16S pan-bacterial PCR can accurately identify patients with ventilator-associated pneumonia


Published in:
Thorax

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
© 2016 The Authors.
This is an Open Access article
distributed in accordance with the terms of the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited. See: http://creativecommons.org/licenses/by/4.0/

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.
16S pan-bacterial PCR can accurately identify patients with ventilator-associated pneumonia

Ventilator-associated pneumonia (VAP) remains a challenge to intensive care units, with secure diagnosis relying on microbiological cultures that take up to 72 hours to provide a result. We sought to derive and validate a novel, real-time 16S rRNA gene PCR for rapid exclusion of VAP. Bronchoalveolar lavage (BAL) was obtained from two independent cohorts of patients with suspected VAP. Patients were recruited in a 2-centre derivation cohort and a 12-centre confirmation cohort. Confirmed VAP was defined as growth of >10⁴ colony forming units/ml on semiquantitative culture and compared with a 16S PCR assay. Samples were tested from 67 patients in the derivation cohort, 10 (15%) of whom had confirmed VAP. Using cycles to cross threshold (Ct) values as the result of the 16S PCR test, the area under the receiver operating characteristic (ROC) curve (AUROC) was 0.94 (95% CI 0.86 to 1.0, p<0.0001). Samples from 92 patients were available from the confirmation cohort, 26 (28%) of whom had confirmed VAP. The AUROC for Ct in this cohort was 0.89 (95% CI 0.83 to 0.95, p<0.0001). This study has derived and assessed the diagnostic accuracy of a novel application for 16S PCR. This suggests that 16S PCR in BAL could be used as a rapid test in suspected VAP and may allow better stewardship of antibiotics.

**Trial registration** VAPRAPID trial ref NCT01972425.

**INTRODUCTION** Ventilator-associated pneumonia (VAP) remains a significant problem in intensive care units (ICUs) and despite reductions in reported VAP rates antibiotic use remains high. The most common indication for antibiotic use remains suspected respiratory infections. VAP is associated with significant morbidity and mortality especially when antibiotics are delayed or are inadequate. However, due to the various conditions that can mimic VAP commonly only 30% of those suspected of having VAP subsequently have this diagnosis confirmed. The delays in obtaining results from conventional microbiological cultures lead to empirical use of broad-spectrum antibiotics of which a significant proportion is later deemed unnecessary. The excessive use of antibiotics is associated with increased antimicrobial resistance and mortality.

The ubiquitous presence of a 16S ribosomal RNA gene in bacteria offers the possibility of detecting a wide range of bacteria in a single PCR. Amplification of the 16S rRNA gene in a PCR assay results in amplification of all bacteria in a sample. Therefore, this offers potential as a screening test for suspected VAP. The aim of this study was to derive and validate a real-time 16S PCR assay for diagnosing confirmed VAP.

**METHODS** Samples from two previously described cohorts of adult patients with clinically suspected VAP recruited from UK ICUs formed the derivation and confirmation cohorts respectively. Brieﬂy, patients were recruited if they met criteria for suspected VAP namely new or worsening chest X-ray changes following at least 48 hours of ventilation, accompanied by two or more of: temperature >38°C; white cell count >11×10⁹/L; or mucopurulent sputum. In the derivation cohort patients were excluded if they had received new antibiotics within the 3 days prior to recruitment; no such exclusion was applied to the conﬁrmation cohort. Patients underwent protocolised broncho-scopic bronchoalveolar lavage (BAL) and an aliquot of BAL ﬂuid was processed using a semiquantitative culture method. This culture was used as our reference diagnostic standard, with growth at >10⁴ colony forming units/ml (CFU/mL) of BAL ﬂuid being deﬁned as ‘VAP positive’ and growth <10⁴ CFU/mL as ‘VAP negative’, these cut-offs being in line with established standards.

Full details of sample processing are described in the online supplementary section. Brieﬂy, the fraction of lavage not used for conventional culture was centrifuged to produce a cell-free supernatant, followed by nucleic acid extraction. The 16S PCR assays are described below; assay 1 and assay 2 were conducted in geographically separate laboratories.

