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A Novel Air-Dried Multiplex High Resolution Melt Assay for the Detection of Extended Spectrum Beta-Lactamase and Carbapenemase Genes.

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A NOVEL AIR-DRIED MULTIPLEX HIGH RESOLUTION MELT ASSAY FOR THE DETECTION OF EXTENDED SPECTRUM BETA-LACTAMASE AND CARBAPENEMASE GENES

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Highlights

- We describe here the development and evaluation of a novel air-dried HRM assay to detect eight major ESBL and carbapenemase genes
- The sensitivity and specificity of this novel air-dried HRM assay in comparison to the reference molecular test was 94.7%-98.5% and 98.5%-99.2%
- The assay had a phenotypic agreement of 91.1% when predicting phenotypic resistance to cefotaxime and meropenem among Enterobacteriaceae isolates
- Cross platform validation showed almost perfect reproducibility in 5 different q-PCR platforms
- No loss of sensitivity was observed after 8-months storage at room (20.4°C ± 0.7), refrigerator (6.2°C ± 0.9) and oven (29.7°C ± 1.4) temperatures.

A NOVEL AIR-DRIED MULTIPLEX HIGH RESOLUTION MELT ASSAY FOR THE DETECTION OF EXTENDED SPECTRUM BETA-LACTAMASE AND CARBAPENEMASE GENES

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Short running title: A novel ESBL-Carb air-dried HRM assay

ABSTRACT

Objectives: This study aimed to develop and evaluate a novel air-dried high-resolution melt (HRM) assay to detect eight major extended spectrum beta-Lactamase (ESBL) (*bla*$_{SHV}$ and *bla*$_{CTXM}$ groups 1 and 9) and carbapenemase (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{VIM}$ and *bla*$_{OXA-48-like}$) genes that cause antimicrobial resistance to cephalosporins and carbapenems.

Methods: The assay was evaluated using 439 DNA samples extracted from bacterial isolates from Nepal, Malawi and UK and 390 clinical isolates from Nepal with known antimicrobial susceptibility results. Assay reproducibility was evaluated across five different q-PCR instruments (Rotor-Gene Q, QuantStudio^{TM} 5, CFX96, LightCycler® 480 and MIC). Assay stability was also assessed upon the assay storage in the refrigerator (6.2°C±0.9), room temperature (20.4°C±0.7) and oven (29.7°C±1.4) at six time points for eight months.

Results: The sensitivity and specificity for detecting the ESBL and carbapenemase genes in comparison to the reference gel-base PCR and sequencing was 94.7% (95%CI: 92.5%-96.5%) and 99.2% (95%CI: 98.8%-99.5%), and 98.5% (95%CI: 97.0%-99.4%) and 98.5% (95%CI: 98.0%-98.9%) when compared to the original HRM wet PCR mix format. The overall agreement was 91.1% (95%CI: 90.0%-92.9%) when predicting phenotypic resistance to cefotaxime and meropenem among Enterobacteriaceae isolates. We observed almost perfect inter-machine reproducibility of the air-dried HRM assay and no loss of sensitivity occurred under all storage conditions and time points.

Conclusions: We present here a ready-to-use air-dried HRM-PCR assay that offers an easy, thermostable, fast and accurate tool for the detection of ESBL and carbapenemase genes in DNA samples to improve AMR.

Word count: 245/250

Keywords: antimicrobial resistance, extended spectrum beta-lactamase, carbapenemase, high resolution melting, molecular diagnostics

INTRODUCTION

Antimicrobial resistance (AMR) is a major global cause of death and the development of new antibiotics is considered a public health priority [1]. An estimated 700,000 deaths are attributable to AMR globally each year, and this number is predicted to rise to 10 million by 2050 [2]. Identification of AMR is typically by culture-based phenotypic antimicrobial susceptibility testing (AST) which requires incubation, from primary sample, for 48 to 96 hours. As clinical management decisions are often taken rapidly, the lack of timeliness of AST leads to empirical treatment, which is often inappropriate [3,4]. First line or broad-spectrum antibiotics are often used in large doses to ensure their efficacy on the suspected but unknown aetiological pathogens [5,6]. Empirical treatment facilitates the emergence of AMR, increases the duration of hospitalisation, damages the patient microbiota and increases the cost of therapy [7–9]. Rapid diagnosis of AMR can enable targeted usage of antibiotics, improved patient outcomes and antimicrobial stewardship [4,6,10,11].

The most common mechanism of drug resistance in Gram-negative bacteria is the production of β-lactamases, including the extended-spectrum β-lactamases (ESBLs) and carbapenemases [12], which provide resistance to the β-lactam antibiotics. Polymerase Chain Reaction (PCR) based detection of ESBLs and carbapenemase genes provide a faster diagnosis of AMR than phenotypic methods, which might in turn generate more timely information for treatment decisions [13,14]. Whist molecular methods for the detection and characterisation of microbial resistance genes are becoming increasingly established, with good agreement with phenotypic methods, producing faster results [15–17], their use in clinical settings is hampered by the high degree of multiplexing needed due to the many genes involved in an AMR phenotype. Additionally, PCR requires a cold chain to maintain the integrity of reagents, equipment, and trained staff, which are often unavailable in low- and middle-income countries (LMICs). One approach that could facilitate the implementation of PCR

assays in LMICs would be to provide the PCR primers, Taq enzyme and buffer components dry in the PCR vessels. This process eliminates the need for a cold chain and simplify preparation, as only the addition of nuclease-free water and the DNA template is needed resuspend the PCR reagents [18–20]. Typically, this process would be done by lyophilisation of the reagents. Lyophilisation, also called freeze-drying, is the process of the removal of water from a product by volatilization and desorption to increase the lifespan of a product. However, lyophilisation is costly and requires the addition of excipients, such as cryoprotectants and bulking agents [19,21]. There are commercially available ready to use thermostable PCR kits for the detection of AMR genes such as GeneXpert CarbaR (Cepheid, USA) and FilmArray Blood culture ID (BioFire, USA), however these are expensive and require proprietary equipment making them difficult to use routinely and difficult to implement in some low-resource settings [22].

We report here the development and validation of an air dried HRM-PCR mix to detect the most frequent ESBLs and carbapenemase enzymes based on a previously validated in-house AMR HRM-PCR assay [23].

METHODS

Air-dried HRM assay optimisation

We adapted an in-house 9-plex HRM PCR [23] into a dry format to detect three major ESBL genes (bla_{SHV} and bla_{CTXM} groups 1 and 9) and five carbapenemase genes (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{VIM}$ and *bla*$_{OXA\text{-}48\text{-}like}$). For the dry-out process, AmpDRY™ (Biofortuna, UK) was used, which is a PCR reaction mix that allows direct air drying of the whole reaction including primers and reporter molecules and removes the need for lyophilisation systems and reagents. The composition of each HRM reaction included a mixture of 1x EvaGreen® dye (Biotium, Canada), primers for detecting ESBL groups and carbapenemase genes, [23] the proprietary air-drying PCR buffer AmpDRY™ (Biofortuna, UK) and PCR grade water to

