Animal & Plant Health Agency

FINAL REPORT

Evaluation and recommendation of a screening protocol for the detection of Extended Spectrum Beta Lactamases of Enterobacteriaceae in food.

FS241023

31st of March 2015

Animal and Plant Health Agency
Woodham Lane
New Haw
Surrey
KT15 3NB.

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1. Liability statement

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2. Executive summary

The aim of this work was to develop and validate phenotypic and molecular methods for isolation, detection and partial characterisation of extended-spectrum β-lactamase (ESBL)–producing Enterobacteriaceae inselected matrices, to produce a Standard Operating Procedure (SOP) for these methods in a suitable format for use by industry, and for industry to trial these methods in the form of a proof of concept study and ring trial.

For the non-industry based side of the work, loop-mediated isothermal amplification (LAMP) assays were developed for the detection of ESBL–producing Enterobacteriaceae from meat, and these were compared with different isolation agars. LAMP assays were developed for CTX-M groups 1, 2 and 9 and OXA-10-like genes. Chicken, lamb, beef, pork and turkey samples were artificially 'spiked' with bacteria at 10, 100 and 1,000 cfu/gram, using 10 different strains of ESBL-producing Enterobacteriaceae (producing CTX-M sequence types 1, 2, 3, 14, 15, OXA-11, SHV-2 or TEM-52) +/- a mix of competitor organisms. Samples were enriched overnight in buffered peptone water (BPW) +/- antibacterials before plating to CHROMagar CTX, OXOID ESBL Brilliance agar and MacConkey agar with 1 mg/L cefotaxime. Selected BPW broths were also tested using LAMP assays, microarrays and sub-cultured onto agar onto which cefpodoxime discs were subsequently applied. For isolation / detection of ESBL-producers from beef, pork, lamb and turkey spiked with 10 or 100 cfu/gram ESBL (natural flora only), all agars and the LAMP assays showed 100% sensitivity and specificity for ESBL spike strains. For chicken samples, both LAMP and chromogenic agars showed improved sensitivity and specificity for detection / isolation of ESBLs compared with MacConkey agar, particularly with competitor bacteria added. In comparison to chromogenic agars, the cefpodoxime disc method and microarray showed reduced sensitivity.

The SOPs developed by APHA were transferred to Leatherhead Food Research for evaluation and field trials. The specificity, sensitivity and precision were all found to be within acceptable limits.

In a test evaluation exercise designed to calculate relevant test statistics, 300 poultry abattoir samples (neck flap and caecal samples), sourced over approximately 5 months (from October 2014 to February 2015) from an existing ongoing FSA study (FS241051) were evaluated by both phenotypic (culture) and genotypic (LAMP) tests. Abattoir

samples were stratified by month, randomised and weighted by abattoir throughput. Additionally, 30 randomly selected retail meat samples were also evaluated to show proof of principle of the method on a further test matrix.

Bayesian approaches without assuming a gold standard were used to analyse the results from the test validation study performed on 300 neck flaps and caecal contents, in order to determine sensitivity, specificity and positive and negative predictive values. For these samples, the culture and CTX-M LAMP sensitivity was 91.7 and 97.1% respectively, while the specificity was 94.8% and 93.4%, respectively. The culture and CTX-M LAMP positive predictive values were 93.1 and 91.5% respectively, while the negative predictive values were 93.7 and 97.6%, respectively.

A small ring trial between Leatherhead and two independent operators showed the method to be transferable and robust.

In addition, LAMP assays were developed and validated for detection of the following antimicrobial resistance genes: *bla*$_{\text{CMY}}$ gene, which encodes an AmpC enzyme; New Delhi Metallo-ß-lactamase-1 (*bla*$_{\text{NDM-1}}$), which confers resistance to carbapenems in addition to most other beta-lactam antibiotics; *bla*$_{\text{VIM}}$, a carbapenem-hydrolysing class B β-lactamase; *bla*$_{\text{OXA-48}}$, a carbapenem-hydrolysing class D β-lactamase. The sensitivity and specificity of these LAMP assays are reported.

Practical application: Isolation and detection of ESBL–producing Enterobacteriaceae from meat and other relevant matrices is an important part of food safety monitoring as some such organisms have potential to cause disease in humans. The LAMP assays developed in this study combined with use of chromogenic agars have the potential to provide robust, rapid detection, isolation and preliminary characterisation of ESBL-producing bacteria in meat**.**

To our knowledge, this is the first time that the use of LAMP assays combined with chromogenic agars has been evaluated for testing for ESBLs from food samples. A dual approach that combines both genetic and phenotypic methods has additional strengths over one method used alone.

3. Non-technical executive summary

Consumers expect that the meat they purchase will be safe to eat. Whilst adequate cooking will kill harmful bacteria that could be present on meat, if products are mishandled, such bacteria can potentially infect individuals who prepare the food or others indirectly via contaminated surfaces or other food products that become contaminated.

In recent years, there has been considerable concern about the bacteria *Escherichia coli (E. coli)* with a certain type of antibiotic resistance profile being present in farm animals such as cattle, chickens, pigs and turkeys. Such bacteria can sometimes subsequently be found in some meat, particularly chicken meat. This type of antibiotic resistance primarily under investigation in this study is caused by enzymes called extended-spectrum β-lactamases (ESBLs), which confer resistance to a group of antibiotics known as the cephalosporins, which are important antibiotics used to treat human infections. Whilst many strains of *E. coli* bacteria live harmlessly in the intestines of most mammals, some strains can cause mild to serious diseases in humans (for example gastro-enteritis, urinary tract infections and systemic infections of the blood and internal organs). If the *E. coli* strains are resistant to antibiotics the infections become more difficult to treat effectively, and the bacteria can be considered more dangerous.

Whilst there have been many studies that have investigated the presence of ESBL-positive bacteria in meat, the methods used in these studies have varied, although there have been some common aspects. Also, to date, there has been a lack of data to validate the sensitivity of the methods used. For example, will such tests be able to detect low numbers of ESBL-producing bacteria from meats, when there might be lots of other bacteria present that could confuse the results? To this end, we sought to develop and validate methods to (1) isolate ESBL-producing bacteria from meats using different agars and (2) to detect ESBL-producing bacteria from meats using genetic methods.

Laboratory studies were performed to determine that the different isolation and detection tests were both sensitive (able to isolate and detect low numbers of ESBL-producing bacteria on meats) and specific (correctly isolated and detected ESBL-producing bacteria). These laboratory studies included a total of 10 different ESBL-producing strains added at different concentrations to chicken, beef, pork, lamb and turkey meats as well as limited work with ready meals. As a result of these laboratory studies an agar isolation method was developed capable of detecting as few as 10

ESBL-producing bacteria per gram in all meat types for all the ESBL-producing bacteria tested (lower levels were not tested). This agar method was shown to be an improvement on some previously used agar methods. The genetic methods performed in a similar manner to the agar tests, with good sensitivity and specificity.

Both agar methods and genetic methods have different advantages and disadvantages, for example the genetic methods can be quicker and give some indication of the type of ESBL gene present, whilst the agar methods are able to isolate the bacteria of interest for further work if needed. When both agar and genetic tests are used together, they present a powerful combination.

Once the agar and genetic tests were developed and tested, a Standard Operation Procedure or SOP was written and passed on to Industry, along with training in all the methodologies involved, so that they could perform a small scale trial of the methods.

The SOPs were passed to Leatherhead Food Research for independent evaluation and trialling. The test evaluation work comprised of three sections: (1) in house validation – using confirmed ESBL-producing strains and non-target strains to produce data on sensitivity, specificity and repeatability; (2) methodology evaluation – a total of 300 abattoir caecal and neck flap poultry samples were used to assess the performance of the screening methods and calculate relevant test statistics (measures of how well the test performs) in detecting ESBLs under field conditions; and (3) a small ring trial of the SOP – 10 blind samples were analysed by Leatherhead and two independent operators to assess the transferability and robustness of the SOP and experimental procedure. Proof of principle of the method was also established for an additional test matrix, using 30 retail meat samples.All of this work was completed with satisfactory results.

