'Sample-in, answer-out'? Evaluation and comprehensive analysis of the Unyvero P50 pneumonia assay

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ABSTRACT

This study aimed to evaluate the performance of the Unyvero P50 pneumonia assay, the first 'sample-in, answer-out' system for rapid identification of pathogens and antibiotic resistance markers directly from clinical specimens. Overall, Unyvero P50 displayed very good sensitivity (>95%); however, specificity was low (33%) mainly due to the fact that 40% of the specimens were reported as normal flora. Specifically, one or more pathogens were identified in 28 of them. From a detailed analysis of 42 specimens selected at random, 76% of the additionally reported pathogens were confirmed present in primary specimens. Detection of selected resistance markers was compared to routine phenotypic susceptibility testing, supplemented with Checkpoints microarray system, PCR and sequencing. Concordance was mixed, primarily due to issues with panel's choice of markers and detection of some intrinsic beta-lactamases. Finally, we offer a critical analysis of the assay's microbial panel and resistance markers and provide suggestions for improvement.

Keywords: Pneumonia, rapid diagnostics, antimicrobial resistance, beta-lactamase, PCR

INTRODUCTION

Pneumonia is defined as consolidative infection of the lower respiratory tract causing significant morbidity and mortality worldwide. In the UK, (infectious and non-infectious) respiratory diseases accounts for 20% of deaths [1] and in 2006, the British Thoracic Society reported that pneumonia alone accounted for over 1/3 of these [1]. Pneumonia can be categorised as community-acquired (CAP) if acquired outside of the healthcare setting, or as hospital-acquired (HAP), when the onset of disease/clinical presentation occurs >48h after hospital admission [2]. In the clinical setting, of particular concern are patients undergoing intensive or critical care, who develop HAP or ventilator-associated pneumonia (VAP), often as a consequence of aspiration and prolonged hospital stay, or related to mechanical ventilation [3]. This prolonged stay along with the use of empirical broad-spectrum antibiotics may result in infection with multi-drug resistant organisms often associated with high mortality [4].

Pneumonia can be caused by a wide variety of bacteria, viruses or fungi that cannot easily be distinguished by clinical presentation [5]. Current routine diagnostic methods are mainly culture-based, which are limited by low sensitivity and unsuitability for detecting atypical pathogens. At present, turnaround times for routine culture and antimicrobial susceptibility testing range from 48-72h; in the meantime, the patient receives empirical antimicrobial therapy [6]. Such empirical therapy may be compromised by antimicrobial resistance or be used unnecessarily to treat infections caused by viruses or susceptible bacteria, thus driving the development of antimicrobial resistance [7,8]. Hence, a rapid test for detecting microorganisms

and their associated susceptibility profiles to direct therapy in pneumonia is urgently needed; both for better prognosis of patients [9] and improved antimicrobial stewardship [10].

Although there has been an emergence of real-time PCR assays targeted towards respiratory diagnosis, a single method available for rapidly identifying the variety of pathogenic causes of pneumonia is lacking. Accordingly, we evaluated the Curetis Unyvero P50 Pneumonia assay, the first 'sample-in and answer-out' system capable of diagnosing pneumonia aetiology directly from clinical specimens. This test combines automated sample preparation with multiplex PCR for selected targets and microarray hybridisation for amplicon detection. It promises to detect 16 bacteria and one fungus as well as 18 antibiotic resistance markers in around five hours (Table 1).

MATERIALS AND METHODS

Specimen Collection and Analysis

We collected anonymised respiratory specimens surplus to clinical requirements from adult in-patients with suspected pneumonia at two tertiary care hospitals in London: the Royal Free (RFH) and University College London Hospitals (UCLH), from December 2014 to June 2015. Duplicate specimens from the same patient were excluded unless collected >6 days apart. Fresh specimens from patients with radiological confirmation of pneumonia were stored at 4°C until processing (within 48h). Curetis Unyvero P50 Pneumonia assay was run as per manufacturer's instructions with a turnaround time of approximately 5h (30 min for mechanical and chemical sample lysis and homogenisation followed by 4h30 for DNA purification, multiplex PCR and microarray detection). Detailed information of the system and method can be found on the manufacturer's website (www.curetis.com).

Routine Clinical Microbiology

Results were compared to those released by the routine clinical microbiology laboratories of the two participating hospitals. For the RFH, this comprised 1:1 v/v dilution with dithiothreitol, semi-quantitative cultures onto three agar plates (Columbia Blood Agar (CBA), Colombia agar with chocolated horse blood (CHOC) and cystine lactose electrolyte deficient agar (CLED)); identification MALDI-TOF MS (Bruker Microflex™ LT) and antimicrobial susceptibility testing (AST) with the BD Phoenix system or by disc diffusion following EUCAST guidelines [11]. For UCLH, undiluted specimens were cultured onto CBA, CHOC and CLED, organisms were identified

using MALDI-TOF or the BioMerieux VITEK2 system and AST was performed using the VITEK 2 or BSAC (British Society for Antimicrobial Chemotherapy) standardised disc susceptibility testing. Atypical species *Chlamydophila pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* are screened using an in house qPCR assay at RFH and by antigen testing or serology at UCLH. MycAssay® Pneumocystis (Myconostica) is used to detect *Pneumocystis jirovecii* at RFH, at UCLH it is detected by Grocott-Gomori's methenamine silver stain.