a final volume of 6.25µl. The reaction mixture was added into each of the wells of a 96-well PCR plate (Starlab, Germany) and was dried in an oven-drier (ElextriQ, UK) at 35°C for 17 hours. PCR was performed by adding 2.5µl of bacterial DNA and 500 mM Betaine (Sigma Aldrich, UK) in PCR grade water to each PCR well containing the dried reagents for a final reaction volume of 12.5µl. When plates were not compatible with the thermocycler used (Rotor-Gene-Q), PCR plates were briefly centrifugated before PCR amplification and the mixture was transferred to the appropriate reaction vessels (Rotor-Gene-Q strip tubes). The optimised PCR amplification protocol consisted of an initial incubation step at 80°C for 15 minutes, followed by 30 cycles of denaturation for 10 seconds at 95°C, annealing for 60 seconds at 66°C and elongation for 10 seconds at 72°C monitoring the fluorescence in the FAM/SYBR channel. HRM analysis was carried out over a temperature range of 75°C to 95°C taking a reading in the HRM/SYBR channel every 0.1°C, with a 2 second stabilisation between each step. Peak calling was automated and indicated by a peak at the predictive melting temperature (Tm) of the target visualised as the negative first derivative of the melting curve in the Rotor-Gene Q software. The Rotor-Gene Q (Qiagen, UK) was used for all the experiments except where stated otherwise. Optimal conditions of the assay were achieved by titration of individual reaction components, optimisation of amplification conditions and drying time. The original primer mix and their concentrations were as previously described [23], except that *bla*$_{\text{TEM}}$ was removed as it is ubiquitous in *Escherichia coli* and the most common variants are narrow spectrum.

Stored bacterial DNA and reference molecular tests

A panel of 439 DNA samples from well documented multidrug resistant (MDR) bacterial isolates from Nepal (n=293), the UK (n=103) and Malawi (n=43) was used to optimise and evaluate the air-dried HRM assay.

Bacterial DNA from Nepal: this comprises isolates collected from 2012 to 2016 at Patan Hospital, Kathmandu, and includes strains of *E. coli* (n=112), *Acinetobacter* spp. (n=72), *Klebsiella pneumoniae* (n=54), *Enterobacter* spp. (n=32), *Pseudomonas aeruginosa* (n=20), *Klebsiella oxytoca* (n=4), *Proteus* spp. (n=1), *Providencia retgerii* (n=1), and *Serratia rubidaea* (n=1). Isolates were collected during routine diagnostic testing from clinical samples.

Bacterial DNA from Malawi: isolates were collected between 1996 and 2012 at Queen Elizabeth Central Hospital during routine diagnostic testing and comprised *E. coli* (n=25) and *K. pneumoniae* (n=18). The collection of isolates was approved by the University of Malawi College of Medicine Research and Ethics Committee (COMREC), Blantyre, under study number P.08/14/1614.

Bacterial DNA from the UK: isolates were collected between 2012 and 2017 from the UK National Health Service hospitals and included *E. coli* (n=40), *K. pneumoniae* (n=27), *Klebsiella aerogenes* (n=12), *Enterobacter cloacae* (n=10), *Citrobacter freundii* (n=4), *P. aeruginosa* (n=4), *Morganella morganii* (n=2), and *K. oxytoca* (n=1).

Further details of all isolates in the sample collection are available in Supplementary Materials.

DNA from the Nepal and Malawi isolates was extracted using the boilate [24] method and isolates from the UK were extracted using the DNeasy Blood and Tissue kit (Qiagen). The isolates sourced in the UK and Nepal were screened for ESBL and carbapenemase markers using reference gel-based PCR published protocols [13,14] and the air-dried HRM assay. The reference PCR reaction mix was performed using DreamTaq PCR reaction mix (Thermo Fisher, UK), 2.5µl of DNA and nuclease free water to a final volume of 12.5µl. PCR amplification was visualised with PicoGreen™ (Life Technologies, USA) staining on a 1% TBE (Tris-borate-EDTA) gel with 1% to 2% of agarose depending on the fragment size to

resolve. This reference gel-based PCR was not performed with the Malawian isolates as next generation sequencing data was available from previous studies [8,23]. In addition, the 439 isolates were screened using the in-house 9-plex HRM PCR assay originally developed in our laboratory [23] using the commercially available Type-it® HRM kit (Qiagen).

Bacterial strains for phenotype prediction evaluation in Nepal

A set of 390 Gram negative bacteria with known phenotypes were chosen based on their resistance profile from a collection of characterised clinical isolates banked at Patan Hospital in Nepal. Bacterial phenotypes were determined using the disk diffusion test following the Clinical & Laboratory Standards Institute (CLSI) guidelines. Banked isolates were selected based on their resistance to meropenem (37%) and cefotaxime (85%) and were resuscitated on MacConkey agar (Thermo Fisher Scientific, USA) and DNA extracted by a boiling lysis method as described elsewhere [24]. Intermediate phenotypic profiles were not selected for the study. To evaluate the agreement between the phenotype and HRM result (genotype), isolates positive for any (one or more) ESBL groups and carbapenemase genes were considered resistant to cefotaxime and isolates positive for any (one or more) carbapenemase genes were considered resistant to meropenem.

Isolates included strains of *E. coli* (n=72), *K. pneumoniae* (n=107), *Acinetobacter* spp. (n=73), *Enterobacter* (n=63), *Salmonella* Typhi (n=25), *K. oxytoca* (n=16), *P. aeruginosa* (n=13), *Salmonella* Paratyphi (n=7), *M. morganii* (n=3), *C. freundii* (n=2), *Serratia* spp. (n=3), *Proteus* spp. (n=2) and *P. rettgeri* (n=1).

Limit of detection

Limit of detection (LOD) of the air-dried assay was evaluated for the ESBL genes bla_{CTXM}-1 and bla_{SHV}, one *E. coli* isolate positive for $bla_{\text{CTXM-1}}$ (isolate 1), one *K. pneumoniae* positive for bla_{SHV} (isolate 2), and one *K. pneumoniae* isolate harbouring for both genes $bla_{\text{CTXM-1}}$ and bla_{SHV} (isolate 3) to estimate the LOD in isolates coproducing multiple genes. The LOD was

performed following a published protocol [25]. Briefly, a single colony of each isolate was incubated at 37 °C for three hours in 5 ml of Luria-Bertani (LB) broth (ThermoFisher Scientific, UK), cultures were then sequentially diluted 1:10 in LB broth, and 10µl of each dilution was plated in triplicate on LB agar. The plates were then incubated overnight at 37 °C and the colonies counted to quantify the CFU/ml in the suspension. Two aliquots of 200µl of each of the suspensions were taken and processed following two extraction methodologies: DNeasy Blood and Tissue kit (Qiagen) and the boilate technique. DNA samples for each dilution series were tested in triplicate using the HRM assay. The LOD was defined as the lowest concentration at which the AMR genes were detected in all three replicates.

Cross-platform validation

To evaluate the compatibility of the air-dried HRM assay in a wide range of platforms, a set of 94 samples comprising all the resistance genes were tested using different qPCR systems including the Rotor-Gene Q, QuantStudio™ 5 (Thermofisher, USA), CFX96 (BioRad, USA), LightCycler® 480 (Roche Life Sciences, Germany) and MIC (Bio Molecular Systems, Australia). Amplification of the markers was assessed together with changes in Tms between platforms.

Evaluation of the stability upon storage at different temperatures

Stability of the dried-HRM assay was evaluated over time under different storage temperatures. A set of 89 samples comprising all the markers and isolates 1-3 at the dilution of the LOD and previous dilution were tested with plates stored at different conditions. One PCR plate with the dried reaction mix was stored for each of the following periods of time; one week (T1), two weeks (T2), one (T3), three (T4) and eight months (T5) and at fridge (5°C), room (20°C) and oven temperature (30°C). PCR plates were sealed with foil adhesive film and individually wrapped in heat sealed aluminium foil laminated pouches containing one desiccant sachet (Merck, USA). Temperature and humidity were recorded weekly.