Practical application: Isolation and detection of ESBL–producing Enterobacteriaceae from meat and other foodstuffs is an important part of monitoring for food safety as some such organisms have potential to cause disease in humans. The LAMP assays developed in this study combined with use of chromogenic agars have the potential to provide robust, rapid detection, isolation and preliminary characterisation of ESBL-producing bacteria in meat**.**

To our knowledge, this is the first time that the use of LAMP assays combined with chromogenic agars has been evaluated for testing for ESBLs from food samples. A dual approach that combines both genetic and phenotypic methods has additional strengths over one method used alone.

Glossary

APHA – Animal and Plant Health Agency.

BPW – Buffered Peptone broth, a liquid media widely used to grow bacteria.

BZ – benzo[b]thiophene-2-boronic acid

CAZ – ceftazidime

cfu – Colony forming units, used to describe the numbers of bacteria, typically assessed by performing counts on an agar plate.

Ct – Cycle threshold

CTX-M – group of ESBL enzymes that confer resistance to cephalosporin antibiotics.

DNA – Deoxyribonucleic acid

Enterobacteriaceae – Family of bacteria including many common gut bacteria such as *Escherichia coli* or *E. coli*

ESBL – Extended Spectrum β-lactamase. Enzymes that are capable of breaking down many penicillin type antibiotics, including cephalosporins.

LAMP – Loop-mediated isothermal amplification, in this study a genetic test to detect ESBL genes.

LFR – Leatherhead Food Research

NDM-1 – New Delhi Metallo-ß-lactamase-1

OXA – group of genes that give bacteria resistance to penicillin antibiotics. Some specific gene variants of OXA also confer resistance to third or higher generation cephalosporin antibiotics, and such variants are known as ESBLs.

PHE – Public Health England

SHV – group of genes that give bacteria resistance to penicillin antibiotics. Some specific gene variants of SHV also confer resistance to third or higher generation cephalosporin antibiotics, and such variants are known as ESBLs.

SOP – Standard Operating Procedure

TEM – group of genes that give bacteria resistance to penicillin antibiotics. Some specific gene variants of TEM also confer resistance to third or higher generation cephalosporin antibiotics, and such variants are known as ESBLs.

4. Introduction

Scientific background to the aims and objectives

Bacteria producing extended-spectrum β-lactamases (ESBL) occur in farm animals both in the UK[1; 2; 3; 4; 5] and other countries.[6; 7; 8; 9; 10; 11; 12] Farm animals can harbour different types of ESBL, with the CTX-M types currently being the most prevalent. ESBL enzymes confer resistance to third or higher generation cephalosporin antibiotics, which are important for human therapy, and so the bacteria that produce them pose a possible public health risk if transmitted from animals to man via ingestion of raw, undercooked or improperly prepared contaminated meat products.[13]

In the UK, one study investigating ESBL producers from chicken, isolated *E. coli* with CTX-M -1, -2 and -14 from UK and imported chicken breast fillets,[13] whilst in another study *E. coli* producing CTX-M groups 1 or 8 were obtained from imported chicken.[14] In a study conducted in Portugal, ESBL-producing bacteria were isolated from 60% of uncooked chicken carcasses.[15] In a study in the Netherlands, 94% of chicken meat samples contained at least one *E. coli* isolate with an ESBL phenotype, most often producing CTX-M -1, -2, -14, SHV -2 and -12 and TEM-20 enzymes.[16]

There is no internationally agreed standard method that sensitively, specifically and rapidly detects ESBL-producing Enterobacteriaceae from meat, although a number of different culture-based techniques have been used. In a UK study, meat samples were enriched in BPW before plating on Cystine Lactose Electrolyte Deficient (CLED) agar + 8 mg/L ciprofloxacin then looking for growth inside the zone of inhibition caused by a cefpodoxime disc,[13] however this approach lacks sensitivity for any ESBL producers that are susceptible to fluoroquinolones. In another study, meat samples were again enriched in BPW then plated on MacConkey agar with and without cefotaxime or ceftazidime at 1 mg/L,[15] whilst in a further study, meat samples were first enriched in peptone water, then in MacConkey broth containing cefotaxime, with final selection on MacConkey agar containing cefotaxime.[17] The disadvantage of such an approach is that neither cefotaxime or ceftazidime on their own are specific for ESBLs, as many AmpC producing bacteria can grow in the presence of these antibiotics,[18] and a total of three days is required to complete the test. Finally, an alternative study involved two steps of selection on agar plates, plating to BCIG (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide) agar to isolate *E. coli* and Iso-sensitest agar with cefotaxime and

amoxicillin/ clavulanate discs to screen for ESBL, following an initial enrichment in BPW,[14] thus taking at least 72 h to obtain results. Although these studies use different approaches for selection of ESBLs they have a common stage of enrichment in a peptone broth, usually BPW, to increase sensitivity.

Although selective agars are able to differentiate ESBL producers from ESBL non-producers, they do not give any indication of the ESBL type and take at least 48 h to obtain results. Molecular approaches may be more rapid and will give an indication of ESBL type, but these only detect specific ESBL types and their sensitivity is dependent on the numbers of ESBL bacteria present.

In recent years Loop-mediated isothermal amplification (LAMP) has been shown to be a sensitive and rapid molecular method of detecting pathogens such as *Mycoplasma pneumoniae*[19] and *Burkholderia pseudomallei*,[20] as well as for specific detection of antibiotic resistance genes such as the gene that encodes New Delhi metallo-β-lactamase - NDM-1.[21; 22] The method uses 6 primer sets designed to recognize independent regions of the target gene, which increases the specificity as well as the rapidity of the reaction. The technique relies on an isothermal DNA polymerase and is performed at a single incubation temperature (at 65ºC) using a simple incubator, such as a water bath or heating block. LAMP results can be visualized by addition of fluorescent dyes and the assay can easily be performed in "real-time" (RT) in LightCycler machines.[23]

The main aim of this work was to develop, evaluate and then recommend a suitable screening method for detecting, isolating and partially characterising ESBLs Enterobacteriaceae in foods. This involved, developing proof-of-principle LAMP assays for selected ESBL types (CTX-M groups 1, 2 and 9 and OXA 10-like genes) likely to be present in bacteria in meat, and comparing these assays with other methods, including a cefpodoxime disc method,[14] two commercially-available chromogenic agars, MacConkey agar containing cefotaxime, and an ESBL microarray, for different meat types spiked with different ESBLs at 10 or 100 or 1,000 cfu/gram, with and without added competitor bacteria. Once these methods had been developed and / or evaluated in the laboratory, an SOP was written which was supplied to industry for further independent evaluation of the analytical performance. This included a field test evaluation exercise using statistically significant numbers (n=300) of abattoir poultry samples (caecal contents and neck flap), as well as a proof of principle study performed

on 30 retail meat samples, and assessment of the robustness of the method by a small ring-trial.

In addition, beyond the scope of the original proposal, we developed and validated LAMP assays for four other important resistance genes that may affect administration of human therapeutics: bla_{CMY}, bla_{NDM-1}, bla_{VIM} and $bla_{OXA-48.}$

5. Aims and objectives of the investigation

There were three overall objectives to this study with various sub-tasks. These have each been written up as separate sections with their own introduction, materials and methods section and discussion, and these sections are followed by an overall discussion assessing the study.

The three main objectives were as below:-

OBJECTIVE 01: Evaluation of methods for the extraction, detection and confirmation of ESBLs from food.

OBJECTIVE 02: Comparison of procedures for bacterial speciation and genetic screening approaches.

OBJECTIVE 03: Selection, evaluation and trial of a method of screening food for ESBLs.

6. OBJECTIVE 01 – Task 1.1

Evaluating a range of extraction approaches, in different food matrices, using molecular and phenotypic methods. A collection of ESBL-producing Enterobacteriaceae, including a number of *E. coli* strains, will be tested. The *E. coli* strains will include at least one AmpC *E. coli* and a field *E. coli* (non-resistant).[1]

Introduction and discussion

There are perhaps three main ways to break down food / meat samples and release bacteria from them, prior to assessing the presence of bacteria in / on them. These three methods are the stomacher, which uses paddles to break down meat / food samples in a bag with diluent, some form of homogenisation with a blender using blades and homogenisation with a blender that uses beads, such as the Bullet blender. In addition to these methods, various swabbing techniques can be used to sample bacteria from the carcass of animals, but swabbing techniques are not applicable to retail meat samples which may be minced or diced.