Comprehensive Microbiological Analysis

For a full comprehensive analysis, 42 specimens were chosen at random. A cross-sectional sweep of growth was taken from a fresh primary culture of the specimen on CHOC and stored in Microbank™ vials at -80°C until analysis. Ten µL of neat and a 10^{-5} dilution in saline solution were plated onto CHOC, CBA, Brilliance UTI agar (UTI) and Columbia colistin-nalidixic acid agar (C-CNA) (Oxoid). CBA, UTI and C-CNA plates were incubated at 37°C in air for 18h while CHOC plates were incubated in 5% CO2 at 37°C for 18h. Representative bacterial colonies of different morphologies on each medium were identified using MALDI-TOF MS.

For bacterial isolates identified during the comprehensive microbiological analysis, susceptibility to beta-lactam antibiotics was evaluated using the disk diffusion method on Mueller-Hinton agar following EUCAST recommendations [11]. The following antibacterial agents (Oxoid) were tested: Aztreonam (30µg), Piperacillin-tazobactam (10-6µg), Ceftazidime (10µg), Imipenem (10µg), Meropenem (10µg), Temocillin (30µg) for Enterobacteriaceae, *Acinetobacter spp*. and *Pseudomonas spp*.; Ertapenem (10µg), Ampicillin (10 µg), Amoxiclav (20-10µg), Cefoxitin (30µg), Cefotaxime (5µg) were also tested for Enterobacteriaceae. Cefoxitin

(30µg) discs were used for identification of potential methicillin resistant *Staphylococcus aureus* (MRSA). Ciprofloxacin susceptibility testing was performed on *P. aeruginosa* and *Escherichia coli* using the gradient diffusion method (Etest®, Biomérieux), interpreted according to EUCAST guidelines (http://www.eucast.org/clinical breakpoints/). Both laboratories report predominant growth of potentially pathogenic species equivalent to 10^5 CFU/ml or above.

Double disc diffusion for detection of beta-lactamases was performed using ROSCO Diagnostica kits. KPC/Metallo-beta-lactamase and OXA-48 Confirm Kit*;* KPC/MBL in *P. aeruginosa*/Acinetobacter and Total ESBL+AmpC Confirm kits were used according to manufacturer's instructions.

Sequence-based Detection of Resistance Mechanisms

We extracted DNA from resistant bacteria using QIAmp DNA Mini Kit (Qiagen) following manufacturer's instructions. The Check-MDR CT103XL test (Checkpoints, NL) was used for molecular detection and identification of genes encoding carbapenemase, AmpC and ESBL enzymes according to manufacturer's instructions. All suspected ESBL, AmpC and carbapenemase positives were confirmed by PCR (HotStart Taq Mastermix, Qiagen). The presence of *mecA* among suspected MRSA and the quinolone resistance-determining regions (QRDR) of the *gyrA* and *parC* genes from fluoroquinolone resistant *E. coli* or *P. aeruginosa* were amplified by PCR. All PCR amplicons were sent for DNA sequencing using the Sanger method at Beckman Coulter Genomics and analysed using BioNumerics (Applied Maths) software and NCBI's BLAST. All primers used in this study are listed in Table S1.

Data analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive and negative likelihood ratios were calculated using MedCalc for Windows. Overall sensitivity and specificity were calculated considering a test result as true positive when both routine culture reported an organism and Unyvero P50 identified the same organism, regardless of additional organisms that may have been identified by Unyvero P50. False positives were specimens where one or more organisms detected by Unyvero P50 were not found by routine microbiology. False negatives were specimens where routine microbiology detected an organism that the Unyvero P50 missed and true negatives were specimens where neither method reported significant organisms.

During analysis of resistance determinants, only genes considered potentially significant (Table 1) were included; *mecA* was only considered significant when detected simultaneously with *S. aureus,* in such cases presence of MRSA was presumed. During comprehensive culture analysis, detections of *S. mitis* group bacteria other than *S. pneumoniae* were ignored.

RESULTS

A total of 103 respiratory clinical specimens from hospital in-patients with pneumonia were tested using the CE-marked Unyvero P50 Pneumonia assay (Unyvero P50) and results were compared to those generated by the clinical microbiology laboratories.

Unyvero P50 targets (Table 1) are distributed across eight independent PCR chambers. Complete test failure occurred for 6 specimens while partial test failures (where one or more of the chambers failed) occurred in 7 specimens. These specimens were excluded leaving a total of 90 specimens for analysis from 84 patients; comprising 55 sputa, 32 endotracheal tubes (ETT) aspirates and 3 bronchoalveolar lavage (BAL). Radiologic and clinical confirmation of pneumonia was sought and the type of pneumonia was classified into HAP, VAP or CAP using standard definitions [2]. The vast majority of our specimens came from patients with HAP (n=49), while 21 and 20 specimens were from VAP and CAP patients respectively.