**Real-time 16S PCR assay 1** The primer and probe sequences targeting the 16S rRNA gene have been described previously. The probe contained a carboxyfluorescein (FAM) label on the 5′ end with a Black Hole Quencher 1 (BHQ1) on the 3′ end. Primers and probe were synthesised by Eurogentec (Liège, Belgium). The ﬁnal 16S PCR reaction mix contained 1.25U HotStarTaq polymerase and 1 × reaction buffer (Qiagen, Manchester, UK), 4 μM MgCl₂, 0.2 mM deoxynucleotide mix (dNTP), 0.25 μM primer 27-F, 0.75 μM primer 16S 1RR-B, 0.3 μM probe 514-S, nuclease-free water (Promega, Southampton, UK) and 10 μL nucleic acid extract to a ﬁnal volume of 25 μL. Real-time PCR was carried out on the ABI 7500 instrument (Applied Biosystems, Life Technologies, Paisley, UK). The primer and hybridisation probe sequences targeting the 16S rRNA gene have been described previously. The hybridisation probe contained a FAM label on the 5′ end with a BHQ1 on the 3′ end. Primers and hybridisation probe were synthesised by Sigma Genosys (Sigma-Aldrich, Ebersberg, Germany).

The ﬁnal 16S PCR reaction mix contained 1X Platinum uracil DNA glycosylase Mastermix (Life Technologies, Paisley, UK), 0.2 μM bovine serum albumin (Sigma, Dorset, UK), a total of 4 mmol/L MgCl₂, 0.4 μM forward and reverse primers, 0.1 μM hybridisation probe, nuclease-free water (Promega, Southampton, UK) and 2 μL of target template for a ﬁnal reaction volume of 10 μL. Real-time qPCR was carried out on a Light Cycler 480 instrument (Roche, Indianapolis, Indiana, USA). This assay was used on samples from the conﬁrmation cohort only.

For the purposes of analysis, the metric was cycles to cross threshold (Ct) as a measure of 16 s rRNA gene load and hence bacterial burden. A higher bacterial load will result in a lower time to cross threshold, that is, a lower Ct value. Details of statistical analyses used can be found in the online supplementary methods section. Both studies had approvals from relevant research ethics committees; full details are in the online supplementary section.

**RESULTS** In the derivation cohort, samples from 67 patients were available, of whom 10 (15%) had ‘microbiologically confirmed VAP’. In the ‘conﬁrmation’ cohort samples from 92 patients were available for analysis; 26 (28%) met the culture criteria for ‘microbiologically confirmed VAP’. The demographic details and organisms cultured are shown in the online supplementary section (see online supplementary tables S1 and S2).

16S PCR assay 1 demonstrated that patients with conﬁrmed VAP had a higher bacterial burden, as signiﬁed by a lower Ct value, than those without VAP (ﬁgure 1A). When evaluated for diagnostic ability by ROC curve, assay 1 demonstrated excellent...
diagnostic ability (see table 1 and figure S1A) with an area under the ROC curve (AUROC) of 0.94 (95% CI 0.86 to 1.00), sensitivity of 100% and specificity 72% at the most optimal cut-off.

In the confirmation cohort, patients with confirmed VAP had significantly lower 16S Ct values (figure 1B), and a similar diagnostic performance was demonstrated (table 1 and figure S1B), with sensitivity of 100% and specificity of 67% at the most optimal cut-off. The difference between the AUROC of the cohorts was not statistically significant (p=0.56).

Samples from the confirmation cohort were also tested using 16S assay 2. As seen in figure 1C, although the absolute Ct values differed between the two assays, the same relationship between VAP and non-VAP samples was observed. ROC analysis (table 1 and figure S1C) demonstrated good diagnostic ability (area under the curve 0.84 95% CI 0.75 to 0.94) with sensitivity 89% and specificity 80% at the optimal cut-off. Although the point estimates of AUROC were higher for assay 1, the difference did not achieve statistical significance (p=0.4). However if the assays are compared at maximal sensitivity (100%), the specificity of assay 1 is significantly higher (table 1). Using the Youden Index to define optimal Ct value cut-offs on the ROC curve, a ‘positive’ result for 16S would be a value below this cut-off (indicating high bacterial load) and a ‘negative’ result would be a value above this cut-off (indicating low bacterial load).

