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

Andrew Conway Morris[1,2] Naomi Gadsby[3], James P McKenna[4] Thomas P Hellyer[5], Paul Dark[6,7], Suveer Singh[8], Timothy S Walsh[2], Danny F McAuley[9,10], Kate Templeton[3], A John Simpson[5], Ronan McMullan[4,9]

1 Division of Anaesthesia, Department of Medicine, University of Cambridge, Cambridge, UK
2 Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK
3 Clinical Microbiology, NHS Lothian, Edinburgh, UK
4 Department of Microbiology, Belfast Health & Social Care Trust, Belfast,
5 Institute of Cellular Medicine, Newcastle University, Newcastle, UK
6 Institute of Inflammation and Repair, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, UK
7 Intensive Care Unit, Salford Royal NHS Foundation Trust, Greater Manchester, UK
8 Intensive Care Unit, Chelsea and Westminster Hospital, Imperial College London, London, UK
9 Centre for Infection and Immunity, Queen's University Belfast, UK
10 Intensive Care Unit, Royal Victoria Infirmary, Belfast, UK

Address for correspondence
Professor John Simpson
3rd Floor, William Leech Building,
Institute of Cellular Medicine,
Medical School,
Newcastle University,
Framlington Place,
Newcastle upon Tyne NE2 4HH
United Kingdom
Phone 00-44-191-208-7770
Fax 00-44-191-208-0723
j.simpson@newcastle.ac.uk

Word count 1590

Funding statement
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 Wellcome Trust through the Health Innovation Challenge (HIC) Fund; and the Sir Jules Thorn Charitable Trust.

Take home message
Conventional microbiological culture diagnosis of ventilator-acquired pneumonia can take 2-3 days. We present a 16S PCR based assay that can allow rapid identification of patients with and without VAP.

140 character summary
16S PCR assay can rapidly identify patients with ventilator-acquired pneumonia, and may aid antibiotic stewardship.

Abstract
Ventilator-acquired pneumonia (VAP) remains a challenge to intensive care units, with secure diagnosis relying on microbiological cultures that take up to 72 hours to result. We sought to derive and validate a novel, real-time 16S rRNA gene polymerase chain reaction (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 two-centre derivation cohort and a 12-centre confirmation cohort. Confirmed VAP was defined as growth of $>10^4$ colony forming units/ml on semi-quantitative culture and compared to 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 (C_t) values as the result of the 16S PCR test, the area under ROC curve (AUROC) was 0.94 (95% CI 0.86-1.0 $p<0.0001$). Samples from 92 patients were available from the confirmation cohort, 26 (28%) of whom had confirmed VAP. The AUROC for C_t in this cohort was 0.89 (95% CI 0.83-0.95 $p<0.0001$). 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.

Introduction

Ventilator-acquired pneumonia (VAP) remains a significant problem in intensive care units (ICU) [1], and despite reductions in reported VAP rates antibiotic use remains high [2]. The commonest indication for antibiotic use remains suspected respiratory infections [3]. VAP is associated with a significant morbidity and mortality [1] especially when antibiotics are delayed or are inadequate [4]. However, due to the various conditions that can mimic VAP, commonly only 30% of those suspected of having VAP subsequently have this diagnosis confirmed [4]. The delays in obtaining results from conventional microbiological cultures lead to empiric use of broad-spectrum antibiotics of which a significant proportion is later deemed unnecessary. The excessive use of antibiotics is associated with increased anti-microbial resistance [5] and mortality [6].
The ubiquitous presence of a 16S ribosomal RNA gene in bacteria offers the possibility of detecting a wide range of bacteria in a single polymerase chain reaction (PCR) [7]. 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. This 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 [8,9] cohorts of adult patients with clinically suspected VAP recruited from UK ICUs formed the derivation [8] and confirmation [9] 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 >$11x10^9$/L; or mucopurulent sputum. In the derivation cohort patients were excluded if they had received new antibiotics within the 3 days prior to recruitment[8], no such exclusion was applied to the confirmation cohort[9]. Patients underwent protocolised bronchoscopic bronchoalveolar lavage (BAL) [8,9] and an aliquot of BAL fluid was processed using a semi-quantitative culture method. This culture was used as our reference diagnostic standard, with growth at >10^4 colony forming units/ml lavage being defined as 'VAP positive' and growth <10^4 CFU/ml as 'VAP negative', these cut-offs being in line with established standards [1,4].