On average Unyvero P50 identified a greater number of potential pathogens than routine microbiology per specimen (1.59 vs 0.59). The most common organisms reported by the culture laboratories were *P. aeruginosa* (n=13), *S. maltophilia* (n=6) and *S. marcescens* (n=6) whereas the most common organisms detected by Unyvero P50 were *S. maltophilia* (n=27), *P. aeruginosa* (n=19) and the *S. mitis* group (n=13) (Table 2, Table S2).

The number of organisms detected per specimen varied, with routine clinical laboratory reporting more than one organism in only 5 specimens, whereas Unyvero P50 detected

polymicrobial flora in 44 specimens (48.9%) (Figure 1). Normal respiratory flora (NRF), non-significant growth (NSG) or mixed growth of doubtful significance (MGODS) was reported for 39 specimens (43%), whereas 3 specimens (3.3%) produced no growth. Unyvero P50, which is not a quantitative test, identified at least one organism in 74 specimens (82.2%) and was negative for 16 specimens (17.8%) including the 3 that produced no growth. Complete results for all specimens are shown in Table S2.

Results from Unyvero P50 and standard microbiology culture were concordant in 59 specimens (65.5%) (Figure 2). Of these, negative results were concordant in 14 specimens, Unyvero P50 identified only the same pathogen(s) as routine culture in 23 specimens, and the same pathogen and at least one additional species in 22 specimens. Non-concordant results occurred in the remaining 31 specimens, which included 28 specimens reported as NRF, NSG or MGODS. On the other hand, two specimens described negative by Unyvero P50 were found to contain a pathogen by the clinical laboratory: one specimen contained *H. influenzae* while the other was positive for *E. faecalis,* an organism not associated with pneumonia and not a target of Unyvero P50. A third specimen was reported by the laboratory as containing *H. influenzae,* whereas Unyvero P50 detected *K. pneumoniae*, *P. aeruginosa* and *S. maltophilia*.

Overall clinical diagnostic accuracy metrics for Unyvero P50 indicates a sensitivity of 95.7% while specificity was 32.6% mainly due to the fact that over 40% of samples were reported as normal flora whereas Unyvero P50 reported an organism in the majority of samples. Positive predictive value was 60.8% while negative predictive value was 87.5%.

Detection of antimicrobial resistance

The clinical laboratories reported a total of 53 organisms (Table S3), 36% of these were fully susceptible, 60% resistant to one or more antimicrobial classes and 39.6% multi-drug resistant (MDR) [12]. Unyvero P50, capable of detecting 18 antibiotic resistance markers, reported 71% of specimens with at least one resistance marker (including 6 from specimens where no organism was detected). Many of these markers (e.g. *bla*$_{TEM}$, *ermB* and *sul1*) are highly prevalent, if not ubiquitous, among both pathogenic and commensal bacterial populations [13], hence their detection in mixed specimens, such as those from the respiratory tract, becomes extremely common.

For this reason, we restricted our analysis to ESBLs, AmpC beta-lactamases, carbapenemases, presumptive MRSA, and fluoroquinolone resistance (FQ^R) among *E. coli* and *P. aeruginosa* only. Unyvero P50 identified 17 occurrences of these resistance markers whilst routine microbiology identified corresponding resistance phenotypes in 14 isolates. In 4 specimens where significant pathogens were detected by routine microbiology and a target of Unyvero P50 was confirmed present by independent molecular analysis, the test had identified the resistance marker correctly in 3 cases (Table 3). An additional 9 clinical bacterial isolates had phenotypic AmpC or carbapenem resistance not detected by Unyvero P50. In 6 cases the additional molecular analysis did not identify a cause for resistance (presumably due to overexpression of chromosomal AmpC enzymes or mutation of porins [14,15]) while *A. baumannii* producing OXA-23 carbapenemase was detected in 3 specimens.

Conversely Unyvero P50 identified several resistance markers, which were not detected by routine microbiology (Table 3). Two putative MRSA that had been missed by routine methods were detected (one sample was reported as NRF, the other was reported as containing *A. baumannii*). Unyvero P50 also identified a $bla_{\text{CTX-M}}$ in a specimen containing *K. pneumoniae* and *S. maltophilia,* whereas routine microbiology reported the specimen as NRF. For AmpCs, Unyvero P50 identified 3 bla_{EBC} and 2 bla_{DHA} genes. In 4 of the specimens, the clinical laboratory reported NRF and in the final specimen the clinical laboratory identified an *E. cloacae* isolate. For carbapenemases, Unyvero P50 identified 5 specimens with $bla_{\text{OXA-51}}$, all containing *A. baumannii* whereas routine microbiology reported NRF for two of the specimens and OXA-23 producing *A. baumannii* for the remaining three. For fluoroquinolone resistance, routine microbiology and Unyvero P50 both identified 2 *E. coli* with *gyrA* mutations resulting in ciprofloxacin resistance. For *P. aeruginosa* one FQ^R isolate with confirmed mutations in *gyrA* was however missed by Unyvero P50, whereas Unyvero P50 identified one *P. aeruginosa* with *gyrA* and *parC* mutations in a specimen reported as NRF.

