For the C. difficile outbreaks. What infection control measures were introduced at the time and how might the NGS information have modified practice?

A reference to a previous description of measures in place has been added.

Do the authors have any other molecular typing data on the C. difficile isolates (MLST, ribotyping , MLVA) to compare with NGS.

MLST data has been provided for more of the *C. difficile* cases throughout the results.

Page 9 bottom line "Figure 2B"

Thank you. The text relating to the individual panels of figures 2 and 3 has been corrected, to reflect the four panels in each, sorry for this error.

Page 8 1st paragraph. Although prepared and sequenced together, samples were analysed sequentially to mimic availability of data in a real situation" Not clear what the authors mean

This sentence has been expanded to clarify what was meant.

Page 13 bottom paragraph. "If this set of cases could have been sequenced in real time....." This is a discussion comment.

This point has been moved to the discussion as suggested.

Page 14 Discussion first line. Too strong a statement to say this paper demonstrates that "bench-top sequencing can transform hospital infection control" particularly since infection control practice is not mentioned throughout and there is no suggestion that NGS was used to introduce or de-introduce an intervention.

Changed from “transform” to “enhance” to tone down the statement. Additionally the impact of NGS on interventions is now discussed in the next paragraph.

Page 14 last paragraph. "...in two outbreaks described were used to inform the hospital's response". It is not clear in what way practice was informed.

This paragraph and the results have been expanded to explain how practice was informed.

Page 15. Of note the authors state the value of NGS is because " widely used current typing strategies are unable to distinguish isolates belonging to a prevalent strain type". Yet for MRSA they chose to apply NGS to two rare clones, which were identified as an outbreak clinically and using spa typing (confirmed by NGS) and not the current prevalent strain types, ST22 and ST36, for which widely used current typing strategies are ineffective.

We would accept this point, and the related manuscript by Didelot *et al* (submitted for publication and provided with this submission) demonstrates the extra resolution available from NGS across a range of *C. difficile* sequence types. However, it is also important to be able to demonstrate that this technology is able to detect closely related isolates as well as distinguishing between those that are distinct. Additionally, in order to provide a timely evaluation of the benchtop sequencing technology, the outbreaks chosen for investigation were the first of this size occurring in our hospital group after availability of the MiSeq machines, together with a recent HPA reported outbreak of significant clinical importance and uncertain aetiology.

Page 15 middle top paragraph "strong evidence in favour of a putative transmission event, justifying infection control intervention." again because infection control interventions are not mentioned throughout this statement seems out of context with the data presented. Need to state which intervention.

This sentence now refers back to a new paragraph above which outlines some of the interventions that occurred in our outbreaks.

Page 15. last sentence "..bench-top format applied to routine hospital infection control". Surely better to say applied to hospital pathogens. To say routine hospital infection control there has to be some mention of practice and how NGS informed it.

The wording here has been changed to reflect this is the first application to routine patient care, and then to healthcare-associated pathogens as suggested.

Page 16 top. Interesting the authors present PCR, MLST (and presumably spa) as a technique that needs to be sent away because it is not organism specific whereas NGS is something that "individual hospital laboratories" will undertake. I think there is currently at least as great a technical and financial hurdle for introducing NGS as there is for other molecular platforms - other potential benefits notwithstanding.

We would agree there are technical and financial hurdles to local implementation of benchtop sequencing, and are actively working as a group to overcome them. We have added to the sentence in the final paragraph of the discussion to make these hurdles more explicit.

Reviewer: Michael Metzker, Ph.D.

Control samples/sequences in conducting phylogenetic studies are absence from the study.

Understanding the level of background diversity is an important concern, and we are grateful to the reviewer for raising this. Although not called controls in the manuscript the eight *S. aureus* isolates sequenced from patients in the ICU involved in MRSA cluster 1, a month after then end of the outbreak, provide important control data regarding the background level of diversity. We have added to the results paragraphs for this cluster to make clearer to reader the level of diversity present. The 7 *C. difficile* cases sequenced from a single hospital in the surveillance reconstruction also provide insight into the level of diversity present and the mean SNVs present between all pairs of these samples has been added to the text. Clearly larger studies are required to provide further background,

and the manuscript by Didelot *et al* (submitted for publication) provided as related material provides significant data on the level of diversity present in *C. difficile*.

Moreover, no statistical framework is provided to assess the strenght of the differences between samples.

Rates of evolution in *S. aureus* and *C. difficile* form the basis for assessments of differences made between samples in this study. Maximum likelihood phylogenies are used to provide a statistically acceptable point estimate for the difference between samples, that can be relatively quickly calculated. We acknowledge that we should have included more information on the uncertainty in the 'molecular clock' estimates, therefore 95% credibility intervals and confidence intervals have been added to the manuscript. A small amount of further information has been included on the basis for the *C. difficile* clock, as although the data have been submitted, they have not yet been accepted for publication.

