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Routine Metagenomics Service for Intensive Care Unit Patients with Respiratory Infection

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Author Contributions: TC, AAM, LBS and JDE: study design. Sample collection and RMg processing: MT, NAY and GH. RMg bioinformatics analysis and interpretation: TC and CA. Reporting of RMg results to physicians LBS and AAM. Clinical interpretation of RMg: JDE, DLAW, CISM and NAB. Review of RMg results at bi-weekly meetings: MW, PR, MC, SG, NAB, CISM and GN. Manuscript write-up: TC and JDE. All authors have read and approved the manuscript.

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At a Glance Commentary

Scientific Knowledge on the Subject:

Respiratory metagenomics (RMg) holds promise as a first-line diagnostic test for lower respiratory tract infections (LRTI). In principle, it rapidly detects all potential pathogens along with antimicrobial resistance determinants and provides sequence typing for infection control or public health actions. Questions however remain on feasibility providing regular same day testing, and whether findings are sufficiently frequent and informative to justify translation into routine service.

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What This Study Adds to the Field:

We implemented a previously validated rapid RMg workflow into a daily pilot service for patients with community- and hospital-acquired LRTIs on a general and specialist respiratory intensive care unit, performed alongside other routinely requested tests. RMg performance was comparable with preceding research studies. Antibiotic treatment was optimised in almost half of patients, but perhaps more significantly, RMg revealed a hidden infectious burden in both ICU settings not reported by routine tests. Reasons included specific tests not being requested, fastidious culture requirements, or because detection required sequencing information, of either virulence factors or typing for more unusual and emerging infections. While multi-centre comparative studies are required this study provides real-world evidence of how RMg could improve the initial management of LRTIs.

Some of the results of these studies have been previously reported in the form of a preprint (medRxiv, 16 May 2023, www.medrxiv.org/content/10.1101/2023.05.15.23289731v1).

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

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Abstract

Rationale:

Respiratory metagenomics (RMg) needs evaluation in a pilot service setting to determine utility and inform implementation into routine clinical practice.

Objectives:

Feasibility, performance and clinical impacts on antimicrobial prescribing and infection control were recorded during a pilot RMg service.

Methods:

RMg was performed on 128 samples from 87 patients with suspected lower respiratory tract infection (LRTI) on two general and one specialist respiratory intensive care units (ICU) at Guy's & St Thomas NHS foundation Trust, London.

Measurements and Main Results:

During the first 15-weeks RMg provided same-day results for 110 samples (86%) with median turnaround time of 6.7hrs (IQR 6.1-7.5 hrs). RMg was 93% sensitive and 81% specific for clinically-relevant pathogens compared with routine testing. 48% of RMg results informed antimicrobial prescribing changes (22% escalation; 26% de-escalation) with escalation based on speciation in 20/24 cases and detection of acquired-resistance genes in 4/24 cases. Fastidious or unexpected organisms were reported in 21 samples including anaerobes (n=12), *Mycobacterium tuberculosis*, *Tropheryma whipplei*, cytomegalovirus and *Legionella pneumophila* ST1326, which was subsequently isolated from the bed-side water outlet. Application to consecutive severe community-acquired LRTI cases identified *Staphylococcus aureus* (two with *SCCmec* and three with *luk* F/S virulence determinants),

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Streptococcus pyogenes (*emm1*-M1uk clone)*, S. dysgalactiae* subspecies equisimilis (STG62647A) and *Aspergillus fumigatus* with multiple treatments and public-health impacts.

Conclusions:

This pilot study illustrates the potential of RMg testing to provide benefits for antimicrobial treatment, infection control and public health, when provided in a real-world critical care setting. Multi-centre studies are now required to inform future translation into routine service.

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Introduction

Community and hospital acquired lower-respiratory tract infections (LRTI) are caused by an expanding range of mono-microbial and polymicrobial infections. This presents significant challenges identifying or excluding microbial cause(s). Typically, samples are tested by different methodologies with results returned at different times over subsequent days (1). Culture is routinely used for bacterial identification, despite being slow, having suboptimal sensitivity particularly after antibiotic treatment and inability to detect many fastidious organisms. Multiplex PCR tests detect various pathogens and can add value to early decision-making, but they only target a restricted repertoire and cannot provide genomic detail or exclude infection.

RMg has potential to become a first-line test for severe pneumonia, given its ability to identify essentially any microbe in a clinical sample along with antimicrobial resistance and virulence determinants (2). Retrospective and prospective proof-of-concept studies and case series have been published (3-9) but none have demonstrated feasibility and the breadth of impact from a single test as a routine daily service. We developed a 6-hour nanopore sequencing workflow (6) and determined performance characteristics and potential utility in a research setting for ventilated patients with COVID-19 (2). Here we moved forward to determine the feasibility and clinical impact of a pilot RMg service, providing daily results to clinicians alongside routinely requested tests. Scientific and clinical oversight of this service improvement project was provided jointly by members of the diagnostic microbiology laboratory, the infectious diseases consult team and the intensive care unit.

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Methods

Setting and sample collection

RMg testing was offered to a 41-bed medical, surgical and specialist respiratory ICU that included an Extracorporeal Membrane Oxygenation (ECMO) service, Monday to Friday between November 22nd-December 15th 2021 and January 4th-March 25th 2022. Pilot service provision was agreed by the Critical Care Governance & Audit Committee under the NHS Quality Improvement and Patient Safety (QIPS) governance process as previously described (10) (QIPS reference 2021:13023). Duty intensivists selected mechanically ventilated (MV) patients to have additional RMg testing alongside culture and 16S rRNA sequencing that was performed at a referral clinical laboratory, with results returned within 4-6 days after acute treatment decisions had been made. Sample selection criteria were based on the potential for a rapid result to assist with diagnosis or exclusion of LRTI and antibiotic prescribing. Respiratory samples were retrieved from ICU at 8.30am with results aimed before 5pm. Representative severe CA-LRTI cases are also presented from the first 8 weeks of the following influenza season (winter 2022), given CA-LRTIs were rare during the pilot conducted during the COVID-19 pandemic.

Respiratory metagenomic sequencing workflow

In total 128 samples were processed for RMg sequencing which included 111 bronchoalveolar lavages (BAL), 3 tracheal aspirates, 8 non-direct bronchoalveolar lavages (NDL) and 6 pleural fluids. RMg testing involved saponin-based host depletion, microbial extraction, library preparation and nanopore sequencing as previously described (2, 6, 11) (Figure 1). Every RMg run also included quality controls (no template control (NTC),

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positive control (PC) and a competitive spiked-in internal control (IC) to identify run or single-sample failures and contamination. Method detailed in supplementary material.

Time-point data analysis was performed using an in-house pipeline (https://github.com/GSTT-CIDR/RespiratoryCmg) incorporating taxonomic classification, antimicrobial resistance (AMR) gene detection and sequence-based typing (SBT). The k-mer based classifier Centrifuge (12), was used for microbial identification using the FDA-ARGOS database (13), curated with only respiratory organisms containing 673 microbial sequences.

Reporting of RMg results

Sequencing reports generated at 30 minutes and 2 hours were interpreted following the Standard Operating Procedure (SOP). Bacteria were reported using 30 minutes sequence-data when representing ≥1% total microbial reads and fungi if ≥5 reads were detected, and with Centrifuge score ≥8000. Centrifuge score is defined as the aggregated score of the number *k*-mers from a sequencing read matching perfectly an organism's reference sequence in the database (12). Microorganisms reported from the RMg workflow in this study were referred to as 'respiratory pathogens' or 'pathogens' and they were defined as microorganisms causing respiratory infection in ICU patients. A list of reportable organisms was compiled and followed for reporting. The list was based on previous LRTI studies (1, 2, 12-14) and previous findings from the archives of microbiological culture in the last 5 years collected from the clinical laboratory (Table E1). Organism-specific reporting criteria were also set for certain organisms (such as *E. faecium* and anaerobes) - reporting is described in detail in supplementary material.

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Acquired resistance genes reporting restricted to extended-spectrum beta lactamases in *Enterobacterales*, SCCmec in *Staphylococcus aureus* and Vancomycin-resistance gene clusters in *Enterococcus faecium* were included in 2 hour sequence reports (2).

Clinical sequence reports were uploaded as a pdf-file to the ICU electronic health record after scientific and medical review. Results were also communicated by email or verbally to the duty intensivist and infectious diseases doctor (Figure 1C). RMg performance was compared with routinely requested tests. Discrepant results were investigated using routine 16S rRNA sequencing testing or an in-house pathogen-specific targeted qPCR.

Data Availability

Sequencing data presented in this study are available to the European Nucleotide Archive (ENA) under project number PRJEB59568.

Patient management and impacts of RMg

Clinical, microbiology and antimicrobial prescribing data were collected prospectively from all MV-ICU patients having at least one LRT sample collected during the pilot, alongside RMg. Samples from patients in non-pilot critical care areas were excluded from downstream analysis and data collection. RMg-based antimicrobial treatment changes and findings of infection control or public health importance communicated by email or phone calls the same day. RMg results and impacts were reviewed biweekly by multidisciplinary team of three intensivists, an infectious disease doctor, two microbiologists and a pharmacist. Clinical implications of unexpected or discrepant results or any adverse impacts of interventions in response to RMg results were reviewed.

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Results

Clinical and microbiological characteristics of ventilated ICU patients

172 ventilated patients admitted to ICU during the 15-week period had 422 LRT samples cultured. In total 128/422 (30%) LRT samples from 87/172 (51%) patients had additional RMg testing (Figure 1A). Clinical characteristics of RMg tested patients was similar to non-tested patients apart from more COVID-19 infections (33% vs 21%) and ECMO therapy (38% vs 5%). (Table 1). 9/172 (5%) patients had any LRT sample growing Gram-negative bacteria (GNB) phenotypically-resistant to first-line empiric treatment of hospital-acquired LRTI (piperacillin-tazobactam) and 3 patients had vancomycin-resistant *E. faecium* (VRE) or carbapenem resistant *P. aeruginosa.* No cases of MRSA or carbapenem-resistant Enterobacterales were reported (Table E2).

RMg performance against routine testing

RMg was performed on 128 samples sent for new suspected community-acquired (CA)-LRTI (23%), at the start (29%) or during (34%) an episode of suspected hospital-acquired (HA)-LRTI or for other reasons (14%) (Table 2). 15/128 samples from 7 patients failed QC (Table E3) and so were excluded from further analysis along with 3 samples from 3 patients taken while on non-ICU acute wards. All remaining 110 samples from 77 patients (96 bronchoalveolar lavages (BAL), 3 tracheal aspirates, 6 non-direct bronchoalveolar lavages (NBL) and 5 pleural fluids) were included in clinical impact evaluations, but 11/110 samples (3 NBL and 8 BALs) that were repeat LRT samples during the same infection episode were excluded from RMg performance calculations. Therefore, RMg performance was calculated on the remaining 99 LRT samples (Figure 1A and Data File E1 and E2).

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The median TAT from sample receipt to RMg reporting was 6.7hrs (interquartile range 6.1-7.5 hrs: maximum 30.5 hrs), with 90% having same-day final reports. This compared with verbal communication of interim culture results when available the following afternoon (median 29hrs) and final reports generated at median of 40hrs.

In 39/42 culture-positive samples, RMg was in agreement with culture findings. RMg missed culture-reported organisms in 3/42 samples. All missed organisms were reported as scanty growth by culture (*S. aureus* [P2 and P77] and *K. pneumoniae* [P7] (Figure E1). RMg did not detect clinically-relevant organisms in 46/57 samples reported as 'negative for pathogens' by culture. These included 23/46 samples reported as 'negative' or 'no growth' by culture and 23/46 samples reported positive for commensal or non-clinically-relevant organisms (such as *Candida* spp.) (Figure E1). Clinically-relevant organisms were detected by RMg in 11/57 samples – these included *S. aureus* (n=3), *E. faecium* (n=2), anaerobic bacteria (n=4), *C. striatum* (n=1) and *C. koseri* (n=1).

Based on these findings RMg was 93% (95% CI, 81-99%) sensitive and 81% (95% CI, 68%-90%) specific on a per-sample basis compared with culture, as per UK Standards for Microbiology Investigations guidelines (14). Only 3/11 findings were not confirmed via confirmatory testing (Table E4) which increased specificity of RMg to 94% (95% CI, 83-99%). Overall, 26/99 (26%) samples contained organisms only reported by RMg, of which 15 were otherwise culture positive and 11 culture negative. These included DNA viruses (HSV-1 (n=4) and CMV (n=1)), bacteria (n=8) and bacteria and HSV-1 (n=1) (data file S1)). Considering additional findings reported by RMg only as 'false-positive detections', specificity was 74% (95% CI, 64-82%). Additionally, on a per sample-type basis RMg was

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92% sensitive (95% CI, 79%-98%) and 81% specific (95% CI, 68%-91%) for BALs and NDLs only (n=92/99).

RMg findings were also compared with routine referral 16S rRNA sequencing. In total 75/99 RMg samples also had 16S rRNA sequencing of which 46/75 (61%) were concordant. These included 29/46 true-negative RMg samples for which neither test reported any pathogens. From the remaining samples 21/75 (28%) were discordant and 8/75 (11%) samples both test were in agreement for ≥1 microbial detection (data file E2).

Acquired resistance genes were also reported in 5 samples, *vanA* [P88], bla$_{SHV}$ [P96, P115 and P122] and bla$_{CXT\text{-}M}$ [P117]. RMg missed *K. pneumoniae* bla$_{CXT\text{-}M}$ in one patient [Figure 2 patient D and sample P110) but it was identified by repeat sequencing using MinION flow-cell that has higher sequencing yields (15)(data-file E1).

Impacts on antibiotic treatment

RMg contributed to prescribing decisions in 88/110 (80%) cases (Table 2). In 24 (22%) cases, antibiotics were started (n=10) or escalated (n=14) based on detecting organisms with intrinsic (n=20) or acquired resistance (n=4) to current therapy, the majority (87%) that day (Table 2). In 29 (26%) cases, antibiotics were de-escalated or stopped predominantly the following morning ward round (n=22/29 cases (76%)). De-escalation occurred in 66% (19/29) of cases when RMg detected no clinically-relevant organisms (13 no organisms, 3 upper respiratory tract commensals, 3 *Candida spp*). All de-escalation cases were followed up. One patient had antibiotics re-started to treat *P. aeruginosa* cultured from a respiratory sample taken 3 days after RMg informed stopping of antibiotics [P78]. The patient was

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otherwise progressing well and left ICU few days later. Clinical details of all cases are in data-file E2, with representative timelines for 3 HA-LRTI cases in Figure 2 cases A-C.

For 35 (32%) patients, antimicrobials were not changed but contributions to prescribing decisions were recorded, mostly by reassuring clinicians of no unexpected pathogens. Thereby preventing escalation, particularly in heavily immunosuppressed patients and with persistent inflammation on antimicrobial treatment (n=11). RMg results also prompted early immunomodulation for suspected inflammatory lung conditions (n=7) after excluding pathogens (Table 3).

Anaerobes detected in 12 samples (10 BALs and 2 PFs) were deemed clinically-relevant based on clinical findings and absence of alternative plausible pathogens. Patients with CA-LRTI (n=8) had history of aspiration and patients with HA-LRTI (n=4) had received antimicrobials for between 5-12 days, some of which lacked anaerobic activity. Antibiotics were started (n=4), de-escalated (n=3) or continued (n=5). Conversely their exclusion from a clinically-suspected lung abscess and empyema prompted a diagnosis of lung infarction with hydro-pneumothorax and shortening of planned antibiotic course from 6 weeks to 5 days (Table E5).

