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

Cubas-Atienzar Ana I.\textsuperscript{1}, Williams Christopher T.\textsuperscript{1}, Karkey Abhilasha\textsuperscript{2}, Dongol Sabina\textsuperscript{2}, Sulochana Manandhar\textsuperscript{2}, Rajendra Shrestha\textsuperscript{2}, Hobbs Glyn\textsuperscript{3}, Evans Katie\textsuperscript{3}, Musicha Patrick\textsuperscript{4}, Feasey Nicholas\textsuperscript{1,5}, Cuevas Luis E.\textsuperscript{1}, Adams Emily R.\textsuperscript{1} and Edwards Thomas\textsuperscript{1}

\textsuperscript{1}Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, UK
\textsuperscript{2}Oxford Clinical Research Unit, Patan Academy of Health Sciences, Kathmandu, Nepal
\textsuperscript{3}Liverpool John Moores University, UK
\textsuperscript{4}Wellcome Sanger Institute, Cambridge, UK
\textsuperscript{5}Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi

**Corresponding author:** Thomas Edwards. Research Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, UK. thomas.edwards@lstmed.ac.uk

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

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ABSTRACT
Here we describe the development and evaluation of a novel air-dried high-resolution melt (HRM) assay to detect eight major extended spectrum beta-Lactamase (ESBL) (SHV and CTXM groups 1 and 9) and Carbapenemase (NDM, IMP, KPC, VIM and OXA-48) genes that cause antimicrobial resistance. The assay was evaluated using 440 DNA samples extracted from bacterial isolates from Nepal, Malawi and UK and 390 clinical Enterobacteriaceae isolates with known resistance phenotypes from Nepal. 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 wet format. The overall phenotypic agreement was 91.1% (95%CI: 90.0%-92.9%) on predicting resistance to cefotaxime and carbapenems. We observed good inter-machine reproducibility of the air-dried HRM assay using the Rotor-Gene Q, QuantStudio™ 5, CFX96, LightCycler® 480 and MIC. Assay stability upon storage in the fridge (6.2°C ± 0.9), room temperature (20.35°C ± 0.7) and oven (29.7°C ± 1.4) were assessed at six time points for eight months and no loss of sensitivity occurred under all conditions. 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 Carbapenamase genes to improve AMR diagnosis and treatment.

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INTRODUCTION

Antimicrobial resistance (AMR) is a major global cause of death and the development of new antibiotics is considered a public health priority. AMR causes an estimated 700,000 deaths globally each year, and this number is predicted to rise to 10 million by 2050. Identification of AMR is typically by culture-based phenotypic antimicrobial susceptibility testing (AST) which require 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 an inaccurate diagnosis and inappropriate treatment. First line or broad-spectrum antibiotics are often used in large doses to ensure their efficacy on the suspected but unknown aetiological pathogens. Empirical treatment facilitates the emergence of AMR, increases the duration of hospitalisation, damages the patient microbiota and increases the cost of therapy. Rapid diagnosis of AMR can enable targeted usage of antibiotics, improved patient outcomes and antimicrobial stewardship. Improved use of antibiotics through the development of rapid diagnostics is an important approach to tackle AMR.

The most common mechanism of drug resistance in Gram-negative bacteria is mediated by the production of β-lactamases, including the extended-spectrum β-lactamases (ESBLs) and Carbapenemases, which provide resistance to the β-lactam antibiotics. Polymerase Chain Reaction (PCR) based detection of ESBLs and Carbapenemase genes may provide a faster diagnosis of AMR than phenotypic methods, which might in turn generate more timely information for treatment decisions. 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, their use in clinical settings is, however, hampered by the high degree of multiplexing needed due to the many genes involved in resistance. In addition, PCR requires a cold chain to maintain the integrity of reagents, equipment, and trained staff, which are often unavailable in low-resource
settings, especially 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 plastics. This process would eliminate the need for the cold chain, ensure their biological integrity and simplify preparation, as only nuclease-free water and the DNA template would need to be added to resuspend the PCR reagents. 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 process is costly and requires the addition of excipients, such as cryoprotectants and bulking agents.

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.

