Evaluating the use of a 22-pathogen TaqMan array card for rapid diagnosis of respiratory pathogens in Intensive Care

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Abstract

Introduction

Pneumonia is highly prevalent in intensive care units (ICU), with high associated mortality. Empirical treatment prioritises breadth of coverage while awaiting laboratory diagnosis, often at the expense of antimicrobial stewardship. Microarrays use multiple, parallel polymerase chain reactions to enable a rapid, syndromic approach to laboratory diagnosis.

Aim

To evaluate the clinical and laboratory implications of introducing a bespoke 22-pathogen TaqMan Array Card (TAC) for rapid pathogen detection in deep respiratory samples from adult ICU.

Methodology

TAC results from all ICU patients prospectively tested over a 9-month period at Cambridge's Clinical Microbiology & Public Health Laboratory were compared to those of corresponding conventional microbiological assays (culture-, PCR- or serology-based), in terms of result agreement and time-to-result availability. Clinical impact was assessed by retrospective review of medical records.

Results

Seventy-one patients were included (45 (63%) male, median age 59). Overall result agreement was 94%, with TAC detecting more pathogens than conventional methods. TAC detected *Streptococcus pneumoniae* more readily than culture (7 vs 0 cases; p=0.02). TAC did not detect *Aspergillus* spp. in eight culture- or Galactomannan-positive cases. Median turnaround time (1 day) was significantly shorter than that of bacterial/fungal culture, *Pneumocystis jirovecii* PCR and Galactomannan testing

(each 3 days; p<0.001), atypical bacteria serology (13 days; p<0.001), and *M. tuberculosis* culture (46 days; p<0.001). Earlier result availability prompted discontinuation of unnecessary antimicrobials in 15/71 (21%) cases, but had no bearing on patient isolation/de-isolation.

Conclusion

TAC provided greater overall yield of pathogen detection and faster turnaround times, permitting earlier discontinuation of unnecessary antimicrobials.

Introduction

Pneumonia is highly prevalent in the intensive care unit (ICU), contributing to 12.5% of adult ICU admissions in England, Wales and Northern Ireland[1], and is acquired in up to 15% of ventilated patients[2]. Associated mortality in this context is high[1], and is strongly affected by the timeliness and appropriateness of antimicrobial therapy[3-6]. A major challenge in the early selection of adequately targeted antimicrobials is the time taken to accurately identify an underlying pathogen, particularly with the heavy reliance on culture-based methods common to most diagnostic laboratories. Delay in reaching a definitive diagnosis leaves clinicians dependent on broad-spectrum empirical approaches to treatment, at the expense of antimicrobial stewardship (AMS). It can also delay initiation of appropriate infection control precautions, if highly infectious or resistant pathogens have not been pre-emptively considered. Additionally, yield of positive results is highly dependent on the range of laboratory tests performed, meaning difficult-to-culture pathogens may be missed when additional testing is not undertaken. The use of molecular techniques to enable a syndromic, rather than organism-centred, approach to laboratory diagnosis is a strategy that aims to reduce this risk.

Microarray technology uses multiple, parallel polymerase chain reactions (PCR) to allow the simultaneous, rapid detection of a wide range of potential pathogens from a single clinical specimen. It consists of a multitude of primer pairs and fluorescence-labelled probes, preloaded and dried onto a surface, that bind with high specificity to complementary sequences of nucleic acid[7]. While microarrays have been used extensively in the fields of cancer research and clinical genetics[8-13], more recently they have drawn growing attention for their potential application to infectious diseases[14-19]. While several studies have explored the comparative sensitivity and specificity of diagnostic microarrays in identifying a broad range of pathogens[20-32], the value and implications of their introduction to clinical practice remain undetermined. This study sought to evaluate the use of a 22-pathogen TaqMan Array Card (TAC) for diagnosing bacterial, viral and fungal pathogens in deep

respiratory samples from adult ICU patients, in terms of comparative diagnostic yield, turnaround time (TAT), and influence on clinical decision-making.

Methods

Following successful clinical validation (pathogen-specific sensitivity ranging from 89.1% to 100%, specificity 96.7% to 100%; see supplementary tables 1 and 2), a 22-pathogen TAC was introduced for use on clinical samples at Cambridge's Clinical Microbiology & Public Health Laboratory (CMPHL) in 2015. The card layout and range of pathogen targets are shown in figure 1. The card's use was limited to immunocompromised and critically ill patients, forming an adjunct to conventional laboratory testing on an ad hoc, clinician-directed basis. The ICU is a 20-bedded mixed medical-surgical unit, which supports tertiary liver failure, solid organ transplant, hepatobiliary surgery and haematology-oncology services (including stem cell transplant). It does not admit neurotrauma or cardiothoracic surgical patients.