Resolution of discrepant results

Culture of respiratory specimens is considered the 'gold standard' to identify the microbial aetiology of pneumonia caused by fungi and bacteria. Limitations of this method include the cut-off loads (typically 10^5 CFU/ml) and the subjective interpretation of results, which may vary among and between laboratories and individual staff members. For this reason, we performed a more comprehensive analysis for 42 specimens selected at random by identifying all organisms included on the Unyvero P50 panel that grew on the primary chocolate agar plate. Our comprehensive investigative culturing method detected one organism in 27 specimens and

2 organisms in 13 specimens, the remaining two specimens had 4 and 0 organisms respectively. In comparison, the routine laboratory reported one organism for only 23 of them, and two organisms for 1 specimen. The main species under-reported by the clinical laboratory were *S. maltophilia* (3 vs 12), *P. aeruginosa* (7 vs 15) and *K. pneumoniae* (0 vs 4).

Of the 42 specimens analysed, results were concordant with Unyvero P50 in 36 specimens (85.7%) including an exact match for 25 specimens while Unyvero P50 detected extra organism(s) in 11 specimens. Conversely comprehensive culture revealed the presence of additional organisms for 4 specimens: *K. oxytoca*, *S. maltophilia*, *S. marcescens* and *E. cloacae* were not detected in one specimen each. Two specimens were found to contain polymicrobial flora with both methods but some of the reported organisms were discordant (Table S4).

All isolated organisms were screened for relevant resistance phenotypes in order to verify concordance and control for the possibility of resistant organisms missed by both methods. It was unfortunately only possible to verify a portion of the discrepant resistance results. Comprehensive culture confirmed the presence of a CTX-M producing *K. pneumoniae*, a DHA producing *M. morganii*, and a FQ^R *P. aeruginosa* in specimens where routine microbiology reported only NRF. One detection of bla_{DHA} was not verified by comprehensive analysis of the same specimen. Additionally, comprehensive culture detected an EBC producing *E. cloacae* and an MRSA, which had been missed by both routine microbiology and Unyvero P50. Two detections of EBC and two detections of MRSA, allegedly missed by the routine laboratory, could not be verified because these specimens were not included in the random selection (Table S5).

DISCUSSION

Accurate microbiological diagnosis of lower respiratory tract infections (LRTIs) is notoriously difficult with as many as 70% of patients never receiving a microbiological diagnosis [16]. Deep lung specimens such as BAL have less contamination from the upper respiratory microflora and are therefore preferable for diagnosis, but due to economic and practical issues, sputa and ETT aspirates are most common in the UK. This study was conducted in order to evaluate the performance of the Curetis Unyvero P50 diagnostic test, the first "sample-in, answer-out" test available on the market for rapid diagnosis of LRTIs. The preceding prototype system was evaluated in a multi-centre study [17] and the full commercial system has been evaluated in Kuwait [18] and Germany [19]. However, this constitutes the first performance evaluation for this test in the UK, and more importantly, is the first study to include a detailed analysis of antimicrobial resistance detection and the first to use an additional method to resolve discrepancies between routine culture and Unyvero P50.

The Unyvero P50 test successfully detected almost all organisms reported as significant by routine microbiology from 90 surplus specimens of patients with confirmed severe LRTI (overall sensitivity=95.7%). The exceptions were 2 organisms (*E. faecalis* and *C. koserii*) not included on the detection panel and 2 instances of *H. influenzae*. Conversely, the headline specificity of the test for pathogen detection was poor, with many specimens described as normal flora (NRF, NSG, MGODS) by routine microbiology.

Test or system failures occurred for 12.6% of specimens, which is of concern. Approximately half of these were partial failures, whereby the test failed because of errors in one or more

reaction chambers. In such cases a result is still available but will exclude targets from the failed chamber(s). Currently, the system does not list these unreliable targets to the user who cannot therefore judge whether or not to make use of the valid results.

A more in-depth culture-based analysis method was used for 42 randomly selected specimens to gain a better understanding of the reasons for discrepant results. This analysis revealed that, in this selection, 76% of cases where Unyvero P50 had reported additional organisms, these were genuinely present and viable in primary specimens. This still leaves a number of detections that cannot be explained this way. There are several possible reasons for this; such as presence of nucleic acid from non-viable organisms, uneven distribution of bacteria within the specimens or technical issues with the specificity and sensitivity of detection (i.e. errors relating to the sensitivity and specificity of the PCR assays or microarray detection). We found the comprehensive culture method a good way of further probing the specimens and would recommend its use in other similar evaluations.