In the derivation cohort, 35 (52%) patients were receiving antibiotics on the day of recruitment. In the confirmation cohort, 69 (75%) were receiving antibiotics and 14 (15%) had undergone a change of antibiotics within the past 3 days. Receipt of antibiotics and recent change in antibiotics were not associated with changes in 16S Ct values (see online supplementary results and table S3).

Figure S2 shows the relationship between Ct values for the two 16S assays, demonstrating a non-linear association.

**DISCUSSION**

To our knowledge, this is the first report of the use of real-time 16S PCR for diagnosing VAP. Although 16S rRNA gene sequencing has been used to explore the microbiome of ventilated patients, data on its diagnostic potential have been absent. In deriving and confirming a test, with a high agreement in test performance between the two cohorts, we demonstrate clear potential for the clinical utility of this test. Turnaround time is 4–6 hours; therefore, this test could impact on antibiotic use, which may otherwise only be rationalised following the results of conventional cultures at 48–72 hours.

This study has a number of strengths. First, we were able to perform derivation and confirmation in two distinct cohorts, with confirmation in a cohort recruited from a diverse group of 12 ICUs. The results are therefore likely to be widely applicable;

---

**Table 1 Diagnostic performance of the two 16S assays**

<table>
<thead>
<tr>
<th>Curve</th>
<th>Assay 1 derivation</th>
<th>Assay 1 confirmation</th>
<th>Assay 2 confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC ROC</td>
<td>0.94 (0.86 to 1.0)</td>
<td>0.89 (0.83 to 0.95)</td>
<td>0.84 (0.75 to 0.94)</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Youden optimum cut-off (Ct)</td>
<td>29.85</td>
<td>29.43</td>
<td>21.59</td>
</tr>
<tr>
<td>Youden optimum sensitivity/specificty (95% CIs)</td>
<td>100 (69 to 100)/72 (58 to 83)</td>
<td>100 (87 to 99)/67 (54 to 78)</td>
<td>89 (70 to 98)/80 (69 to 89)</td>
</tr>
<tr>
<td>Maximum sensitivity optimum cut-off (Ct)</td>
<td>29.85</td>
<td>29.43</td>
<td>22.02</td>
</tr>
<tr>
<td>Maximum sensitivity/specificty (95% CIs)</td>
<td>100 (69 to 100)/72 (58 to 83)</td>
<td>100 (87 to 100)/67 (54 to 78)</td>
<td>100 (86 to 100)/15 (8 to 26)</td>
</tr>
</tbody>
</table>

(ROC curves displayed in online supplementary figure S1).

As avoiding false-negative results is important in rapid tests for VAP, we also report the specificity at maximum (100%) sensitivity.

AUC, area under the curve; Ct, cycles to crossing threshold; VAP, ventilator-associated pneumonia.
indeed, the microbiological spectrum found is similar to reports from other countries. Second, by using consistent diagnostic procedures within each cohort, we avoided some of the problems which occur with the diagnosis of VAP. Our rate of microbiologically confirmed VAP in both cohorts (23%) is at the lower end of the reported range but not out of keeping with other reports and we believe this may, in part, reflect the use of highly standardised BAL protocols.

A disadvantage of this study is that samples were obtained bronchoscopically, requiring resource and exposing patients to a small but definite risk, and the applicability of this test to other sample types cannot be inferred. The assays we describe here are also limited to bacterial detection. The differences between the two assays tested, and the use of stored samples, highlight the need for external prospective validation before this measure could be implemented in routine clinical practice. Further refinements of assays may also improve diagnostic performance. The reference standard of growth of organisms on conventional culture remains imperfect, and recent changes to the standard of growth of organisms on conventional culture, remains imperfect, and recent changes to the standard of growth of organisms on conventional culture remain the established standard and is used but within 6 hours rather than the 48 hours taken for the conventional cultures. Our rate of microbiologically confirmed VAP in both cohorts (23%) is at the lower end of the reported range but not out of keeping with other reports and we believe this may, in part, reflect the use of highly standardised BAL protocols.