At APHA Weybridge all the above three forms of homogenisation / extraction equipment are available. However, the blender using blades and the Bullet blender using beads are generally only suitable for smaller pieces of sample (up to a maximum of ~ 10 grams for the Bullet blender at APHA), whilst stomachers can typically process 25 grams of sample / meat. One of the disadvantages with performing an extraction on smaller samples will be a reduction in sensitivity of isolation of bacteria. For example, if a method of extraction used 1 gram of sample / meat and enrichment, then in theory the limit of detection is 1 bacteria per gram of meat. However, if the method uses 25 grams of meat and enrichment, the method is potentially 25x more sensitive.

With respect to swabbing, there are several studies available in the literature that compare swabbing to other methods of estimating bacterial populations in food / meat samples, but these are generally applied to animal carcasses and not to food. In one study, the Belgian swabbing sampling method for pig carcasses was compared with the reference destructive method with regard to *Escherichia coli* and aerobic plate counts, *Salmonella* and *Campylobacter* prevalence and their relationship. Recovery was

[1] A brief literature review was performed at APHA Weybridge. Discussions concerning methodologies were held at APHA Weybridge on 13th June 2011 with people from all institutes and the FSA present. Recommended extraction procedure was performed at APHA Weybridge and Leatherhead Foods.

significantly lower for the swabbing method and corresponded to a recovery of 36% for *E. coli* counts and 81% for aerobic plate counts in comparison with the destructive method. There was no significant difference between the swabbing and destructive sampling methods for the prevalence of *Salmonella* or *Campylobacter*.[24; 25] In another study, where swabbing was compared to excision, average bacterial recoveries by swabbing, expressed as a percentage of the appropriate recoveries achieved by excision, varied widely from 2 to 100%.[26] Finally, in a study where swabbing was compared to excision for samples from 1,352 bovine, 188 ovine, and 176 porcine carcasses from 70 separate visits to commercial slaughterhouses operating under normal conditions, the mean total aerobic viable bacterial counts for all species sampled by excision was 5.36 log units, which was significantly greater than the 4.35 log units measured for swabbing.[27] Whilst there are some studies that show swabbing that can at best, give similar results to excision,[28] in view of the negative studies, and the inappropriateness of swabbing for the type of samples covered in this project, swabbing was not investigated as a method of recovery.

There is limited data available in the literature that extraction by stomaching and other methods yields similar results. In a comparative study involving 51 food samples, treatment with a new spindle extraction process and stomaching showed that recovery of total aerobic micro-organisms (naturally occurring mesophilic microflora) and foodborne pathogens (from samples inoculated with *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes*) for both methods was highly correlated.[29] In another study, in which coliforms and *E. coli* were isolated from food samples and where results from a Pulsifier and a Stomacher 400 were compared, overall counts of pulsificates and stomachates did not differ significantly.[30] Also, where extraction by use of a blender and a stomacher, were compared to an unrelated method, in the stomacher homogenization, all 100 samples showed no significant difference between Sanita-kun sheet and AOAC Method 966.24, and in the blender homogenization, out of 100 samples tested, 99 showed no significant difference between Sanita-kun sheet and AOAC Method 966.24.[31]

These results collectively show two interesting things. Firstly, the authors considered stomaching a suitable “gold standard” method to compare other extraction methods to, and secondly that all methods of extraction gave similar results.

The food industry standard method for extraction is a stomacher, using 25 grams of meat + diluent, which after homogenisation, is enriched in Buffered Peptone water

(BPW). For the food industry, meat samples are generally prepared according to ISO 6887-2:2003 "*Microbiology of food and animal feeding stuffs – preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 2: Specific rules for the preparation of meat and meat productions; Reference number ISO 6887-2:2003(E).*" This covers the testing of different cuts of meat, surface/skin and whole carcass where appropriate (e.g. poultry). The approved method for most meat samples involves homogenization using a stomacher with the sample placed in a sterile plastic bag and made up to the final volume of diluent. For whole carcass non-destructive sampling the carcass is manually shaken in the diluent for 30 s before pouring off the rinse diluent into a sterile container.

Therefore, in view of the studies that have already been performed and the major methodologies used in food laboratories, it was considered inappropriate to spend time and resources on investigating other extraction procedures that would not be available to many food laboratories and would have reduced sensitivity of detection. It was considered that time would be better spent on investigating different additives to BPW to increase isolation of ESBLs. The required work with different ESBLs and AmpC *E. coli* and a field *E. coli* as competitor strains has been completed and is discussed in other parts of this document, but using the single extraction method, which was considered most sensitive and suitable.

Conclusions

Based on the evidence of the above publications, the methodology used by most food laboratories, the type of methodology suitable for the samples, and in view of the increased sensitivity afforded by processing larger quantities of sample using excision methods, the following extraction procedure was recommended.

Recommended method of extraction

<u>If performing bacterial counts on samples</u> - If performing bacterial counts on foods, then it is important to perform extraction / stomaching in cold Phosphate Buffered Saline (PBS) rather than in BPW, since performing extraction in BPW at room temperature could result in bacteria in meat growing before dilutions of PBS are plated out to agars to perform viable counts.[32] This could result in inaccurate counts.

As such, if performing extraction for bacterial counts, stomach 25 grams of meat with 100 mls of cold (~ 2°C to 8°C) PBS to give a 1/5 dilution of meat to diluent. If dilutions for counts can not be performed immediately, maintain the homogenate at ~ 2°C to 8°C prior to performing dilutions and counts. Once counts have been performed, add the 125 mls of chilled homogenate to 125 mls of double strength BPW (250 mls total) and incubate overnight at 37°C to enrich those bacteria present.

General extraction procedure - Wear newsterile gloves for each food sample processed and spray these gloves with 70% ethanol before starting work as an added precaution to maintain sterility and prevent any possibility of cross contamination between positive samples.

Place a sterile stomacher bag on a top pan balance and open the top of the stomacher bag aseptically to receive samples (meat). Tare the balance so reading is zero. Using sterile scissors cut off pieces of meat / sample (if needed) into small pieces of about 3 to 5 grams into the stomacher bag on the balance until 25 grams (+/- 0.5 grams) has been weighed into the stomacher bag. If the meat is minced or already in small pieces, use sterile forceps to transfer to the stomacher bag until 25 grams (+/- 0.5 grams) is weighed.

From relevant marked sterile BPW, pour ~ 100 mls (use PBS as above if performing counts) into the stomacher bag. Place the stomacher bag + meat sample + BPW into the stomacher and stomach for 4 minutes at 260 beats per minute until most of the meat forms a homogenous suspension. Pour the meat / BPW homogenate back into the original marked BPW container. Incubate overnight at 37°C to enrich those bacteria present.

7. OBJECTIVE 01: Tasks 1.2 and 1.3[1]

Task 1.2: Evaluating the ability of molecular methods to detect ESBL genes directly from food extracts and after enrichment of bacteria in food extracts following overnight culture in Buffered Peptone water (BPW). A range of methods will be applied to food extracts from Task 1.1, including PCR-based methods, isothermal amplification methods, and screening methods using nano-arrays. The molecular techniques would also be used for ESBL gene characterisation from screening agar media.

Phenotypic and molecular methods will also be compared for sensitivity by the analysis of diluted samples. Similar approaches will be used to determine and compare the limit of detection of molecular and phenotypic tests for different ESBL-producing strains.

Task 1.3: Evaluating commercial agars and cefpodoxime (CFP) disks for screening for ESBLs.[2] A range of agars will be used to screen for resistance using a large panel of ESBL *Enterobacteriaceae*, focusing on CTX-M and certain TEM ESBL genes. The use of cefpodoxime (10µg) disks will also be evaluated using food extracts. This evaluation will include determining the sensitivity and specificity of these methods for ESBL detection.

These tasks 1.2 and 1.3 have been written up as one section, as the work inter-relates.[3]

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[1] Most work performed as part of these two sections was performed APHA Weybridge, including preparation of the enrichments from food extracts with standard and selective enrichment, the use of different selective agars, standard PCR tests and the development and validation of the LAMP assays.

[2] The work evaluating the use of cefpodoxime disks and nano-arrays (ESBL arrays) was performed at Public Health England, London (PHE). Both methods performed at PHE used enrichment broths were prepared at APHA, and sent to PHE as frozen samples, with 10% glycerol as cryo-protectant.

[3] Aspects of the work performed in this section have been published as below:

1. Optimisation of isolation of Extended Spectrum Beta-Lactamase Enterobacteriacceae from meat Lemma, F. et al. (2013). In Proceedings of the 23rd European Congress of Clinical Microbiology and Infectious Disease. Abstract and poster. Berlin, Germany.