Analysis of the resistance results was more complex. Many of the resistance markers included on the Unyvero P50 panel are common among commensals of the respiratory tract. We therefore restricted our analysis to markers where resistance could reasonably be linked to a particular species (MRSA and FQ^R) or where we felt that their presence might impact treatment, regardless of the species of origin (ESBLS, plasmidic AmpCs and carbapenemases) (Table 1). A relatively large number of discrepancies in resistance detection were still noted. For example the Unyvero P50 detected 2 putative MRSA isolates, and a CTX-M producer in specimens reported to only contain normal respiratory flora. Comprehensive culture confirmed

a CTX-M producing *K. pneumoniae* was present in the latter, but unfortunately the presumptive MRSA specimens were not available for further study. It should be noted that the *mecA* assay of Unyvero P50 is not species specific and it is possible that the *mecA* genes in question originated from *S. epidermidis* rather than *S. aureus* [20]. During analysis, we only considered specimens where Unyvero P50 reported both *S. aureus* and *mecA* as potentially containing MRSA.

Conversely, other discrepancies were potentially confusing. The majority of these related to the detection of chromosomal beta-lactamases. We suspect detection of chromosomal variants of AmpC enzymes (DHA in *M. morganii* and EBC (aka ACT/MIR) in *Enterobacter spp* [15]) in several cases; this is because there was no evidence for plasmidic AmpC enzymes in these specimens although the natural host species of these enzyme types were detected. Indeed, it can be difficult to develop PCR assays able to reliably distinguish certain plasmidic and chromosomal AmpC variants in their species of origin [21]. Five OXA-51 producing *A. baumaniii* were also detected; the OXA-51 enzyme is however intrinsic to *A. baumannii* and does not confer carbapenem resistance without an additional promoter provided by the insertion sequence IS*Aba1* [22]. Conversely, several *A. baumannii* isolated by routine microbiology carried $bla_{\text{OXA-23}}$ which is not a target of Unyvero P50.

In our opinion, the composition of the resistance panel should be substantially redesigned to account for the common microflora of the respiratory tract and global distribution of beta-lactamases. Several resistance genes, such as bla_{TEM}, *sul1* and *ermB,* are common among both pathogenic and commensal species found in the respiratory tract, and are therefore unusable unless their species of origin within the specimen is known. On the other hand, other resistance

genes causing concern globally, such as those encoding OXA-48, NDM and VIM type carbapenemases [23] are not included.

Although the organism panel from the test is rather comprehensive, it could be further improved. *Mycoplasma pneumoniae* is not included as a target, and the test cannot differentiate between *S. pneumoniae* and other members of *S. mitis* group not relevant for respiratory tract infections [24] and should be replaced with an assay capable of detecting *S. pneumoniae* only. In addition, the complete lack of detection of viruses is a concern as viruses can account for a substantial amount of respiratory infections, especially during winter months. The manufacturer has recently released a new cartridge, the P55, addressing some of these issues.

In summary, we find the sensitivity of detection of this test to be good, and therefore the treating clinician can be reasonably certain that if one of the targets of the test is absent, it is unlikely to be present, at least in significant numbers. Deciding which of the multiple organisms often detected in one specimen should be treated is another matter. As the specimens in this study all came from patients with known severe infections (42% were intensive care patients) it may be argued that many of the "additional" organisms detected by the test would have warranted treatment which could have improved outcomes for these patients, particular as the test is considerably faster than routine culture [19]. On the other hand, too many reported pathogens may unnecessarily confuse the physician's choice of antimicrobial therapy, and may inadvertently lead to over-prescription of antimicrobials which would be detrimental to current efforts to improve antimicrobial stewardship worldwide [25]. Clinical studies evaluating the

potential effect on patient outcomes from use of technology such as the Curetis Unyvero P50 are urgently required to establish the role this technology may play in the future microbiology laboratory.

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CONFLICTS OF INTEREST: None

ETHICAL APPROVAL

We adhered to a Governance framework with an overarching ethics agreement for the UCL Infection DNA Bank (Reference: 12/LO/1089), relating to the use of patient specimens surplus to clinical needs and anonymised patient data without consent.