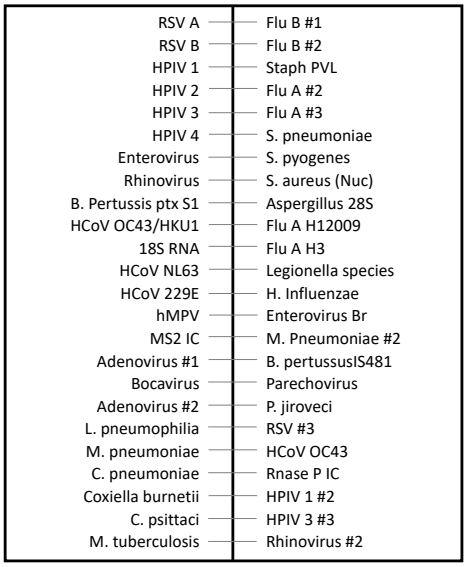

Figure 1: TAC plate layout design. *RSV = respiratory syncytial virus; HPIV = human parainfluenza virus; HCoV = human coronavirus; hMPV = human metapneumovirus; Flu B = influenza B; Flu A = influenza A.*

Nucleic acid extraction

Nucleic acid extraction was undertaken using the NUCLISENS easyMAG platform (Biomerieux, Marcy L-Etoile), in accordance with manufacturers' instructions. Nucleic acids were extracted from 500µL of sample, with a dilution of MS2 bacteriophage added pre-extraction to act as an internal extraction and inhibition control.

TaqMan Low-Density Array

TaqMan Array Cards (Applied Biosystems, Foster City, CA) are microfluidic cards with 8 specimen loading ports leading to 48 inter-connected wells, each of which can be preloaded with the primers and TaqMan MGB probes necessary for independent simplex PCR reactions (33). Following completion of specimen loading, wells are sealed to create a closed system for each reaction to occur in parallel. Our 22-pathogen TAC used a collection of in-house primers, with more than one

pan-specific or type-specific primer set included to increase overall specificity for some pathogens. The card also included primers targeting the genes of endogenous control RNase P, the internal control MS2, and the known marker of *Staphylococcus aureus* virulence, Panton–Valentine leukocidin (PVL).

Cards were run on either the Vii A7 or QuantStudio 7 Flex platform (Thermo Fisher Scientific, Life Technologies, Carlsbad, CA), following a modified version of the method described in Steensels *et al*[26]. Briefly, 20µL of nucleic acid extract was mixed with 26µL of TaqMan Fast Virus 1-step mastermix (Thermo Fisher Scientific, Life Technologies, Carlsbad, CA) and 58µL of RNase free water, before being loaded onto the card. Reverse transcriptase real-time PCR was undertaken according to the following amplification protocol: 50˚C for 5 minutes (reverse transcription step), 95˚C for 20 seconds, then 45 cycles of 95˚C for 1 second followed by 60˚C for 20 seconds, with a fluorescence reading taken on the FAM channel at each cycle. Detection of a clear exponential amplification curve with a cycle threshold (CT) value ≤38 for any single gene target was reported as a positive result for the relevant pathogen.

Conventional microbiological testing

A single in-house multiplex PCR assay formed the basis of conventional testing for common respiratory viruses (adenovirus, enterovirus, human metapneumovirus, influenza A virus, influenza B virus, parainfluenza virus, rhinovirus, and respiratory syncytial virus (RSV)). In-house monoplex PCR was used for detection of *Bordetella pertussis* and *Pneumocystis jirovecii* (PCP). *Aspergillus* spp. were tested for by culture on Sabouraud Dextrose Agar with Chloramphenicol, with or without testing for galactomannan antigen in serum (serum GM) and/or bronchoalveolar lavage (BAL GM) by Platelia™ *Aspergillus* enzyme immunoassay (Bio-Rad Laboratories, Hercules, CA). Conventional testing for *Mycobacterium tuberculosis* consisted of microscopy using auramine-phenol or Ziehl-Neelsen stains, and culture in a Mycobacteria Growth Indicator Tube (MGIT), with or without PCR (GeneXpert,

Cepheid, Sunnyvale, CA). *Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, and Streptococcus pyogenes* were tested for by culture on blood and chocolate agars. Infection with *Chlamydia* spp., *Coxiella burnetii,* or *Mycoplasma pneumoniae* was tested for by serology at the national reference laboratory (Public Health England, Colindale, London, UK). *Legionella pneumophila* serogroup 1 was tested for by detection of antigen in urine, using the Alere BinaxNOW™ Legionella Urinary Antigen Card (Thermo Fisher Scientific, Life Technologies, Carlsbad, CA), and buffered charcoal yeast extract media was used for *Legionella* spp. culture when suspicion of infection was high. Conventional laboratory methods were not routinely available to detect bocavirus or coronavirus.