22/110 (20%) RMg findings had no recordable impact either because infection was diagnosed at another site (n=8), results were not acted upon (n=6) or decisions were made before results were returned (n=4) (Table 3).

Information for infection control

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Same-day communication of one VRE and two ESBL cases prompted early institution of barrier precautions. The VRE (P88 and Figure 2C) was only cultured from a rectal swab requested during follow up, in response to RMg results. Additionally, *K. variicola* (3 patients) transmission based on overlapping ward-stays was investigated using RMg data. *K. variicola* from 2/3 patients had 18% of genome shared with 99.9999% genetic similarity patient-to-patient transmission (genetic similarity also confirmed by whole genome sequencing of the isolate where 90% of the genome was shared between samples).

Unexpected organisms reported by RMg

RMg reported organisms not generally detectable by tests requested during initial patient investigation in 9 samples that were classified as unexpected. *L. pneumophila* ST1326 (serogroup 10) was an ICU-acquired infection 13 days post-cardiothoracic surgery [P123], confirmed by PCR but not urinary antigen testing. The same sequence type was isolated from the adjacent hand-basin tap water (Figure 2 case A), so new water filters were fitted to prevent further cases. *M. tuberculosis* was detected in a patient admitted with haemoptysis few months after starting anti-tuberculous therapy that was interpret as dead organisms so no action was taken. That sample and 4 further samples were auramine and culture negative. *Tropheryma whipplei* [P103] was detected in a patient with HA-LRTI post thymoma resection prompting ceftriaxone treatment and follow up by infectious diseases but the significance remained uncertain. CMV detection in a patient with Jo-1 anti-synthetase deficiency prompted plasma viral load testing which was positive (log 3.5-3.6). It was considered clinically-relevant and ganciclovir was commenced [P35]. HSV-1 was detected in 5 samples, but all were considered non-pathogenic reactivation.

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Representative cases from 2021/2022 winter season

8 severe CA-LRTI cases were admitted over an 8 week period of which 6 were co-infections with influenza (Table E6A). RMg identified Panton Valentine Leukocin (PVL)-*S. aureus* (3), *S. pyogenes* (2), *S. pneumoniae* (2), *S. dysgalactiae* (1), *L. pneumophila* (1) and *A. fumigatus* (1). Only one streptococcus (*S. pneumoniae*) was cultured. Treatment was escalated in 3 cases that day (addition of linezolid, intravenous immunoglobulin or ambisome (Figure 2E-2H). PVL-*S. aureus* and *S. pyogenes* cases were reported to public health that day. Subsequent analysis of RMg data identified one *S. pyogenes* as *emm1*-M1uk clone and the *S. dysgalactiae* as subspecies *equisimilis* (STG62647A).

16 additional patients had RMg testing of which the most important result was unexpected HSV-2 in a patient with new hepatitis and suspected drug rash post-vascular surgery (Figure 2 case D and table E6). High dose acyclovir was started that day. Subsequent plasma and rash swab samples were HSV-2 positive.

Discussion

There are frequent calls to expedite evaluation of metagenomic testing for acutely unwell patient (16). We need to determine how early comprehensive pathogen information can be made clinically-valuable by improving antimicrobial prescribing and other infection control or public health interventions, rather than the dominant outcome being frequent treatment of clinically-irrelevant components of the microbiome that promotes further antimicrobial resistance. We therefore evaluated RMg here after detailed assessment of performance characteristics, incorporation of quality controls and positivity-thresholds chosen to avoid

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major errors (2, 6). We also provided RMg prospectively alongside the routine microbiology service, interpreted by the ICU infectious diseases consult team and the duty intensivist, so results were incorporated into daily clinical decision making. Microbiology laboratory, infectious diseases, antimicrobial pharmacy, infection control and ICU were all part of the new-service evaluation team to ensure seamless communication of all relevant information between the laboratory and clinical teams. This was combined with oversight from a bi-weekly multidisciplinary review group that monitored prescribing and any unexpected safety signals. Thus, given the complex behavioural framework around interpreting novel molecular tests, balancing concerns to ensure treatment with appropriate antimicrobials without adverse societal impact of driving AMR (17), we concluded it was now both appropriate and necessary to begin evaluating metagenomics in a structured real-world setting.

There were five overlapping categories impacted by RMg. Firstly, earlier provision of results that culture usually provides (median 40 hours vs 6.7 hours from RMg) to improve initial antimicrobial treatment. This occurred in almost half of patients and was predominantly due to species identification rather than acquired resistance genes, which were uncommon in this cohort. Secondly, identification of organisms that are hard to identify by culture or are suppressed by prior antibiotics. Anaerobes with or without *S. milleri* were found in 10% of samples, similar to a previous study using the same service framework (10) and have been identified in other RMg studies (7). Anaerobes have been considered respiratory pathogens (7, 18-20) although their significance has more recently been questioned (21), given recognition they are part of the healthy respiratory microbiome (22, 23). Their absence from microbiology reports due to fastidious culture requirements has prevented detailed clinic-pathological correlation, so this metagenomic approach can prompt reassessment of their significance when identified above reporting thresholds. In cases where they are considered the causative agent there would be opportunity for useful antibiotic de-escalation rather than

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continuing on broad-spectrum antibiotics without an identified pathogen. Thirdly, reporting no (significant) organisms to provide an actionable "negative" result in appropriate clinical contexts, which RMg is uniquely placed to provide. No adverse consequences were identified when de-escalation took place in response to "negative" results, however safe de-escalation always requires close monitoring, confidence in sample quality and an understanding of methods limitations and reporting thresholds. This is particularly relevant in acutely unwell patients or when considering immunomodulation for clinically-suspected inflammatory lung conditions. The fourth category was identifying AMR organisms for infection control. One VRE and 2 ESBL cases prompted early institution of barrier precautions. Detection of the VRE was unlikely without RMg testing. Furthermore, clinical adjudication of discordant RMg cases during bi-weekly review meetings, concluded that no adverse consequences were caused. In particular, RMg false-negative cases (missed bacteria reported as scanty growth in polymicrobial samples) did not cause adverse outcomes. Also, adjudication concluded that the significance of not reporting these organisms was unclear.

These first four categories represent potential improvements to the routine culture pathway, but the final category was identifying organisms currently requiring targeted molecular tests that were not requested by intensivists. Some proved clinically-important such as the CMV, HSV-2 and *L. pneumophila* cases, with the latter prompting interventions to prevent further cases. In contrast, the 5 HSV-1, *M. tuberculosis* and probably the *T. whipplei* cases were not significant; although, all would be in different clinical contexts (24-27). Providing unexpected or un-requested results can prompt unnecessary investigation and treatment; however, identifying benefits in about a third of cases as found here could be considered an acceptable yield.

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Utility of this fifth "molecular" category is extended when the additional information provided by pathogen sequencing is considered. Targeting the water supply as the source of *L. pneumophila*-ST1326 required sequence-based typing, not just organism identification, as did confirming *K. variicola* transmission (2). RMg applied to severe CA-LRTI cases identified virulence factors (*luk f/S*) and emerging virulent clones missed by culture (*S. dysgalactiae* STG62647A and *S. pyogenes emm1*-M1uk) (28). The latter is of particular public health significance given its link with severe paediatric infections and deaths, first announced by UKHSA 8 weeks after this case (29, 30). Finally, demonstrating ability to assess genotypic azole resistance in *A. fumigatus* sequence from RMg data would be a significant improvement to current fungal AMR testing.

This study has limitations. Only 3 samples maximum were processed per day due to limited operator availability so the proportionate impact may reduce, when extended to all respiratory samples. Reported sample-failure rate (12%) was mostly due to a defective DNA-extraction batch or operator-introduced contamination which is a recognised current limitation of RMg (31, 32). This can be addressed by reducing hands-on-time via automation. Only phenotypes from acquired-resistance elements were reported, however, expansion to phenotypic prediction caused by other AMR mechanisms (mutational) to increase RMg-usability in settings with higher AMR rates is required. This workflow is not designed to detect RNA viruses. Therefore assessing a modified version of this workflow that detects RNA viruses, or assessing other workflows that additionally detect RNA viruses are necessary to extract the full value from RMg (33, 34). Finally, due to the lack of a non-infectious control group, the pathogenicity of certain organisms could not formally assessed, beyond considering all available clinical information at the bed-side during their stay on ICU.

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In conclusion, this pilot service demonstrates the clinical utility of RMg testing in a routine setting, reporting organisms usually detected by culture alongside fastidious and/or uncultivable organisms while also providing genomic information for AMR prediction and/or identifying hospital-acquired infections and emerging hyper-virulent community clones aiding infection controls decisions. Realising all these benefits for individual patients and the wider healthcare system will require change to current practice by many professional groups working more closely together in the same acute timeframe (35). Clinical implementation still requires further technology refinement, addressing accreditation and regulatory requirements, along with gathering data from larger multi-centre studies and health-economic analyses. These studies should include comparisons with usual practice and application of RMg to infectious and non-infectious patients to better inform distinction between colonisation and infection and quantify clinical impacts. (32, 36). Nevertheless, given recognised gaps in preparedness highlighted by the COVID-19 pandemic (37-39) and increasing AMR (40, 41), this study gives both encouragement and urgency to introduce metagenomics for evaluation as standard of care in acute care pathways (16, 42).

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Figures

Figure 1. Schematic overview of the study.

Overview of the patient cohort and sample set included in the RMg pilot service (A). The metagenomics regime followed on a daily basis when samples were requested for RMg service. Steps outlined include sample collection until reporting results to ICU physicians (B). Respiratory metagenomic end-to-end clinical pathway (C). MV= Mechanically-ventilated; LRT= lower respiratory tract; RMg= Respiratory metagenomics; QC= quality control.

Figure 2. Patient ICU-timelines illustrating integration of RMg results into antimicrobial treatment and infection control decisions.

Hospital-acquired LRTI

A) ICU acquired *L. pneumophila* ST1326 pneumonia. Unexpected bacteria prompting antibiotic escalation, infection control and public health interventions. B) *P. aeruginosa* VAP-LRTI. New bacterial pathogen in patient with severe COVID-19 pneumonitis prompting antibiotic escalation. C) ICU-acquired vancomycin resistant *E. faecium.* Unexpected AMR-bacteria with patient & infection control impact. D) Unexpected disseminated reactivation of HSV-2.

Community-acquired LRTI

E) Influenza with secondary *S. pyogenes* infection F) Influenza with secondary PVL-MRSA and *S. pyogenes* infection. G) Influenza with secondary PVL-MSSA and *S. dysgalactiae* infection. H) Influenza with secondary invasive aspergillosis prompting urgent treatment. Details or each case are presented in supplementary material.

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Tables

Table 1. Clinical characteristics and routine microbiological testing of the patient cohort during 2021-2022 winter season.

	Patients with RMg (n=87[1])	Patients without RMg (n=85)
PATIENT DETAILS		
Age	53 (39-65)	62 (53-71)
Sex (F)	30 (34%)	27 (32%)
ECMO	33 (38%)	4 (5%)
Reason for admission		
Respiratory infection		
COVID	29 (33%)	18 (21%)
LRTI (other)	20 (23%)	17 (20%)
CAP	7 (8%)	4 (5%)
Medical (non-respiratory)	13 (15%)	14 (17%)
Cardiothoracic surgery	7 (8%)	16 (19%)
Other surgery	7 (8%)	11 (13%)
Septic shock	4 (5%)	5 (6%)
RESPIRATORY CULTURE[2]		
Number of LRT samples	242	180
Gram negative organisms	99 (41%)	76 (42%)
Gram positive organisms	35 (14%)	31 (17%)
Candida spp	85 (35%)	67 (37%)
Candida spp only	50 (21%)	36 (20%)
Aspergillus spp.	2 (1%)	0
No growth or URTF	81 (33%)	48 (27%)

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[1]Clinical details at time of each RMg test presented in data-file E2. These include patients excluded post-downstream data analysis. [2]Organisms cultured from all LRT samples collected during the 15-week study period are in Table E2.

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Table 2. Antimicrobial treatment changes in response to RMg results

Indication for RMg testing	N=128
CA-LRTI	30 (23%)
HA- LRTI	80 (63%)
- Start of episode	37 (29%)
- During episode	43 (34%)
- Other[1]	18 (14%)
Antibiotic prescribing[2]	N=110[3]
Receiving antibiotics start of RMg test day	89 (81%)
Receiving antibiotics end of RMg test day	98 (89%)
De-escalation: antibiotics stopped with RMg[4]	29
- Same day	7 (24%)
- Next day[5]	22 (76%)
Meropenem	15
Piperacillin-Tazobactam	6
Linezolid	5
Other	3
Escalation: antibiotics started with RMg[6]	24
- Same day	21 (87%)
- Next day[7]	3 (13%)
Meropenem	10
Linezolid	4
Other	10

[1] Includes where the focus of infection was uncertain at time of testing, to exclude respiratory infection pre-immunomodulation or after completing therapy for LRTI. [2]15 patients had antibiotics started or stopped for reasons not linked with the RMg result. [3]Excludes samples failed at QC (n=15) or sent from non-pilot critical care areas (n=3). [4]Antifungals were stopped in 3 patients in response to RMg results [5]2 patients had antibiotics stopped on second

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day after testing. [6]Antifungals were started for 5 patients in response to RMg results [7]3 patients had antibiotics started in response to RMg results two days after receipt of the result

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Table 3. Categorising impact of respiratory metagenomic results on antibiotic prescribing

Treatment category	Organisms identified by RMg[1]	Antibiotic changes
START OR ESCALATE TO ACTIVE ANTIMICROBIALS N=24 **[22%]**	AmpC Enterobacterales (9); Anaerobes (3); *Candida* spp (2); P. *aeruginosa* (3); VRE (1); ESBL K. pneumoniae (1); *H. influenza* and *K. variicola* (1)[2]; *H. influenza* and *M. morganii* (1); *E. faecium* (2); C. *striatum* (1)	STARTED: Meropenem (10); Linezolid (3); Linezolid & Anidulafungin (1); Ciprofloxacin (1); Piperacillin-Tazobactam (2); Ciprofloxacin (1); Other (6)
DE-ESCALATE OR STOP N=29 **[26%]**	No significant organisms (29) (comprising no organisms (13), *Candida* spp (3), URTF (3) Anaerobes (3); MSSA (2); *K. variicola* & *H. influenzae* (3); *E. faecalis* (1); *E. coli* (1)	STOPPED: Meropenem (6); Piperacillin-Tazobactam (4); Piperacillin-Tazobactam & Gentamicin (2); Linezolid (1); Meropenem & Linezolid (4); Meropenem & Other (5) ; Co-amoxiclav (1); Ciprofloxacin (1); Levofloxacin (2); Other (3)
PREVENT ESCALATION, REASSURE OR INFORM OTHER THERAPY N=35 **[32%]**	No significant organisms: No organisms (10), *Candida* spp only (3), URTF (2); Anaerobes (6); *K. pneumoniae* (3); Other Enterobacterales (4); MSSA (4); *Candida* spp. [with MSSA] (1), *C. striatum* (1), *E. faecium* (1)	Reassure correct antimicrobial chosen (17); Exclude organisms to bring forward immunomodulation (7). Exclude untreated organisms in complex or immunosuppressed patients (8) Persistent inflammation on current antibiotics (3)

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NO TREATMENT IMPACT N=22 **[20%]**	No significant organisms: No organisms (11), *C. albicans* (2), *K. pneumoniae* (3), other Enterobacterales (3) and 1 each of *P. aeruginosa, E. faecalis,* MSSA (2)	Clinical concern prevented de-escalation in response to RMg result (6); Potential or proven infection at other site (8); Delay returning result (4); Missed information [resistance] (2); quantity of organism (1) organism missed (1)

Impact categories were: I) earlier appropriate antimicrobials where the result prompted changes to existing therapy to target the identified organism(s), II) de-escalating antimicrobials where the result contributed to stopping or narrowing antimicrobial spectrum III) prevent antimicrobial escalation, reassuring that current therapy was appropriate or informing non-antimicrobial treatment therapy and IV) no identified benefit for range of reasons.