The cases deemed genetically unrelated in this study, are sufficiently different, given the molecular clock rates, that a formal statistical framework for interpreting the differences was not required. Similarly many related cases were genetically indistinguishable. However it is clear that cases may arise where the genetic distance between them leaves uncertainty as to whether transmission is possible. To appropriately interpret such situations a method that jointly accounts for uncertainty in the molecular clock and the phylogeny is required. Such a method, using Bayesian statistics, is described in the manuscript by Didelot *et al*. However in this paper we chose a method that facilitated rapid analysis of samples, such that results could be returned in clinically relevant timescales using existing computational resources.

Regions of genomic alignment are missing from the method section.

The methods have been clarified to state the maximum likelihood trees drawn are based on the mapped whole genome data. The sentence dealing with this in the methods has been moved above the section detailing the analysis of individual genes to aid clarity.

Selection of appropriate (i.e., unrelated) controls is missing as well.

Please see comments above regarding controls. The first paragraph in the methods section has been amended to make clearer that potentially unrelated *S. aureus* samples were also deliberately sequenced.

The authors state using a JC method in modeling genetic changes, but why this model? Many studies use multiple models to remove model bias from the analysis. No description is provided regarding a statistical method, such as the widely used Bayesian posterior probability method.

Within this pilot study, the goal of the phylogenetic analysis was to identify those isolates so closely related that transmission was plausible, not to re-construct the evolutionary relationships between the entire group of isolates. The choice of phylogenetic model made was a compromise between providing the best possible phylogenetic reconstruction congruent with this aim, and balancing this with the time taken and the expertise required to conduct the analysis. We aimed to present a technique that could be used to provide timely results to a routine laboratory, and therefore chose a maximum likelihood approach. The particular software tool chosen for the analysis is well established with over 5800 citations. The choice of JC substitution model was guided both by the speed of the analysis and also the relatively few variable sites identified in some outbreaks, where there may be too few samples and variable sites to support using more complex models with more parameters.

Without appropriate controls, drawing conclusions such as "NGS confirms transmission..." (title on page 10) and "NGS refutes transmission..." (title on page 11) are baseless. Moreover, even with appropriate controls, a statistical framework is necessary to determine the significance of sequence clusters. No statistical methods are provided. The results are incomplete due to the lack of appropriate controls and statistical framework. The study could benefit from analyzing more complex evolutionary models. The problems associated with the above makes the intrepretation of the results no credible.

We accept that the use of “confirms transmission” may be too strong, and so have amended this where it occurs, to reflect that NGS data support rather than confirm transmission. As set out above use of a molecular clock rather than controls provides the framework for interpreting the differences seen. Where observed SNV differences between isolates are in the 1000s, and rates of evolution of the order of 1-10 SNVs/genome/year, transmission can be refuted even without a statistical model, and is already done so in practice by reference laboratories refuting transmission across different strain types such as different multi-locus sequence types. We are very aware that where the number of SNVs is low, but not zero, this uncertainty should be formally evaluated and this is an area of on-going work within our group.

Figures 2, 3, and 4 could be better illustrated to show the genomic regions that resulted in the phylogenetic trees.

This is a good point and plots showing the location of variable sites, coding sequences and portions of the reference genome called have been included as new supplementary material for the 3 of the 4 clusters with multiple SNVs present.

Interpretation is limited by the problems identified above

Please see responses above.

The authors should provide the reader with guidance as to why they chose the MiSeq system over the Ion Torrent PGM (another benchtop sequencer). Where does the PacBio system fit in rapid analysis.

We have added that we only evaluated one of these technologies as a limitation in our discussion, and suggest some other alternatives. The PacBio system offers rapid analysis, but in contrast to the other technologies requires considerably more space, which may make it less suitable for use in a routine hospital laboratory.

The authors note that they provide the "...first demonstration of rapid sequencing in a benchtop format...", but reference 8 (Holger et al.) makes the same claim. Please reconcile this statement.

This sentence has had an ‘and’ removed to clarify that this is the first demonstration of this technology applied to routine patient care and healthcare associated pathogens specifically. Sorry for this confusion.

Can the authors also provide more description as to why with 77.6x and 50.4x coverage (page 9), only 80% of the reads mapped to their respective genomes?

This sentence has been clarified to explain these percentages are after quality filtering – details of which are given in the methods. New supplementary figures illustrate the regions of the genome called. The percentages are for the percentage of the reference genome called, rather than the percentage of reads mapped which were 94.8% and 90.0% for *S. aureus* and *C. difficile* respectively. Uncalled regions of the (Sanger-sequenced) references include repetitive regions which 150bp reads

cannot cover and mobile elements, as well as other non-core genome. This clarification has been added to the manuscript.

VERSION 2 – REVIEW

REVIEWER	Dr Jonathan Edgeworth Director, Centre for Clinical Infection and Diagnostics Research Department of Infectious Diseases Kings College London St Thomas' Hospital London SE1 7EH UK Competing interests. I am involved in similar studies to determine the clinical utility of NGS for improving infection control practice. No other known conflicts or competing interests
REVIEW RETURNED	02/05/2012

GENERAL COMMENTS	I am happy with the responses

REVIEWER	Michael Metzker, Ph.D. Associate Professor Baylor College of Medicine USA
REVIEW RETURNED	08/05/2012

GENERAL COMMENTS	The authors have done a great job addressing our concerns.