Data extraction

CMPHL's electronic health records were retrospectively searched for the results of all TAC tests performed on Addenbrooke's Hospital respiratory samples between 1st March and 31st December 2016. TAC results from patients in the following categories were excluded: age <18 years, inpatients in non-ICU settings at the time of respiratory sampling, outpatients at the time of respiratory sampling, TAC performed on sample types other than deep respiratory specimens (bronchoalveolar lavage (BAL), tracheal aspirate or pleural fluid). Basic patient data (date of birth, age, gender, date of admission) and TAC test data (specimen collection date, final report date, pathogens identified, CT values) were retrieved from hospital and laboratory records. Laboratory records relating to each eligible TAC sample were manually examined to ascertain details of conventional microbiological tests undertaken during the same hospital episode (tests undertaken, results, CT values, dates of specimen collection, dates of final report). In the event of multiple conventional tests, details of the closest positive result in time to the result of TAC were recorded. If all conventional tests for a given pathogen were negative, the closest negative result in time to the TAC result was recorded, with tests undertaken on the same clinical sample chosen for comparison whenever possible.

Clinical impact

Actual and potential clinical impact assessments were performed by an ICU consultant (ACM), who reviewed clinical details and laboratory results independently of the laboratory-based team. Cases were classified as community-acquired pneumonia (CAP; onset ≤48 hours into hospital admission), hospital-acquired pneumonia (HAP; onset >48 hours into hospital admission) or ventilator-associated pneumonia (VAP; onset >48 hours into mechanical ventilation). Actual clinical impact was measured as the proportion of cases in which changes to antimicrobial prescriptions (initiation or discontinuation of any agent) or movement of patients (into or out of isolation) occurred within 24 hours of TAC result availability. Whether or not these changes were deemed likely to have arisen in direct response to TAC results was recorded. Potential clinical impact was measured as the proportion of cases in which patient movement or rationalisation of antimicrobial regimes could reasonably have been expected in response to TAC results, but had not been made in practice. The number of cases of detection by conventional methods of pathogens not included in TAC's targets was also recorded, as well as the impact of these pathogens on antimicrobial prescriptions.

Statistical analysis

Data were collated on Microsoft Excel (patient age and gender distributions, number and identity of pathogens detected overall and per sample, times of specimen collection relative to hospital admission), before being transferred to GraphPad Prism (GraphPad Software, La Jolla, California, USA) and Stata 14 (StataCorp, College Station, Texas, USA) for statistical analysis (Chi-squared test for comparison of proportions of positive patients, McNemar's test for analysis of result agreement between test methods, Willcoxon's signed-rank test for comparison of CT values, Mann–Whitney U test for comparison of median TATs, log-rank test for likelihood of earlier result availability).

Ethics Committee approval was not sought for this Service Evaluation, as there was no deviation from established laboratory or clinical practice.

Results

Seventy-one adult ICU patients met the inclusion criteria (figure 2); 45 (63%) male, median age 59 (IQR 43.5-69). Sampling was undertaken >48 hours from admission in 66 (93%) cases. Retrospective clinical review found the number of cases with CAP, HAP and VAP to be 18 (25%), 30 (42%) and 21 (30%) respectively. The remaining two cases were deemed to have been non-infectious in nature; one with pulmonary oedema and one with autoimmune pneumonitis.

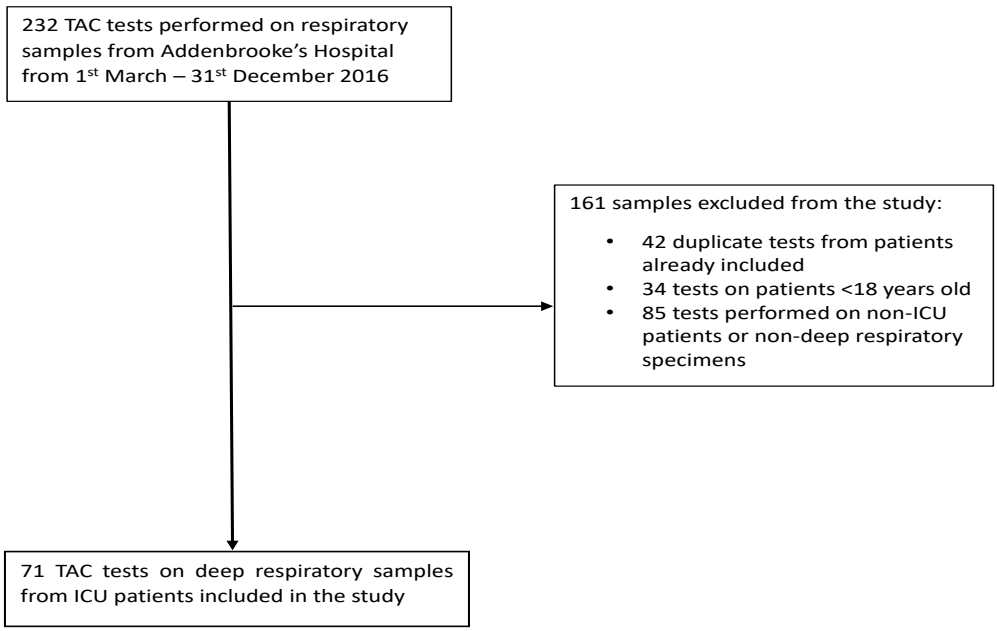

Figure 2. Summary of samples included.