[1]Organisms identified by RMg in monomicrobial and polymicrobial LRT samples. [2]*K. variicola* and *H. influenza* grown but susceptibilities not available. *H. influenza* resistant to coamoxiclav but patient improving and extubated so patient completed 5 day course. Coamoxiclav started 2 days later. ESBL= Extended spectrum b-lactamases, MSSA=methicillin-sensitive *S. aureus*, URTF= Upper respiratory tract flora. VRE= Vancomycin-resistant *E. faecium*

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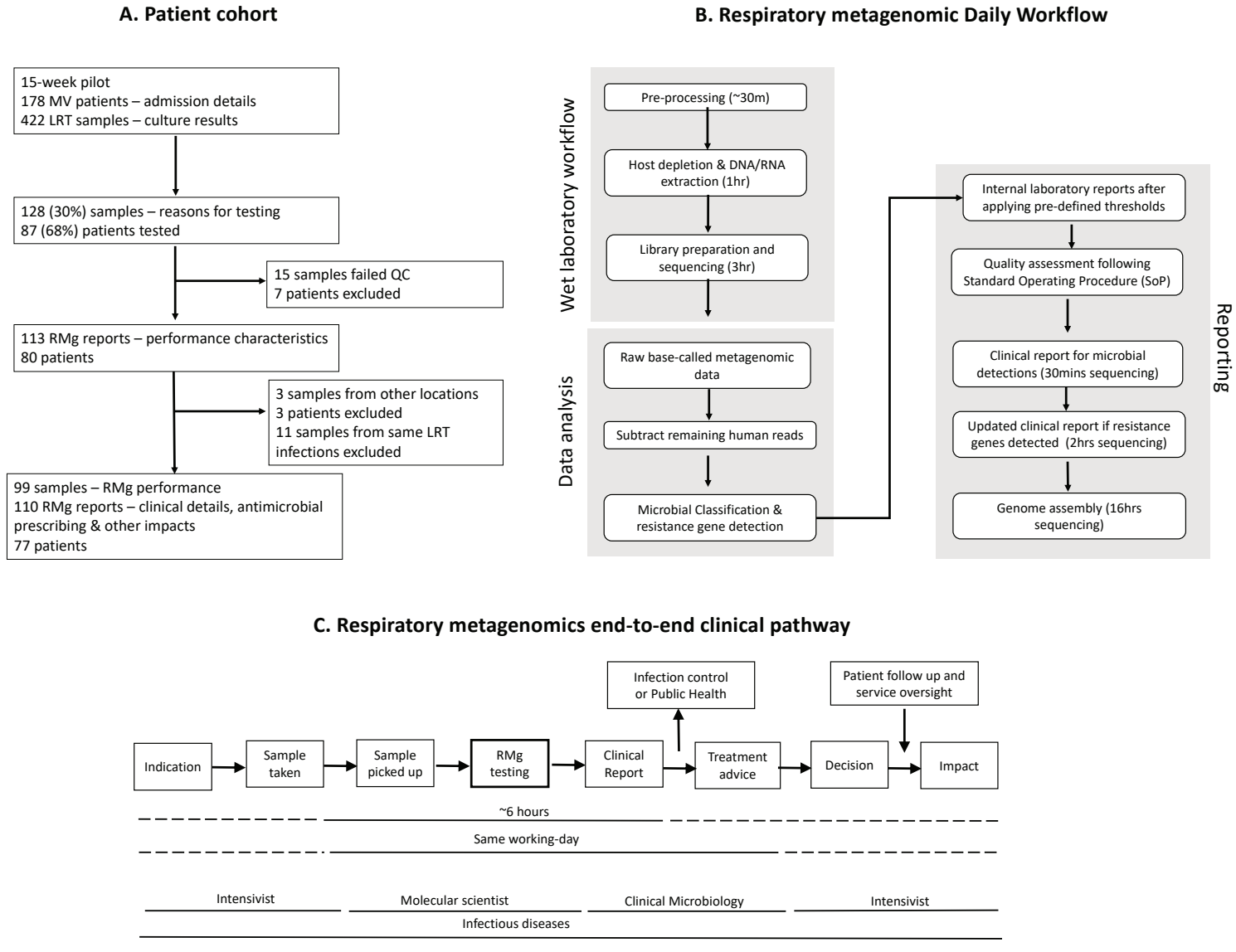

Figure 1

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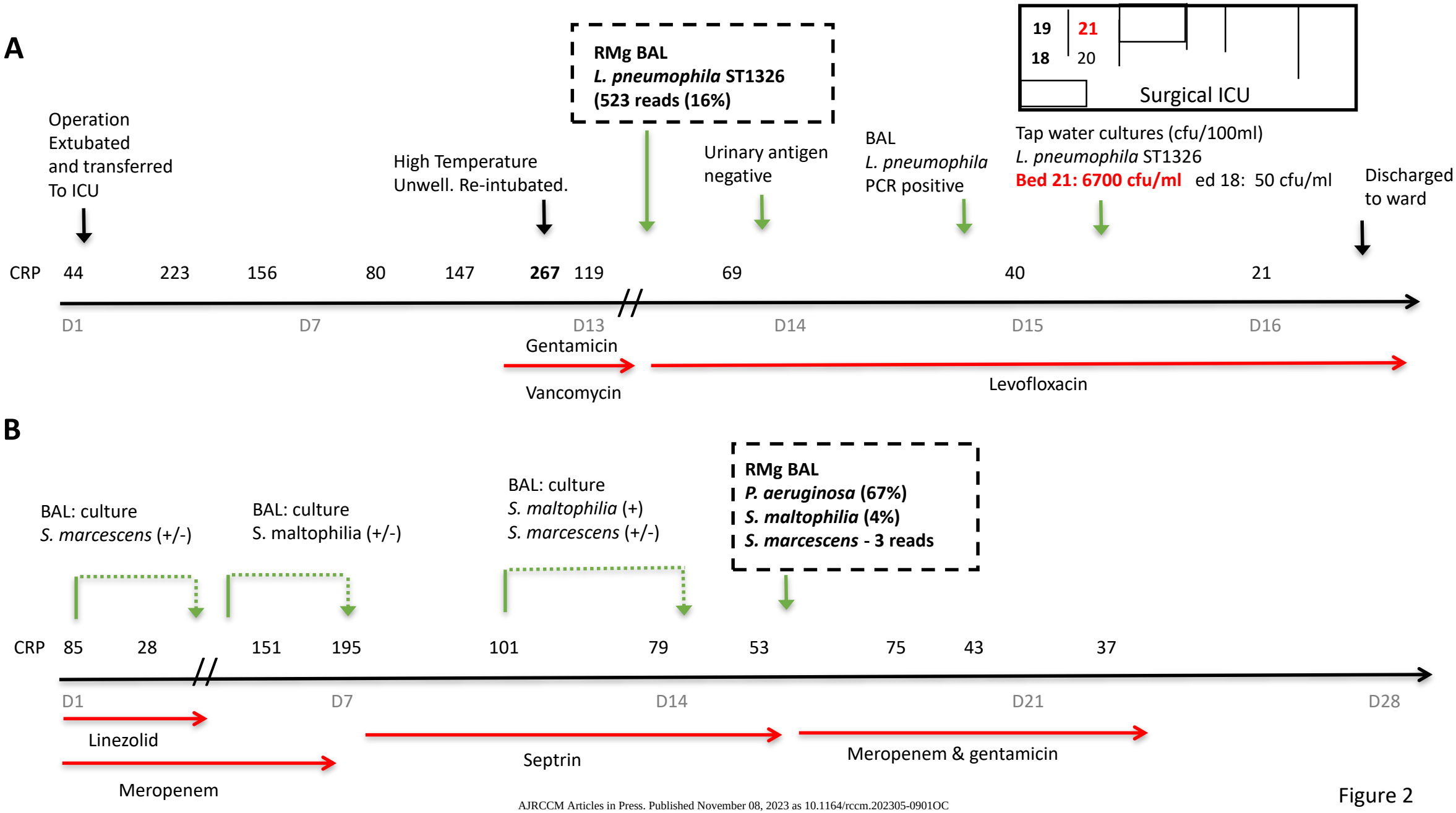

Figure 2

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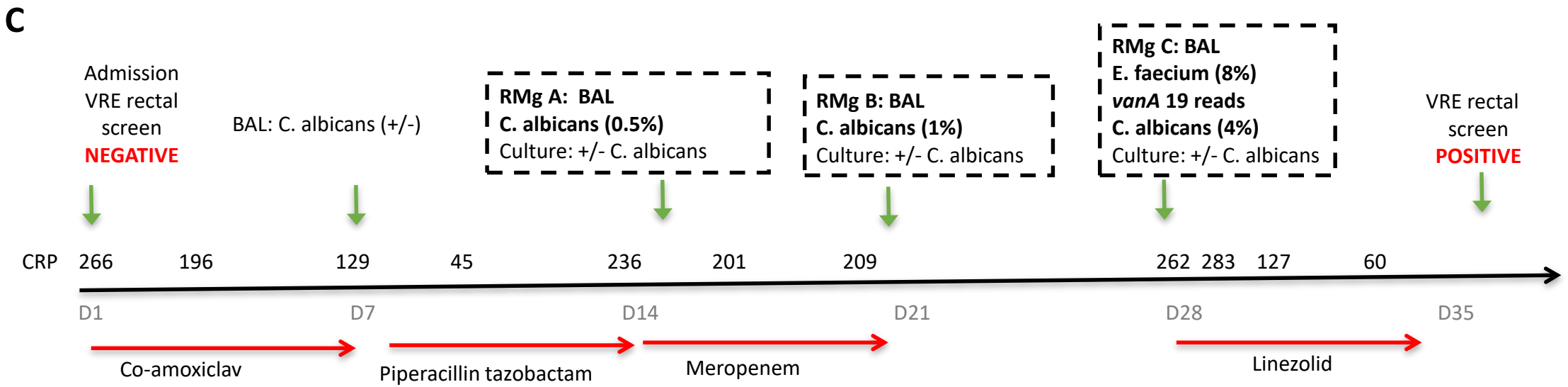

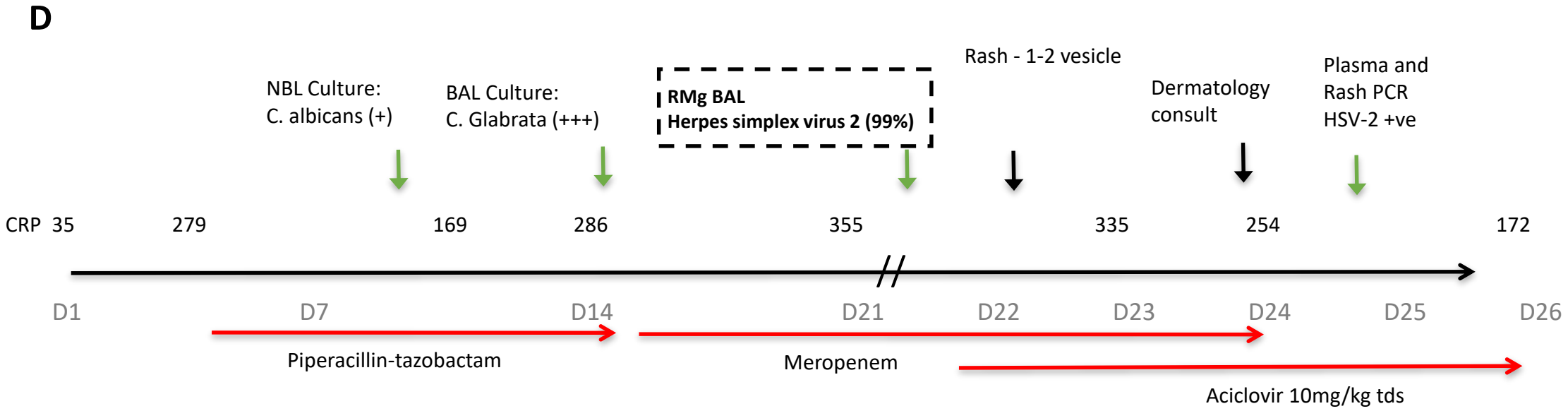

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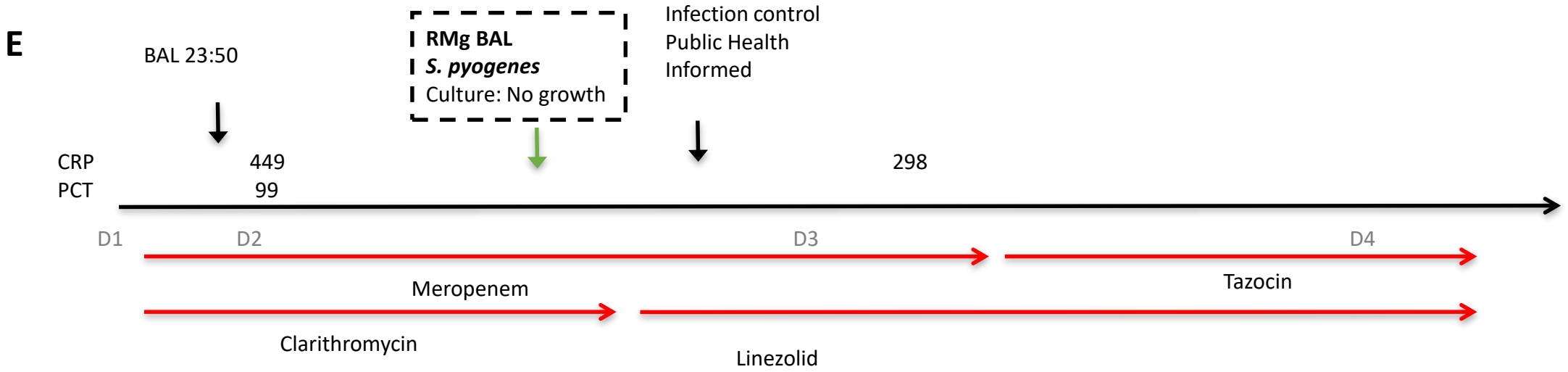