2. Isolation and Detection of Extended Spectrum β-Lactamase (ESBL)-Producing Enterobacteriaceae from Meat using Chromogenic Agars and Isothermal Loop-Mediated Amplification (LAMP) Assays. Anjum, M. et al. (2013). Journal of Food Science. Volume 78, Issue 12, pages M1892–M1898.

Introduction

This introduction is taken from part of the main introduction due to commonality

There is no internationally agreed standard method that sensitively, specifically and rapidly detects ESBL-producing *Enterobacteriaceae* from meat or other foodstuffs, although a number of different culture-based techniques have been used. In a UK study, meat samples were enriched in BPW before plating on Cystine Lactose Electrolyte Deficient (CLED) agar + 8 mg/L ciprofloxacin then looking for growth inside the zone of inhibition caused by a cefpodoxime disc,[13] however this approach lacks sensitivity for any ESBL producers that are susceptible to fluoroquinolones. In another study, meat samples were again enriched in BPW then plated on MacConkey agar with and without cefotaxime or ceftazidime at 1 mg/L,[15] whilst in a further study, meat samples were first enriched in peptone water, then in MacConkey broth containing cefotaxime, with final selection on MacConkey agar containing cefotaxime.[17] The disadvantage of such an approach is that neither cefotaxime nor ceftazidime on their own are specific for ESBLs, as many AmpC-producing bacteria can grow in the presence of these antibiotics,[18] and a total of three days is required to complete the test. Finally, an alternative study involved two steps of selection on agar plates, plating to BCIG (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide) agar to isolate *E. coli* and Iso-sensitest agar with cefotaxime and amoxicillin/ clavulanate discs to screen for ESBL, following an initial enrichment in BPW,[14] thus taking at least 72 h to obtain results. Although these studies use different approaches for selection of ESBLs, they have a common stage of enrichment in a peptone broth, usually BPW, to increase sensitivity.

Although selective agars are able to differentiate ESBL-producers from ESBL non-producers, they do not give any indication of the ESBL type and take at least 48 h to obtain results. Molecular approaches may be more rapid and will give an indication of ESBL type, but these only detect specific ESBL types and their sensitivity is dependent on the numbers of ESBL present.

In recent years Loop-mediated isothermal amplification (LAMP) has been shown to be a sensitive and rapid molecular method of detecting pathogens such as *Mycoplasma pneumoniae*[19] and *Burkholderia pseudomallei*,[20] as well as for specific detection of antibiotic resistance genes such as the gene that encodes New Delhi metallo-β-lactamase (NDM-1).[21; 22] The method uses 6 primer sets designed to recognize

independent regions of the target gene, which increases the specificity as well as the rapidity of the reaction. The technique relies on an isothermal DNA polymerase and is performed at a single incubation temperature (at 65ºC) using a simple incubator, such as a water bath or heating block. LAMP results can be visualized by addition of fluorescent dyes and the assay can easily be performed in "real-time" (RT) in LightCycler machines.[23]

Materials and methods

Initial strategy. At the first meeting of collaborating partners, it was agreed that the bulk of work to be done with chicken meat, then small amounts of validation with other meats. The types of agars to use and the ESBL and competitor bacteria to use were also agreed. Also, that "ethanol sterilisation of meat was deemed suitable" to provide uniformity for different batches of chicken meat, which would be spiked with ESBLs and with competitor bacteria. The competitor bacteria for the other meat samples would be their own natural micro-flora. In this way the isolation and identification methods would be tested and validated not only for different meat types, with different ESBLs types at different levels, with different types of enrichment, but also with the meat's own "natural" flora, and a very robust challenge of added artificial challenge bacteria, to represent a thorough evaluation of the isolation and identification methods used.

As a general strategy to eliminate possible media variation, it was agreed that the APHA would supply PHE with frozen BPW broths with 10% glycerol (as cryo-protectant) for aspects of work to be performed at PHE, such as the arrays and the cefpodoxime disk isolation work. This strategy meant that a common set of enrichment broths could be used for all the different methodologies study, although PHE were only supplied with a subset of the enrichment broths.

Gold standard. The initial proposal stated that "A series of PCRs, run as multiplex or simplex in RT format, will act as standards against which isothermal amplification methods (02/02) will be evaluated for sensitivity, specificity and cost". In Appendix III Tables 1 and 2, the raw data results for all the meat samples evaluated are presented. It can be seen that most of these samples have results for multiplex PCR for CTX, OXA, SHV and TEM genes.[33] However, it is inappropriate to compare the results from isothermal amplification (LAMP) assays to results from these PCRs for several reasons. Firstly, the correct gold standard to refer all results to is the known presence or absence

of ESBLs in the meat samples, and this is known in all spiking experiments. Secondly, the LAMP assays have not been targeted to detect SHV and TEM genes, for reasons that will be given. Thirdly, the multiplex PCR can only detect general CTX-M, and cannot detect CTX-M groups, making comparison impossible, and fourthly, we have limited evidence on how sensitive the multiplex PCR would be at detecting accurately the CTX, OXA, SHV and TEM from a mixed culture. It is likely that the sensitivity of the multiplex PCR would be markedly reduced by the presence of competitor organisms, and therefore it would not be an appropriate "gold standard" against which to compare the LAMP or other detection methods. However, we have performed single PCR reactions on all genes used for amplification by LAMP: CTX, OXA, SHV, TEM, CMY, NDM, VIM and AmpC; on average the time taken to perform these PCR assays is 90 mins which is much longer than the LAMP assay.

Selection of agars for the study. Rambach, CHROMagar CTX and Oxoid ESBL Brilliance agar, were used in this study for ESBL detection. As MacConkey agar is widely used for isolation of Enterobacteriaceae, this was added as a comparator agar. Since two commercial ESBL agars were already being used, it was decided not to use ChromID ESBL or CHROMagar containing ceftazidime, since previous publications have shown that these agars do not have as good sensitivity and specificity at selecting ESBLs as CHROMagar CTX.[18]

Agars and chemicals used for selective plating. Antibiotics and chemicals were purchased from Sigma-Aldrich. CHROMagar CTX supplement (CHROMagar, France) was added to CHROMagar ECC agar base to make CHROMagar CTX. This same supplement was also added to Rambach agar to make Rambach CTX agar for specific isolation of ESBL *Salmonella*. ESBL Brilliance agar plates (Oxoid, UK) were purchased as pre-poured plates. MacConkey agar (Oxoid) was prepared with 1 mg/L cefotaxime. Each batch of agar was tested prior to use with suitable control organisms.

Bacterial isolates used to spike meat samples and as competitor bacteria. ESBL-producing strains used to spike meat samples, and competitor strains used to provide uniform challenge to spike strains (added to chicken meat only) are listed in Table 1. In addition to these isolates, two CTX-M *Salmonella enterica* strains, S04327-09 (pig) which was a monophasic *Salmonella* Typhimurium DT193 (*S.* 4,5,12:i:-) strain and

S04330-09 (pig), which was *Salmonella* Bovismorbificans strain, were used to spike meat samples. These were mainly investigated with respect to using "Rambach CXT" agar only. All isolates were grown overnight in LB-G broth at 37°C for use.

Human and animal isolates. Most of the isolates used in this work were isolates from animals, as othese strains were considered those likely to be contaminating meat samples and as such most relevant to study. However, the OXA-11 and SHV-2 strains were both obtained from PHE and were of human origin, since at the time of the study, suitable animal isolates with these genes were not available. From the point of view of the isolation and detection techniques used, the selective characteristics of the agars or the genetic specificity of the LAMP assays, will not be affected by the human or animal origin of the isolates. As such, it was not deemed necessary in any parts of this study to perform additional work specifically with human isolates.

Meat samples used in spiking experiments (chicken, beef, pork, lamb and turkey). All meats used in spiking experiments were sourced from local retail outlets. All meat samples were portioned into 25 g samples, placed in sterile plastic bags and stored frozen until required. Chicken samples were the only meat type to which artificial competitor organisms were added. To ensure the background flora was roughly uniform between different batches of chicken, the chicken breast were placed in 70% ethanol for 15 mins, and then air dried for 30 mins on foil, to ensure minimum background flora including ESBL-producing bacteria.