Full details of sample processing are described in the supplemental 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 2 were conducted in geographically separate laboratories.

Real-time 16S PCR assay 1

The primer and probe sequences targeting the16S rRNA gene have been described previously[10]. The probe contained a 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 1x reaction buffer (Qiagen, Manchester, UK), 4 µM $MgCl_2$, 0.2mM dNTP, 0.25µM primer 27-F, 0.75µM primer 16S 1RR-B, 0.3µM probe 514-S, nuclease-free water (Promega, Southampton, UK) and 10µl nucleic acid extract to a final volume of 25µl. Real-time PCR was carried out on the ABI 7500 instrument (Applied Biosystems, Life Technologies Ltd, Paisley, UK). This assay was used for samples from the derivation cohort, to establish proof-in-principle of the diagnostic utility of this approach, and was also used for samples from the confirmation cohort.

Real-time 16S PCR assay 2

The primer and hybridisation probe sequences targeting the 16S rRNA gene have been described previously [S1]. The hybridisation probe contained a FAM label on the 5' end with a Black Hole Quencher 1 (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® UDG Mastermix (Life Sciences, Paisley, UK), 0.2 µM bovine serum albumin (Sigma, Dorset, UK), a total of 4 mmol/L $MgCl_2$, 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, IN, USA). This assay was used on samples from the confirmation cohort only.

For the purposes of analysis, the metric was cycles to cross threshold (C_t) as a measure of 16s rRNA gene load and hence bacterial burden. A higher bacterial load will result in a lower time to cross threshold, i.e. a lower C_t value. Details of statistical analyses used can be found in the supplemental methods section. Both studies had approvals from relevant research ethics committees, full details are in the supplemental 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 supplemental section (tables S1 and S2).

16S PCR assay 1 demonstrated that patients with confirmed VAP had a higher bacterial burden, as signified by a lower C_t 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 AUROC of 0.94 (95% confidence interval 0.86-1.00) and sensitivity of 100% and specificity 72% at the most optimal cut-off.

In the confirmation cohort, patients with confirmed VAP had significantly lower 16S C_t 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 significant (p=0.56).

Samples from the confirmation cohort were also tested using 16S assay 2. As seen in figure 1C, although the absolute C_t 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 (AUC 0.84 95% CI 0.75-0.94) with sensitivity 89%, specificity 80% at the optimal cut-off. Although the point estimates of AUROC were higher for assay 1, the difference did not achieve 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 C_t 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%) of patients were receiving antibiotics on the day of recruitment. In the confirmation cohort, 69 (75%) were receiving antibiotics and 14 (15%) had undergone change of antibiotics within the past 3 days. Receipt of antibiotics and recent change in antibiotics were not associated with changes in 16S C_t values (see supplemental results and table S3).

Figure S2 shows the relationship between C_t 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. Turn-around-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. Firstly, we were able to perform derivation and confirmation in two distinct cohorts, with confirmation in a cohort recruited from a diverse group of 12 intensive care units. The results are therefore likely to be widely applicable; indeed, the microbiological spectrum found is similar to reports from other countries [4]. Secondly, by using consistent diagnostic procedures within each cohort, we avoided some of the problems which occur with the diagnosis of VAP [1,4]. Our rate of microbiologically confirmed VAP in both cohorts (23%) is at the lower end of the reported range[4] 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 indeed may well be influenced by inter-current antibiotics generally, and recent changes in antibiotics specifically[11]. However this remains the established standard[4], and is used routinely for clinical decision-making. As such, the 16S assay described here can predict the results of a clinically relevant test, but within 6 hours rather than the 48-72 hours taken for the conventional cultures.