METHODS

Air-dried HRM assay optimisation

We adapted an in-house 9-plex HRM PCR that detects nine major ESBL genes (TEM, SHV and CTXM groups 1 and 9) and Carbapenemase genes (NDM, IMP, KPC, VIM and OXA-48) developed in our laboratory to a dry format. 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, 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. PCR plates were briefly centrifuged before PCR amplification and, when plates were not compatible with the thermocycler used, the mixture was transferred to the appropriate reaction vessels. 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. Positivity was indicated by a peak at the predictive melting temperature (Tm) of the target visualised as the negative first derivative of the melting curve. 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, however the TEM was removed. ²⁰

**Stored bacterial DNA and reference molecular tests**

A panel of 440 DNA samples from well documented multidrug resistant (MDR) bacterial isolates from Nepal (n=294), the UK (n=103) and Malawi (n=43) was used to optimise and evaluate the air-dried HRM assay.

DNA from Nepal: this comprises isolates collected from 2012 to 2016 at Patan Hospital and includes strains of *Escherichia coli* (n=112), *Acinetobacter* spp. (n=72), *Klebsiella pneumoniae* (n=59), *Enterobacter* spp. (n=34), *Pseudomonas aeruginosa* (n=10), *Klebsiella oxytoca* (n=4), *Proteus* spp. (n=1), *Providencia retgerii* (n=1), and *Serratia rubidaea* (n=1).
DNA from Malawi: isolates were collected between 1998 and 2016 at Queen Elizabeth Central Hospital 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.

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), *Enterobacter aerogenes* (n=12), *Enterobacter cloacae* (n=13), *Citrobacter freundii* (n=4), *P. aeruginosa* (n=4), *Morganella morgani* (n=2), and *K. oxytoca* (n=1).

DNA from the Nepal and Malawi isolates was extracted using the boilate \(^{21}\) 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 PCR published protocols\(^ {11,12}\) with some modifications and the air-dried HRM assay. The reference PCR reaction mix was performed using DreamTaq PCR reaction mix (Thermo Fisher, UK), 2.5\(\mu\)l of DNA and nuclease free water to a final volume of 12.5\(\mu\)l.

PCR amplification was visualised with PicoGreen\(^ {\text{TM}}\) (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.\(^ {7,20}\) In addition, the 440 isolates were screened using the in-house 9-plex HRM PCR assay originally developed in our laboratory\(^ {20}\) using the commercially available Type-it\(^ {\circledR}\) HRM kit (Qiagen).

**Bacterial strains for phenotype prediction evaluation from Nepal**

A set of 390 Gram negative bacteria with known phenotypes were chosen based on their resistance to imipenem (34%), meropenem (37%) and cefotaxime (85%) from a collection of characterised clinical isolates banked at Patan Hospital in Nepal. Isolates included strains of *E. coli* (n=72), *K. pneumoniae* (n=107), *Acinetobacter* spp. (n=73), *Enterobacter* (n=63), *K.*
oxytoca (n=16), P. aeruginosa (n=13), M. morganii (n=3), P. rettgeri (n=1), Proteus spp. (n=2), Serratia spp. (n=3), Salmonella Typhi (n=25) and Salmonella Paratyphi (n=7).

Isolates were resuscitated on MacConkey or nutrient agar and DNA extracted by a boiling lysis method as described elsewhere. 21

Limit of detection

Limit of detection (LOD) of the air-dried assay was evaluated for the ESBL genes CTXM-1 and SHV, one positive for CTXM-1 (isolate 1), one positive for SHV (isolate 2), and a positive isolate for both genes (isolate 3) to estimate the LOD in isolates coproducing multiple genes. 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. 22 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 isolates co-producers of 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\textsuperscript{20} 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, the air-dried HRM assay had almost perfect agreement (κ = 0.94-1) for the ESBL CTXM group and Carbapenemase markers and moderate agreement (κ = 0.79) for SHV. SHV was often found in coproducers of multiple genes and the sensitivity of SHV was lower in isolates carrying two (76.7%) and three genes (59.3%) than single producers of SHV (92.6%).

**Bacterial strains for phenotype prediction evaluation from Nepal**

The overall agreement of the air-dried HRM result and phenotype was 91.1% (95% CI: 89.0%-92.9%) for Enterobacteriaceae isolates and 59.0% (95% CI: 52.8%-64.1%) for non-Enterobacteriaceae isolates (Acinetobacter spp., P. aeruginosa and H. influenzae). The air-dried HRM assay had strong agreement with the phenotype (κ = 0.820) among Enterobacteriaceae isolates with a sensitivity on predicting resistance to cefotaxime of 92.7% (95% CI: 88.9%-95.4%) and on predicting resistance to carbapenems 83.9% (95% CI: 76.2%-87.9%). However, the phenotype was poorly predicted among non-Enterobacteriaceae isolates using the air-dried HRM assay (κ = 0.251).