Pathogens detected

One or more pathogens were detected in 33 (46%) patients using TAC, compared to 29 (41%) by conventional methods (p=0.5). TAC detected bacterial pathogens that were not cultured in 11 cases.

Co-infection was identified in 11 (33%) positive TAC tests, compared to five (17%) positives by conventional methods (p=0.03). A summary of co-infecting pathogen combinations is shown in supplementary table 3. Twelve cases were identified in which conventional methods diagnosed potentially pathogenic organisms that were not included in the TAC targets; Gram-negative bacteria in 11 and herpes simplex virus in one.

Agreement with conventional methods

Overall result agreement was 94%. Thirteen pathogens had 100% test agreement, eight of which had no positive results. Of the 56 patients tested by conventional viral PCR, 100% test agreement was observed for influenza A (six positive), adenovirus (two positive), RSV (two positive) and parainfluenza virus (one positive). None of the 14 patients tested with atypical bacteria serology gave positive results (100% agreement with TAC). Similarly, none of the 67 patients tested for *M. tuberculosis* by conventional methods had positive results by culture, conventional PCR or TAC. Of the 31 patients tested for *Legionella* by urinary antigen test, 1 was positive (100% agreement with TAC). Samples from all 71 patents underwent routine bacterial culture; agreement with TAC was 100% for *S. pyogenes* only (no cases). *B. pertussis* was not tested for by routine methods in any patient. Table 1 shows comparative yields of pathogen detection for those with test agreement <100%. *Aspergillus niger* was detected by culture in two cases (both <10,000 cfu/ml), neither of which were detected by TAC.

Table 1. Comparative yields of pathogen detection in instances of <100% test agreement. *TAC = TaqMan Array Card; serum GM = serum galactomannan antigen; BAL GM = bronchoalveolar lavage galactomannan antigen.*

Target pathogen	Features	TAC		Percentage agreement	P-value
		Positive	Negative		
***Aspergillus* spp.**	**Any marker positive**	2	8	89%	p=0.078
	No markers positive	0	61		
***Aspergillus* spp.**	**Culture positive**	0	2	94%	p=1
	Culture negative	2	67		
***Aspergillus* spp.**	**BAL GM positive**	2	4	89%	p=0.12
	BAL GM negative	0	29		
***Aspergillus* Spp.**	**Serum GM positive**	1	2	87%	p=0.5
	Serum GM negative	0	12		
H. influenzae	**Culture positive**	4	0	94%	p=0.125
	Culture negative	4	63		
Human metapneumovirus	**PCR positive**	0	1	98%	p=1
	PCR negative	0	55		
P. jirovecii	**PCR positive**	2	0	94%	p=0.5
	PCR negative	2	31		
Rhinovirus	**PCR positive**	3	1	98%	p=1
	PCR negative	0	52		
S. aureus	**Culture positive**	2	0	94%	p=0.125
	Culture negative	4	65		
S. pneumoniae	**Culture positive**	0	0	90%	**p=0.0156**
	Culture negative	7	64		

Relative quantitation

Paired analysis of CT values for results obtained by TAC and conventional PCR revealed no significant differences (figure 3).

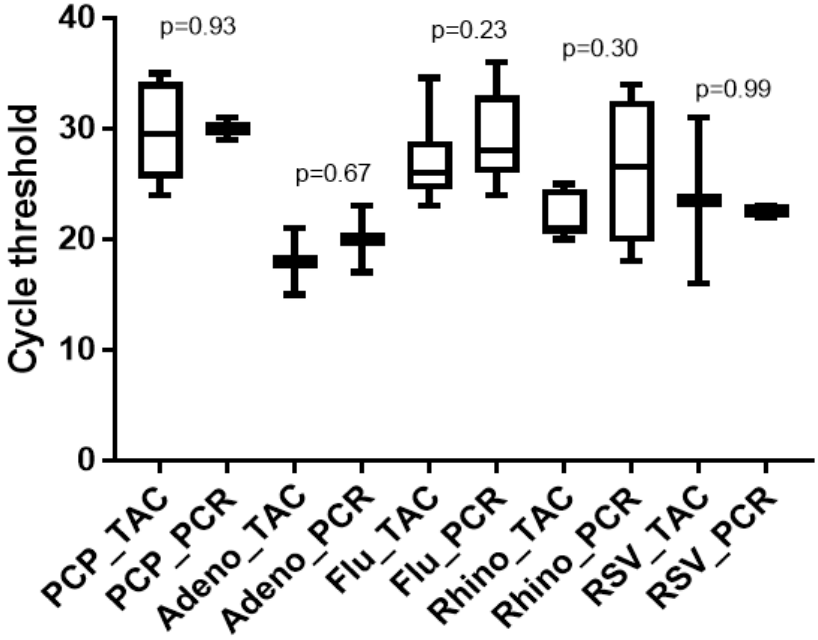
Cycle threshold
40
30
20
10
0
p=0.93
p=0.67
p=0.23
p=0.30
p=0.99
PCP_TAC
PCP_PCR
Adeno_TAC
Adeno_PCR
Flu_TAC
Flu_PCR
Rhino_TAC
Rhino_PCR
RSV_TAC
RSV_PCR