Data analysis

Statistical evaluations were performed with SPSS v.19 (2010, US). The outcome of all tests was labelled as 0 when negative or 1 when positive. The level of agreement between tests was determined using Cohen's Kappa. Kappa coefficients (κ) with values between 0 and 0.20, 0.21 and 0.39, 0.40 and 0.59, 0.60 and 0.79, 0.80 and 0.90 and 91 to 1 were interpreted as no agreement, minimal, weak, moderate, strong, and almost perfect agreement, respectively. [26] Statistical significance of differences in Tms between platforms was measured using One-Way-ANOVA and differences of peak height between different storage conditions using One-Way-ANOVA with Tukey's test for Post-Hoc analysis. Statistical significance was set at a p-value < 0.05.

Results

Air-dried HRM assay evaluation using banked DNA

The air-dried HRM assay was capable of identifying the eight markers, each of which was characterised by the presence of a single peak at the expected Tm (Fig. 1a). The assay was also able to identify co-producers of up to four AMR markers (Fig. 1b). There was no overlap between adjacent peaks with a minimum separation of peak Tm of 0.8 °C allowing easy identification of multiple genes within the same sample.

Measures of diagnostic accuracy and agreement of the air-dried HRM assay for detecting individual genes compared to the reference tests are detailed in Table 1 (PCR and WGS) and Table 2 (original 9-Plex HRM assay). The overall sensitivity and specificity of the air-dried HRM assay for all genetic markers in comparison with the reference gel-based PCR and sequencing were 94.7% (95%CI: 92.5%-96.5%) and 99.2% (95%CI: 98.8%-99.5%) and, in comparison with the original 9-plex HRM PCR assay [23] were 98.5% (95%CI: 97.0%-99.4%) and 98.5% (95%CI: 98.0%-98.9%). When compared with the reference gel-based PCR and sequencing, the air-dried HRM assay had almost perfect agreement (κ=0.94-1) for

the ESBL *bla*$_{CTXM}$ group and carbapenemase markers and moderate agreement (κ=0.79) for *bla*$_{SHV}$. *bla*$_{SHV}$ was often (77/102) found in coproducers of multiple genes and the sensitivity of bla$_{SHV}$ was lower in isolates carrying two (76.7%) and three genes (59.3%) than single producers of bla$_{SHV}$ (92.6%). The air-dried HRM assay was 100% sensitive and 99.3%-99.7% for detecting carbapenemase co-producer isolates in comparison to the reference gel-based/sequencing and original 9-plex HRM PCR assay respectively.

Bacterial strains for phenotype prediction evaluation from Nepal

The overall percentage of agreement of the air-dried HRM result and phenotype was 92.4% (95%CI: 89.9%-94.4%) for Enterobacteriaceae isolates and 57.1% (95%CI: 49.6%-64.4%) for non-Enterobacteriaceae isolates. The air-dried HRM assay had strong agreement with the phenotype (κ=0.845) among Enterobacteriaceae isolates with a sensitivity on predicting resistance to cefotaxime of 92.1% (88.0%-95.1%) and on predicting resistance to carbapenems 84.2% (75.3%-90.9%). However, the phenotype was poorly predicted among non-Enterobacteriaceae isolates using the air-dried HRM assay (Table 3). The sensitivity to meropenem was stratified by gene detected to investigate if the presence a particular carbapenemase gene was associated with false positivity as with the carriage of *bla*$_{OXA-48-like}$ genes does not always provide resistance to meropenem [27]. In this study the presence of *bla*$_{OXA-48-like}$ or any other carbapenemase gene was not associated with an increase of false positive rate in meropenem sensitive isolates ($\chi 2$ test $p > 0.05$).

Cross-platform validation

Almost perfect reproducibility was obtained on all instruments. Cut-off was established for each instrument by evaluating five threshold values set as 20%, 10%, 7.5%, 5% and 3% of the fluorescence of the highest peak. The optimal cut-off for the Rotor-Gene Q, QuantStudio and MIC was 5% of the fluoresce of the highest peak and for CFX96 and LightCycler® 480

it was 10%. These cut-offs produced almost perfect agreement with the reference tests (κ=0.935).

The amplicon Tm (°C) shifted across platforms (Fig. 2) and ranged from±0.013°C to±0.99°C for *bla*$_{\text{CTXM-1}}$,±0.07-1.09°C for *bla*$_{\text{CTXM-9}}$,±0.08-1.15°C for *bla*$_{\text{IMP}}$, ±0.02-1.26°C for *bla*$_{\text{KPC}}$, ±0.01-1.38°C for *bla*$_{\text{NDM}}$, ±0.19-1.5°C for *bla*$_{\text{OXA-48-like}}$, ±0.08-0.94°C for *bla*$_{\text{SHV}}$ and ±0.12-1.27°C depending on the platform used. The Tm differences within the same peak and neighbouring peaks is shown in Tables 4a and 4b for each of the platforms. The Tm difference was not statistically significant for any of the platforms for either the type of peak, peaks within the same cluster (p=0.318) and neighbouring clusters (p=1.00).

Limit of detection

The limit of detection was 11.5, 102 and 960 cfu/reaction using DNeasy kit and 2.3, 20.4 and 192 cfu/reaction by the boilate method for isolates carrying the *bla*$_{\text{CTXM-1}}$, *bla*$_{\text{SHV}}$ and both *bla*$_{\text{CTXM-1}}$ and *bla*$_{\text{SHV}}$ genes, respectively.

Stability upon different storage conditions

The effect of storage time and temperature on the air-dried HRM assay was assessed by analysing the plate mean fluorescence peak height and amplification of isolates, including at the LOD dilution. The average temperature for room storage, fridge and oven was 20.4°C±0.7, 6.2°C±0.9 and 29.7°C±1.4 respectively, the humidity of the room was at 36.5%±9.34. Overall, room temperature was the best storage condition compared to fridge and oven. The difference of mean fluoresce peak hight was not statistically significant within the same time point but was statistically significant between different time points (Fig. 3). The peak height started decreasing after storage time T3 for room and oven storage, and at T2 for fridge storage (Fig. 3). Nonetheless, the difference of mean peak height produced with the air-dried HRM assay stored at time T3 (one month) was not statistically significant to the produced at T0, T1 and T2 at all storage conditions. The air-dried HRM assay recovered at

T4 and T5 (fridge only) produced significantly lower peak heights when compared to T1, T2 and T3 (room temperature only). The mean peak height produced with the air-dried HRM assay stored at time T5 at room temperature, was comparable to all time points at all storage conditions and timepoints except at T1 for fridge storage (Fig. 3).

Isolate 1 was negative at the LOD dilution at T3 under oven storage; isolate 2 was negative at the LOD dilution at T3 under room temperature and oven storage, and isolate 3 was positive in all runs tested (Fig 4). Of the 89 isolates tested, 100% were positive for all markers at all storage times and conditions, except for one sample that had one of three marker peaks below the cut-off (bla_{NDM}) at T4 fridge storage (data not shown).