**Cross-platform validation**

A good reproducibility was obtained on all instruments. Peak calling was performed by visual observation by the presence of a peak at the expected Tm and 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 CTXM-1, ± 0.07-1.09°C for CTXM-9, ± 0.08-1.15°C for IMP, ±0.02-1.26°C for KPC, ±0.01-1.38°C for NDM, ±0.19-1.5°C for OXA-48, ±0.08-0.94°C for 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 3a and 3b 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 CTXM-1, SHV and both CTXM-1 and SHV genes, respectively.

**Stability upon different storage conditions**

The effect of storage time and temperature was assessed on the AmpDry mix by analysing the plate mean fluorescence peak height and amplification of isolates, including isolates at LOD dilutions. The average temperature for room storage, fridge and oven was 20.35°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 height 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 AmpDry mix stored at time T3 (one month) was not statistically significant to the produced at T0, T1 and T2 at all storage conditions. The AmpDry mix 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 AmpDry mix 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 (NDM) at T4 fridge storage (data not shown).

Discussion
In this study, we evaluated the performance of an 8-plex HRM PCR assay in dry format 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.23,24 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) and its stability
during handling in warm conditions enables easy storage and transportation of the assay at
ambient temperature for long periods of time. This would be of particular importance in
LMICs where laboratories face insufficient and suboptimal cold chain capacity.25

As this assay is easy to set-up and interpretation of results with analysis of the melting data
can be automated, it may be straightforward to implement 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 assay has good performance using the boilate extraction
method, which is fast, simple and easy to implement with minimal resources.
This is timely as global capacity in molecular diagnostics has recently surged in response to the COVID-19 pandemic. 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, which require a panel of PCR assays. Molecular detection of AMR genes can provide useful epidemiological data and enable the tracking of particular resistance genes at a hospital or national level.26

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, which was not statistically significant. 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 can be applied with simple instructions.

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.27,28

The overall agreement to predict bacterial phenotypes was strong (κ = 0.82) 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 many other mechanisms of resistance such as efflux pumps, chromosomal mediated AmpC enzymes, permeability defects, and modifications of target sites that are less common in the family Enterobacteriaceae.29,30 Possible causes of false negative results amongst Enterobacteriaceae isolates include the carriage of less common β-lactamase genes that are not covered by the HRM assay, such as plasmid mediated AmpC enzymes31, or GES-1.32
Other reasons for phenotype-genotype mismatches include enzyme modifications that change the spectrum of activity and susceptibility profile \(^{33}\), and also isolates with MICs close to the breakpoint being incorrectly classified during phenotypic susceptibility testing. \(^{34}\)

To summarise, the air-dried HRM assay detected ESBL and Carbapenemase genes fast, effectively and with 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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**Authors’ contributions**

A.I.C.A., T.E., E.R.A. and L.E.C. contributed to the conception and design of the study; A.I.C.A., C.W., S.M. and R.S. carried out the experimental work. A.I.C.A., T.E. and C.W. analysed the data. A.I.C.A. 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: WHO Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics, 2017. https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf

2. O’Neill J: Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. Rev Antimicrob Resist, 2016. https://amr-review.org/, accessed on 11.02.21

3. Kerremans JJ, Verboom P, Stijnen T, Hakkaart-van Rijn L, Goessens W, Verbrugh HA, Vos MC: 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

4. Saha SK, Darmstadt GL, Baqui AH, Hanif M, Ruhulamin M, Santosham M, Nagatake T, Black RE: Rapid identification and antibiotic susceptibility testing of Salmonella enterica serovar typhi isolated from blood: Implications for therapy. J Clin Microbiol, 2001. https://doi.org/10.1128/JCM.39.10.3583-3585.2001

5. Avesar J, Rosenfeld D, Truman-Rosentsvit M, Ben-Arye T, Geffen Y, Bercovici M, Levenberg S: Rapid phenotypic antimicrobial susceptibility testing using nanoliter arrays. Proc Natl Acad Sci USA, 2017. https://doi.org/10.1073/pnas.1703736114

6. Barenfanger J, Drake C, Kacich G: Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. J Clin Microbiol, 1999, 1;37(5):1415-8.