Figure 3. Comparison of cycle threshold (CT) values between TAC and conventional PCR. *PCR = results of real-time PCR; TAC = results generated using the TaqMan Array Card; PCP =* P. jirovecii*; Adeno = adenovirus; Flu = influenza A; Rhino = rhinovirus; RSV = respiratory syncytial virus. P-values generated by paired comparison of CT values for PCR and TAC tests, using the Willcoxon signed-rank test.*

Time to result availability

Median TAC TAT (1 day) was comparable to that of viral PCR and *Legionella* antigen (1 day; p=0.93 and p=0.07 respectively), but significantly shorter than the TAT of bacterial/fungal culture, PCP PCR, BAL GM and serum GM (3 days; each p<0.001), atypical bacteria serology (13 days; p<0.001), and *M. tuberculosis* culture (46 days; p<0.001). Median TAT for *M. tuberculosis* PCR (0 days) was faster than that of TAC (p=0.04). Hazard ratios for earlier result availability are shown in figure 4, comparing each of the conventional assays to TAC.

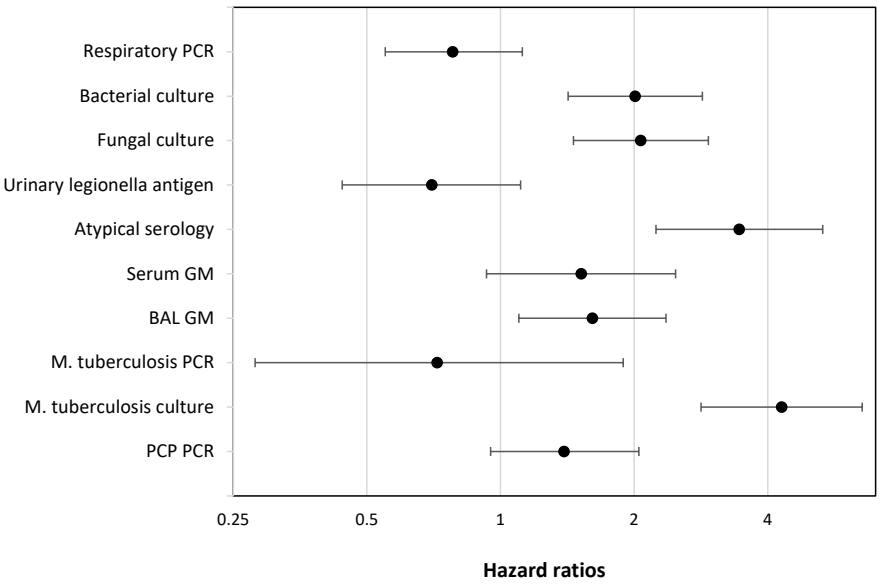
Respiratory PCR
Bacterial culture
Fungal culture
Urinary legionella antigen
Atypical serology
Serum GM
BAL GM
M. tuberculosis PCR
M. tuberculosis culture
PCP PCR
0.25
0.5
1
2
4
Hazard ratios

Figure 4. Hazard ratios for earlier result availability, comparing conventional assays to TAC. *Hazard ratios for earlier result availability, comparing conventional assays to TAC: HR <1 suggests higher likelihood of earlier result availability, HR >1 suggests lower likelihood of earlier result availability. TAC = TaqMan Array Card; Respiratory PCR = viral PCR; serum GM = serum galactomannan antigen; BAL GM = bronchoalveolar lavage galactomannan antigen; TB PCR =* M. tuberculosis *PCR; Urine legionella = Alere BinaxNOW™ Legionella Urinary Antigen Card; PCP PCR =* P. jirovecii *PCR.*

Clinical impact

Antimicrobial agents were introduced within 24 hours of TAC results in 12 cases, four directly attributable to TAC results (figure 5); co-trimoxazole was introduced to treat two TAC-detected PCP cases, and piperacillin-tazobactam was switched to co-amoxiclav for the treatment of two TAC-detected *H. influenzae* cases. The other eight cases of antimicrobial introductions resulted from conventional detection of pathogens not targeted by TAC, or as empirical treatment changes. Antimicrobial agents were discontinued within 24 hours in 33 cases; eight cases of clarithromycin discontinuation based on negative TAC results for atypical bacteria, five cases of co-trimoxazole discontinuation because of negative TAC PCP results, and the two instances of piperacillin-tazobactam discontinuation due to *H. influenzae* detection. None of the 69 patients for which bed movement data was available were moved into or out of infection control isolation within 24 hours of TAC result availability.