Prior to use each batch of meat samples was checked to be free of ESBL-producing bacteria by enriching in BPW and then plating to CHROMagar CTX (ESBL Brilliance agar and MacConkey agar + 1 mg/L cefotaxime were also used for some samples) for sensitive detection of ESBLs.[18]

Spiking of meat samples (chicken, diced beef, minced beef, pork, lamb and turkey). In order to contaminate meat samples with known levels of different ESBL bacteria, the meat samples were spiked with ESBL bacteria (Table 1) as follows**.**

Viable counts were performed on CHROMagar ECC of overnight cultures of spike strains (Table 1 - ESBLs and / or competitors) to check that growth was ~ 10^9 cfu/ml.[32] Competitor isolates were mixed in a 1:1 ratio and suitable dilutions of all isolates were made in sterile normal saline such that meat samples could be spiked with 10, 100 or

1,000 cfu/g of meat with ESBL-producing bacteria (Table 1) and 10^6 cfu/g of meat with competitor bacteria, as required (Table 1).
Chicken samples were spiked after homogenisation with 10, 100 or 1,000 cfu/g of each ESBL spike organism tested (Table 1) with and without competitor organism.
For beef, minced beef, lamb, pork and turkey only three different ESBL-producing bacteria were evaluated to represent CTX-M groups 1, 2 and 9 (isolates LREC 147, 245 and 261, Table 1), and these strains in separate meat samples, were spiked at 10 or 100 cfu ESBL-producing bacteria/g meat (or control), enriched in BPW and with their own natural flora only, and no added competitor bacteria.

Numbers of samples used in development and evaluation studies. All of the individual samples that were used in method development and evaluation studies, with results for growth on different agars and the multiplex PCR for CTX, OXA, SHV and TEM genes, are shown in Appendix III and Table 1 for chicken meat, and Appendix III Table 2 for other meats tested. As such, 290 different broths from chicken meat were plated to 4 different agars.

Chicken enrichment broths used for non agar methods. A subset of the above BPW broths (see Appendix III, Table 1 for all the broths) for spiked chicken meat only were tested for the presence of ESBLs using the developed LAMP assays, the cefpodoxime disc method, and the Check-MDR CT102 microarray (Check-Points, Netherlands, mainly for ESBL genes).

Homogenisation and spiking of meat samples (see Task 1.1 also). For all meat samples tested, 25 g of meat sample was placed in a sterile stomacher bag with ~ 50 ml of sterile BPW and homogenised in a stomacher (Seward 400 circulator) for 4 min at 260 beats per min. After homogenisation, additional BPW was added to the meat / BPW mix to a final BPW volume of 225 ml, and the meat samples were spiked with ESBL and competitor bacteria at this stage.

Different enrichment types, spike levels, and presence or absence of competitor bacteria. For chicken samples, Table 2 gives an example of all the different conditions that were examined for two ESBL spike strains in two experiments. A total of 16

similar experiments were performed for all ESBL spike strains shown in Table 1 (see Appendix III and Tables 1 and 2 for full details).

For chicken, for each of the ESBL-producing bacteria, seven 25 g chicken samples were homogenised, to provide seven chicken / BPW mixes that were then spiked at 0 and ~ 10, 100, and 1,000 cfu/g of chicken meat with and without the cocktail of competitor isolates (Table 1) at 10^6 cfu/g chicken meat. These seven (per ESBL strain tested) 250 ml chicken / BPW mixes were then split into 3 x 80 ml aliquots as follows to provide normal (no additives) and selective enrichment (1 mg/L cefotaxime or 1 mg/L + 64 mg/L BZ compound - benzo[b]thiophene-2-boronic acid), reported to inhibit AmpC type bacteria.[18] Both of these additives alone and combined were expected to inhibit many non-ESBL producing bacteria. Thus for chicken meat, for each of the eight different ESBL-producing bacteria tested, there were a total of 28 different conditions.

All BPWs resulting from the above experiments were incubated at 37°C overnight prior to plating to agars. However, samples were also plated to agars and used in LAMP assays prior to enrichment, to determine if detection of ESBLs by phenotypic and genotypic methods could be achieved prior to enrichment.

Plating to agars and recording results. All BPW broths after incubation (and for some before incubation also to determine of detection could be achieved without enrichment) were plated to the different agars which were incubated for 24 hours at 37°C with the exception of CHROMagar CTX and Rambach CTX, which were incubated for 48 hours. Presumptive ESBL *E. coli* colonies grew as blue / green colonies on CHROMagar CTX and as deep blue colonies on Oxoid ESBL Brilliance agar. On the type of MacConkey agar used, lactose fermenters such as *E. coli* grew as pink colonies, whilst non lactose fermenters grew as terracotta coloured colonies. See also Figure 1 which shows purple (in this instance of ESBL) and blue green colonies on CHROMagar CTX. *Salmonella* grow as white colonies on CHROMagar ECC and CTX, as terracotta coloured colonies on MacConkey agar and as pink / red colonies on Rambach agar.

Preliminary RPA assay development. Recombinase Polymerase Amplification (RPA) assay was developed for *bla*TEM and *bla*SHV. The assay was tested with three different primer sets and a negative control (no DNA) and a water only reaction. For each test assay 1 µl template DNA from strain B2309 was included. The assay was performed using the TwistAmpTM kit, according to manufacturer's instructions. The

assay was performed on a MxPro real-time PCR machine (Agilent) for 1 h, with fluorescent readings taken every minute.

Primer design, strains and LAMP assays. LAMP assays were developed for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-11}$, *bla*$_{CTX-M}$ group 1, 2 and 9 ESBL genes using EIKEN Software (Eiken Chemical Co. Ltd) and LAMP Designer software (Premier Biosoft) for primer design (Table 3). Strains used specifically for the development of the LAMP assays for CTX-M groups 1, 2 and 9 and OXA-10-like are provided in Table 4. Details on the *bla*$_{TEM}$, and *bla*$_{SHV}$LAMP assays have not been provided as these assays were either non-specific or too long.

Each LAMP assay was performed in 25 µl reactions and included: 1 µM of the backward loop (BLOOP) and forward loop (FLOOP) primers, 0.2 µM of the forward (F3) and backward (B3) primers, and 2 µM forward internal primer (FIP) and backward internal primer (BIP) primers, 15 µl of OptiGene Isothermal Mastermix (containing fluorescent dye) and 1 µl of template DNA. DNA template was either the BPW broth, or a crude DNA extract made from 0.5 ml BPW broth as described elsewhere.[34] The assay was run on a MxPro real-time PCR machine (Agilent) at 65ºC for 1 h, with fluorescent readings taken every minute, followed by 95ºC for 1 min, with continuous reading to determine the point at which the products of amplification dissociated. A blank negative control (no DNA) was included for each assay, as were suitable positive controls. All LAMP assays were performed at least in duplicate with three biological replicates per test.

In addition, LAMP assays for *bla*$_{CMY}$, *bla*$_{NDM-1}$, *bla*$_{VIM}$ and *bla*$_{OXA-48}$ were also developed and tested as described above. See Appendices IV, V, VI and VII for details of strains used in each LAMP assay.

Sensitivity and specificity of LAMP primer sets. The specificity of each primer set was evaluated for the corresponding LAMP assay by using a panel of strains harbouring different ESBL genes (Table 4). To determine the sensitivity of each LAMP assay, the *bla*$_{CTXM-1, -2, -14}$ and *bla*$_{OXA-11}$ genes were amplified by PCR from positive control strains using PCR primers previously described.[35] The number of gene copies in 1µl of the gel purified PCR product, whose DNA concentration was measured using a Nanodrop spectrometer at 280 nm, was calculated using Avogadro's number and the molar concentration of the product with use of an on-line tool.[36] Serial dilutions (in DNase

free water) of the template were prepared and tested, in triplicate, using each LAMP assay. The last dilution at which all three reactions amplified was determined as the limit of detection of the assay. The limit of detection for each assay when testing bacterial culture was determined by using serial dilutions of a crude lysate made from an overnight culture after determining the cfu/ml of the bacterial culture using the method of Miles, Misra & Irwin.[32]

Additional LAMP assays. The sensitivity and specificity of the LAMP primer sets for the four additional LAMP assays are given in Appendices VI, VII, VIII and IX.

Validation meat samples tested by the LAMP assays. For the LAMP assays, 18 un-spiked BPW broth enrichment samples and 54 spiked BPW broth enrichment samples were tested.