7. Musicha P, Feasey NA, Cain AK, Kallonon T, Chaguza C, Peno C, Khonga M, Thompson S, Gray KJ, Mather AE, Heyderman RS, Everett DB, Thomson NR, Msefula CL: Genomic landscape of extended-spectrum β-lactamase resistance in Escherichia coli from an urban African setting. J Antimicrob Chemother, 2017.
8. Panda S, El Khader I, Casellas F, López Vivancos J, García Cors M, Santiago A, Cuenca S, Guarner F, Manichanh C: Short-term effect of antibiotics on human gut microbiota. PLoS One, 2014. https://doi.org/10.1371/journal.pone.0095476

9. 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

10. 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

11. 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

12. 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

13. 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

14. 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

15. 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

16. 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

17. Babonneau J, Bernard C, Marion E, Chauty A, Kempf M, Robert R, Vincent QB, Ab L, Johnson C, Alcaïs A, Marion E, Kempf M, Cottin J, Saint-André JP, Marsollier L, Ardant MF, Adeye A, Goundote A, Chauty A, Agossadou D: 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

18. Okeke IN, Feasey N, Parkhill J, Turner P, Limmathurosakul D, Georgiou P, Holmes A, Peacock SJ: Leapfrogging laboratories: the promise and pitfalls of high-tech solutions for antimicrobial resistance surveillance in low-income settings. BMJ Glob Heal, 2020, 5:e003622

19. 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

20. Edwards T, Williams C, Teethaisong Y, Sealey J, Sasaki S, Hobbs G, Cuevas LE, Evans K, Adams ER: 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

21. 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, 1;41(2):117-22.

22. McHugh ML: Interrater reliability: The kappa statistic. Biochem Medica, 2012. https://doi.org/10.11613/bm.2012.031

23. 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

24. 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

25. 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

26. Castagnola E, Tatarelli P, Mesini A, Baldelli I, La Masa D, Biassoni R, Bandettini R: 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

27. 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

28. Wain J, Diep TS, Ho VA, Walsh AM, Hoa NTT, Parry CM, White NJ: 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

29. Lee CR, Lee JH, Park M, Park KS, Bae IK, Kim YB, Cha CJ, Jeong BC, Lee SH: Biology of Acinetobacter baumannii: Pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front Cell Infect Microbiol, 2017.
30. 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

31. Reuland EA, Hays JP, De Jongh DMC, Abdelrehim E, Willemsen I, Kluytmans JA JW, Savelkoul PHM, Vandenbroucke-Grauls CMJE, Naiemi N 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

32. Polotto M, Casella T, de Lucca Oliveira MG, Rúbio FG, Nogueira ML, de Almeida MTG, Nogueira MCL: 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

33. Edwards T, Heinz E, Aartsen J van, Howard A, Roberts P, Corless C, Fraser AJ, Williams CT, Bulgasim I, Cuevas LE, Parry CM, Roberts AP, Adams ER, Mason J, Hubbard ATM: Piperacillin/tazobactam resistant, cephalosporin susceptible Escherichia coli bloodstream infections driven by multiple resistance mechanisms across diverse sequence types. BioRxiv, 2020:2020.09.18.302992

34. Mensah N, Tang Y, Cawthraw S, Abuoun M, Fenner J, Thomson NR, Mather AE, Petrovska-Holmes L: 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 and, 1b) simultaneous detection of two (pink), three (yellow) and four genes (blue) in isolates coproducers of ESBL and carbapenemases genes.

**Figure 2.** Melting temperatures of the eight amplicons of the air-dried HRM assay ran in the CFX96, QuanStudioTM 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 (20.35°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 (CTX-M-1 positive), 2 (SHV positive) and 3 (CTX-M-1 and 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 |
|-------------------|-----------|-----------|------------|-------------|--------------|---|
| CTX-M-1           | 242       | 10        | 99.2%      | 94.9%       | 97.3%        | 0.94 |
|                   | 2         | 185       | 97.7%-100% | 91.7%-97.5%| 95.3%-98.6%  |    |
| CTX-M-9           | 14        | 1         | 100%       | 99.8%       | 99.8%        | 0.96 |
|                   | 0         | 422       | 76.8%-100% | 98.3%-99.6%| 98.7%-99.9%  |    |
| SHV               | 94        | 8         | 79.7%      | 97.5%       | 92.7%        | 0.79 |
|                   | 24        | 314       | 71.3%-86.5%| 95.2%-98.9%| 89.9% -95%   |    |
| NDM               | 112       | 3         | 99.7%      | 99.7%       | 99.1%        | 0.98 |
|                   | 1         | 321       | 90.2%-98.6%| 98.3%-100%  | 97%-99.5%    |    |
| IMP               | 2         | 0         | 100%       | 100%        | 100%         | 1.00 |
|                   | 0         | 438       | 15.8% -100%| 99.2%-100%  | 99.2%-100%   |    |
| KPC               | 8         | 0         | 100%       | 100%        | 100%         | 1.00 |
|                   | 0         | 432       | 63.1%-100% | 99%-100%    | 99.2% -100%  |    |
| OXA-48            | 13        | 0         | 92.9%      | 99%         | 99.8%        | 0.96 |
|                   | 1         | 426       | 66.1%-99.8%| 99.2%-100%  | 98.8% - 100% |    |
| VIM               | 17        | 2         | 100%       | 99.5%       | 99.6%        | 0.94 |
|                   | 0         | 421       | 80.5%-100% | 98.3%-99.9%| 98.4%-99.9%  |    |
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\textsuperscript{20} using Type-it\textsuperscript{®} HRM buffer (Qiagen).