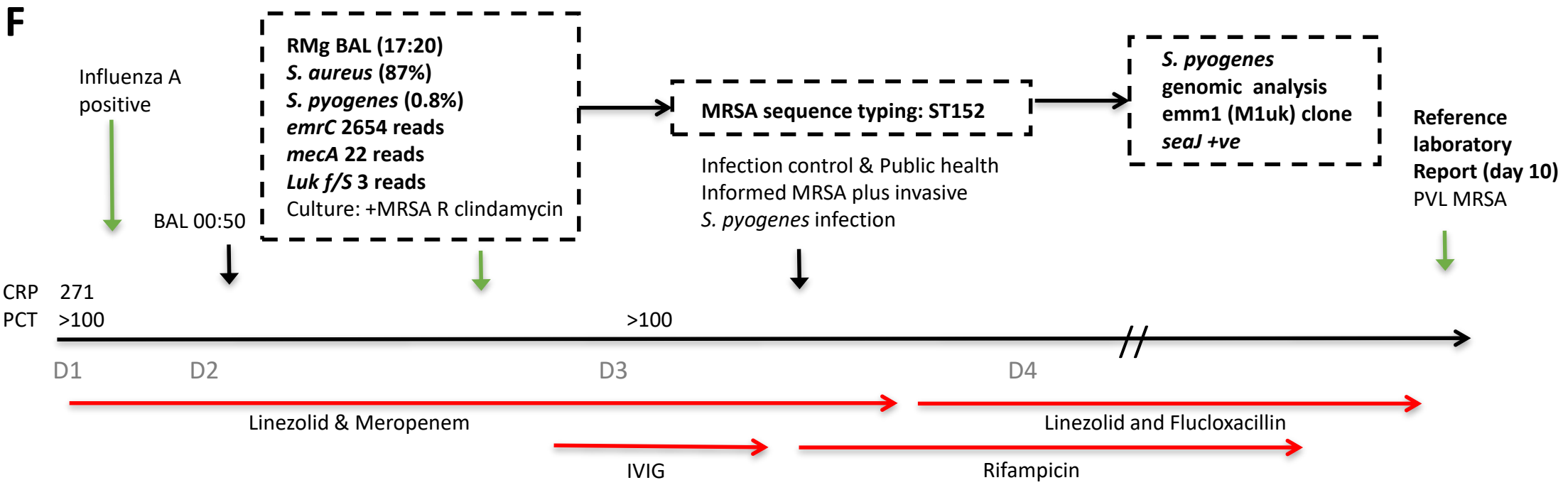

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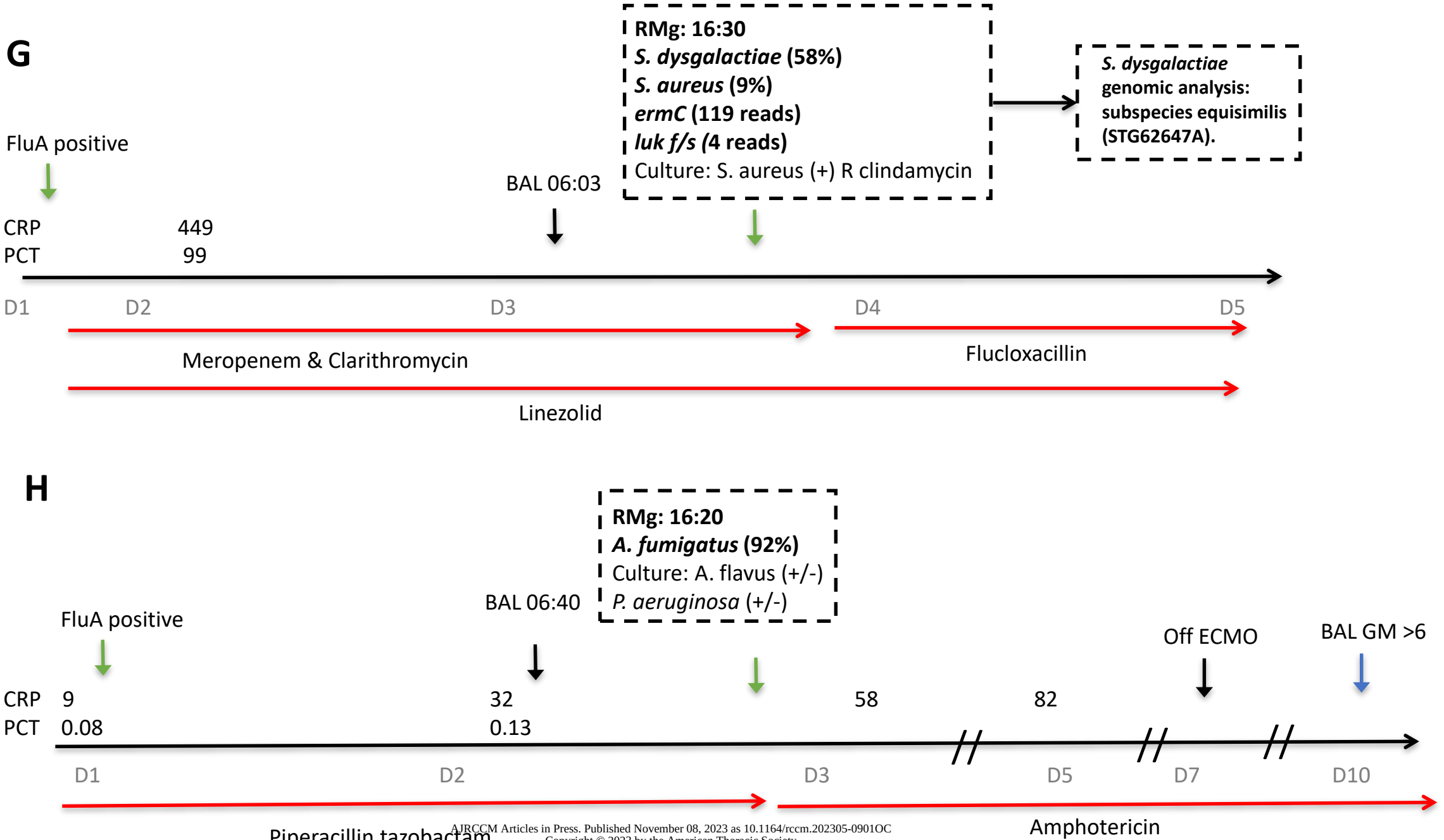

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Routine metagenomics service for intensive care unit patients with respiratory infection

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ONLINE DATA SUPPLEMENT

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Supplementary methods

Hospital governance processes for pilot service provision

A new service evaluation proposal for Respiratory metagenomics (RMg) testing for intensive care unit & ECMO patients) was submitted to the critical care governance committee in September 2021 under the NHS Quality Improvement and Patients Safety (QIPS) initiative. It was submitted by the ICU clinical lead, microbiology diagnostic laboratory and infectious diseases consultant. It included service objectives, supporting published data (1, 2) and experience providing a same-day 16S rRNA sequencing service for respiratory infections during the 2019/2020 winter season (3). It included a Standard Operating Procedure (SOP), data from a pre-pilot testing phase when surplus routine samples were analysed by RMg under research ethics framework and results compared with routine culture and other microbiology tests. The application included description of the end-to-end testing pathway (Figure 1C), result reporting methods, oversight processes including review by a group meeting bi-weekly including intensivists and internal and external clinical microbiologists, and with criteria for halting the service in response to incidents or service failings. Seminars on respiratory metagenomics were given at infectious diseases and intensive care educational meetings prior to starting and questionnaires were conducted for 10 representative scientists and clinicians from the clinical laboratory and the intensive care to understand their views on unmet needs that RMg could meet, their understanding of the methodology and their views on interpretation and potential utility. The service was agreed and commenced on November 22nd 2021. Follow up meetings were held after the first 4 weeks before recommencing in January 4th 2022 and continuing through to 25th March 2022. Surplus samples taken as part of routine clinical care used for confirmatory testing or assay development were used without

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requiring written informed consent with approval from the UK HRA & REC committee (UK HRA & REC reference 20/SC/0310)(E1).

Recommencing pilot service provision during the 2022 winter season

Data from the first winter season service was reported back to the ICU governance group. Absence of severe seasonal CA-LRTI cases was noted due to provision during the unusual setting of the COVID-19 pandemic. They were an important target group identified in the QUIPS submission. The service was therefore restarted on 3rd October 2022 and provided for testing all lower respiratory tract (LRT) samples taken from the ICU admitting severe CA-LRTI patients and that provided ECMO support. Service processes were the same as the previous season but without the bi-weekly oversight group. Samples were multiplexed on MinION flowcells.

Microbiological culture

Microbiological culture for all respiratory samples was performed in an ISO15189-accredited laboratory following standard operating procedures (4). Tracheal aspirates were streaked directly (10 µl of sample) onto blood agar and chocolate agar plates without any prior centrifugation. BALs and NDLs were initially spun down at 1200g for 10 min, with supernatant decanted and sample sediment was retained (~500 µl of sample). Residual sample was re-suspended by vortexing (10 sec), and 10 µl of sample streaked onto blood agar, chocolate agar and fastidious anaerobic agar (FAA). All plates were incubated at 37 °C in an aerobic or an anaerobic environment for 48 h for initial examination. For the detection of *Candida* spp. and *Aspergillus* spp. Sabouraud (SAB) plates were set up and incubated for 5 days at 37 °C in

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aerobic conditions. MALDI-TOF (Bruker) was used to confirm bacterial colonies and microscopy for *Aspergillus* spp. Sample reported 'normal respiratory flora (NRF)' where no pathogenic organism was reported or as 'no growth (NG)' when no organisms were observed after 48 h of incubation were considered as culture-negative samples in this study. Antibiotic susceptibility for any detected pathogens was performed using agar diffusion, following guidelines set up by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology (5).

Routine detection of viral and atypical pathogens

For routine detection of SARS-CoV-2, reverse transcriptase (RT) PCR using the Highplex 24 system (AusDiaganostics Pty Ltd.),was performed by the clinical laboratory according to the manufacturer's instructions (SARS-CoV-2, Influenza and RSV 8-well, Catalogue number: 20081, version: 08). This assay utilises 200 µl of decanted supernatant from respiratory clinical samples targets the Orf1ab and Orf8 of SARS-CoV-2. For the detection of respiratory viruses and atypical pathogens such as *Legionella pneumophila*, multiplex RT-PCR was performed by the clinical laboratory upon request. Summary of the viral PCR testing results are in Table E7.

Routine testing of 16S bacterial rRNA gene sequencing

Samples requested for 16S bacterial rRNA gene sequencing were referred to the Great Osmond Street Hospital (GOSH). Briefly, extracted bacterial DNA was subjected to PCR amplification of the 16S rRNA bacterial gene followed by sequencing of the PCR product using an Illumina platform.

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Galactomannan

For galactomannan (GM) requests the clinical laboratory referred samples to the Mycology Reference Laboratory National Infection Services, UKHSA at Southmead Hospital, Bristol where the Platelia *Aspergillus* Antigen kit (BIO-RAD –62794) is used. Sera and BALs were subjected for GM detection following manufacturer's instructions.

RMg Sequencing workflow

RMg workflow consisted of three aspects: host DNA depletion, microbial DNA extraction and sequencing was performed based on previously published (1, 2) and patented methods (6). In total 128 samples were processed for RMg sequencing which included 111 bronchoalveolar lavages (BAL), 3 tracheal aspirates, 8 non-direct bronchoalveolar lavages (NDL) and 6 pleural fluids (PF). Sample anonymization was done prior to RMg processing (winter 2021-2022 samples = P1-P128 and winter 2022-2023 samples= CS016, CS026, CS034, CS045, and CS053).

Initially, mucoid respiratory samples only, were sputasol-treated (SR0233 - Oxoid) in a 1:1 ratio for 15 min at 37°C to homogenise samples. For non-mucoid samples, 1 ml of sample was used for RMg sequencing.

At this step a competitive spiked-in internal control (IC) was introduced in each sample at the beginning of each run along with a no-template-control (NTC) and a positive control (PC) with all controls processed through the full pipeline. For NTC, sample was replaced with nuclease free water (NFW) and introduced to monitor barcode cross-talk and laboratory and/or reagent contamination. For the IC, 10^4 cfu/ml of *Jonesia denitrificans* (JD)) (NCTC

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10816) was introduced into each patient sample – this allowed identifications of individual sample failures. Spiked quantity of JD was predetermined after serial dilutions of the organism were spiked in culture-positive and culture-negative samples. Chosen quantity allowed identification of no-growth samples without affecting detection of potential pathogens in positive samples (data not shown). For the PC, 10^4 cfu/ml of JD was introduced in a PBS control which acted as a positive control to monitor whole run failures. An external positive control (EPC) was also introduced when a new batch of reagents was used and was processed in parallel with patient samples. For the EPC 10^6 cfu/ml of environmental Gram-positive (*Aneuribacillus aneurinilyticous*: ATCC 12856) and Gram-negative bacteria (*Listonella pellagium*: NCTC 11316) and yeast (*Zygosaccharomyces rouxi*: NCPF 3879) were introduced into a PBS control. The EPC was used to ensure efficiency of new reagents. Following sputasol treatment, samples were centrifuged at 12,000xg for 5 min and supernatant was then carefully decanted leaving ~50 µl and pellet for host depletion step. For host depletion, 200 µl of PBS and 200 µl of HL-SAN buffer (5M of NACL and 100mM of $MgCl_2$) were added along with 40 µl of saponin (working stock of 1% saponin (Sigma – 47036-50G-F)) and 10 µl of HL-SAN DNase (Articzymes – 70910-202). Samples were incubated on a thermomixer at 37°C shaking at 1000 rpm for 10 min to induce host cell lysis and digest of newly-released host DNA. Samples were then washed with 800 ml of PBS and centrifuged (12,000 xg for 3min) to pellet microbial organisms.

For microbial cell lysis pellet was re-suspended in lysis buffer (800 µl; Roche UK) followed by bead-beating (Lysis Matrix E beads (MP Biomedicals) and 3 min at 50 o/s on Human Tissue Lyser, Qiagen) to release microbial DNA. Centrifugation (1 min at 18,000xg) and removal of ~200 µl supernatant were then followed. Next, supernatant was treated with proteinase K (20 µl; Qiagen UK) for 5 min at 65°C shaking at 1000 rpm; on a thermomixer to

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digest residual proteins followed by DNA extraction using the Fast Pathogen 200 protocol on a MagNA Pure 24 System (Roche UK).

Prior to library preparation samples and controls were subjected to individual 1.2X AMPure XP bead wash and eluted in 15 µl of NFW. For each daily run (which included ≤3 samples plus controls) library preparation was performed using the Rapid PCR Barcoding Kit (Oxford Nanopore Technologies (ONT)) as previously described (1, 2) with following alterations; for the PCR reaction Taq DNA polymerase (PrimeSTAR® GXL DNA Polymerase TaKaRa) with double reaction volumes was used with the following conditions; initial denaturation at 98 °C for 2 min, cycling conditions for 35 cycles were denaturation at 98 °C for 15sec, annealing at 56 °C for 15sec, extension at 68 °C for 45 sec °C and final extension at 68 for 4 min. Following PCR reaction, samples and controls were subjected to individual 0.6X AMPure XP bead wash and eluted in 14 µl of buffer recommended by the manufacturer. Finally, samples and controls were prepared for singleplex sequencing using nanopore flongles (ONT) with sequencing performed on the GridION platform (ONT). Samples processed during the 2022-2023 winter season were multiplexed (≤ 4 samples plus controls) and sequenced on a MinION flowcell on the GridION platform. Raw sequence data were acquired using the ONT MinKNOW software (version 21.05.12) with live basecalling by ONT Guppy (version 5.0.16+b9fcd7b) using the barcode-at-both-ends parameter to ensure high quality barcode assortment.