Discussion

In this study, we evaluated the performance of a dry format 8-plex HRM PCR assay to detect ESBL and carbapenemase genes. The assay showed high sensitivity, specificity and measures of agreement for all markers when compared to the reference tests. In addition, the drying process did not result in loss of performance, with all the resistance genes of the 89 clinical isolates correctly classified after 6 months of storage.

The dry format of the assay overcomes key real-world challenges relating to transport, storage, and freezing/thawing issues, which can substantially lower the sensitivity of PCR.[28,29] This HRM assay presents several major advantages over fresh qPCR mixes as its resistant to long periods of storage at relatively warm temperatures (30 °C). Additionally, the HRM assay is more economic than fluorescent probes-based assays and has good performance using the boilate extraction methodThis would be of particular importance in LMICs where laboratories face insufficient and suboptimal cold chain capacity and scarcity of funds.[30]

The air-dried HRM assay mix recovered at T4 and T5 stored in the fridge had lower peak heights that at oven and room temperature. This directs that fridge storage is the less suitable

for this assay than room temperature and oven. However, the detection of *bla*$_{\text{CTXM-1}}$ and *bla*$_{\text{SHV}}$ was compromised at LOD dilution in isolate 1 and 2 at T3 for oven and room storage what suggests that detection of genes at very low concentrations can be compromised after one-month storage.

The interpretation of results via analysis of the melting data can be automated in the systems' software, which reduces subjectivity and intra-operator variation. The assay would be implementable in laboratories with access to qPCR facilities, but otherwise moderate resources, as all that is required is to reconstitute the mix and add template DNA. The level of multiplexing enables detection of the 8 major carbapenemase and ESBL gene families in a single tube with a sensitivity and specificity compared to reference molecular tests. Molecular detection of AMR genes can provide useful epidemiological data and enable the tracking of particular resistance genes at a hospital or national level.[31]

Cross-platform validation illustrates a remarkably good performance on all 5 q-PCR systems (Rotor-Gene Q, QuantStudio™ 5, CFX96, LightCycler® 480 and MIC) evaluated, with minimal variation on the peak Tms. The cut-offs however required slight adjustment (5% or 10% of the highest peak) to achieve the best performance, nevertheless this is straightforward correction that is automated for peak calling.

The protocol has some constraints as a 24h incubation from primary sample to grow the isolates is still required prior DNA extraction. The assay has not been evaluated using direct clinical samples but the LOD obtained here indicates sensitivity to be insufficient to detect the low CFU/ml (>1/ml) possible in bacterial bloodstream infections. [32,33]. Since an internal amplification control has not been included to maximise sensitivity, the essay should be used with caution on samples that might contain PCR inhibitors such as stool or soil

Another constraint of the air-dried HRM assay is the limitation to distinguish between narrow-spectrum and extended-spectrum resistance genes. This is particularly important for

bla$_{SHV}$, however, as many *bla*$_{SHV}$ found in non-*Klebsiella* spp. are ESBL [34] all bla$_{SHV}$ were considered ESBL to maximise sensitivity of the test. This may over estimate resistance if is not interpretated with knowledge of the local epidemiology of the area.

The overall agreement to predict bacterial phenotypes was strong amongst Enterobacteriaceae isolates but weak in non-Enterobacterial isolates. Thus, we do not recommend the use of the assay in non-Enterobacterial isolates. The high discrepancy among non-Enterobacteriaceae isolates can be explained as *Acinetobacter* spp. and *Pseudomas* spp. have other frequent mechanisms of resistance such as efflux pumps, permeability defects, modifications of target sites that are less common in the family Enterobacteriaceae [35,36], and chromosomal mediated AmpC enzymes[37] or *bla*$_{GES-1}$[38]. An HRM assay for the detection of AmpC enzymes has already been developed[23] and could be easily adapted to a two tube AmpC and ESBL-Carb air-dried HRM assay using the methodology detailed here. Other reasons for phenotype-genotype mismatches include enzyme modifications that change the spectrum of activity and susceptibility profile [39], and also isolates with MICs close to the breakpoint being incorrectly classified during phenotypic susceptibility testing. [40].

To summarise, the air-dried HRM assay rapidly detected ESBL and carbapenemase genes high specificity and sensitivity and maintained performance after six months of storage at room temperatures. This 8-plex dry HRM assay was also successfully transferred to 5 different PCR platforms indicating that can be reliably implemented in many laboratories. The assay can become a useful tool for AMR diagnosis and surveillance.

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Competing interests

None to declare

Ethical approval

Not required

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Authors' contributions

AICA, TE, ERA and LEC contributed to the conception and design of the study; AICA, CTW, SM and RS carried out the experimental work. TE, AK, DS, HG, EK, MP and NK obtained the bacterial isolates used in the study.

AICA, TE, and CW analysed the data. AICA wrote the first draft of the manuscript. All authors were involved in the manuscript preparation and revision, and approval of the final version of the manuscript.

References

[1] Tacconelli E, Magrini N. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Organ. Mund. la Salud, 2017.

[2] O'Neill J. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. Rev Antimicrob Resist 2016.

[3] De Waele JJ, Dhaese S. Antibiotic stewardship in sepsis management: toward a balanced use of antibiotics for the severely ill patient. Expert Rev Anti Infect Ther 2019;17:89–97. https://doi.org/10.1080/14787210.2019.1568239.

[4] Kerremans JJ, Verboom P, Stijnen T, Hakkaart-van Roijen L, Goessens W, Verbrugh HA, et al. Rapid identification and antimicrobial susceptibility testing reduce antibiotic use and accelerate pathogen-directed antibiotic use. J Antimicrob Chemother 2008. https://doi.org/10.1093/jac/dkm497.

[5] Seok H, Jeon JH, Park DW. Antimicrobial Therapy and Antimicrobial Stewardship in Sepsis. Infect Chemother 2020;52:19. https://doi.org/10.3947/ic.2020.52.1.19.

[6] Avesar J, Rosenfeld D, Truman-Rosentsvit M, Ben-Arye T, Geffen Y, Bercovici M, et al. Rapid phenotypic antimicrobial susceptibility testing using nanoliter arrays. Proc Natl Acad Sci U S A 2017. https://doi.org/10.1073/pnas.1703736114.

[7] Lambregts MMC, Wijnakker R, Bernards AT, Visser LG, le Cessie S, de Boer MGJ. Mortality After Delay of Adequate Empiric Antimicrobial Treatment of Bloodstream Infection. J Clin Med 2020;9:1378. https://doi.org/10.3390/jcm9051378.

[8] Musicha P, Feasey NA, Cain AK, Kallonen T, Chaguza C, Peno C, et al. Genomic landscape of extended-spectrum β-lactamase resistance in Escherichia coli from an urban African setting. J Antimicrob Chemother 2017. https://doi.org/10.1093/jac/dkx058.

[9] Panda S, El Khader I, Casellas F, López Vivancos J, García Cors M, Santiago A, et al.

Short-term effect of antibiotics on human gut microbiota. PLoS One 2014. https://doi.org/10.1371/journal.pone.0095476.

[10] Cole KA, Rivard KR, Dumkow LE. Antimicrobial Stewardship Interventions to Combat Antibiotic Resistance: an Update on Targeted Strategies. Curr Infect Dis Rep 2019;21:33. https://doi.org/10.1007/s11908-019-0689-2.

[11] Llor C, Bjerrum L. Antimicrobial resistance: Risk associated with antibiotic overuse and initiatives to reduce the problem. Ther Adv Drug Saf 2014. https://doi.org/10.1177/2042098614554919.