In conclusion, we have derived and confirmed the diagnostic utility of a rapid laboratory test for VAP in a multicentre setting. We propose that this test has the potential to permit rapid decisions to direct antimicrobial therapy in patients with suspected VAP thus improving stewardship of antibiotics in the ICU.

References

1. Hunter JD. Ventilator associated pneumonia. *BMJ*. 2012;344:e3325.

2. Behnke M, Hansen S, Leistner R, et al. Nosocomial infection and antibiotic use: a second national prevalence study in Germany. *Dtsch Arztebl Int*. 2013;110:627–633.

3. Vincent J-L, Rello J, Marshall J, et al. International study of the prevalence and outcomes of infection in intensive care units. *JAMA*. 2009;302:2323–2329.

4. Chastre J, Fagon J-Y. Ventilator-associated pneumonia. *Am J Respir Crit Care Med*. 2002;165:867–903.

5. McGowan JE. Antimicrobial Resistance in Hospital Organisms and Its Relation to Antibiotic Use. *Clin Infect Dis*. 1983;5:1033–1048.

6. Kett DH, Cano E, Quartin AA, et al. Implementation of guidelines for management of possible multidrug-resistant pneumonia in intensive care: an observational, multicentre cohort study. *Lancet Infect Dis* 2011;11:181–189.

7. Renvoisé A, Brossier F, Sougakoff W, Jarlier V, Aubry A. Broad-range PCR: Past, present, or future of bacteriology? *Med Mal Infect*. 2013;43:322–330.

8. Conway Morris A, Kefala K, Wilkinson TS, et al. Diagnostic importance of pulmonary interleukin-1 beta and interleukin-8 in ventilator-associated pneumonia. *Thorax*. 2010;65:201–207.

9. Hellyer TP, Conway Morris A, McAuley DF, et al. Diagnostic accuracy of pulmonary host inflammatory mediators in the exclusion of ventilator-acquired pneumonia. *Thorax*. 2015;70:41-47.

10. Schabereiter-Gurtner C, Nehr M, Apfalter P, Makristathis A, Rotter ML, Hirschl AM. Evaluation of a protocol for molecular broad-range diagnosis of culture-negative bacterial infections in clinical routine diagnosis. *J Appl Microbiol*. 2008;104:1228–1237.

11. Montravers P, Fagon JY, Chastre J, et al. Follow-up protected specimen brushes to assess treatment in nosocomial pneumonia. *Am Rev Respir Dis*.1993;147:38–44

Acknowledgements:
The authors wish to acknowledge the work of the 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 Dimmick, Ian F Laurenson, Helen Walsh, Sarah Nutbrown, Charley Higham, Teresa Melody, Keith Couper, Jacqueline Baldwin, Alexandra Williams, Kylie Norrie, Julie Furneval, Tracey Evans, Heidi Dawson, Griania White, Lia McNamee, Leona Bannon, Laura Evans, Neil Young, Alasdair Hay, Ross Paterson, Stuart McLellan, Peter Kelleher, Berge Azadian, Masao Takata, Ildiko Kustos, John Cheesborough, and Roland Koerne, in recruiting patients to the confirmation cohort. We are also grateful for the work of David Swann, Pam Ramsay, Gordon McNeil and Kallirroi Kefala in recruiting patients into the derivation cohort. We also wish to record our gratitude to the patients and their relatives who allowed this research to occur.

Conflicts of interest
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 SelexRM 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 for Gilead Sciences Ltd. All other authors declare no conflicts of interest.