Sequencing was run for 24 hours with the first 30 min data used for pathogen identification and 2 hours data used for AMR gene detection using an in-house developed bioinformatics pipeline (https://github.com/GSTT-CIDR/RespiratoryCMg). Prior to analysis, human reads were discarded by alignment with genome reference (GCA_000001405.15, assembly GRCh38.p13 version) and non-human reads were exported and used for pathogen

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identification and AMR gene detection (described below). See Figure 1 for a schematic workflow of the RMg method.

Library preparation PCR products and 0.6X bead wash eluates were assessed by DNA quantification using the high sensitivity dsDNA assay kit (Thermo Fisher) on the Qubit 3.0 Fluorometer (Thermo Fisher) prior to nanopore sequencing.

Analysis of metagenomic data

Determining parameters for microbial classification

Microbial reads were classified against a custom microbial database using Centrifuge ((7)v1.0.4) with default parameters. Reads mapping to a single organism were binned into species-level taxonomic groups for abundance estimation. Reads mapping to multiple organisms were aligned to their respective genome assemblies with minimap2 ((8), v2.18) with the read assigned to the taxonomic group with the best alignment based on BLAST identity score.

Classification score and abundance thresholds used in this study were determined using the dataset published by Charalampous *et al*. 2021 (3) as the training set. A series of abundance thresholds (≥ 0.1%, 1%, 2%, 5%, 10%) and classification scores provided by Centrifuge (≥2000, 8000, 16000) were tested to determine the best thresholds for bacterial detection to use in order to ensure the best performance for detection of true-positive findings whilst reducing identification of false positive and false negative findings.

For fungal and yeast detection a more sensitive threshold was required. A threshold of ≥5 for *Aspergillus* and *Candida* spp. classified reads was used to determine any sample positive for yeast or fungal organisms. This was based on previous knowledge that the clinical microbiology laboratory tests utilise more sensitive detection limits for fungal and yeast

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organisms – if a single *Aspergillus* colony is identified on the cultivated plate then sample is considered positive for *Aspergillus*. This differs from the more stringent detection of microbial culture used for bacterial organisms (~10^2 bacterial cfu/ml – i.e. one colony identified on a plate previously streaked with a swab taken from an undiluted LRT sample). Applying a less-stringent threshold provided the highest sensitivity while avoiding false positive detections.

The parameters chosen were then tested on a number of samples (n=18) and quality controls were processed in a pre-pilot phase prior to the pilot study and results were compared to routine culture results (Table E8). Sensitivity and specificity was calculated on a per sample basis (14) using the Clopper–Pearson exact method (https://www.medcalc.org/calc/diagnostic_test.php) (Table E9).

Based on these findings in order to not compromise sensitivity and specificity of the method the chosen single-read centrifuge classification score was >7999 with reads with <8000 centrifuge score removed from further analysis. Similarly, bacterial organisms represent ≥1% of total passed microbial reads. Fungi and yeast were reported if ≥ 5 passed reads with a >7999 centrifuge score were identified. In addition to this, *S. pyogenes, M. tuberculosis* and *L. pneumophila* were reported if ≥ 10 passed reads with a >7999 centrifuge score were identified.

Database for microbial classification

The FDA-ARGOS microbial reference database (9), was used to create the Centrifuge index used for microbial identification. The FDA-ARGOS genomic database utilises numerous quality-control metrics based on the regulatory-grade reference genome criteria that a successful genome entry would require. For example, required coverage for genome assembly is 95% with 20X depth at every position across the assembly (9). A list of

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clinically-relevant organisms was compiled based on previous microbiology results reported in the past 5 years. Based on this list the database was further curated with organisms either added or removed from the database (https://github.com/GSTT-CIDR/RespiratoryCMg). Genome assembly data retrieved from Refseq database (9, 10) and Dustmasker (version 2.10.1) was used to mask low complexity regions. The "centrifuge_build" tool from Centrifuge was used on default parameters to create the index. The database consists of 673 entries which includes 624 bacteria, 3 DNA viruses, 45 fungi and yeast and 1 protozoa ((https://github.com/GSTT-CIDR/RespiratoryCMg).

Misclassification rate

The misclassification of reads to closely-related species is a common bioinformatics problem in metagenomics. To address this, a selection of commonly-found organisms in LRT samples (*Enterobacter cloacae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, Klebsiella variicola, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mitis, Streptococcus oralis, Streptococcus pneumoniae*) were chosen and data from isolate whole genome sequencing (WGS) were retrieved from NCBI (Table E10). Data was subsampled to 200mbases of sequencing reads and then processed using the in-house metagenomics pipeline to assess the True positive (TP) and false positive (FP) rate.

A truth set was derived from mapping reads to reference genomes using minimap2. Reads were given a classification category based on the concordance (TP, True Negative (TN)) or discordance (FP, and False Negative (FN)) between the in-house metagenomics pipeline and minimap2 (Table E10). The percentage of TP classified reads was ≥ 93% for *E. cloacae* (94.6%), *E. faecalis* (95.1%), *E. faecium* (94.5%), *E. coli* (94.0%), *K. aerogenes* (94.5%), *K.*

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pneumoniae (93.7%)*, K. variicola* (96.1%)*, S. aureus* (96.6%)*, S. epidermidis* (94.2%) and *S. pneumoniae* (99.0%), whilst a lower percentage of TP classified reads of ≥70% was achieved for *S. mitis* (70.0%), *S. oralis* (79.9%) and *K. oxytoca* (77.9%). For *S. mitis, S. oralis* the percentage of TP reads at the genus level increases to 90.2% and 98.2% respectively, indicating that the majority of misclassified reads happen at an intra-genus level. *K. oxytoca* contained a high number of reads classified as TN (12.1%) indicating a large level of contamination in the dataset.

Therefore in order to address the misclassification rate observed, the rate of TP classified reads were used in the algorithm development for realignment of misclassified reads within our metagenomic datasets. Reads were first binned into genus-level groups and the abundance of each species calculated within these bins. The most abundant species for each genus bin were identified, and assigned the reads for that genus if its genus-level abundance was higher above the threshold determined above. If the genus-level abundance for the top species fell below this threshold, then the next most abundance specie(s) were added until the threshold was met. Reads within the bin were then distributed proportionally across these organisms.

Reads were determined to be TP if they mapped to the correct species using the in-house metagenomics pipeline and mapped in minimap2. TN reads were identified if they were classified as a different species with the in-house metagenomics pipeline and not mapped in minimap2 and reads were determined as FN if they mapped to a non-target species in with the in-house metagenomics pipeline but mapped in minimap2. Finally, reads were determined to be FP if they mapped to target species with the in-house metagenomics pipeline but not mapped in minimap2)**.**

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Phenotypic prediction based on detected resistance genes

Phenotypic prediction was performed based on the detection of resistance genes using 2 hours of sequencing data. For the detection of antibacterial resistance genes Abricate (11) and Scagaire (12) were used both with default parameters. Briefly, basecalled and demultiplexed FASTQ files were converted into FASTA files and were analysed using Abricate, to detect resistance genes against the Comprehensive Antibiotic Resistance Database (CARD) (13). Next, based on the Abricate output, Scagaire was used to predict and include only clinically-relevant genes based on the pathogen identified by RMg. Scagaire uses a bundled database consisted of the 40 most common-sequenced bacterial species present in the RefSeq database and is designed to only report clinically-relevant resistance genes. Clinically-relevant gene alignments were reported only if >1 gene alignment was detected with <90% gene coverage to ensure exclusion of any possible bioinformatics errors.

This analysis was only carried out to determine presence or absence of acquired genotypic determinants conferring resistance to antibiotics used on the ICU. These included (i) genes conferring resistance to beta-lactams in Enterobacterales (ii) presence of *mecA* genes in *S. aureus*-positive samples which would confer resistance to Methicillin and (iii) presence of *van* gene clusters in *E. faecium*-positive samples which would confer resistance to Vancomycin (Table E11).

Reporting of detected organisms and resistance gene

The pipeline generated sequencing reports at three time intervals (30min, 2 hrs and 16 hrs of sequencing) which were interpreted following the Standard Operation Procedure (SOP) for reporting metagenomics results previously formulated by clinicians and scientists.

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The sequencing report was divided into three sections, (i) sample identifiers, (ii) quality control measurements, (iii) microbial organisms and resistance genes detected above pre-defined thresholds and (iv) full list of classified organisms and AMR genes detected before applying pre-defined thresholds.

The reporting SOP described quality control thresholds and positivity thresholds established using pre-existing RMg data produced from our workflow. Initially, each sample was assessed based on performance, quality and contamination rate. Samples with <100 total reads and/or <10 microbial classified reads after 2hrs of sequencing were failed and repeated on the next day if residual sample was available. Samples with common contaminant reads representing ≥10% of total microbial classified reads were considered contaminated and also failed. Samples with ≥100 reads after 2hrs and <10% contamination rate were passed and reported accordingly based on the organisms and resistance genes detected. For samples where only the IC control organism (JD) was detected were reported as negative.

Quality controls introduced at the beginning of each sequencing run were also assessed. For the PC, *J. denitrificans* reads were recorded after every daily run during the pre-pilot and pilot phase in order to establish a cut-off demonstrating a successful run. This was used as quality control on the 30 min reports. As per latest analysis, PC control with ≥100 classified *J. denitrificans* reads and were consisted 90% of the total microbial classified reads would pass the quality control (QC) check. For the NTC, any reads identified in the NTC were considered contaminants and if were present in samples were also considered contaminant reads and were excluded. Common contaminants in our dataset mainly included environmental organisms such as: *Moraxella osloensis, Cutibacterium acnes* and *Acinetobacter johnosonii* and *E. coli.* As *E. coli* was a common contaminant in our dataset, a sample was only considered *E. coli*-positive only if *E. coli* reads were more abundant than the

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reads assigned to the IC. This allowed the identification of true-positive findings whilst eliminating contaminant reads.

A list of reportable organisms was compiled and followed for reporting. The list was based on previous lower respiratory tract infections studies (1, 2, 14-16) and previous findings from the archives of microbiological culture in the last 5 years collected from the clinical laboratory (Table E1). Microorganisms reported from the respiratory RMg workflow in this study were referred to as 'respiratory pathogens' or 'pathogens' and they were defined as microorganisms causing respiratory infection in ICU patients.

Detected respiratory pathogens samples processed in the pilot study were: *Aspergillus flavus*, *Bulkhoderia* spp., *Citrobacter koseri*, *Citrobacter freundii*, *Escherichia coli*, *Enterobacter cloacae* complex, *Fusobacterium nucleatum*, *Hafnia alvei*, *Klebsiella aerogenes*, *Klebsiella michiganensis, Klebsiella oxytoca*, *Klebsiella pneumoniae, Klebsiella variicola, Legionella pneumophila, Morganella morganii*, *Mycobacterium tuberculosis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Stenotrophomona maltophilia*, *Staphylococcus aureus*, *Streptococcus pneumoniae and Tropheryma whipplei*.

Reporting criteria for certain organisms were also followed based on criteria set by the clinical laboratory for microbiological cultures. Anaerobes were only reported if above pre-defined thresholds in BALs and PFs. *Candida* spp. is often detected in LRT samples but considered a common lung coloniser and not an infectious agent (17). Hence, *Candida* spp. were reported following fungal-specific thresholds but were not defined as a 'pathogen' in this study. Clinical-relevance of certain organisms such as *Corynebacterium striatum* and *Enterococcus faecium,* was also unclear. Hence, *C. striatum* and *Enterococci spp.* along with other organisms with the "if predominant" flag in Table E1 were only reported to the clinical team if they were the highest abundant organism with respect to normal flora detected within the sample.

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Sequence-based typing of organisms for infection control purposes using sequencing data

Downstream analysis was performed for organisms identified from metagenomic sequencing that were suspected in transmission events. The analysis included reference-based alignment against a representative genome based on organism of interest, generation of consensus sequence using aligned reads, SNP distance calculation between the consensus sequences (1). For this analysis 24hrs of sequencing RMg data were used and genomes were considered related if a genetic similarity of ≥99.99% was reported https://nanoporetech.com/accuracy. Pathogens from patients not included in the study but were suspected to be part of an outbreak were isolated, extracted and subjected to whole genome sequencing as previously described (1) to investigate genetic similarity from pathogens identified in samples processed with RMg.

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Supplementary Results

Limit of Detection (LoD) of the RMg workflow

We previously reported that the analytical LoD of the RMg workflow is dependable on the human/microbial DNA levels present in the each sample (LoD=range of 10^3-10^5 c.f.u/ml for respiratory samples) (2). However, we sought to ensure that the introduction of the IC organism did not affect the analytical LoD of the method. Therefore, to determine the analytical LoD of the method, an uninfected 'upper respiratory tract flora' (URTF) BAL sample was used. Serial tenfold dilutions of Gram negative (*K. pneumoniae*), Gram positive (*S. aureus*) and a yeast (*Candida albicans*) were introduced to the aliquoted triplicates of the BAL sample. The IC organism was also introduced in pre-determined cell-quantities (10^4 c.f.u/ml) in each replicate. The LoD (≥2/3 positive replicates) for both bacterial organisms (*K. pneumoniae* and *S. aureus*) and the yeast (*C.* albicans) in the BAL sample was determined to be 1000 (10^3) cells after applying pre-defined thresholds. Hence, the analytical LoD of the method was determined to be 1000 c.f.u/ml for bacteria and yeast (Table E12).

Pre-pilot runs

In total 18 samples spiked with the IC organism were depleted and sequenced along with QC as previously described. The pre-pilot was carried out to ensure all aspects of the workflow which includes host depletion, library preparation, nanopore sequencing and data analysis were performing well (see figure 1).

In this sample set 13/18 samples were concordant with the culture including 11 culture-positive samples and 2/14 reported as culture-negative or with not-clinically significant

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organisms by culture (Table E8A). The remaining samples (n=5) were deemed discordant. These included two samples were RMg missed the culture-reported organism (PR9 and PR12) and two culture-negative samples where metagenomics identified additional organisms – Vancomycin-resistant Enterococcus faecium (VRE) in PR6 and Streptococcus pneumoniae in PR14. Both additional findings were confirmed by additional testing. The last discordant sample did not produce enough reads during RMg sequencing so it was deemed as a failure.

The pre-pilot dataset was used as the testing dataset to test positivity thresholds and quality-control rules. Also, analysis of the quality controls revealed that *E. coli* contamination was common in the pre-pilot sample set, hence the reporting rule for *E. coli* was used as described previously – *E. coli* would only reported if is present at a higher abundance from the internal control organism in clinical samples (Table E8B). Additionally, applying QC rules, allowed identification of failed sample (PR13 had <100 total reads after 2hrs of sequencing) and failed positive control. Only one PC failed – PR-C7 had <100 total reads after 2hrs of sequencing.

Technical failures and excluded samples

In total there were 128 respiratory samples processed with the RMg workflow. However, 15/128 (12%) samples failed during QC and where excluded from the study (Table E3). The majority of the samples failed due to microbial reads being ≤10 reads after 2hrs of sequencing and hence failed QC checks and were excluded from reporting and downstream analysis (n=12/15 samples). The increased failure rate was caused by using a faulty batch of extraction kit (confirmed by the manufacturer) that resulted to a tenfold microbial loss after extraction. The batch was used during the pilot for the first two months which possibly led to the failure of 9/15 samples. Majority of the samples were also reported as ‘no organisms

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detected' by microbiological testing which reflected that microbial loss caused from faulty extraction batch could have led to the samples below the LoD of the RMg workflow. Also, samples considered as part of the same infection episode collected from the same patient were excluded for RMg performance characteristics. A new infection episode was designated based on clinical judgement having resolved and antibiotics stopped. Based on this, 11/128 samples were excluded from RMg performance calculations.