| 9-Plex HRM | Positives | Negatives | S (95% CI) | SP (95% CI) | ACC (95% CI) | κ |
|------------|-----------|-----------|------------|-------------|--------------|---|
| CTXM-1     | 237       | 13        | 99.17%     | 97.7%-100%  | 96.59%       | 0.93 |
|            | 2         | 185       |            |             |              |     |
| CTXM-9     | 14        | 1         | 100%       | 76.8%-100%  | 99.8%        | 0.96 |
|            | 0         | 422       |            |             |              |     |
| SHV        | 83        | 16        | 97.65%     | 91.7%-99.7% | 95.88%       | 0.88 |
|            | 2         | 336       |            |             |              |     |
| NDM        | 106       | 8         | 97.25%     | 92.1%-99.4% | 97.49%       | 0.93 |
|            | 3         | 322       |            |             |              |     |
| IMP        | 1         | 1         | 100%       | 2.5%-100%   | 99.77%       | 0.67 |
|            | 0         | 436       |            |             |              |     |
| KPC        | 8         | 0         | 100%       | 63.1%-100%  | 100%         | 1.00 |
|            | 0         | 429       |            |             |              |     |
| OXA-48     | 12        | 1         | 100%       | 73.5%-100%  | 99.8%        | 0.96 |
|            | 0         | 424       |            |             |              |     |
| VIM        | 13        | 5         | 100%       | 75.3%-100%  | 98.7%        | 0.85 |
|            | 0         | 421       |            |             |              |     |
**Table 3.** Variability of melting temperature within the same and between neighbouring cluster obtained in the validated platforms.

### 3a. Standard deviation of the melting temperatures within the same cluster (±°C).

|                | CFX96 | LightCycler® 480 | MIC | QuanStudio™ 5 | Rotor-Gene Q |
|----------------|-------|------------------|-----|---------------|--------------|
| CTXM-1         | 0.27  | 0.19             | 0.15| 0.25          | 0.08         |
| CTXM-9         | 0.09  | 0.25             | 0.28| 0.11          | 0.08         |
| NDM            | 0.33  | 0.20             | 0.31| 0.15          | 0.14         |
| SHV            | 0.08  | 0.14             | 0.03| 0.17          | 0.03         |
| KPC            | 0.26  | 0.19             | 0.07| 0.16          | 0.20         |
| OXA-48         | 0.06  | 0.18             | 0.02| 0.10          | 0.18         |
| VIM            | 0.27  | 0.19             | 0.15| 0.25          | 0.08         |

### 3b. Mean difference of the melting temperatures within neighbouring clusters (°C).

|                | CFX96 | LightCycler® 480 | MIC | QuanStudio™ 5 | Rotor-Gene Q |
|----------------|-------|------------------|-----|---------------|--------------|
| OXA-48 & IMP   | 0.83  | 0.95             | 1.18| 0.84          | 1.12         |
| KPC & OXA-48   | 1.74  | 1.62             | 1.51| 1.62          | 1.78         |
| VIM & KPC      | 1.78  | 1.80             | 1.79| 1.86          | 1.29         |
| NDM & VIM      | 1.95  | 2.00             | 2.05| 2.14          | 2.48         |
| CTXM1 & NDM    | 1.91  | 1.61             | 1.52| 1.66          | 1.60         |
| SHV & CTXM-1   | 1.20  | 1.15             | 1.14| 1.16          | 1.27         |
| CTXM-9 & SHV   | 0.96  | 1.02             | 1.11| 1.01          | 0.88         |