Curve	Assay 1 Derivation	Assay 1 Confirmation	Assay 2 Confirmation
AUC ROC	0.94 (0.86-1.0) p<0.0001	0.89 (0.83-0.95) p<0.0001	0.84 (0.75-0.94) p<0.0001
Youden optimum cut off (C_t)	29.85	29.43	21.59
Youden optimum sensitivity/specificity (95% confidence intervals)	100(69-100)/72 (58-83)	100(87-99)/67 (54-78)	89(70-98)/80 (69-89)
Maximum sensitivity optimum cut off (C_t)	29.85	29.43 Ct	22.02 Ct
Maximum sensitivity /specificity (95% confidence intervals)	100(69-100)/72 (58-83)	100(87-100)/67 (54-78)	100(86-100)/15 (8-26)

Table 1: Diagnostic performance of the two 16S assays
(ROC curves displayed in figure S1). AUC- Area under curve. C_t –Cycles to crossing threshold. As avoiding false-negative results is important in rapid tests for VAP, we also report the specificity at maximum (100%) sensitivity.

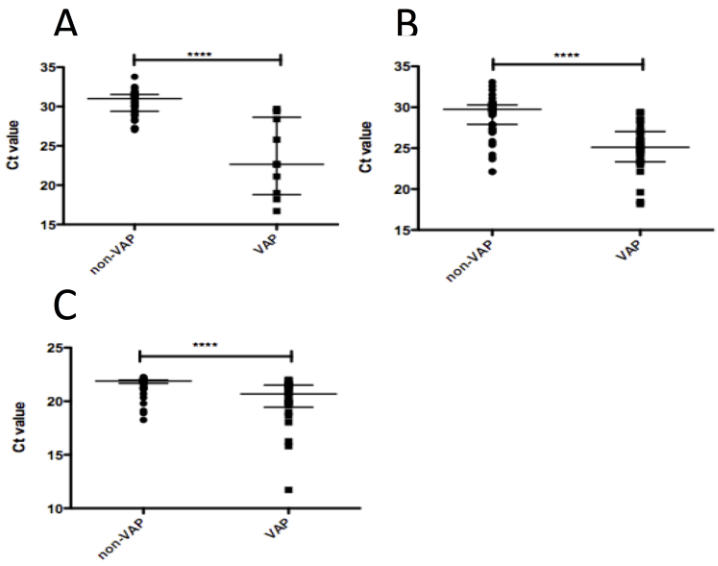

Figure 1: Real-time 16S PCR results as expressed by cycles to cross threshold (C_t) for samples from patients.
Panel A. C_t values from assay 1 amongst derivation cohort patients with and without confirmed VAP. N=67, 57 non-VAP and 10 VAP, error bars show median and inter-quartile range. **** p<0.0001 by Mann-Whitney U test.
Panel B. C_t values from assay 1 amongst confirmation cohort patients with and without confirmed VAP. N=92, 66 non-VAP and 26 VAP, error bars show median and inter-quartile range. **** p<0.0001 by Mann-Whitney U test.
Panel C. Ct values from assay 2 amongst confirmation cohort patients with and without confirmed VAP. N=92, 66 non-VAP and 26 VAP, error bars show median and inter-quartile range. **** p<0.0001 by Mann-Whitney U test.

Supplemental methods – Marked up version

Semi-quantitative culture of BAL fluid

After thorough mixing, 10μl of whole BAL fluid was inoculated and evenly spread onto solid culture media and incubated for 40-48 hours. The resulting number of colonies of bacterial pathogens was counted, with fewer than 10 taken to indicate $<10^3$ colony-forming units (CFU) per mL, 10-100 colonies indicating 10^3-10^4 CFU/mL, and greater than 100 colonies indicating $>10^4$ CFU/mL. Confirmed VAP was defined as growth of bacteria at $>10^4$ CFU/ml [1,4].

Sample processing

BAL fluid was prepared as described previously [8,9] with the cell-free supernatant being stored at -80˚C. BAL fluid from the derivation cohort underwent nucleic acid extraction using the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) with a pre-treatment protocol for Gram positive bacteria according to the manufacturer's instructions. Nucleic acid extraction on samples from the validation cohort was completed using MagNA pure 96 DNA and viral NA small volume kit (Roche, Indianapolis, IN, USA), by different staff, in a separate laboratory. Negative control samples comprising sterile saline underwent identical extraction to give a measure of 'background' 16S rRNA gene DNA in assay reagents.