Supplementary figure footnotes

Supplementary footnote to patients' timelines for Figure 2.

A) ICU acquired *L. pneumophila* ST1326 pneumonia. Unexpected bacteria prompting antibiotic escalation, infection control and public health interventions.

55-60 year old woman post aortic and mitral valve replacement. Extubated post operation but increasing secretions and heart failure requiring re-intubation. Sepsis was considered a contributory factor, with empiric vancomycin and gentamicin started due to penicillin allergy. Two days later temperature had increased to 38.5, inotropes started and CT chest showed new consolidation. RMg identified *L. pneumophila* at 30 minutes sequencing. The patient had been started on levofloxacin prior to communication due to continued deterioration. There was sufficient sequence for MLST at 8 hours to identify a non-serogroup strain based on detecting 5 of 7 MLST gene (flaA[3], PilE [10], asd [-], Mip [28], mompS [-], proA [9], neuA [207]). Sample was confirmed by local PCR and culture, but not urinary legionella antigen testing, with typing confirmed on a cultured colony from the respiratory sample as

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ST1326 by reference laboratory after 7 days. Infection control was informed on day of RMg result leading to declaration of a serious incident and urgent water sampling. *L. pneumophila* ST1326 found in surgical ICU tap water sent to the public health reference laboratory with highest concentration in water from tap adjacent to patient bed space [21]. Water filters were fitted onto all ICU water taps pending definitive resolution.

B) *P. aeruginosa* VAP. New bacterial pathogen in patient with severe COVID-19 pneumonitis prompting antibiotic escalation

40-45 year old man who was SARS-CoV-2 unvaccinated with no past medical history or risk factors for severe COVID-19. Referred for ECMO few days after ICU admission with severe COVID-19 pneumonitis and a right hydro-pneumothorax and 2 intercostal drains. Admission BAL grew only scanty *S. marcescens* so linezolid was stopped and meropenem continued. A repeat BAL taken few days after ICU admission reported scanty S. maltophilia on Day 6 and with CRP increasing meropenem was changed to septrin (the *S. marcescens* was also reported as septrin susceptible). Tracheostomy performed and further intercostal drain inserted and patient made little progress with drains intermittently blocked. Repeat BAL for culture again identified the *S. maltophilia* and *S. marcescens*. RMg was performed two weeks post-admission to inform whether to either prolong the septrin course or escalate treatment. Identification of *P. aeruginosa* by RMg informed change to meropenem and gentamicin. *P. aeruginosa* was not grown from the contemporaneous sample submitted for culture that day but was grown from a follow up BAL. The patient made steady progress over a prolonged ICU stay leading to successful discharge. Escalation to appropriate antibiotics informed by same day RMg identification of *P. aeruginosa* was recorded as a significant benefit by the MDT at the time.

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C) ICU acquired vancomycin resistant *E. faecium.* Unexpected AMR-bacteria with patient & infection control impact.

80-85 year old woman with emergency abdominal aortic aneurysm. Long term smoker, transferred to ICU for ventilation and renal replacement therapy required post-operation. Commenced on co-amoxiclav escalated to tazocin after CT chest showed bronchiectasis and consolidation. Inflammatory markers increased on tazocin so escalated to meropenem despite culture and RMg-A only showing *C. albicans*. Repeat testing (RMg-B) showing only *C. albicans* informed stopping meropenem, but patient failed to progress with CRP increasing. Chest was again considered the most likely focus so RMg-C was performed to guide therapy. Detection of VRE prompted monotherapy with linezolid associated with CRP fall from 262 to 60 and clinical improvement. The significance of VRE as a cause of VAP was debated but for this patient the RMg result was considered to have a positive impact based on the clinical response to linezolid. Infection control were informed of the result and contact precautions were instituted along with repeat VRE rectal screening that was positive.

D) Unexpected disseminated reactivation of HSV-2

65-70 year old man was admitted for elective thoraco-abdominal aorta replacement. He had cardiac arrest in theatre and massive blood transfusion. He had no history of immunosuppression. He was initially extubated but then re-intubated in the surgical recovery unit around day 5 and commenced empirically on piperacillin-tazobactam for presumed HA-LRTI. A BAL taken at intubation grew *C. albicans*. He initially improved but then deteriorated ~two-weeks post-surgery with new pyrexia (40^{o}C) and was transferred to the ICU where a repeat BAL was taken and he was empirically started on meropenem and anidulafungin. The BAL grew *C. glabrata*. He did not progress and was reported as being

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obtunded when sedation was lightened and had a new maculopapular rash on abdomen and thighs. He also had persistent temperature with increasing inflammatory markers with bilirubin raised at 113 and ALT at 106. A BAL was sent for RMg as part of a septic screen to investigate an infectious cause, although HA-LRTI was not considered the most likely explanation. When HSV-2 was identified (99% of reads), a decision was made to start high dose acyclovir and a plasma HSV PCR was requested. Dermatology review suspected a drug rash and meropenem was stopped. The rash was reviewed by virology and a vesicle identified which was swabbed along with the surgical wound. Plasma, wound and vesicle swabs were all HSV-2 PCR positive. The patient gradually improved over the following days and CRP falling from 335 to 172.

E) Influenza with secondary *S. pyogenes* infection not detected by culture

65-70 year old man with no past medical history presented with history of cough and sore throat. Intubated on arrival in emergency department (ED) and transferred to referral hospital ICU but failed conventional mechanical ventilation that day so transferred to St Thomas' for ECMO. Upon arrival he was shocked but passing urine and had high inflammatory markers. An influenza PCR was positive. He was stabilised, treated with zanamavir, meropenem, clarithromycin and anidulafungin and a BAL was performed at 08:50 for RMg and culture. The RMg report was communicated at 17:20 with 13 reads of *S. pyogenes*. It was reported to intensivists who started linezolid that night and stopped clarithromycin and anidulafungin. Infection control and public health was told of an invasive *S. pyogenes* infection that day. The BAL and blood cultures taken from admitting hospital did not grow *S. pyogenes*. There were insufficient reads after 16 hour sequencing to perform genomic typing.

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F) Influenza with secondary PVL-MRSA and *S. pyogenes* infection, the latter not detected by culture

50-55 year old man with no past medical history presented with breathlessness, cough and chest pain. Intubated on arrival in ED and transferred to referral hospital ICU but failed conventional mechanical ventilation that day so transferred to St Thomas' for VVA ECMO. He arrived in septic shock and multi-organ failure with rising INR and low platelets. An influenza PCR was positive. He was stabilised and treated with zanamavir, meropenem, linezolid, clarithromycin and anidulafungin. A BAL performed on next day in the morning was sent for RMg and culture. The RMg report was communicated in the afternoon identifying *S. aureus* and *S. pyogenes*. There were 22 reads of mecA that informed communication of an MRSA and 3 reads of luk F/S which informed communication of the MRSA being PVL positive. These results were communicated to hospital infection control team that day. ermC and FusC were noted but not communicated to the clinical team in line with the SOP. The patient was commenced on intravenous immunoglobin that day with further modifications to therapy over the following days. The following morning after 16 hour sequencing the MRSA was identified as ST152 and the full results were communicated to public health. Final culture result was communicated on day 5 as MRSA resistant to clindamycin and fucidic acid. The MRSA was sent to the reference laboratory for PVL typing that confirmed on day 15. 8 weeks later UKHSA reported an increase in invasive *S. pyogenes* infections. *S. pyogenes* sequence from the 16 hour report were therefore reviewed and it was

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identified as an emm1-M1uk clone, which has been associated with severe disease in this outbreak.

G) Influenza with secondary PVL MSSA and S. dysgalactiae infection the latter not detected by culture

50-55 year old man with Crohn's disease on azathioprine was unwell while on holiday and transferred by ambulance. On arrival in the UK he was initially transferred to an ICU for conventional mechanical ventilation but when this failed he was referred for VVA ECMO. On arrival he was shocked with multi-organ failure. An influenza PCR was positive. He was treated with Zanamavir, Meropenem, Linezolid, Clarithromycin and Anidulafungin. A BAL could not be taken on the first full day of admission due to continued haemodynamic instability but was performed the following day in the morning and sent for RMg and culture. The RMg report was interpreted as containing *S. dysgalactiae* and *S. aureus*. No *mecA* genes were detected but there were of luk F/S gene alignments were detected. PVL MSSA and *S. dysgalactiae* and were communicated in the afternoon. 119 ermC reads were noted but not reported. Meropenem and Clarithromycin were stopped the following morning and the patient was started on Flucloxacillin in addition to continuing the linezolid and anidulafungin. The PVL–MSSA was communicated to infection control and public health. Culture of the sample reported only MSSA resistant to clarithromycin. Following identification of *S. pyogenes* in case G as an emm1-M1uk clone, the S. dysgalactiae reads from the 16 hour report were reviewed and identified as subspecies equisimilis (STG62647A), which has been identified as associated with severe disease.

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H) Influenza with secondary invasive aspergillosis prompting urgent treatment

30-35 year old woman with a history of asthma presented to local hospital with history of cough and green/rusty sputum and influenza PCR-positive on admission. She was treated with co-amoxiclav and clarithromycin but progressed to mechanical ventilation which failed after few days prompting referral for ECMO. Culture of respiratory sample at referral hospital was negative. On arrival for ECMO, she was haemodynamically stable and commenced on piperacillin-tazobactam and zanamavir. Secondary infection was not clinically suspected. A BAL was taken on the morning of the first full day on ICU. RMg results were communicated in the afternoon as containing *Aspergillus fumigatus* based on presence of 9852 reads after 30 minutes sequencing. She was commenced on ambisome. And piperacillin-tazobactam was stopped the following morning. The following morning full *Aspergillus* genome was assembled with 82% coverage at 10x depth. Two mutations in Cyp51A (R279T and L272I) not associated with azole resistance were detected (https://sbi.hki-jena.de/FunResDb/) however this was not communicated to the clinical team. On the 5th day the culture report was scanty *Aspergillus flavus* and scanty *P. aeruginosa*. The significance of *P. aeruginosa* was unclear and the patient had continued to improve with de-cannulation targeted for few days later but piperacillin-tazobactam was started empirically. Voriconazole was commenced and the ambisome stopped when voriconazole levels were in the therapeutic range and the patient had been decannulated from ECMO. The reference laboratory confirmed *A. fumigatus* susceptible to Ambisome and Voriconazole.

Footnote to clinical metadata file E2 columns U-Z, AA and AB

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Column Q: CA-LRTI was designated when RMg samples were taken as part of investigation for the community-onset infection episode (Mostly day 1-3 of ICU admission but up to day 5). HA-LRTI (new) was designated when RMg was performed at the start of a new infection episode or HA-LRTI (D), when RMg was performed during an episode of HA-LRTI to investigate suspected failure of therapy or to inform whether to stop or change antimicrobials. The "unclear or other" focus was where CA-LRTI or HA-LRTI were not the primary clinically suspected focus but was part of the differential and when results from RMg would be taken into account when deciding on treatment.

Columns R-T were the antimicrobials prescribed at the start of the day and those that were either started or stopped in response to the RMg result either that day or the next day. Antimicrobials stopped or started for other reasons were not included. There was usually no consensus on the reasons for commencing antifungal therapy linked with identification of *Candida spp* in respiratory samples and their prescription was only recorded where there was a majority agreement that they were significant and the RMg result was the main reason for commencing or stopping treatment.

Column U: Brief details from discussion at time of result communication or as recorded in clinical notes at that time. Reasons for submitting samples for RMg and prescribing decisions made were not assessed against formal criteria for diagnosing CA or HA-LRTI. Interpretation of the significance of RMg identified organisms was discussed with the infectious diseases team when the result was returned but the final decision including prescribing decisions were made by the intensivist. Their decisions made in real-time were recognised to be challenging and based on sometimes incomplete information in acutely unwell patients whose condition changed rapidly. This was particularly relevant to COVID-19 patients who were often heavily immunosuppressed and had prolonged stay on ICU. Serum CRP levels are presented

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because they were frequently used to assess patient trajectory during a septic episode and part of the decision on interpreting significance of microbiological results.

Column V: Escalate (E) and de-escalate (D) was based on starting (column S) or stopping (column T) antibiotics based on RMg results. It was recognised that some opportunities for de-escalation were not taken given this was a new test under evaluation. Any bacteria subsequently identified in a respiratory sample on ICU after de-escalation was in column Q or column W. Reassure (R) was based on there either being a plan to escalate that was prevented by the RMg result or when intensivists stated when requesting RMg testing that they wanted confidence antimicrobials were active against the organisms in the LRT, or to ensure there was no new organism when the patient was not responding as expected. No impact was recorded when RMg result were not taken into account in making decisions, either because of delay or when the result was not acted on or did not address the need.

Column W: Comments made after follow up or at the bi-weekly service review group that considered subsequent course of patients who had antibiotics de-escalated or where there was discrepancy between RMg and culture results. Intensivists also had opportunity to explain reasons why they or their colleagues concluded that results were either reassuring (R) or of no benefit (N). There was opportunity for external microbiologists to challenge conclusions and categories. Consensus was reached in most cases and where not an explanation was recorded. Examples of discussion on how results informed the potential utility beyond antimicrobial prescribing and for future implementation were also recorded.

Figure E1. Performance of RMg workflow against conventional testing

Performance of RMg workflow against microbiological findings from the routine laboratory and breakdown of organisms detected by metagenomics in the 2021-2022 sample cohort.

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Table E1: List of pre-defined organisms* and reference for each organism

Bacteria	Report	Reference
Acinetobacter baumannii	Y	(14-16)
Burkholderia spp	Y	(16)
Burkholderia cepacia	Y	(16)
Citrobacter fruendii[1]	Y	(14, 15)
Citrobacter koseri[1]	Y	(14, 15)
Klebsiella aerogenes[1]	Y	(1, 14-16)
Enterobacter cloacae[1]	Y	(1, 14-16)
Enterococcus faecium	Only if predominant	Microbiological archives
Enterococcus faecalis	Only if predominant	Microbiological archives
Escherichia coli[1]	Y	(1, 14-16)
Fusobacterium necrophorum[2]	Only if predominant	Microbiological archives & (16)
Fusobacterium nucleatum[2]	Only if predominant	Microbiological archives &(16)
Haemophilus influenzae[1]	Y	(1, 14, 16)
Hafnia alvei[1]	Y	Microbiological archives
Klebsiella oxytoca[1]	Y	(1, 14-16)
Klebsiella pneumoniae[1]	Y	(1, 14-16)
Klebsiella variicola[1]	Y	(14-16)
Legionella pneumophila	Y	(14-16)
Moraxella catarrhalis[1]	Y	(1, 14-16)
Morganella morganii[1]	Y	(14-16)
Proteus mirabilis[1]	Y	(1, 14-16)
Pseudomonas aeruginosa	Y	(1, 14-16)
Serratia marcescens[1]	Y	(1, 14-16)

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Stenotrophomonas maltophilia	Only if predominant	(1, 2, 16)
Streptococcus agalactiae	Only if predominant	(14-16)
Streptococcus pneumoniae[1]	Y	(2, 14-16)
Streptococcus pyogenes	Y	(1, 14-16)
Streptococcus viridans group[2] *(S. anginosus, S. intermedius, S. constellatus)*	Y	Microbiological archives & (16)
Staphylococcus aureus[1]	Y	(1, 14-16)
Tropheryma whipplei	Only if predominant	Microbiological archives
Mycobacterium tuberculosis	Y	Microbiological archives
Mycobacterium non-tuberculosis	Y	Microbiological archives
Corynebacterium striatum	Only if predominant	Microbiological archives
Fungi		
A. fumigatus	Y	(16)
A.niger	Y	Microbiological archives
A. flavus	Y	Microbiological archives
A. versicolor	Y	Microbiological archives
A. nidulans	Y	Microbiological archives
A. glaucus	Y	Microbiological archives
A. terreus	Y	Microbiological archives

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A. clavatus	Y	Microbiological archives

*All organisms listed were also identified in the archives of reportable organisms by microbiological culture in the last 5 years in the clinical laboratory.
[1] For tracheal aspirates these organisms were only reported if were predominant in comparison to normal respiratory flora (NRF). Organisms considered part of the NRF were: *Neisseria* spp., haemolytic *Streptococci*, diphtheroid-like bacteria and coagulase negative *Staphylococci.*
[2] These organisms were only reported if identified in BALs, NDLs and PFs.