[12] Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ V. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 2015. https://doi.org/10.1038/nrmicro3380.

[13] Monteiro J, Widen RH, Pignatari ACC, Kubasek C, Silbert S. Rapid detection of carbapenemase genes by multiplex real-time PCR. J Antimicrob Chemother 2012. https://doi.org/10.1093/jac/dkr563.

[14] Dallenne C, da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. J Antimicrob Chemother 2010. https://doi.org/10.1093/jac/dkp498.

[15] Luo J, Yu J, Yang H, Wei H. Parallel susceptibility testing of bacteria through culture-quantitative PCR in 96-well plates. Int J Infect Dis 2018. https://doi.org/10.1016/j.ijid.2018.03.014.

[16] Williams CT, Musicha P, Feasey NA, Adams ER, Edwards T. ChloS-HRM, a novel assay to identify chloramphenicol-susceptible Escherichia coli and Klebsiella pneumoniae in Malawi. J Antimicrob Chemother 2019. https://doi.org/10.1093/jac/dky563.

[17] Martineau F, Picard FJ, Grenier L, Roy PH, Ouellette M, Bergeron MG. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. J Antimicrob Chemother 2000. https://doi.org/10.1093/jac/46.4.527.

[18] Chen HW, Ching WM. Evaluation of the stability of lyophilized loop-mediated isothermal amplification reagents for the detection of Coxiella burnetii. Heliyon 2017. https://doi.org/10.1016/j.heliyon.2017.e00415.

[19] Babonneau J, Bernard C, Marion E, Chauty A, Kempf M, Robert R, et al. Development of a Dry-Reagent-Based qPCR to Facilitate the Diagnosis of Mycobacterium ulcerans Infection in Endemic Countries. PLoS Negl Trop Dis 2015. https://doi.org/10.1371/journal.pntd.0003606.

[20] Okeke IN, Feasey N, Parkhill J, Turner P, Limmathurotsakul D, Georgiou P, et al. Leapfrogging laboratories: the promise and pitfalls of high-tech solutions for antimicrobial resistance surveillance in low-income settings. BMJ Glob Heal 2020;5:e003622.

[21] Arunrut N, Kiatpathomchai W, Ananchaipattana C. Multiplex PCR assay and lyophilization for detection of Salmonella spp., Staphylococcus aureus and Bacillus cereus in pork products. Food Sci Biotechnol 2018. https://doi.org/10.1007/s10068-017-0286-9.

[22] Okeke IN, Feasey N, Parkhill J, Turner P, Limmathurotsakul D, Georgiou P, et al. Leapfrogging laboratories: the promise and pitfalls of high-tech solutions for antimicrobial resistance surveillance in low-income settings. BMJ Glob Heal 2020;5:e003622. https://doi.org/10.1136/bmjgh-2020-003622.

[23] Edwards T, Williams C, Teethaisong Y, Sealey J, Sasaki S, Hobbs G, et al. A highly multiplexed melt-curve assay for detecting the most prevalent carbapenemase, ESBL,

and AmpC genes. Diagn Microbiol Infect Dis 2020. https://doi.org/10.1016/j.diagmicrobio.2020.115076.

[24] Dashti AA, Jadaon MM, Abdulsamad AM, Dashti HM. Heat treatment of bacteria: A simple method of DNA extraction for molecular techniques. Kuwait Med J 2009.

[25] Edwards T, Sasaki S, Williams C, Hobbs G, Feasey NA, Evans K, et al. Speciation of common Gram-negative pathogens using a highly multiplexed high resolution melt curve assay. Sci Rep 2018. https://doi.org/10.1038/s41598-017-18915-5.

[26] McHugh ML. Interrater reliability: The kappa statistic. Biochem Medica 2012. https://doi.org/10.11613/bm.2012.031.

[27] Bakthavatchalam Y, Anandan S, Veeraraghavan B. Laboratory detection and clinical implication of oxacillinase-48 like carbapenemase: The hidden threat. J Glob Infect Dis 2016;8:41. https://doi.org/10.4103/0974-777X.176149.

[28] Coleman WB, Tsongalis GJ. Laboratory Approaches in Molecular Pathology—The Polymerase Chain Reaction. Diagnostic Mol. Pathol., 2017. https://doi.org/10.1016/b978-0-12-800886-7.00002-9.

[29] Baumforth KRN, Nelson PN, Digby JE, O'Neil JD, Murray PG. The polymerase chain reaction. J Clin Pathol - Mol Pathol 1999. https://doi.org/10.1136/mp.52.1.1.

[30] Ashok A, Brison M, LeTallec Y. Improving cold chain systems: Challenges and solutions. Vaccine 2017. https://doi.org/10.1016/j.vaccine.2016.08.045.

[31] Castagnola E, Tatarelli P, Mesini A, Baldelli I, La Masa D, Biassoni R, et al. Epidemiology of carbapenemase-producing Enterobacteriaceae in a pediatric hospital in a country with high endemicity. J Infect Public Health 2019. https://doi.org/10.1016/j.jiph.2018.11.003.

[32] Opota O, Jaton K, Greub G. Microbial diagnosis of bloodstream infection: Towards molecular diagnosis directly from blood. Clin Microbiol Infect 2015.

https://doi.org/10.1016/j.cmi.2015.02.005.

[33] Wain J, Diep TS, Ho VA, Walsh AM, Hoa NTT, Parry CM, et al. Quantitation of bacteria in blood of typhoid fever patients and relationship between counts and clinical features, transmissibility, and antibiotic resistance. J Clin Microbiol 1998. https://doi.org/10.1128/jcm.36.6.1683-1687.1998.

[34] Liakopoulos A, Mevius D, Ceccarelli D. A Review of SHV Extended-Spectrum β-Lactamases: Neglected Yet Ubiquitous. Front Microbiol 2016;7. https://doi.org/10.3389/fmicb.2016.01374.

[35] Lee CR, Lee JH, Park M, Park KS, Bae IK, Kim YB, et al. Biology of Acinetobacter baumannii: Pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front Cell Infect Microbiol 2017. https://doi.org/10.3389/fcimb.2017.00055.

[36] Lupo A, Haenni M, Madec J-Y. Antimicrobial Resistance in Acinetobacter spp. and Pseudomonas spp. Antimicrob. Resist. Bact. from Livest. Companion Anim., 2018. https://doi.org/10.1128/microbiolspec.arba-0007-2017.

[37] Reuland EA, Hays JP, De Jongh DMC, Abdelrehim E, Willemsen I, Kluytmans JAJW, et al. Detection and occurrence of plasmid-mediated AmpC in highly resistant gram-negative Rods. PLoS One 2014. https://doi.org/10.1371/journal.pone.0091396.

[38] Polotto M, Casella T, de Lucca Oliveira MG, Rúbio FG, Nogueira ML, de Almeida MTG, et al. Detection of P. aeruginosa harboring bla CTX-M-2, bla GES-1 and bla GES-5,bla IMP-1 and bla SPM-1 causing infections in Brazilian tertiary-care hospital. BMC Infect Dis 2012. https://doi.org/10.1186/1471-2334-12-176.

[39] Edwards T, Heinz E, Aartsen J van, Howard A, Roberts P, Corless C, et al. Piperacillin/tazobactam resistant, cephalosporin susceptible Escherichia coli bloodstream infections driven by multiple resistance mechanisms across diverse sequence types. BioRxiv 2020:2020.09.18.302992.