Statistical analysis

Non-normal data are presented as median and inter-quartile range, and analysed by Mann-Whitney U- test. The diagnostic performance of the 16S assays, expressed as cycle number to cross threshold (C_t) was analysed by plotting Receiver Operator Characteristic (ROC) curves, with optimum cut-off defined by the Youden index (the cut off which produces the largest sum of sensitivity and specificity)[S2]. Differences between areas under ROC curves were analysed by the methods of Hanley and McNeil[S3,S4]. $P<0.05$ was taken to indicate statistical significance. Analysis was conducted using Prism (v5f for Mac, Graphpad, Carlsbad, CA, USA).

Ethical permissions

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The samples from patient derivation and validation cohorts were collected in studies approved by Lothian Research Ethics Committee (REC) (LREC/2002/8/19) and NRES North East REC (11/NE/0242) and Scotland A REC (11/SS/0089), respectively, with informed consent/assent from the next of kin.

Supplemental results

Characteristic	Derivation cohort	Validation
N=	67	92
Mean Age (range) years	59 (26-87)	60 (18-87)
% male	65%	71%
Median (IQR) APACHE II Score on admission	21 (16-26)	20 (15-23)
% Surgical admission	50%	41%
Median (IQR) ICU length of stay	23 (15-30)	19 (12-35)
% ICU mortality	28%	28%
% Hospital mortality	33%	37%
% Receiving antibiotic at time of lavage	52%	75%
% undergoing change in antibiotic therapy within 3 days of enrolment	0%	15%

Table S1. Demographic and clinical features of the derivation and validation cohorts.

Type of organism	Derivation	Validation
Enterobacteriaceae	**4**	**9**
***Haemophilus spp*. & *Moraxella spp*.**	**1**	**2**
Pseudomonas aeruginosa	**0**	**5**
Staphylococcus aureus	**4**	**8**
Other bacteria (*Streptococcus* spp., *Acinetobacter baumannii*, coagulase-negative *Staphylococcus* spp.)	**1**	**4**

Table S2: Bacteria grown at $>10^4$ CFU/ml in the derivation and validation cohorts. Two patients in the validation cohort grew more than one organism above the threshold.

Influence of antibiotics on results of the 16s assay

In the derivation cohort, 35 (52%) of patients were receiving antibiotics on the day of recruitment, and none had experienced a change in antibiotics in the preceding three days. In the validation cohort, 69 (75%) of patients were receiving antibiotics on the day of enrolment. As can be seen in table S3 below, there were no significant differences in C_t values between patients receiving antibiotics, and those not receiving antibiotics, in either assay. Recent change in antibiotics is more likely to create 'false negatives' on conventional cultures[11], and (15%) of validation cohort patients had a change of antibiotics in the preceding three days. Again we found no difference in C_t values between these groups, on either assay.

Cohort	Not receiving antibiotics on enrolment	Receiving antibiotics on enrolment	P value	Change of antibiotics within 3 days	No change of antibiotics within 3 days	P value
Derivation	30.5(22.7-31.4)	31.0(28.3-31.5)	0.96	NA	NA	
Validation – assay 1	27.1 (26.2-30.1)	29.5 (26.2-30.1)	0.13	29.5 (27.4-30.0)	29.1 (25.6-30.1)	0.63
Pooled derivation and validation-assay 1	29.5 (26.0-31.2)	29.7 (27.3-30.4)	0.96	NA	NA	
Validation – assay 2	21.9(21.3-22.0)	21.5 (20.4-21.9)	0.23	21.8 (20.9-21.9)	21.9(21.4-22.0)	0.25

Table S3. C_t values for patients by antibiotic status at time of study recruitment. Values shown as median (interquartile range), p value from Mann-Whitney U test comparing the preceding two columns.

Supplemental References

Additional references used solely in the supplemental section are indicated below

S1 Yang S, Lin S, Kelen GD, et al. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J Clin Microbiol*. 2002;40:3449–3454.