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Table E2. Microbiological culture results from all respiratory samples from all patients admitted during the pilot study period

Bacteria in 442 respiratory samples from the whole 172 patient cohort	Number of patients with ≥1 positive sample	Number (%) of patients with ≥1 sample having acquired β-lactam resistance (%)[1]	Phenotypic acquired β-lactam resistance
P. aeruginosa	18	5 (3%)	MEP (2) PTZ, CTZ (1) PTZ, MEP (1) MEP, PTZ, CTZ (1)
E. coli	16	9 (5%)	COAM (7); COAM, PTZ (1) ESBL (1)
K. pneumoniae	16	6 (4%)	COAM; COAM, PTZ (3) ESBL(2)
Other *Klebsiella spp* [2]	8	0	0
C. koseri	8	1	CFX, PTZ (1)
S maltophilia	5	0	0
H. influenza	5	2	COAM (2)
P. mirabilis	1	1	COAM (1)
Other *Enterobacterales*[3]	26	3	ESBL (1) COAM, PTZ (1)

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			CTZ, PTZ (1)
Other GNB[4]	4	N/A	N/A
Patients with Gram negative bacteria (GNB) in any sample	107 (62%)	25 (23%)	COAM (15:9%): PTZ (9:5%)
S. aureus (MRSA)	20 (0)	N/A	N/A
E. faecium (VRE)	10 (3)	N/A	N/A
E. faecalis	6	N/A	N/A
Other Gram positive	5	N/A	N/A
Patients with Gram positive bacteria (GPB) in any sample	38 (23%)	N/A	N/A

88 (21%) samples were taken during the first 48 hours of admission and 334 (79%) taken >48 hours after admission. Other phenotypically reported acquired resistance [1] Quinolone resistance in 6 patients: *P. aeruginosa* (3), *E. coli* (1), *M. morganii* (1) C. koseri (1). Aminoglycoside resistance in 1 patient (*E. col*i) [2] *K. variicola* (5) *K. oxytoca* (3) [3] *Serratia spp.* (9) *Enterobacter spp.* (7); *M. morganii* (2); *H. alvei* (2) [4] 2 *Acinetobacter spp*. (2) *Achromobacter spp.* (1) *Elizabethkingae spp.* (1). Meropenem (MEP), piperacillin-tazobactam (PTZ), ceftazidime (CTZ), co-amoxiclav (COAM), CFX (cefuroxime), Extended-spectrum beta-lactamases ((ESBLs) (2 bla_{SHV} and 2 $bla_{CXT\text{-}M}$))

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Table E3. Pilot samples failed to pass quality checks

Sample ID	Total reads	Human reads (2hrs)	Total Microbial Reads (2hrs)	Microbial classified (2hrs)	Reported Organisms /IC above threshold (2hrs)	Classified reads (2hs)	Routine Microbiological Testing
P10	18309	18294	15	6	*K. pneumoniae* *J. denitrificans*	4 2	*K. pneumoniae* (S)
P12	19085	19074	11	2	None	0	*S. aureus* (S)
P22	14802	14777	25	10	*J. denitrificans*	10	No organisms detected
P36	0	0	0	0	None	0	*C. krusei* C. albicans
P37	0	0	0	0	None	0	No organisms detected
P42	56	40	16	11	None	0	No organisms detected
P43	30	6	24	20	*J. denitrificans*	17	URTF
P48	9846	9787	59	9	None	0	No organisms detected
P51	8401	8366	35	10	None	0	No organisms detected

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P52	35497	2648	32849	32159	*J. denitrificans* *A. johnsonii* *C. acnes* *E. coli*	29272 1573 404 402	*S. marcescens* (S)
P59	579	576	3	3	None	0	No organisms detected
P61	19959	635	19324	18876	*J. denitrificans* *A. johnsonii* *C. acnes* *F. periodonticum* *E. coli*	16068 1214 699 391 281	No organisms detected
P104	103	39	64	51	*J. denitrificans*	16	No organisms detected
P107	10667	10601	66	1	None	0	No organisms detected
P114	347	160	187	184	*J. denitrificans*	176	*K. pneumoniae*

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Table E4. Breakdown of discordant results, confirmatory analysis on discordant findings and outcome.

Detections pre-confirmatory analysis				**Confirmatory Analysis**		
Sample ID	RMg Detected	Culture	Outcome	Additional Clinical Microbiology Testing	Previous culture	Outcome Post-confirmatory testing
P26	URTF Mixed anaerobes *Streptococcus pneumoniae*	*Candida albicans* URTF	FP	*Streptococcus* spp. *Prevotella* spp. identified by 16S rRNA sequencing *S. pneumoniae* detected by culture subsequently	Negative	TP
P40	*Staphylococcus aureus*	*Candida albicans*	FP	*Staphylococcus aureus* 16S rRNA sequencing	*Staphylococcus aureus*	TP
P50	*Staphylococcus aureus*	*Candida albicans*	FP	*Staphylococcus aureus* 16S rRNA sequencing	*Staphylococcus aureus*	TP
P65	Mixed Anaerobes URTF	Negative	FP	*P.micra* detected in PF by culture subsequently	None	TP
P66	*Citrobacter koseri*	Negative	FP	Not requested	*Citrobacter koseri* *Klebsiella pneumoniae* *Enterococcus faecalis*	FP
P83	*Staphylococcus aureus* *Candida albicans*	*Candida albicans*	FP	16S rRNA sequencing detected *Staphylococcus aureus*	*Candida albicans*	TP

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P88	VRE *Candida albicans*	*Candida albicans*	FP	Positive rectal screening for VRE	*Candida albicans*	TP
P94	URTF Mixed anaerobes	URTF and GPC in the gram stain	FP	Not requested	None	FP
P102	*Corynebacterium striatum* *Candida albicans*	*Candida albicans*	FP	16s rRNA sequencing detected *Corynebacterium striatum*	*Candida albicans*	TP
P106	*Enterococcus faecium*	Commensals	FP	*Enterococcus faecium* (positive in-house qPCR assay)	*Staphylococcus. epidermidis*	FP
P128	URTF Mixed anaerobes *Candida albicans*	URTF	FP	Not requested	*Tropheryma whipplei* *C. albicans*	FP
P2	Negative	*Staphylococcus aureus*	FN	positive qPCR assay for *Staphylococcus aureus*	Negative	FN
P7	*Candida albicans* *Candida dubliniensis* *Klebsiella pneumoniae* (below thresholds)	*Klebsiella pneumoniae*	FN	16S rRNA sequencing detected *Enterobacteriaceae*spp.	*Klebsiella pneumoniae* *Candida dubliniensis* VRE *Staphylococcus epidermidis*	FN
P77	Negative	*Staphylococcus aureus*	FN	Not requested	Negative	FN

TP= True Positive, FN= False negative, FP= False Positive, TN=True Negative, VRE= Vancomycin resistant *E. faecium*

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Table E5A. Presenting characteristics and treatment decisions in response to metagenomic results in patients with CA-LRTI and with comparison to culture.

HA-LRTI: Clinical Details	**Metagenomic Result**	**Culture**	**16S rRNA Sequencing**	**Antibiotics Pre-test**
45-50F CAP Empyema CRP >400	**PLEURAL FLUID (P78):** *S. constellatus (44%)* *P. micra (13%)* *S. anginosus (4%)* *S. intermedius (4%)* *F. vaginae (2%)*	*S. constellatus* (+++) *Prevotella buccae* (+++)	Not requested	Empiric co-amoxiclav. Prevented early escalation
20-25M Trauma to chest and abdomen from car accident. ECMO aspiration.	**BAL (P87):** *F. gonidiafoemans* (34%) *P. melaninogenica* (10%)	*S. aureus* (+/-)	*Fusobacterium* spp. *Prevotella* spp.	Piperacillin-tazobactam (Extended from 3-7 days)
40-45F mixed overdose. Found unconscious and aspiration	**BAL (P23):** *P. nigrescens (34%) P. melanogenica (23%) P. micra (5%)*	URTF	*Streptococcus spp* *Enterococcus spp* *Prevotella spp*	Empiric tazocin. Prevented escalation
80-85M Emergency surgery for hip fracture with MI. CRP 206	**BAL (P81)**: *P. micra (47%) S. anginonsus (13%) Bacteroides uniformis (6%)*	*S. milleri* (prolonged culture)	*Bacteroides spp.* *Prevotella spp.* *Pyramidobacter spp.*	Co-amoxiclav course extended from 5 to 7 days. Prevented escalation

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to 370. ? new LRTI				
56-60M Decompensated alcoholic liver disease with empyema	**BAL (P85)**: *P. micra (77%), S. meyeri (9%): F. nucleatum (5%) F. canifelinum (2%) S. intermedius (2%) C. albicans (0.1%)*	*S. milleri* (+/-) *C. albicans* (+/-)	*Fusubacterium nucleatum*	Empiric tazocin de-escalated to co-amoxiclav and fluconazole added
70-75M MI. Reintubated after early self-extubation. T38.4	**BAL (P94):** *R. mucilaginosa (54%), N. mucosa (15%), S. viridans (4%), P. melaninogenica (4%), S. odontolytica (4%), P. aeruginosa (3%) S. sanguinis (2) S. gordonii (1.3%), P nigrescens (1%)*	URTF (+)	Not requested	Empiric co-amoxiclav. Continued. Prevented escalation
40-45F Sore throat followed by CAP with empyema. PCT>100 ?*S. pyogenes*	**BAL (P65)**: *P. micra (44%) P. nigrescens (3%)* **PLEURAL FLUID (P70):** *P. micra (81%), P. nigrescens (8%) P. melaninogenica (2%), F. nucleatum*	BAL: No growth PLEURAL FLUID: *P. micra (*prolonged culture)	P65: Not requested P70: *Prevotella spp*.	Excluded S. pyogenes. De-escalated meropenem & Linezolid to co-amoxiclav (with metronidazole 7 days)

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	(0.2%)			
35-40M COVID-19. ECMO. PCT>100 Suspected lung abscess discharging into pleural space	**BAL (P69)**: *C. dubliniensis* **BAL (P71):** No organisms	BAL & PLEURAL FLUID: *C. dubliniensis* (+/-)	P69: *Corynebacterium spp.* P71: *Corynebacterium jeikeium*	Excluded abscess. Meropenem & Linezolid de-escalated to coamoxiclav for 5 days*

**C. dubliniensis* was considered to have contaminated the lower respiratory tract and was not treated.

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Table E5B. Presenting characteristics and treatment decisions in response to metagenomic results in patients with HA-LRTI of anaerobes and with comparison to culture

HA-LRTI: Clinical Details	**Metagenomic Result**	**Culture**	**16S rRNA Sequencing**	**Antibiotics Pre-test**	**Antimicrobials and Clinical Progress**
40-45M COVID-19 pneumonitis. Day 33 ECMO. CRP 4 to 124 over 2 days CT – Lung infarct or abscess	**BAL (P19):** *F. nucleatum* (66%): *F. canefelinum (29%): C. albicans* – 1 read	*C. albicans* (+/-)	*F. nucleatum & Prevotella spp*	No for 13 days then meropenem for 2 days before RMg	Metronidazole added. Prolonged course.
70-75M Day 17 post thymomectomy. T38.4 and CRP 50 to 279. ?HAP	**BAL (P128)** :*S. oralis (37%): Streptococcus spp (16%); S constellatus (7%): S. anginosis (6%) E. corrodens (3%): Veilonnella parvula (3%) P. melanogenica (2%) F. nucleatum (1%)*	*C. albicans* (+)	Not done	No for 5 days and ceftriaxone before that	Not started day of result. Following day increased oxygen requirement and plugging. More septic. Metronidazole started then co-amoxiclav added. Extubated
45-50M 9 days COVID-19 pneumonitis in hospital before	**BAL (P26):** *N. mucosa (17%): P. melanogenica (11%): S.*	*C. albicans* (+/-); URTF (+/-)	Streptococcus spp, H. parainfluenzae & Prevotella spp	No for 5 days then co-amoxiclav	Linezolid added prior to methylprednisol

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ECMO. CRP 12. RMg to exclude pathogens before treating with methylprednisolone.	*pneumoniae (3%): E. corodens (2%): S. mitis* (1.7%): <10 reads multiple other anaerobes: *C. albicans* – 3 reads.			started 2 days before RMg	one. Good response
55-60M Alcoholic liver disease. Intubated 10 days. Persistent pyrexia and no progress.	**BAL (P98):** *R. mucilaginosa (3%): S. anginosus (3%): S. oralis (0.4%):* <10 reads multiple other anaerobes.	*C. dubliniensis* (+/-); URTF (+/-)	Not done	No for 7 days then ciprofloxacin started day before	More septic on ciprofloxacin. PCT increase from 0.6 to 2.2. Changed to tazocin. Extubated 3 days later

**C. dubliniensis* was considered to have contaminated the lower respiratory tract and was not treated.

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Table E6A. Organisms reported by RMg and routine testing from 8 CA-LRTI infections during the second pilot period

RMg reported	**Number**	**Reported by routine requested tests**
S. pyogenes	2	0
S. aureus[1]	3	3
S. pneumoniae	2	1
S. dysgalactiae	1	0
H. influenzae	1	1
A. fumigatus	1	1[2]
L. pneumophila	1	1 (PCR-detected)
Other[3]	7	5
TOTAL	**18**	**12**

Routine testing was culture apart from *L. pneumohila* (PCR)

[1] Two with *SCCmec* and all with *luk F/S.* [2] Reported as *A. flavus* by routine laboratory but confirmed as *A. fumigatus* by reference laboratory. [3] *Candida spp* (3), *E. coli* (1*), S. marcescens* (1), *K. pneumoniae* (1), HSV-1 (1). The significance of these organisms remained uncertain.