Direct detection (no enrichment) of ESBLs from meat with LAMP assays. For direct detection of ESBLs using LAMP assays, 1 g of chicken meat was spiked with different concentrations of the CTX-M group 2 strain, in the presence or absence of competitor organisms, as described previously in Objective 1, Task 1.

Preliminary test evaluation, detection of ESBLs from retail chicken samples. As part of the initial method development, fifteen chicken breast samples were obtained from retailers and enriched overnight in BPW as previously described. Both chromogenic agars and the LAMP assay were used to detect for presence of ESBLs in the enriched samples as described above.

Isolation of ESBLs using cefpodoxime discs. The method as published elsewhere was used[14] for a total of 51 BPW broth samples. These 51 BPW broth samples included negative controls (n = 13, e.g. not spiked with ESBL strains), samples that were spiked with 10 cfu ESBL bacteria / gram of meat ESBL organisms (n = 26) and samples that were spiked with 100 cfu ESBL bacteria / gram meat (n = 12). Three of the BPWs were not enriched, to determine if the methods could detect ESBLs prior to enrichment.
A total of 4 different ESBL organisms were used to spike chicken samples subsequently used for the cefpodoxime disk method including CTX-M 1, 3, 14 and 15 strains.

Characterisation of ESBLs in enriched BPW from chicken samples by arrays. The Check-MDR CT102 arrays were used on DNA extracted by the Qiagen DNAeasy Blood and Tissue kit from 28 of the spiked chicken samples. This array is able to detect CTX-M (CTX-M group 1, 2, 9, 8/25) ESBLs and critical mutations found in TEM- and SHV- type ESBLs. It also allows detection of five major families of carbapenemase genes. The genes detected by the Check-MDR CT102 arrays are shown in Table 5.
Twenty-five BPWs were tested blind, including a negative control and 24 meat samples spiked with: 10, 100 or 1,000 cfu/g of different CTX-M ESBL-producing strains (harbouring $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ genes), with or without competitor strains. Some of the BPWs included additives to inhibit non-ESBLs as previously described. These additives were present to determine if they increased the sensitivity of detection by the arrays, by inhibiting non-target flora.

Calculation of sensitivity and specificity for different tests. Sensitivity and specificity for different tests was calculated as previously described.[37] In brief, the sensitivity was calculated as (positives by both test and gold standard / positives for gold standard) x 100, whilst the specificity was calculated as (negatives by both test and gold standard / negatives for gold standard) x 100. The gold standard for isolation and detection of ESBLs was the known presence or absence of ESBLs in each sample, since all batches of meats were tested free of ESBLs prior to spiking, and any ESBLs were then added at known levels.

Results

Agar and multiplex PCR results for chicken samples. All of the raw data results for the different agars can be seen in Tables 1 (chicken only) and 2 (other meats) of Appendix 3. In addition, results for two experiments only with multiplex PCR results are presentedin Table 2 and a summary of agar and LAMP results can be seen in Table 6.
For enriched meat samples, both chromogenic agars gave similar results (Table 6), although CHROMagar CTX showed increased sensitivity in some instances when competitor strains were present in the spike mixture, because it was able to inhibit these. MacConkey agar + 1 µg/ml cefotaxime had lower sensitivity and specificity than the chromogenic agars (Table 6), especially for chicken samples with added competitor strains. This was because the major colony type overgrew and / or affected the colour of other colonies and also because the agar was not as inhibitory to non-ESBL producers

as the chromogenic agars. Thus, the chromogenic agars were able to isolate ESBLs of CTX-M types 1, 2, 3, 14 and 15 as well as OXA-11, SHV-2 and TEM52 ESBL types from chicken meat when present at 10 cfu/gram of meat. Without enrichment (see table 1 of appendix III) however, in general ESBLs were not isolated from any of the agars.
The multiplex PCR (Figure 2) was in many cases able to detect the presence of ESBL genes relevant for the spike bacteria, but it lacked the sensitivity of the agar isolation (see grey cells of Table 2). In particular, for lower levels of spike bacteria (10 cfu/gram) or in the presence of competitor bacteria, the multiplex PCR could show reduced sensitivity as compared to isolation on agars.

Agar and multiplex PCR results for beef, lamb, pork and turkey. The raw data results for all beef, lamb, pork and turkey samples spiked with three different ESBL strains, with their own natural flora as competitor bacteria, are shown in shown in Appendix III, Table 2. This table also includes the results for the multiplex PCR for CTX-M, OXA, SHV and TEM genes. For these meat samples, all agar types showed 100% sensitivity for isolation of ESBLs from spiked samples after enrichment in BPW. However, in one instance, there was loss of specificity for the Oxoid ESBL Brilliance agar.
The multiplex PCR was 100% sensitive and 100% specific for detecting the presence of CTX-M genes from ESBL bacteria added to these meat samples.

RPA assays. The results for the RPA assays were presented at the meeting for collaborators and the FSA, held at APHA on September 2011. The results showed that the RPA *bla*TEM assays performed were positive under all conditions, including in the negative control with water only. The assay was repeated several times with different primers but the results remained the same including for negative control. It was concluded that the false positive obtained in the negative control assays was due to *E. coli* DNA contamination, probably in the isothermal DNA polymerase. TwistDx who sell the RPA kits was contacted and confirmed that this may indeed be the case.

LAMP assays – sensitivity, specificity with pure cultures. The results of the primer sets which performed best for each LAMP assay (amplified the target gene in approximately 40 min or less) are shown in Table 4; both the cycle threshold (Ct) value and melting point were taken as indicators of performance and specificity. The time taken to produce a positive result ranged between 6 and 38 min and was both strain and assay dependent.

These assays were also much faster than singleplex PCR assays performed using the same control strains; on average PCR reactions were 90 min long (data not shown). The results show the different LAMP assays to be 100% specific for the isolates included in the validation panel (Table 4).

The sensitivity of the LAMP assays were measured in gene copies per µl, and varied for each assay tested. It was most sensitive for the assay designed to detect CTX-M group 1, which detected at 41 gene copies per µl. For CTX-M group 2 and for CTX-M group 9, the detection limit was 109 and 169 gene copies per µl, respectively. For the OXA-10 like LAMP assay the detection limit was 74 gene copies per µl.

In contrast when overnight bacterial cultures were used the detection limits for all the LAMP assays were similar, being between 10^4 and 10^5 cfu/ml of crude lysate, which may be due to differences in the copy number of plasmids harbouring the target genes.

We were not able to use the LAMP for detection of *bla*TEM due to the contamination of the isothermal polymerase from both these systems with the TEM gene. Also, for detection of *bla*SHV by LAMP assays, we were unable to reduce the time of the LAMP reaction below 51 minutes, despite many attempts being made by re-designing the primers. A decrease in time resulted in loss of specificity of the amplification product (data not shown). Therefore, it was agreed that neither the *bla*TEM nor *bla*SHV LAMP assays would be used for the remainder of the study.

LAMP assays – validation with different meat types following enrichment. The LAMP assays that had been validated to work with specific strains were then used to detect for the presence of ESBLs in spiked meat following enrichment for bacteria. Preliminary results indicated that the LAMP assays were more sensitive and faster if a crude DNA extract of the BPW enrichment broth was used as template rather than the fresh BPW (Table 7). There was no evidence that the ability of LAMP to detect ESBL genes was compromised due to the presence of different meat matrices in the enrichment broth (Tables 7 and 8, summarised in Table 6).

For chicken samples without added competitor bacteria, the LAMP assay showed 100% sensitivity and specificity for all the samples tested and was able to correctly confirm ESBL type, including the CTX-M group (Tables 6 and 7). There was 100% sensitivity for detection of CTX-M groups 2 and 9 genes (Tables 6 and 7) but some loss of sensitivity when competitor organisms were present in samples harbouring strains with CTX-M group 1 and OXA-10 like genes, particularly at the lowest spike level (Table

6). The LAMP assay only detected CTX-M group 1 genes in 7/9 chicken samples and OXA-10 like genes in 0/3 chicken samples when the spike level was 10 cfu/g and competitor bacteria were present, and detected OXA-10 like genes in 1/3 samples when the spike level was 100 cfu/ml and competitor bacteria were present (Table 6). However, this lack of detections was reversed for the CTX-M group 1 assay when the enrichment contained both cefotaxime and the BZ (CTX/BZ) compound (Appendix III Tables 1 and 6). It was also reversed for the OXA-10-like assay at the spike level of 100 cfu/g when the enrichment broth contained CTX/BZ (Appendix III, table 1).