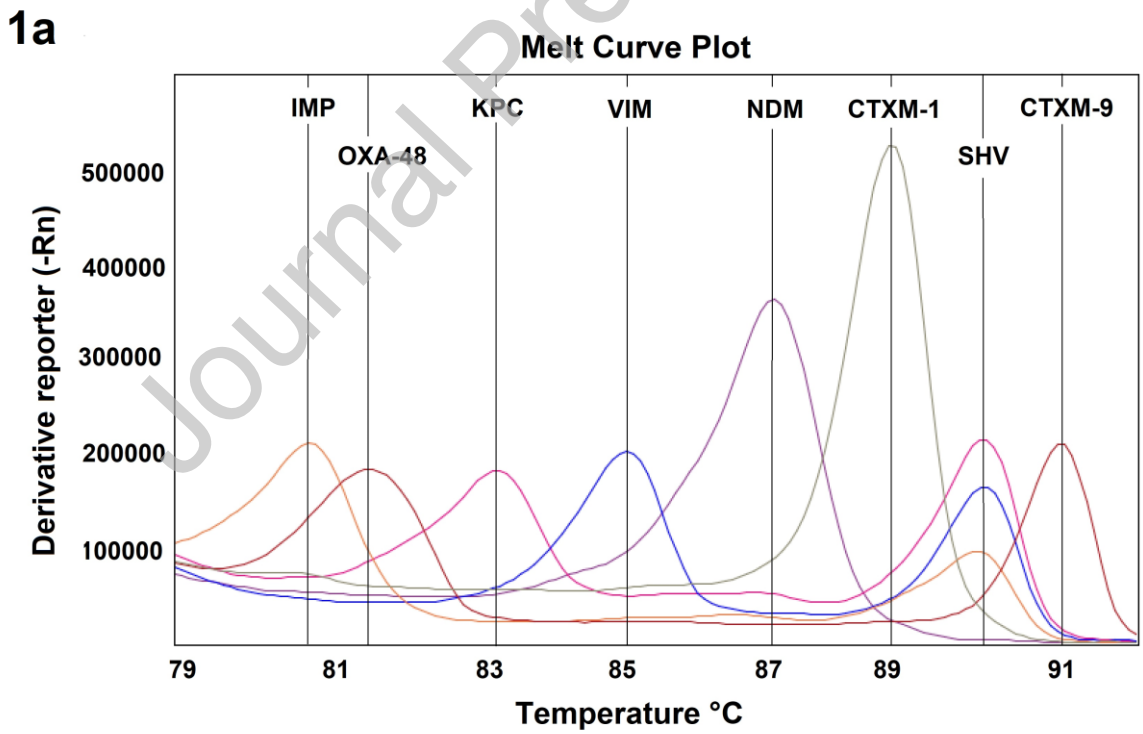
https://doi.org/https://doi.org/10.1101/2020.09.18.302992.

[40] Mensah N, Tang Y, Cawthraw S, Abuoun M, Fenner J, Thomson NR, et al. Determining antimicrobial susceptibility in Salmonella enterica serovar Typhimurium through whole genome sequencing: A comparison against multiple phenotypic susceptibility testing methods. BMC Microbiol 2019. https://doi.org/10.1186/s12866-019-1520-9.

Figure 1. Melt curve profile of the air-dried HRM assay showing, 1a) the panel comprising the eight markers, being some of the isolates also co-producers and, 1b) detail of the simultaneous detection of two (pink), three (yellow) and four genes (blue) in isolates coproducers of ESBL and carbapenemases genes.

1a) Orange: *K. pneumoniae* harbouring bla_{SHV} and bla_{IMP} genes; red: *E. coli* harbouring $bla_{OXA\text{-}48\text{-}like}$ and $bla_{CTXM\text{-}9}$ genes; pink: *K. pneumoniae* harbouring bla_{SHV} and bla_{KPC} genes, blue: *K. pneumoniae* harbouring bla_{SHV} and bla_{VIM} genes; purple: *Acinetobacter* spp. harbouring bla_{NDM} gene; and in grey: P. aeruginosa harbouring bla_{IMP} gene.

2b) Pink: *K. pneumoniae* harbouring bla_{SHV} and $bla_{CTXM\text{-}1}$ genes; yellow: *K. pneumoniae* harbouring $bla_{OXA\text{-}48\text{-}like,}$ bla_{SHV} and $bla_{CTXM\text{-}1}$ genes; and blue: *K. pneumoniae* harbouring $bla_{OXA\text{-}48\text{-}like,}$ $bla_{NDM,}$ bla_{SHV} and $bla_{CTXM\text{-}1}$ genes.



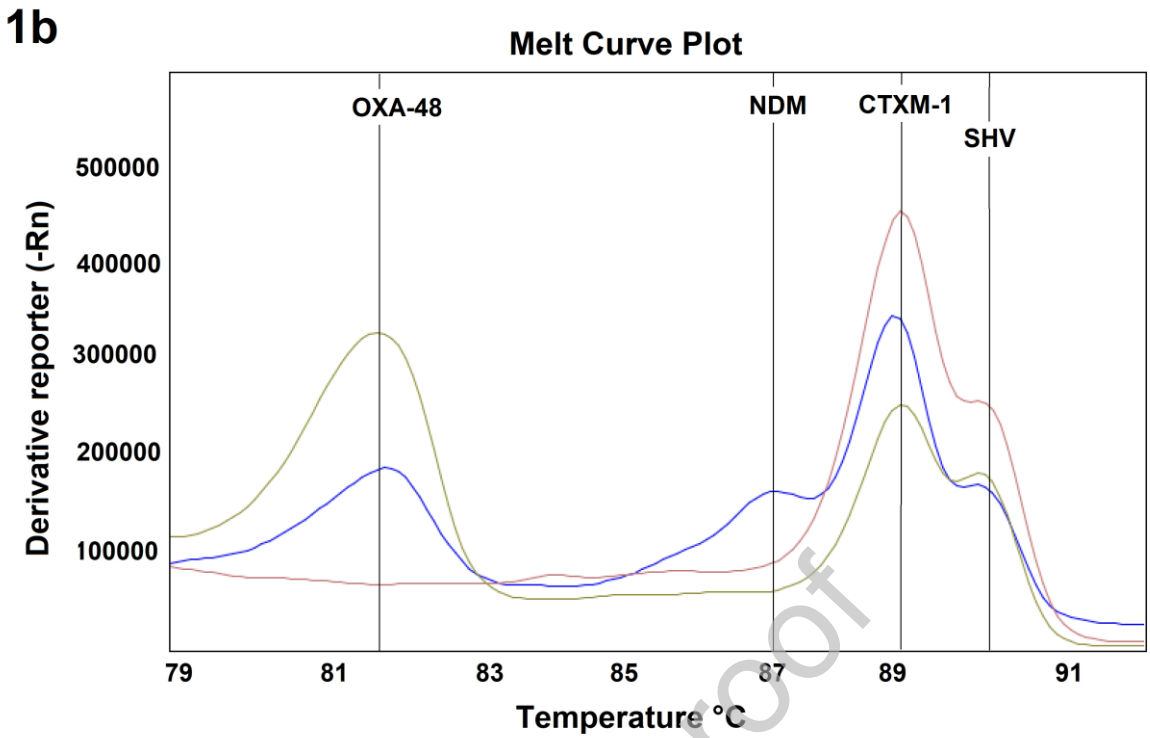


Figure 2. Melting temperatures of the eight amplicons of the air-dried HRM assay ran in the CFX96, QuantStudio™ 5 (QStudio), Rotor-Gene-Q (RotorGene-Q), LightCycler® 480 (LC48) and MIC. The whiskers show the maximum and minimum values, with the exceptions of outliers (circles) and extremes (rhombus).