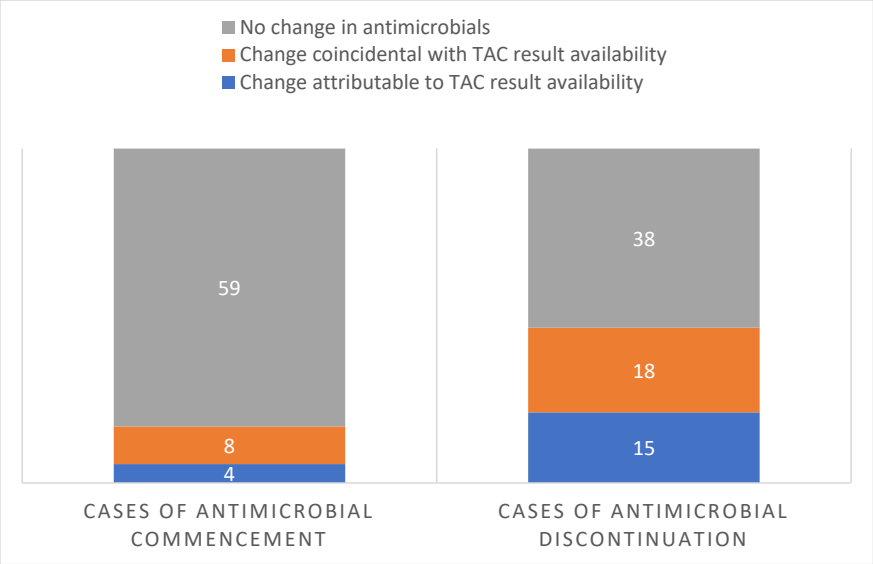
No change in antimicrobials
Change coincidental with TAC result availability
Change attributable to TAC result availability
59
8
4
38
18
15
CASES OF ANTIMICROBIAL COMMENCEMENT
CASES OF ANTIMICROBIAL DISCONTINUATION

Figure 5. Antimicrobial prescription changes made within 24 hours of TAC result availability. *TAC = TaqMan Array Card*.

In addition to the 17 instances of antimicrobial changes in response to TAC (13 cases of discontinuation only, two cases of commencement only, and two cases of both discontinuation and commencement), six cases were identified in which antimicrobial rationalisation could reasonably have been expected. These included four cases of *S. aureus* or *S. pneumoniae* detection, which could have permitted a switch away from piperacillin-tazobactam to more narrow-spectrum agents, and two cases of negative TAC results for atypical bacteria that could have led to clarithromycin discontinuation. No instances of missed opportunities for patient isolation or de-isolation were identified.

Discussion

Using a syndromic approach to laboratory diagnosis, this 22-pathogen TAC enabled greater yield of respiratory pathogen detection and faster result availability than conventional methods alone, leading to direct clinical benefit in terms of treatment optimisation and AMS. However, negative results for *Aspergillus* spp. in several cases with positive culture or Galactomannan tests suggests the current iteration of the card may need optimisation for diagnosing aspergillosis.

In addition to providing faster result availability, TAC detected seven cases of *S. pneumoniae* that were missed by culture, as well as a number of instances of multi-pathogen co-infections. Increased rates of pathogen detection have previously been observed in analyses of commercially available microarrays[30,31], and in comparisons of conventional bacterial PCR with culture methods in patients already started on antimicrobial therapy[34,35]. While these findings may reflect greater sensitivity of

molecular diagnostic assays, they also serve to highlight an important challenge in the interpretation of results; that of accurately differentiating pathogens causative of infection from those constituting colonising flora[35]. There is a need for algorithmic tools to assist in interpreting low-level positive results from extensive multiplex panels, but corroboration with clinical findings and the use of CT values as a proxy measure of likely pathogen burden remain reasonable approaches. Reassuringly, CT values did not differ significantly between TAC and conventional real-time PCR in this study.

A potential assay limitation identified here is TAC's performance in *Aspergillus* detection; eight possible cases were missed, although none of these were positive by more than one conventional *Aspergillus* test. *Aspergillus* infections are notoriously difficult to diagnose, and it is advisable that TAC is not relied upon in isolation for this purpose. Additionally, low overall positivity rates for both TAC and conventional methods in this study make it difficult to draw conclusions on the risk of false-negative TAC results for other, rarer pathogens.