For the non-chicken meat samples (Table 8), the LAMP assays showed 100% sensitivity and specificity for detection of ESBLs after enrichment in BPW at both spike levels in the presence of natural competitors.

Therefore, the LAMP assays were shown to be both sensitive and specific for the main ESBL genes of interest (CTX-M group 8, TEM ESBL and SHV ESBL genes were not included) and had comparable sensitivity to that of the chromogenic agars if used on enriched broths (Table 6).

Direct detection of ESBLs from meat with LAMP assays without enrichment. The LAMP assay for CTX-M group 2 was able to directly detect a CTX-M group 2 strain added to chicken meat but only when the bacteria were present at counts of $\geq 10^4$ cfu/gram (Table 9). The detection limit decreased by 10-fold when competitor organisms were added (Table 9). This provided evidence that LAMP assays could be used directly to detect presence of ESBLs from meat samples, however the method was dependent on the concentration of bacteria present in the sample.

Affect of additives in enrichment broths. For the chicken samples only, selective enrichment (BPW + 1 mg/L cefotaxime or 1 mg/L + 64 mg/L BZ compound - benzo[b]thiophene-2-boronic acid) was evaluated compared to BPW alone. Both of these additives alone and combined were expected to inhibit many non-ESBL producing bacteria, whilst the BZ compound is reported to specifically inhibit AmpC bacteria.[18]

The effect of these additives on inhibiting non-ESBL isolates as shown when cultures were subsequently plated on CHROMagar ECC can be seen in Figure 1. The additives could also be seen, in some instances, to results in increased presence / brightness of PCR bands (Figure 2) for CTX-M bands, presumable by inhibiting some of the non-ESBL bacteria, and thereby allowing more CTX-M gene copies to be available for

amplification. The additives were also able, in limited instances, to increase the sensitivity of detection by LAMP assays (as previously discussed), particularly when the counts of spike ESBL bacteria in meat samples were low (10 cfu ESBL bacteria / gram of meat).

Whilst the additives to the BPW broth meant that many more ESBLs were isolated on MacConkey agar + 1 mg/L cefotaxime, there was little to no effect on increasing the isolation of ESBLs if either of the two chromogenic agars were used as the final isolation agar.

Cefpodoxime disc method. The results for all the BPW broths that were checked by the cefpodoxime disk method are shown in Table 10. Neither the chromogenic agars nor the cefpodoxime disk method were able to detect ESBLs in the two samples that were spiked with 10 cfu ESBL bacteria / gram of meat, but were tested prior to enrichment. Of the remaining 48 samples that were all enriched, the cefpodoxime disk method was able to detect all but four positives, giving the method a sensitivity of 88.6%, but 100% specificity. There were no false positives, but the cefpodoxime disk method failed to detect ESBL producers in five chicken samples spiked with 10 cfu/g (of which four had competitor strains added to them). Addition of discs to each plate made this method slightly more time-consuming than direct plating to agar.

Array results for spiked chicken samples. Microarrays were performed on a subset of chicken meat samples that had been spiked (Table 11). Four of the CTX-M-spiked chicken samples that had been enriched in BPW were not detected by the Check-MDR CT102 array. These four samples had a low spike level (10 cfu/g) and also contained added competitor strains. For the other CTX-M-positive samples, the arrays correctly characterised the CTX-M gene to group level regardless of the level of spiking or presence of competitors.

Interestingly, in one sample, CTX-M-9 group ESBL was detected although no spike was present.

Isolation of ESBL Salmonella. "Rambach CTX" agar was used to test for the isolation of two ESBL-producing strains from spiked chicken samples (Table 12). Using this agar, it was possible to specifically isolate *Salmonella* strains when present at only 10 cfu/gram of meat. The other agars used were also able to isolate *Salmonella* from the spiked chicken samples but results are clearer if Rambach CTX agar is used, as *Salmonella* grow pink on this agar, whilst on CHROMagar CTX for example, several different bacterial species, including *Salmonella,* can grow as white colonies. Additionally, Rambach CTX agar was able to isolate ESBL *Salmonella* pre-enrichment in some instances (Table 12).

Isolation and detection from retail chicken samples. For 15 retail chicken samples, 13 were positive for presumptive ESBLs according to results from CHROMagar CTX and Oxoid BRILL agar (Table 13). Of these 13 positive, 11 were also positive by the LAMP assay for group 1 CTX-M. The two samples that were positive on agar but negative by the LAMP assay had <10 cfu CTX-M group 1 ESBL colonies on CHROMagar CTX even after enrichment.

Discussion

Whilst some previous studies have used MacConkey agar supplemented with antibiotics[15; 17] to isolate ESBLs from meat, there are now a number of commercially-available chromogenic agars for the isolation of ESBL producers. One of these (Oxoid ESBL Brilliance agar, as used in this study) has been reported to give a higher degree of sensitivity and specificity than MacConkey agar with ceftazidime discs,[38] whilst another commercial agar (ESBL-Bx; bioMérieux, Marcy l'Etoile, France) was compared with MacConkey agar supplemented with 2 mg/L ceftazidime, and shown to have superior sensitivity for isolation of ESBL producers.[39] However, a third study showed that CHROMagar CTX (as used in this study) had superior specificity for growth of ESBL-producing bacteria than ESBL-Bx, in particular in relation to its ability to inhibit bla_{AmpC} isolates.[18]

In this study, two chromogenic ESBL agars were compared with MacConkey agar supplemented with cefotaxime and both performed consistently better than MacConkey agar. Although more expensive than MacConkey agar supplemented with suitable cephalosporin antibiotics, the improved sensitivity and specificity of the commercial ESBL agars and ability to indicate likely bacterial species should reduce the number of

colonies that need to be further evaluated to confirm ESBL status, and thus, may be cost-effective. There is also a danger of not detecting ESBLs if MacConkey supplemented with 1 mg/L cefotaxime is used, particularly if the meat is colonized with bacteria expressing AmpC enzymes which may overgrow ESBL bacteria present in lower numbers on this agar. Supplementing BPW enrichment broths with additives (cefotaxime / cefotaxime and benzo[b]thiophene-2-boronic acid) was shown to enrich for the target bacteria on the agar plates, but addition of these substances did not increase the sensitivity of isolation when chromogenic agars were used.

Whilst there was a slight loss of sensitivity of LAMP assays compared with isolation using chromogenic agars for chicken samples in the presence of artificial competitor organisms, the LAMP assays were 100% sensitive and specific for non-chicken meat with natural competitor organisms. Also, for CTX-M group 2 and 9, sensitivity was similar to the chromogenic agar methods, which may be an indication of plasmid copy influencing the sensitivity of the LAMP assays.

The one sample in which CTX-M-9 group ESBL was detected although no spike was present suggests that the chicken sample used to prepare the enriched homogenate may have, in this instance, been positive for a native group 9 CTX-M strain, and that the alcohol "decontamination" was not 100% effective for these samples.

The two additives to the BPW enrichment broths were shown in a few instances to increase the sensitivity of the LAMP assays, presumably by suppressing growth of non target bacteria and allowing higher numbers of target bacteria to grow. As such, the use of such additives may be advantageous if LAMP assays were used in absence of agar isolation. However, we did not recommend the use of the additives for our industry recommended method, as it was felt this would increase the test complexity. For agar isolation using CHROMagar CTX, the additives were not shown to improve isolation of ESBLs from spiked chicken samples.

In addition to the LAMP assays for ESBL genes, further LAMP assays were developed / validated in agreement with the FSA for *bla*$_{\text{CMY}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{OXA-48}}$ or as previously described for *bla*$_{\text{NDM-1}}$[21; 22] The sensitivity of the best performing primers from these LAMP assays were as follows: *bla*$_{\text{CMY}}$ - 500 copies/µl of the target gene; *bla*$_{\text{NDM-1}}$-1,000 copies/µl of target gene; *bla*$_{\text{VIM}}$ -875 copies/µl of the target gene; *bla*$_{\text{OXA-48}}$ -500 copies/µl of the target gene. Further details are given in Appendices VI, VII, VIII and IX.