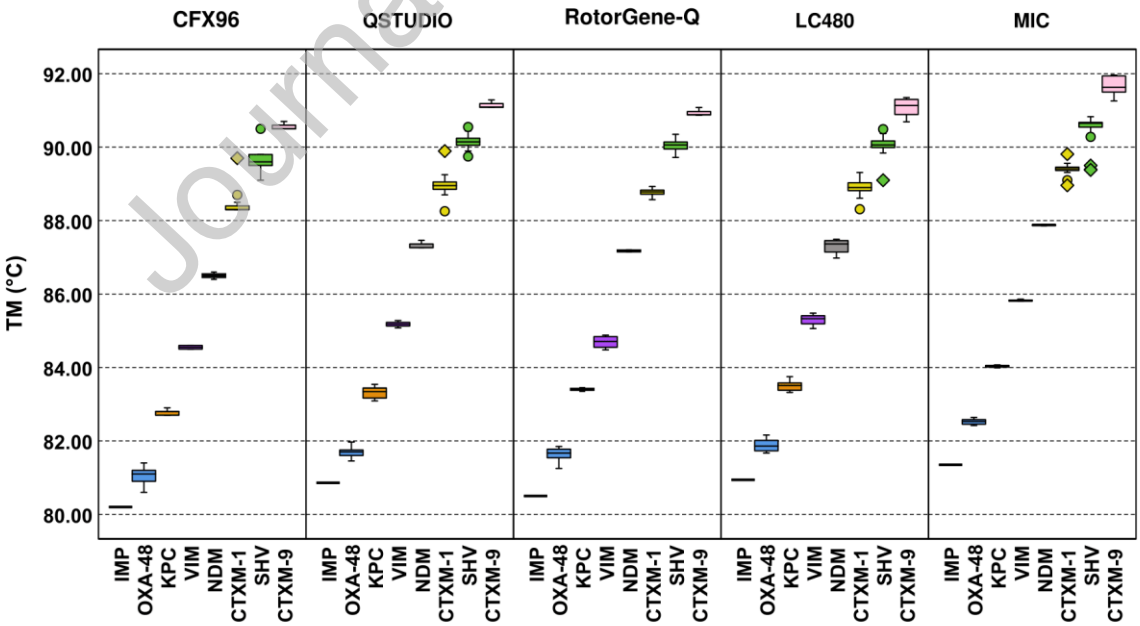


Figure 3. Plate mean fluoresce peak height at the beginning of study (T0), one week (T1), two weeks (T2), one month (T3), three months (T4) and eight months (T5) under fridge storage (6.2°C±0.9), room temperature (20.4°C±0.7) and oven (29.7°C±1.4). Colour of asterisks indicates which storage conditions were statistically different between time points: blue (fridge), orange (room temperature), red (oven), black (all temperature conditions).

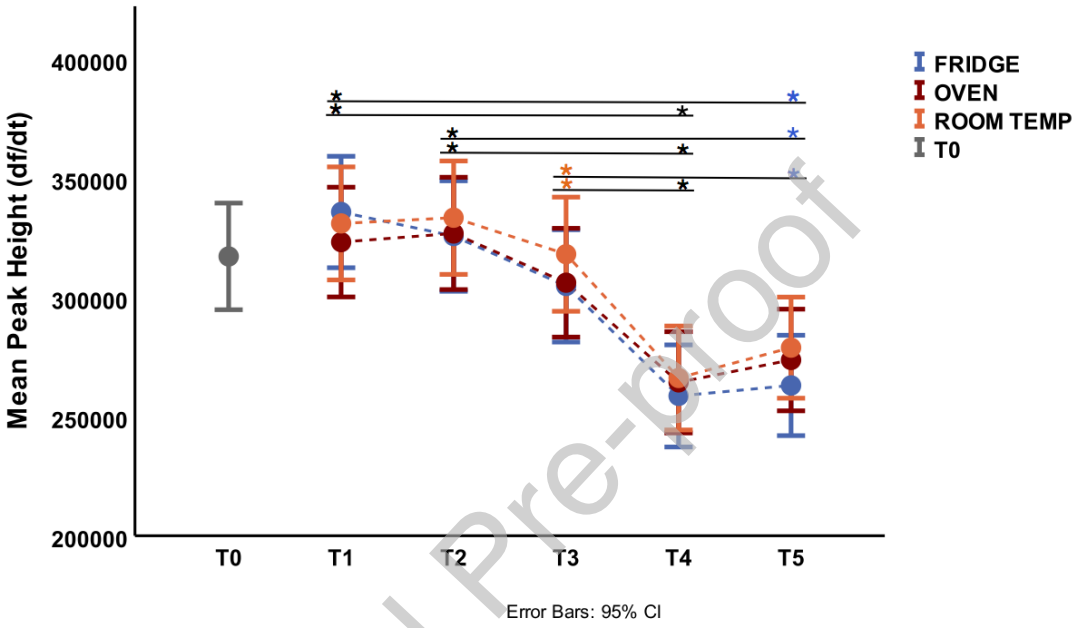


Figure 4. Peak height of the isolates 1 (*bla*$_{CTXM-1}$ positive), 2 (*bla*$_{SHV}$ positive) and 3 (*bla*$_{CTXM-1}$ and bla$_{SHV}$ positive) at LOD dilution at different timepoints and storage conditions.

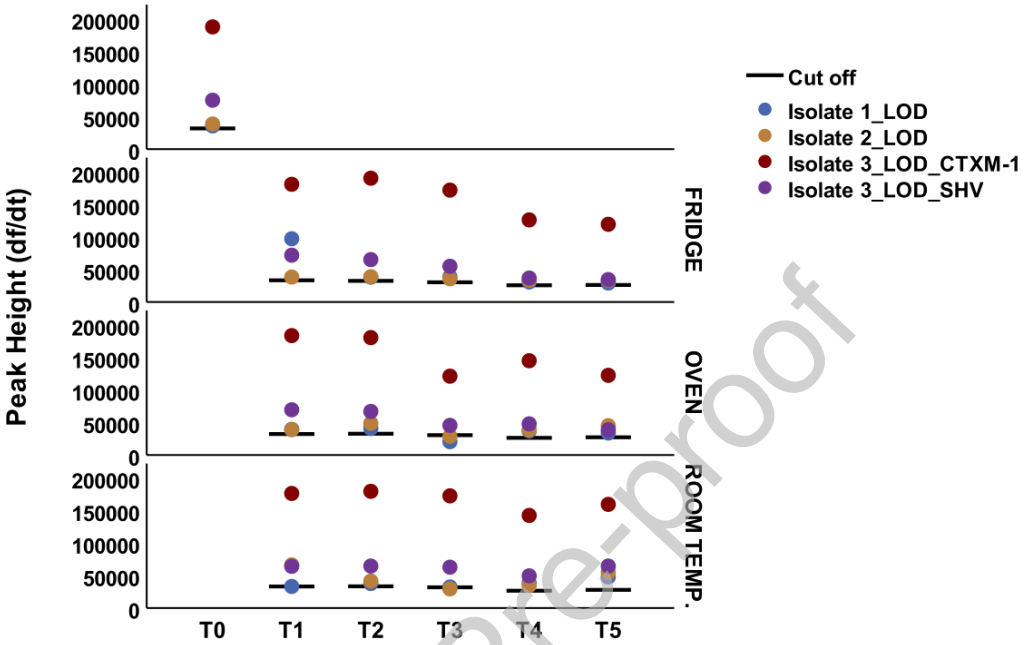


Table 1. Sensitivity (S), specificity (SP), accuracy (ACC) and agreement (κ) of the air-dried HRM assay for detecting individual genes compared to the reference PCR and WGS.