References

1. Hubbard R (2006) The burden of lung disease. Thorax 61 (7):557-558. doi:10.1136/thx.2006.066050
2. Anand N, Kollef MH (2009) The alphabet soup of pneumonia: CAP, HAP, HCAP, NHAP, and VAP. Semin Respir Crit Care Med 30 (1):3-9. doi:10.1055/s-0028-1119803
3. Rotstein C, Evans G, Born A, Grossman R, Light RB, Magder S, McTaggart B, Weiss K, Zhanel GG (2008) Clinical practice guidelines for hospital-acquired pneumonia and ventilator-associated pneumonia in adults. Can J Infect Dis Med Microbiol 19 (1):19-53
4. Brusselaers N, Vogelaers D, Blot S (2011) The rising problem of antimicrobial resistance in the intensive care unit. Ann Intensive Care 1:47. doi:10.1186/2110-5820-1-47
5. Enne VI, Personne Y, Grgic L, Gant V, Zumla A (2014) Aetiology of hospital-acquired pneumonia and trends in antimicrobial resistance. Curr Opin Pulm Med 20 (3):252-258. doi:10.1097/MCP.0000000000000042
6. Masterton RG, Galloway A, French G, Street M, Armstrong J, Brown E, Cleverley J, Dilworth P, Fry C, Gascoigne AD, Knox A, Nathwani D, Spencer R, Wilcox M (2008) Guidelines for the management of hospital-acquired pneumonia in the UK: report of the working party on hospital-acquired pneumonia of the British Society for Antimicrobial Chemotherapy. J Antimicrob Chemother 62 (1):5-34. doi:10.1093/jac/dkn162
7. Cooke J, Stephens P, Ashiru-Oredope D, Johnson AP, Livermore DM, Sharland M, Antimicrobial Stewardship Sub-Group of the Department of Health's Advisory Committee for Antimicrobial R, Healthcare Associated I (2014) Antibacterial usage in English NHS hospitals as part of a national Antimicrobial Stewardship Programme. Public Health 128 (8):693-697. doi:10.1016/j.puhe.2014.06.023
8. Kollef MH (2004) Appropriate empiric antimicrobial therapy of nosocomial pneumonia: the role of the carbapenems. Respir Care 49 (12):1530-1541
9. Niederman MS (2006) Use of broad-spectrum antimicrobials for the treatment of pneumonia in seriously ill patients: maximizing clinical outcomes and minimizing selection of resistant organisms. Clin Infect Dis 42 Suppl 2:S72-81. doi:10.1086/499405
10. Davies S (2011) Annual Report of the Chief Medical Officer; Infections and the rise of antimicrobial resistance. Department of Health; London: 2013. volume 2
11. Matuschek E, Brown DF, Kahlmeter G (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 20 (4):O255-266. doi:10.1111/1469-0691.12373
12. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18 (3):268-281. doi:10.1111/j.1469-0691.2011.03570.x
13. Roger Finch PD, Mark Wilcox, William Irving (2012) Antimicrobial Chemotherapy 6th edition. 6 edn. Oxford university Press. doi:10.1093/med/9780199697656.001.0001
14. Pages JM, James CE, Winterhalter M (2008) The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol 6 (12):893-903. doi:10.1038/nrmicro1994
15. Jacoby GA (2009) AmpC beta-lactamases. Clin Microbiol Rev 22 (1):161-182. doi:10.1128/CMR.00036-08
16. Blasi F, Garau J, Medina J, Avila M, McBride K, Ostermann H, group Rs (2013) Current management of patients hospitalized with community-acquired pneumonia across Europe: outcomes from REACH. Respir Res 14:44. doi:10.1186/1465-9921-14-44

17. Schulte B, Eickmeyer H, Heininger A, Juretzek S, Karrasch M, Denis O, Roisin S, Pletz MW, Klein M, Barth S, Ludke GH, Thews A, Torres A, Cilloniz C, Straube E, Autenrieth IB, Keller PM (2014) Detection of pneumonia associated pathogens using a prototype multiplexed pneumonia test in hospitalized patients with severe pneumonia. PLoS One 9 (11):e110566. doi:10.1371/journal.pone.0110566
18. Jamal W, Al Roomi E, AbdulAziz LR, Rotimi VO (2014) Evaluation of Curetis Unyvero, a multiplex PCR-based testing system, for rapid detection of bacteria and antibiotic resistance and impact of the assay on management of severe nosocomial pneumonia. J Clin Microbiol 52 (7):2487-2492. doi:10.1128/JCM.00325-14
19. Kunze N, Moerer O, Steinmetz N, Schulze MH, Quintel M, Perl T (2015) Point-of-care multiplex PCR promises short turnaround times for microbial testing in hospital-acquired pneumonia--an observational pilot study in critical ill patients. Ann Clin Microbiol Antimicrob 14:33. doi:10.1186/s12941-015-0091-3
20. Ryffel C, Tesch W, Birch-Machin I, Reynolds PE, Barberis-Maino L, Kayser FH, Berger-Bachi B (1990) Sequence comparison of *mecA* genes isolated from methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Gene 94 (1):137-138
21. Perez-Perez FJ, Hanson ND (2002) Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 40 (6):2153-2162
22. Turton JF, Ward ME, Woodford N, Kaufmann ME, Pike R, Livermore DM, Pitt TL (2006) The role of ISAba1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. FEMS Microbiol Lett 258 (1):72-77. doi:10.1111/j.1574-6968.2006.00195.x
23. Canton R, Akova M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Miriagou V, Naas T, Rossolini GM, Samuelsen O, Seifert H, Woodford N, Nordmann P, European Network on C (2012) Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect 18 (5):413-431. doi:10.1111/j.1469-0691.2012.03821.x
24. Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, Tettelin H, Sorensen UB (2008) Evolution of *Streptococcus pneumoniae* and its close commensal relatives. PLoS One 3 (7):e2683. doi:10.1371/journal.pone.0002683
25. Antimicrobial resistance: Tackling a crisis for the health and wealth of nations, The Review on Antimicrobial Resistance Chaired by Jim O'Neill, December 2014 (2014).