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Table E6B. Organisms reported by RMg and routine testing from 16 HA-LRTI* infections during the second pilot period

RMg reported	**Number**	**Reported by routine requested tests**
Candida spp	6	5
K. pneumoniae	3	1
Herpes simplex virus [2]	3	0 [2]
Other	4	4
TOTAL [3]	**16**	**10**

Routine testing was culture apart from Herpes simplex viruses (PCR)

*RMg was performed on 17 samples but only 14 had parallel samples sent for culture and are presented in this table. [2] Two HSV-1 and one HSV-2, the latter clinically significant (Figure 2 case D). Routine PCR testing was not requested prior to reporting RMg result although the test was available. [3] *E. faecium* (VRE), *S. marcescens,* anaerobes, *A. baumanii* - all single detections.

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Table E7: Summary of viral PCR results reported by routine microbiology laboratory

Viral Target detected	**Number of Positive samples**
Sars-CoV-2	49
Enterovirus/Rhinovirus	1
Respiratory Syncytial Virus (RSV)	1
Other[1]	7
Negative/Not requested	62/8
TOTAL [2]	**128**

[1]5 samples were positive for Cytomegalovirus, 1 was positive for Epstein Bar Virus and 1 was positive for *L. pneumophila* (included in the respiratory PCR panel).

[2] Total number includes samples not included in the RMg performance calculations (n=29/128).

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Table E8A. Metagenomic data^ from samples processed during pre-pilot phase

Sample ID	Total No reads	Microbial reads	Human reads	Detected Organisms/IC above threshold	Organism/IC reads	Organism reported by culture	Comments
PR1	6629	5087	512	*P. aeruginosa* *Serratia marcescens* *A. xylosoxidans* *J. denitrificans*	3183 1453 301 75	*P. aeruginosa* *S. marcescens*	Concordant
PR2	25524	4181	20898	*P. aeruginosa* *C. freundii* *S. maltophilia* *J. denitrificans*	3130 569 326 49	*P. aeruginosa* *S. maltophilia*	Concordant
PR3	15634	14951	149	*Pseudomonas aeruginosa*	14792	*P. aeruginosa*	Concordant
PR4	25598	1607	23434	*Rothia mucilaginosa* *S. anginonsus* *S. viridans* *Candida tropicalis* *Jonesia denitrificans*	324 66 52 44 1076	*C. tropicalis*	Concordant
PR5	20507	7080	13072	*H. influenza* *E. coli* *A. fumigatus*	3010 2349 4	*E. coli* *H. influenzae* *A. fumigatus*	Concordant

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PR6	36980	12991	3737	*E. faecium* *J. denitrificans*	6934 1858	Commensals	Discordant – FP
PR7	32767	13779	16701	*P.aeruginosa* *S. marcescens* *A. xylosoxidans* *J. denitrificans*	3902 3570 3856 2263	P. aeruginosa *S. marcescens*	Concordant
PR8	18823	16241	18	*P.aeruginosa* *A. xylosoxidans* *B. cenocepacia* *J. denitrificans*	15545 212 132 26	P. aeruginosa K. pneumoniae *	Discordant – False Negative
PR9	17854	10326	85	*N. mucosa* *P. melaninogenica* *S. viridans* *J. denitrificans* *A. fumigatus*	6378 2715 212 10 13	*A. fumigatus*	Concordant
PR10	20599	11534	5958	*P. aeruginosa* *J. denitrificans* *C. albicans*	6569 333 15	*P. aeruginosa* *C. albicans*	Concordant
PR11	29609	24998	1838	*K. aerogenes* *J. denitrificans*	24165 374	*K. aerogenes*	Concordant
PR12	6828	921	5625	*C. freundii* *P. aeruginosa* *J. denitrificans* *C. koseri* *S. maltophilia*	461 212 94 85 50	*A. fumigatus**	Discordant – False Negative
PR13	50	16	22	*J. denitrificans* *E. coli*	13 2	*P. aeruginosa* *C. albicans*	fail

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PR14	11771	121	11609	*S. pneumoniae* *J. denitrificans*	122 4	Negative	Discordant – False Positive Pneumococcal antigen positive
PR15	1086	244	819	*J. denitrificans*	241	Negative	Concordant
PR16	12803	6868	4300	*P. aeruginosa* *J. denitrificans*	211 6542	*P. aeruginosa*	Concordant
PR17	14532	8891	1082	*N. mucosa* *H. parahaemolyticus*	8409 103	URTF	Concordant
PR18	5658	776	4838	*P. aeruginosa* *J. denitrificans*	11 762	*P. aeruginosa*	Concordant

^ sequencing data after 2hrs of sequencing; *Organism missed by the RMg workflow

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Table E8B: Metagenomic data* of quality controls processed during the pre-pilot phase

Negative control	Total No reads	Microbial reads	Human reads	Organisms Identified	Organisms reads	*J. denitrificans* abundance (%)
PR-PC1	18814	17149	62	*J. denitrificans*	17018	99.2%
PR-PC2	16833	13282	132	*J. denitrificans* *P. aeruginosa*	12904185	97.1%
PR-PC3	4908	3138	55	*J. denitrificans* *E. coli*	2886184	91.9%
PR-PC4	23840	20926	52	*J. denitrificans* *E. coli*	20635190	98.6%

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PR-PC5	21205	19758	71	*J. denitrificans* *E. coli*	19417 228	98%
PR-PC6	22578	21085	139	*J. denitrificans* *C. acnes*	19875 846	94%
PR-PC7	439	392	1	*J. denitrificans* *S. mitis*	363 14	92%
PR-PC8	7	6	0	*J. denitrificans*	6	100%
PR-PC9	9919	8927	15	*J. denitrificans*	8873	99%

*sequencing data after 2hrs of sequencing

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Table E9. Performance reported after testing different parameters on training set for pathogen identification.

Abundance thresholds (≥ 0.1%, 1%, 2%, 5% and 10%) tested against a series of classification scores provided by Centrifuge. (≥ 2000 (A), ≥ 8000 (B), ≥ 16000 (C).

A

	0.1%	**1%**	**2%**	**5%**	**10%**
TP	13	12	11	10	9
FN	1	2	3	4	5
TN	1	2	2	2	2
FP	2	1	1	1	1
Sensitivity	0.93	0.86	0.79	0.71	0.64
Specificity	0.33	0.67	0.67	0.67	0.67

B

	0.1%	**1%**	**2%**	**5%**	**10%**
TP	13	12	11	10	9
FN	1	2	3	4	5
TN	1	2	2	2	2
FP	2	1	1	1	1
Sensitivity	0.93	0.86	0.79	0.71	0.64
Specificity	0.33	0.67	0.67	0.67	0.67

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C

	0.1%	**1%**	**2%**	**5%**	**10%**
TP	13	12	11	10	9
FN	1	2	3	4	5
TN	1	2	2	2	2
FP	2	1	1	1	1
Sensitivity	0.93	0.86	0.79	0.71	0.64
Specificity	0.33	0.67	0.67	0.67	0.67

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Table E10: List of organisms tested to identify the misclassification rate observed using the in-house metagenomics bioinformatics pipeline

Organism	Classification rate (%)	Misclassified organisms (abundance %)
Enterobacter cloacae	94.55	*K. pneumoniae (2.68%), E. coli (0.89%)*
Enterococcus faecalis	98.65	*E. avium (0.6%), P. harei (0.39%)*
Enterococcus faecium	94.68	*E. durans (2.74%), E. faecalis (0.87%), E. hirae (0.69%), S. aureus (0.35%)*
Escherichia coli	97.69	*S. flexneri (0.95%), E. tarda (0.43%), C. fruendii (0.35%)*
Haemophilus parainfluenzae	93.45	*H. influenza (4.57%), H. aegyyptius (0.93%), H. parahaemolyticus (0.60%)*
Klebsiella aerogenes	97.40	*C. koseri (1.21%), K. pneumoniae (0.94%), K. oxytoca (0.23%)*
Klebsiella oxytoca	88.78	*K. pneumoniae (7.15%), R. planticola (1.85%), S. plymuthica (0.92%), E. coli (0.48%)*
Klebsiella pneumoniae	95.61	*K. oxytoca (1.03%), S. aureus (0.95%), R. planticola (0.68%), K. quasipneumoniae (0.35%)*
Klebsiella variicola	96.41	*K. pneumoniae (1.93%), K. quasipneumoniae (0.75%), K. oxytoca (0.31%)*
Staphylococcus aureus	99.71	*N/A*

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Staphylococcus epidermidis	95.96	*S. hominis (2.36%), S. saccharolyticus (0.55%), S. aureus (0.46%)*
Streptococcus mitis	72.28	*S. pneumoniae (12.85%), S. oralis (7.90%), S. ARGOS256 (1.21%)*
Streptococcus oralis	80.29	*S.ARGOS256 (17.15%), S. intermedius (1.15%), S. gordonii (0.70%)*
Streptococcus pneumoniae	99.74	*N/A*
Streptococcus pyogenes	97.00	*S. dysgalactiae (2.27%), S. agalactiae (0.30%)*

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Table E11: List of antimicrobial resistance gene reported in our study

Gene name	Organisms associated	Affected Antibiotics	Mechanism
mecA	*S. aureus*	Methicillin	Plasmid
vanA	*E. faecium*	Vancomycin	Plasmid
bla$_{TEM-1}$	Enterobacterales	Narrow spectrum to beta lactams	Plasmid
bla$_{TEM-4}$	Enterobacterales	ESBL	Plasmid
bla$_{SHV-11}$	Enterobacterales	Narrow spectrum to beta lactams	Plasmid
bla$_{SHV-186}$	Enterobacterales	ESBL	Plasmid
bla$_{OXA-1_1}$	Enterobacterales	Ampicillin	Plasmid
bla$_{CTX}$ (most common is bla$_{CTX-M-15}$ & bla$_{CTX-M-15}$)	Enterobacterales	ESBL	Plasmid

ESBL= Extended spectrum beta-lactamases

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Table E12. Analytical limit of detection of the RMg workflow determined using a culture-negative BAL sample

Sample	Replicate	Pathogen	Approx. number of pathogen cells (CFU)	Total reads (2hrs)	Human reads (2hrs)	Microbial reads	Classified reads* mapped to the organism
SA 10^4	1	***S. aureus***	10,000	28997	53	18171	1469 (8%)
SA 10^4	2		10,000	5946	78	3986	772 (19.3%)
SA 10^4	3		10,000	39907	138	24381	3150 (13%)
SA 10^3	1		1,000	31442	201	19125	211 (1%)
SA 10^3	2		1,000	22459	65	13394	156 (1%)
SA 10^3	3		1,000	35387	157	20797	61 (0.3%)
KP 10^4	1	***K. pneumoniae***	10,000	32092	17113	9713	1412 (15%)
KP 10^4	2		10,000	55750	120	36067	3411 (10%)
KP 10^4	3		10,000	49353	74	26585	1187 (5%)
KP 10^3	1		1,000	33494	594	22096	410 (1.86%)
KP 10^3	2		1,000	59246	629	37031	765 (2%)

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KP 10^3	3		1,000	43532	156	27095	460 (2%)
CA 10^3	1	***C. albicans***	1,000	15623	547	8579	46 (0.5%)
CA 10^3	2		1,000	968	144	414	10 (2%)
CA 10^3	3		1,000	15975	1595	7916	118 (2%)
CA 10^2	1		100	2808	1760	788	3 (0.4%)
CA 10^2	2		100	75719	38280	11372	0
CA 10^2	3		100	1120	952	103	2 (2%)

*The number of reads detected for all samples was above the pre-defined thresholds after 2hrs of sequencing .

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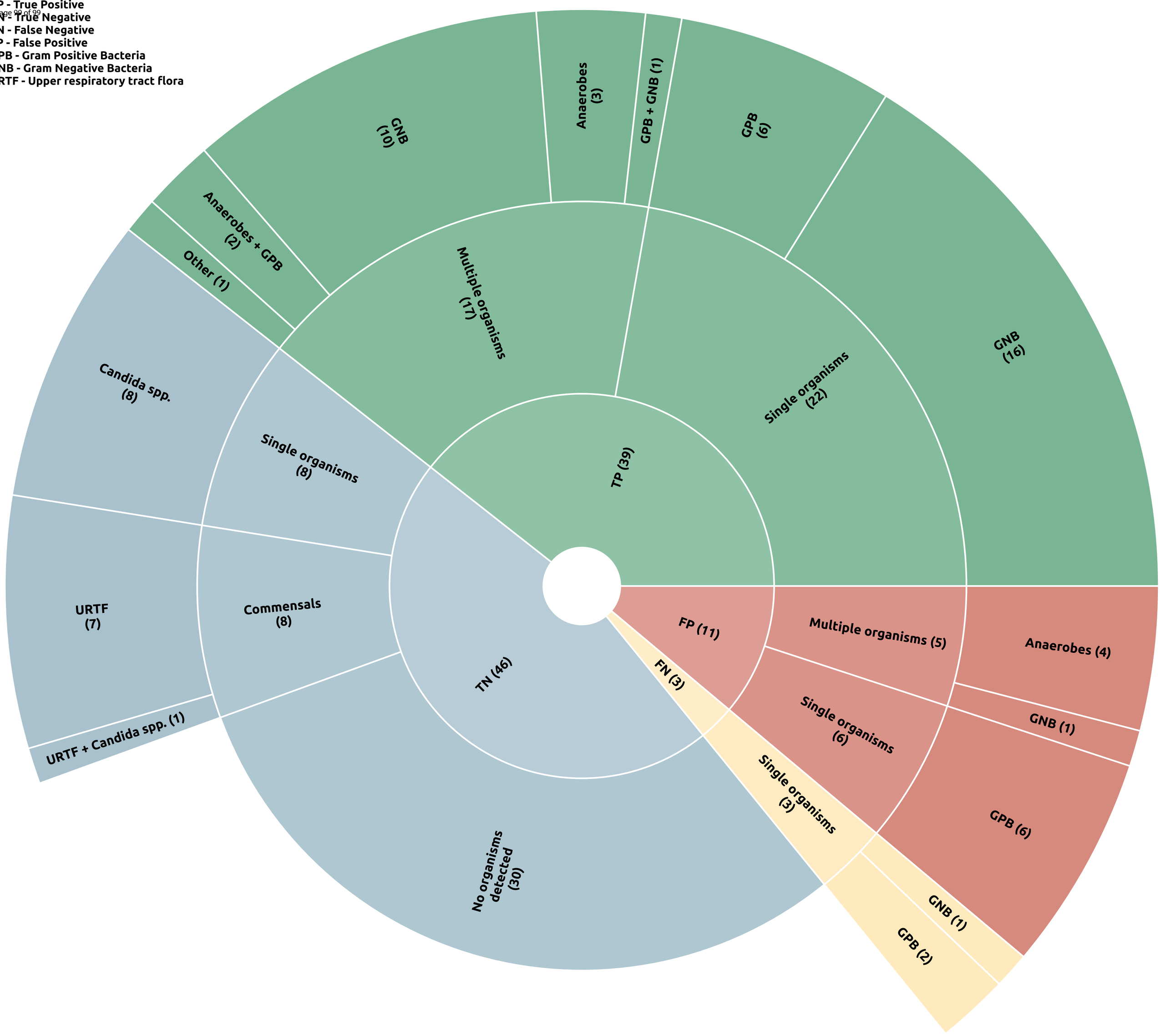
TP - True Positive
TN - True Negative
FN - False Negative
FP - False Positive
GPB - Gram Positive Bacteria
GNB - Gram Negative Bacteria
URTF - Upper respiratory tract flora



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