	Reference PCR/WGS					
	Positives	Negatives	S (95% CI)	SP (95% CI)	ACC (95% CI)	κ
Bla_{CTXM-1}			99.2% 97.7%-100%	94.9% 91.7%-97.5%	97.3% 95.3%-98.6%	
Positives	242	10				0.94
Negatives	2	185				
Bla_{CTXM-9}			100% 76.8%-100%	99.8% 98.3%-99.6%	99.8% 98.7%-99.9%	
Positives	14	1				0.96
Negatives	0	422				
Bla_{SHV}			79.7% 71.3%-86.5%	97.5% 95.2%-98.9%	92.7% 89.9%-95%	
Positives	94	8				0.79
Negatives	24	314				
Bla_{NDM}			99.1% 90.2%-98.6%	99.7% 98.3%-100%	99.1% 97%-99.5%	
Positives	112	3				0.98
Negatives	1	321				
Bla_{IMP}			100% 15.8%-100%	100% 99.2%-100%	100% 99.2%-100%	
Positives	2	0				1.00
Negatives	0	438				
Bla_{KPC}			100% 63.1%-100%	100% 99%-100%	100% 99.2%-100%	
Positives	8	0				1.00
Negatives	0	432				
$Bla_{OXA-48-like}$			92.9% 66.1%-99.8%	100% 99.2%-100%	99.8% 98.8%-100%	
Positives	13	0				0.96
Negatives	1	426				
Bla_{VIM}			100% 80.5%-100%	99.5% 98.3%-99.9%	99.6% 98.4%-99.9%	
Positives	17	2				0.94
Negatives	0	421				

Table 2. Sensitivity (S), specificity (SP), accuracy (ACC) and agreement (κ) of the air-dried HRM for detecting individual genes compared to the original 9- Plex HRM assay [23] using Type-it® HRM buffer (Qiagen).

	9-Plex HRM					
	Positives	Negatives	S (95% CI)	SP (95% CI)	ACC (95% CI)	κ
Bla_{CTXM-1}			99.2% 97.7%-100%	93.4% 89.0%-96.5%	96.6% 94.4%-98.1%	
Positives	237	13				0.93
Negatives	2	185				
Bla_{CTXM-9}			100% 76.8%-100%	99.8% 98.3%-99.6%	99.8% 98.7%- 99.9%	
Positives	14	1				0.96
Negatives	0	422				

Bla_{SHV}			97.7% 91.7%-99.7%	95.5% 92.7%-97.4%	95.9% 93.6%-97.5%	
Positives	83	16				0.88
Negatives	2	336				
Bla_{NDM}			97.3% 92.1%-99.4%	97.6% 95.3%-99.0%	97.5% 95.6%-98.9%	
Positives	106	8				0.93
Negatives	3	322				
Bla_{IMP}			100% 2.5%-100%	99.8% 98.7%-99.9%	99.8% 98.7%-99.9%	
Positives	1	1				0.67
Negatives	0	436				
Bla_{KPC}			100% 63.1%-100%	100% 99%-100%	100% 99.2%-100%	
Positives	8	0				1.00
Negatives	0	429				
$Bla_{OXA-48-like}$			100% 73.5%-100%	99.8% 98.7%-100%	99.8% 98.8%-100%	
Positives	12	1				0.96
Negatives	0	424				
Bla_{VIM}			100% 75.3%-100%	98.6% 96.9%-99.5%	98.7% 98.4%-99.9%	
Positives	13	5				0.85
Negatives	0	421				

Table 3. Sensitivity (S), specificity (SP), accuracy (ACC) and agreement (κ) of the air-dried HRM compared to the phenotype in isolates from Nepal.

Enterobacteriaceae						
Phenotype						
	Meropenem					
HRM Carb	Resistant	Susceptible	S (95% CI)	SP (95% CI)	ACC (95% CI)	**K**
Positives	80	6	84.2% 75.3%-90.9%	97.1% 93.8%-98.2%	93.2% 89.5%-95.6%	0.834
Negatives	15	200				
	Cefotaxime					
HRM Carb/ESBL	Resistant	Susceptible	S (95% CI)	SP (95% CI)	ACC (95% CI)	**K**
Positives	232	5	92.1% 88.0%-95.1%	89.80% 77.8%-96.6%	91.69% 88.0%-94.6%	0.729
Negatives	20	44				
Non-Enterobacteriaceae						
	Meropenem					
HRM Carb	Resistant	Susceptible	S (95% CI)	SP (95% CI)	ACC (95% CI)	**K**
Positives	23	5	46.0% 31.8%-60.7%	87.8% 73.8%-95.9%	64.8% 54.1%-74.6%	0.313
Negatives	27	36				
	Cefotaxime					
HRM Carb/ESBL	Resistant	Susceptible	S (95% CI)	SP (95% CI)	ACC (95% CI)	**K**
Positives	42	4	50.0% 38.9%-61.1%	42.9% 9.9%-81.6%	49.5% 38.8%-60.1%	0.020
Negatives	42	3				

Table 4. Variability of melting temperature within the same and between neighbouring cluster obtained in the validated platforms.

4a. Standard deviation of the melting temperatures within the same cluster (±°C).

	Standard deviation of the melting temperatures within the same cluster (±°C)				
	CFX96	LightCycler® 480	MIC	QuantStudio™ 5	Rotor-Gene Q
Bla_{CTXM-1}	0.27	0.19	0.15	0.25	0.08
Bla_{CTXM-9}	0.09	0.25	0.28	0.11	0.08
Bla_{NDM}	0.33	0.20	0.31	0.15	0.14
Bla_{SHV}	0.08	0.14	0.03	0.17	0.03
Bla_{KPC}	0.26	0.19	0.07	0.16	0.20
$Bla_{OXA-48-like}$	0.06	0.18	0.02	0.10	0.18
Bla_{VIM}	0.27	0.19	0.15	0.25	0.08

4b. Mean difference of the melting temperatures within neighbouring clusters (°C).

	Mean difference of the melting temperatures within neighbouring clusters (°C)				
	CFX96	LightCycler® 480	MIC	QuantStudio™ 5	Rotor-Gene Q
$Bla_{OXA-48-like}$ & bla_{IMP}	0.83	0.95	1.18	0.84	1.12
Bla_{KPC} & $bla_{OXA-48-like}$	1.74	1.62	1.51	1.62	1.78
Bla_{VIM} & bla_{KPC}	1.78	1.80	1.79	1.86	1.29
Bla_{NDM} & bla_{VIM}	1.95	2.00	2.05	2.14	2.48
Bla_{CTXM-1} & bla_{NDM}	1.91	1.61	1.52	1.66	1.60
Bla_{SHV} & bla_{CTXM-1}	1.20	1.15	1.14	1.16	1.27
Bla_{CTXM-9} & bla_{SHV}	0.96	1.02	1.11	1.01	0.88