425 **TABLES**

Gram-positive Bacteria	**Gram-Negative Bacteria**	**Fungus**	**Resistance genes**
Staphylococcus aureus *Streptococcus mitis* group	*Acinetobacter baumanii*, *Escherischia coli*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Stenotrophomonas maltophilia*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, *Enterobacter* spp, *Proteus* spp	*Pneumocystis jirovecii*	***bla*$_{CTX-M}$, *bla*$_{DHA}$, *bla*$_{EBC}$,** *ermA, ermB, ermC* **GyrA83, GyrA87, ParC *bla*$_{KPC}$, *bla*$_{oxa-51}$** *bla*$_{TEM}$, *bla*$_{SHV}$, *mefA*, *msrA*, ***mecA*,** *sul1* *int1*

426 Table 1. Pathogens and resistance markers detected by Unyvero P50. Resistance markers

427 considered during our analyses are in bold.

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Target Organism	Routine laboratory	UnyVero P50	True Positive (Routine and Unyvero P50)	False Positive (Unyvero P50 only)	False Negative (Routine only)
A. baumannii	3	10	3	7	0
Enterobacter spp	3	9	3	6	0
E. coli	5	8	5	3	0
H. influenzae	3	7	1	6	2
K. pneumoniae	3	11	3	8	0
M. catarrhalis	3	6	3	3	0
M. morganii	0	1	0	1	0
Proteus spp	1	5	1	4	0
P. aeruginosa	13	19	13	6	0
S. marcescens	6	9	6	3	0
S. aureus	5	11	5	6	0
S. maltophilia	6	27	6	21	0
*S. mitis group**	0	13	0	13	0
L. pneumophila	0	0	0	0	0
C. pneumoniae	0	0	0	0	0
P. jirovecii	0	0	0	0	0
K. oxytoca	0	0	0	0	0
E. faecalis	1	N/A	0	0	1
C. koseri	1	N/A	0	0	1
Negative specimens	42	16	N/A	N/A	N/A

Table 2. Frequency of organisms detected by routine microbiology and Unyvero P50 (n= 90 specimens). Negative specimens include those classified by routine microbiology as NRF, NSG, MGODS or no growth.

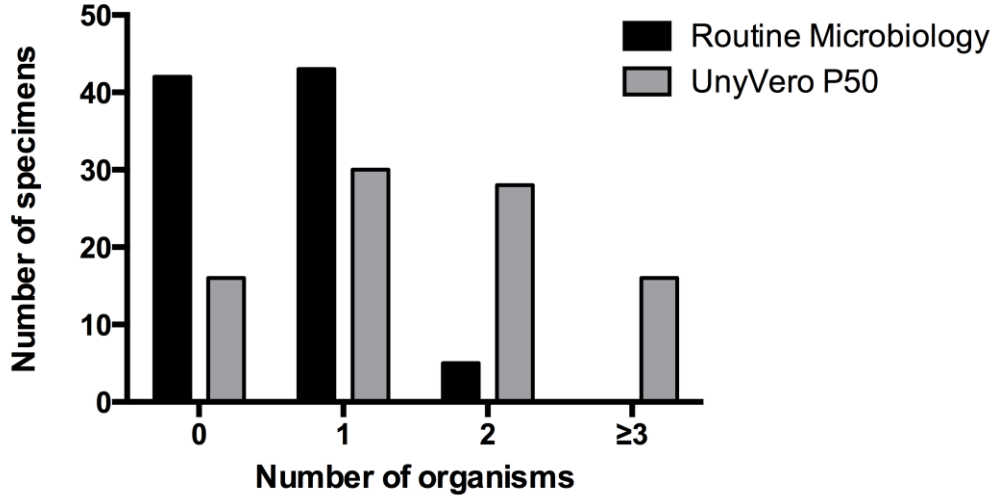
*_S. mitis_ group is not considered significant by the routine microbiology laboratories, only confirmed detections as _S. pneumoniae_ are reported. There were no reports of _S. pneumoniae_ from these specimens.