S2 Youden WJ. Index for rating diagnostic tests. *Cancer*. 1950;3:32–35.

S3 Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology*. 1982;143:29-36.

S4 Hanley JA, McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology*. 1983;148:839-843.

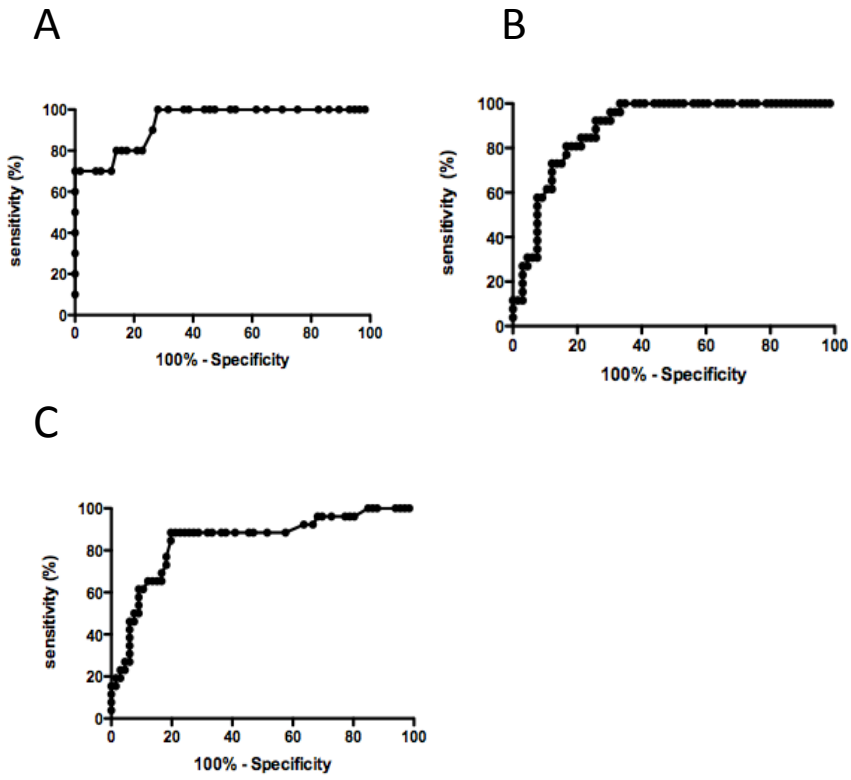

Figure S1: ROC curves for real-time 16S assays. Details of test performance are shown in table 1 of the main manuscript.
Panel A. ROC from assay 1 amongst derivation cohort patients with and without confirmed VAP.
Panel B. ROC from assay 1 amongst validation cohort patients with and without confirmed VAP.
Panel C. ROC from assay 2 amongst validation cohort patients with and without confirmed VAP.

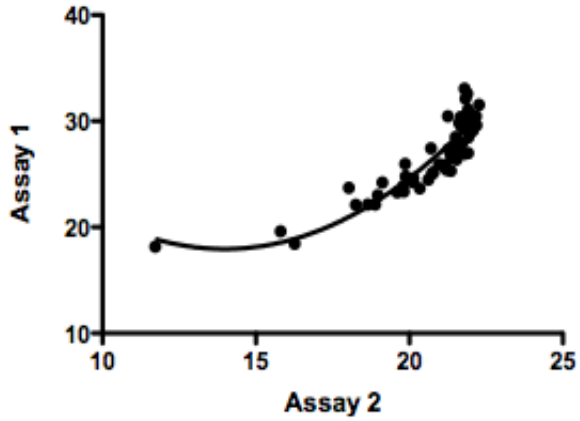

Figure S2: Relationship between results from real-time 16S PCR assays 1 and 2 on the validation cohort samples. The regression line describes a non-linear function ($y=28.96+3.38xX+0.06xX^2$), $r^2=0.91$, results from n=92 samples.