Acknowledgements The authors thank VAP-RAPID collaborators, Niall H Anderson, Alistair I Roy, Simon V Baudouin, Stephen E Wright, Gavin D Perkins, Melinda Jeffels, Cecilia M O’Kane, Craig Spencer, Shondipon Laha, Nicole Robin, Savita Gossain, Kate Gould, Marie-Hélène Ruchaud-Sparagano, Jonathan Scott, Ian Dimmock, Ian F Lauerson, Helen Walsh, Sarah Nutbrown, Charley Higham, Teresa melody, Keith Couper, Jacqueline Baldwin, Alexandra Williams, Kylie Norrie, Julie Furnevel, Tracey Evans, Heidi Dawson, Griania White, Lia McNamee, Leona Bannon, Laura Evans, Neil Young, Alasdair Hay, Ross Paterson, Stuart McLellan, Peter Kellerer, Berge Azadian, Masao Takata, Ildiko Kun during the manuscript. The authors also thank David Swann, Pam Ramsay, Gordon McNeil and Kallirroi Kefala for their work in recruiting patients to the confirmation cohort. The authors also thank the patients and their relatives who allowed this research to take place.

Contributors ACM designed the study, obtained funding, recruited patients, analysed data and wrote the manuscript. NG performed the assays, analysed the data and revised the manuscript. JPMM performed the assays, analysed the data and revised the manuscript. TP designed the study, recruited patients and revised the manuscript. PD recruited patients, obtained samples and revised the manuscript. S’s recruited patients, obtained samples and revised the manuscript. TS recruited patients, obtained samples and revised the manuscript. DFM recruited patients, obtained samples and revised the manuscript. KT obtained the funding, designed and supervised the assays, and wrote the manuscript. AJS designed the study, obtained funding, recruited patients and wrote the manuscript. RM obtained the funding, designed and supervised the assays, and wrote the manuscript.

Funding This study was funded by: the Northern Ireland Health and Social Care Research and Development division; the Hospital Infection Society; the Department of Health and Welcome Trust through the Health Innovation Challenge Fund (HICF)(0510/078); and the Sir Jules Thorn Charitable Trust (03/17A).

Competing interests ACM is a member of the advisory board of Serendex and is chief investigator on a diagnostics study jointly funded by Innovate UK and Becton Dickinson. KT has worked on evaluations of diagnostic systems for Becton Dickinson, Cepheid, Enigma, GenMark and SequxRM has received research grant income from Innovate UK for a diagnostics consortium (with Randox Diagnostics Ltd), investigator-led grant income from Pfizer Ltd and is a consultant/advisor to Gilead Sciences Ltd. All other authors declare no conflicts of interest.

Patient consent Obtained.

Ethics approval Lothian Research Ethics Committee (REC), NRES North East REC, Scotland A REC.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

16S pan-bacterial PCR can accurately identify patients with ventilator-associated pneumonia

Andrew Conway Morris, Naomi Gadsby, James P McKenna, Thomas P Hellyer, Paul Dark, Suveer Singh, Timothy S Walsh, Danny F McAuley, Kate Templeton, A John Simpson and Ronan McMullan

Thorax published online December 14, 2016

Updated information and services can be found at: http://thorax.bmj.com/content/early/2016/12/14/thoraxjnl-2016-209065

These include:

References
This article cites 12 articles, 5 of which you can access for free at: http://thorax.bmj.com/content/early/2016/12/14/thoraxjnl-2016-209065#BIBL

Open Access
This is an Open Access article distributed in accordance with the terms of the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited. See: http://creativecommons.org/licenses/by/4.0/

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Open access (229)

Notes

To request permissions go to: http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to: http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to: http://group.bmj.com/subscribe/