Retrospective review of the clinical implications of earlier result availability revealed direct benefit to patient treatment and AMS; therapy changes were observed in 24% of cases, including several instances of discontinuation of unnecessary treatments. Three cases in which antimicrobial rationalisation had already occurred based on earlier conventional test results, but could have happened sooner if TAC had been performed immediately after specimen collection were also identified. Although TAC results were found to be responsible for a reasonable reduction in clarithromycin prescribing, it should be noted that adjunctive macrolide therapy may be beneficial in the treatment of severe pneumonia, regardless of causative organism[6,36]. The identification of six cases in which TAC result availability failed to prompt appropriate antimicrobial rationalisation suggests that TAC's potential as an AMS tool may be limited by behavioural factors. While this could be due to clinician unfamiliarity with TAC technology, antibiotic prescribing decisions are complex

and not solely based on the results of diagnostic tests. Diagnostic stewardship has recently become an important area of focus in tackling the spread of antimicrobial resistance[37], but clinician education and buy-in will be vital steps in ensuring the effectiveness of this approach.

The assay's impact on broad-spectrum antibiotic prescribing was limited by its inability to test for pathogens expected in HAP and VAP, with only 25% of patients having been diagnosed with CAP; ten cases were identified in which antimicrobial rationalisation was not possible because of uncertainty over the status of pathogens not included on the card, or because antimicrobial susceptibility information was lacking. Similarly, the absence of targets for genetic markers of antimicrobial resistance with potential for nosocomial spread was identified as the main reason for lack of impact on patient isolation and de-isolation. An advantage of TAC technology is that the selection of molecular targets on the card can be tailored according to clinical need. Future card designs for use in ICU patients should prioritise the inclusion of targets for Gram-negative organisms and the carriage or mutation of known antimicrobial resistance genes, in order to maximise its impact on antimicrobial prescribing and infection control practices.

It is important to recognise some study limitations arising from the fact that this was a non-randomised observation of ad hoc TAC use, at the discretion of clinicians. Firstly, separate clinical specimens were used for TAC and conventional tests in some patients, meaning differences in sample quality or specimen type may have influenced results; non-deep respiratory specimens were accepted for some test methods, in line with routine laboratory practice. Similarly, differences in times of specimen collection relative to illness onset may have affected relative positive yields for some tests. A prospective evaluation of the systematic use of TAC in an ICU setting is needed to better address these issues. The study was also not designed to evaluate a number of important laboratory aspects of TAC's introduction, which should be targets of further research; the impact of

reduced specimen handling on risk of sample contamination, the effect of extremely low-volume specimen use on rates of inadequate sampling, and the effect of syndromic multiplex testing on overall laboratory workflow. Finally, a relatively large number of TAC tests were excluded from analysis, as the study focused solely on deep respiratory samples from adult ICU patients, and did not include analysis of repeat sampling. Although this may limit the generalisability of the study, we selected this cohort to focus on a relatively homogenous group that are likely to benefit from rapid identification of pathogens. The use of TAC for rapid diagnosis of respiratory pathogens in paediatric and non-ICU cohorts is worthy of further investigation, and varying compositions of pathogen targets should be explored to maximise the utility of the assay in different patient groups.

Overall, TAC provided greater yield of respiratory pathogen detection, with faster turnaround time than conventional microbiological methods, and demonstrated potential to benefit antimicrobial prescribing in ICU. Future work should focus on customisation of the assay to optimise its use in priority patient populations, with the aim of including molecular targets with greater potential to influence AMS and inform infection control procedures.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Supplementary table 1. Clinical validation based on a comparison of the results of TAC and conventional real-time multiplex viral PCR (parallel testing performed on 417 consecutive clinical samples from December 2014 to January 2015)

Pathogen	True Positive	True Negative	False Positive	False Negative	Sensitivity %	Specificity %
Flu A	15	402	0	1	93.75 (67.71 - 99.67)	100.00 (98.82 - 100.00)
Flu B	4	413	0	0	100.00 (39.58 - 100.00)	100.00 (98.85 - 100.00)
RSV	92	325	11	0	100.00 (95.01 - 100.00)	96.73 (94.05 - 98.27)
HPIV	12	405	1	1	92.31 (62.09 - 99.60)	99.75 (98.42 - 99.99)
Adenovirus	9	408	8	1	90.00 (54.12 - 99.48)	98.08 (96.10 - 99.10)
Rhinovirus	84	333	6	5	94.38 (86.78 - 97.91)	98.23 (96.00 - 99.28)
HMPV	10	407	1	1	90.91 (57.12 - 99.52)	99.75 (98.42 - 99.98)
HCoV	18	399	2	1	94.74 (71.89 - 99.72)	99.50 (98.01 - 99.91)
Overall	209	208	27	10	95.43 (91.76 - 97.79)	88.51 (83.73 - 92.29)

Sensitivity and specificity estimates calculated using Medcalc online statistical software, using the Clopper-Pearson test to calculate 95% confidence intervals.