	ESBL producer	MRSA	Fluoroquinolone resistance	Carbapenemase producer	AmpC producer
Routine Microbiology + Checkpoints/PCR	not detected	n=1	n=3 1x *P. aeruginosa* (GyrA 83), 2x *E. coli* (GyrA 83; GyrA 83 + GyrA 87)	n=4 3 *A. baumannii* (*bla*$_{OXA-23}$) 1 *P. aeruginosa* (no enzyme found)	n=5 3x *S. marcescens* 2x *E. aerogenes* Presumed chromosomal AmpC upregulation
Unyvero P50	n=1 *bla*$_{CTX-M}$	n=3*	n=3 1x *P. aeruginosa* (GyrA83, ParC) 2x *E. coli* (GyrA83, GyrA83 + GyrA87)	n=5 *bla*$_{OXA-51}$ 2x *A. baumannii* *1*x *A. baumanii* + *S. maltophilia* 2x *A. baumannii* + *S. maltophilia* + *S.aureus*	n=5 2 x bla$_{DHA}$ *1*x *M. morganii* + *S. marcescens* *1*x *P. aeruginosa* + *S. maltophilia* 3 x bla$_{EBC}$ 2x Enterobacter spp. 1x Enterobacter spp + *M. catarrhalis*
Concordance	No	1/3	2/3	No	No

Table 3. Number of potentially significant resistance mechanisms detected by routine microbiology versus Unyvero P50

*We assumed presence of MRSA when both *S. aureus* and *mecA* were detected in the specimen

441 **FIGURES**



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443 Figure 1. Distribution of the number of micro-organisms detected per specimen

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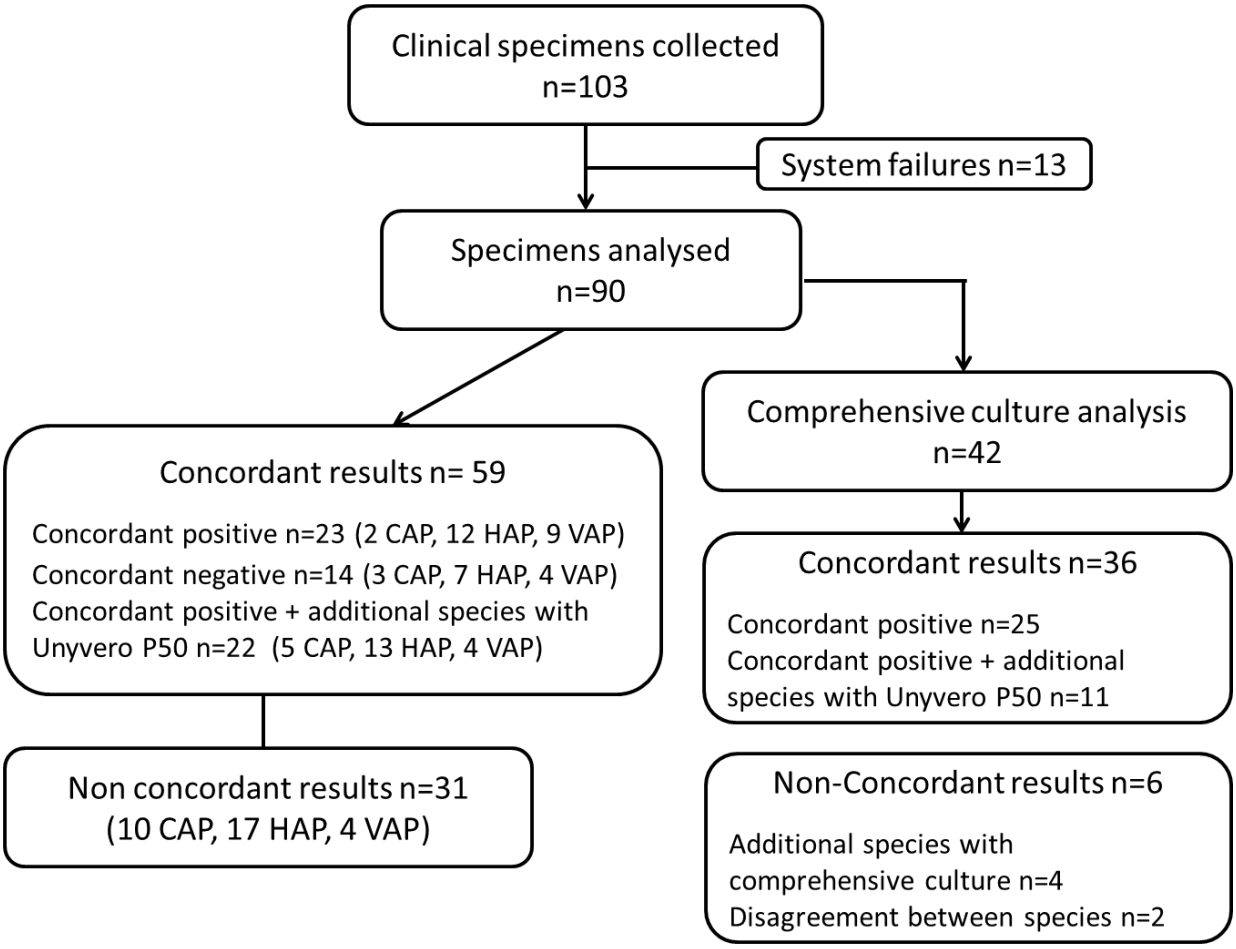

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446 Figure 2 Summary of results