Flu A = influenza A; Flu B = influenza B; RSV = respiratory syncytial virus; HPIV = human parainfluenza virus (genotyes 1-4); HMPV = human metapneumovirus; HCoV = human coronavirus (OC43, HKU1, NL63, 229E). All discrepant results were low titre viral loads with late Ct values. Subsequent analysis of the observed 'false positive' RSV and adenovirus TAC results suggested superior TAC sensitivity, rather than a reduction in sensitivity of the gold standard PCR test or actual false positivity.

Supplementary table 2. Clinical validation based on TAC results for commercially purchased controls, External Quality Assessment (EQA) panels and clinical samples known to be positive by gold standard microbiological tests

Pathogen target(s)	True Positive	True Negative	False Positive	False Negative	Sensitivity (95% CI)	Specificity (95% CI)
Bordetella pertussis	46	21	0	2	95.83 (85.75 - 99.49)	100.00 (83.89 - 100.00)
Bocavirus	28	23	0	0	100.00 (87.66 - 100.00)	100.00 (85.18 - 100.00)
Legionella pneumophila	70	36	0	3	95.89 (88.46 - 99.14)	100.00 (90.26 - 100.00)
Mycoplasma pneumoniae	41	20	0	5	89.13 (76.43 - 96.38)	100.00 (83.16 - 100.00)
Chlamydia psittaci	28	20	0	2	93.33 (77.93 - 99.18	100.00 (83.16 - 100.00)
Chlamydia pneumoniae	11	20	0	1	91.67 (61.52 - 99.79)	100.00 (83.16 - 100.00)
Coxiella burnetii	9	20	0	0	100.00 (66.37 - 100.00)	100.00 (83.16 - 100.00)
Mycobacterium tuberculosis	82	51	0	3	96.47 (90.03 - 99.27)	100.00 (93.02 - 100.00)
Streptococcus pneumoniae	22	20	0	0	100.00 (84.56 - 100.00)	100.00 (83.16 - 100.00)
Streptococcus pyogenes	9	20	0	0	100.00 (66.37 - 100.00)	100.00 (83.16 - 100.00)
Staphylococcus aureus	10	20	0	0	100.00 (69.15 - 100.00)	100.00 (83.16 - 100.00)
Aspergillus	31	28	0	1	96.88 (83.78 - 99.92)	100.00 (87.66 - 100.00)
Haemophilus pneumoniae	16	22	0	0	100.00 (79.41 - 100.00)	100.00 (84.56 - 100.00)
Parechovirus	41	24	0	4	91.11 (78.78 - 97.52)	100.00 (85.75 - 100.00)
Pneumocystis jiroveci	58	20	0	2	96.67 (88.47 - 99.59)	100.00 (83.16 - 100.00)

Sensitivity and specificity estimates calculated using Medcalc online statistical software, using the Clopper-Pearson test to calculate 95% confidence intervals.

The gold standard microbiological tests used to confirm positive clinical samples were culture for L. pneumophilia*,* M. tuberculosis*,* S. pneumoniae*,* S. pyogenes*,* S. aureus*,* Aspergillus *and* H. pneumoniae*, and PCR for* B. pertussis*, bocavirus, parechovirus and* P. jiroveci*. No gold standard microbiological tests were used for* M. pneumoniae*,* C. psittaci*,* C. pneumoniae *or* C. burnetti *because only commercial controls (Vircell, Granada, Spain) and EQA panels (Quality Control for Molecular Diagnostics, Glasgow, UK) were used to validate TAC for the detection of these pathogens.*

Supplementary table 3. TAC-identified co-infections

	Pathogen 1	Pathogen 2	Pathogen 3	Pathogen 4
Sample 1	*H. influenzae*	*S. pneumoniae*	Rhinovirus	*P. jirovecii*
Sample 2	*H. influenzae*	*S. aureus*	Rhinovirus	
Sample 3	Influenza A	Adenovirus	*Aspergillus* sp.	
Sample 4	*H. influenzae*	Influenza A		
Sample 5	*H. influenzae*	Influenza A		
Sample 6	*H. influenzae*	*S. pneumoniae*		
Sample 7	*H. influenzae*	*S. pneumoniae*		
Sample 8	*H. influenzae*	*S. aureus*		
Sample 9	*S. aureus*	*S. pneumoniae*		
Sample 10	Rhinovirus	Respiratory syncytial virus		

Table displaying the collection of pathogens identified in the ten participants with more than one pathogen detected by TAC. Pathogens are presented in no particular order.