The specificity for *bla*$_{CMY}$, *bla*$_{VIM}$ and *bla*$_{NDM-1}$ were 100% against the panel of 41 strains that were tested. For the *bla*$_{OXA-48}$ LAMP primers (both sets) the specificity was 98% due to a cross-reaction with one *bla*$_{OXA-9}$ harbouring strain included in our panel. However, this reaction was intermittent and may havebeen either due to contamination or cross-reaction due to sequence similarity in the OXA-9 gene harboured by this strain. It is noteworthy that 3 other OXA-9 harbouring strains included in the panel were negative by the OXA-48 LAMP assay. Appendices IV, V, VI and VII provide full details.

Whilst it is accepted that these four LAMP assays were not part of the original proposal, and rather than target ESBL genes, target carbapenamase (OXA_{48}, NDM and VIM) and AmpC genes, these are antibiotic resistance genes that may soon be important to detect in meat and other food products. Carbapenem resistant bacteria are extremely serious in certain human infections, and such isolates have been found in pigs.[40] Development of a test for such genes in meat is therefore important. AmpC genes are likely to be abundant in meat already, based on their abundance in farm animals. If isolates harbouring AmpC genes become more predominant in causing disease in humans, it would also be important to detect them in food samples. Thus, the initial validation of these four probes over and above the probes to target ESBL genes will make the work performed in this study relevant in future studies.

In conclusion, this work has successfully validated a phenotypic agar detection method with sensitivity to at least 10 cfu/gram of meat, for five different meat types (chicken, beef, pork, lamb and turkey). This has been validated for bacteria harbouring eight different ESBL genes including CTX-M sequence types 1, 2, 3, 14 and 15, and in addition for OXA-11, SHV-2 and TEM-52 ESBL types. Additionally, LAMP probes have been developed and / or validated for CTX-M group 1, group 2 and group 9 and OXA ESBL genes, as well as to additional carbapenemase and AMPC genes.

A screening method that combines both phenotypic detection and isolation, with genotypic detection and characterisation, has many advantages over tests that involve only agar isolation or genotypic detection. For example, the agar isolation method provides an isolate, should further genetic characterisation be required such as multi-locus sequence typing (MLST) or pulse-field gel electrophoresis (PFGE) to trace outbreaks, and it also has the ability to isolate strains with an ESBL phenotype, which may have an ESBL gene other than those covered by the LAMP assays. Conversely, the LAMP assays provide results more rapidly (48 hours earlier) than the agar method for

the major ESBL types known to be in meat in the UK at present. Additionally, the LAMP assays are able to identify the major ESBL genes, and distinguish between three major CTX-M groups and therefore, for example, between CTX-M sequence types 1 and 14.

Thus a combined approach of agar isolation and genetic screening using LAMP assays, is both a novel and a robust way to screen and characterise ESBLs from food samples.[41]

Tasks 1.2 and 1.3 - Table 1. ESBL isolates used to spike meat samples and artificial competitor strains for chicken samples only

Strain no	Organism [a]	Resistance / mechanism [b]	ESBL group	Use
LREC 147	*E. coli*	CTX-M 2	CTX-M group 2	ESBL Spike strain
LREC 151	*Klebsiella pneumoniae*	SHV-2	*bla*SHV ESBL	ESBL Spike strain
LREC 163	*Pseudomonas aeruginosa*	OXA-11	OXA-10	ESBL Spike strain
LREC 217	*E. coli*	CTX-M-15	CTX-M group 1	ESBL Spike strain
LREC 226	*E. coli*	CTX-M-3	CTX-M group 1	ESBL Spike strain
LREC 245	*E. coli*	CTX-M 1	CTX-M group 1	ESBL Spike strain
LREC 261	*E. coli*	CTX-M 14	CTX-M group 9	ESBL Spike strain
LREC 509	*E. coli*	TEM-52	*bla*TEM ESBL	ESBL Spike strain
LREC 113	*Morganella morganii*	DHA-M (AmpC gene)	Not an ESBL	Non *E. coli* Competitor
LREC 133	*Salmonella* Senftenberg	CIT-M (AmpC)	Not an ESBL	Non *E. coli* Competitor
LREC115	*Citrobacter freundii*	De-repressed AmpC	Not an ESBL	Non *E. coli* Competitor
S630	*Salmonella* Enteritidis PT 4	Amoxicillin resistant	Not an ESBL	Non *E. coli* Competitor
S631	*Salmonella* Enteritidis PT 29	Amoxicillin sensitive	Not an ESBL	Non *E. coli* Competitor
S632	*Salmonella* Typhimurium PT 104	ESBL -ve but multi-resistant	Not an ESBL	Non *E. coli* Competitor
LREC 92	*E. coli*	ESBL negative but CAZ RS [c]	Not an ESBL	*E. coli* Competitor
LREC 645	*E. coli*	CIT-M (AmpC)	Not an ESBL	*E. coli* Competitor
LREC 646	*E. coli*	CIT-M (AmpC)	Not an ESBL	*E. coli* Competitor
LREC 647	*E. coli*	CIT-M (AmpC)	Not an ESBL	*E. coli* Competitor

a Artificial competitor strains were used to provide a uniform challenge to isolation / detection from chicken meat samples only. Where spike organism was an *E. coli* then challenge organisms were non *E. coli* and vice-versa, to simplify isolation of correct colony types on agar.
b Details of ESBL genes and AmpC genes, or resistance phenotype
c ESBL negative strain that showed reduced sensitivity to ceftazidime

Tasks 1.2 and 1.3 – Table 2. Comparative enumeration of ESBLs on CHROMogenic media and by standard multiplex PCR for experiments 4 and 5 only

Exp No	Spike organism	Spike cfu per gram	Competing organisms	Enrichment type	CHROM Agar CTX (ESBLs)	OXOID ESBL Brill (ESBLs)	CTX-PCR	OXA-PCR	SHV-PCR	TEM-PCR
4	CTX-M 14	10	All	BPW	3+	2+	+	-	-	+
4	CTX-M 14	10	All	CTX	3+	3+	+	-	-	+
4	CTX-M 14	10	All	BZ/CTX	3+	3+	+	-	-	+
4	CTX-M 14	10	None	BPW	3+	3+	+	-	-	-
4	CTX-M 14	10	None	CTX	3+	3+	+	-	-	-
4	CTX-M 14	10	None	BZ/CTX	3+	3+	+	-	-	-
4	CTX-M 14	100	All	BPW	3+	3+	+	-	-	+
4	CTX-M 14	100	All	CTX	3+	3+	+	-	-	+
4	CTX-M 14	100	All	BZ/CTX	3+	3+	+	-	-	+
4	CTX-M 14	100	None	BPW	3+	3+	+	-	-	-
4	CTX-M 14	100	None	CTX	3+	3+	+	-	-	-
4	CTX-M 14	100	None	BZ/CTX	3+	3+	+	-	-	-
4	CTX-M 14	0	None	BPW	-	-	-	-	-	+
4	CTX-M 14	0	None	CTX	-	-	-	-	-	-
4	CTX-M 14	0	None	BZ/CTX	-	-	-	-	-	-
4	CTX-M 14	1000	All	BPW	3+	3+	+	-	-	-
4	CTX-M 14	1000	All	CTX	3+	3+	+	-	-	+
4	CTX-M 14	1000	All	BZ/CTX	3+	3+	+	-	-	+
4	CTX-M 14	1000	None	BPW	3+	3+	+	-	-	-
4	CTX-M 14	1000	None	CTX	3+	3+	+	-	-	-
4	CTX-M 14	1000	None	BZ/CTX	3+	3+	+	-	-	-
5	CTX-M 1	10	All	BPW	3+	2+	+	-	-	+
5	CTX-M 1	10	All	CTX	1+	1+	-	-	-	+
5	CTX-M 1	10	All	BZ/CTX	3+	3+	+	-	-	+
5	CTX-M 1	10	None	BPW	3+	3+	-	-	-	-
5	CTX-M 1	10	None	CTX	3+	3+	-	-	-	-
5	CTX-M 1	10	None	BZ/CTX	3+	3+	-	-	-	-
5	CTX-M 1	100	All	BPW	3+	3+	-	-	-	+
5	CTX-M 1	100	All	CTX	3+	3+	+	-	-	+
5	CTX-M 1	100	All	BZ/CTX	3+	3+	+	-	-	+
5	CTX-M 1	100	None	BPW	3+	3+	+	-	-	+
5	CTX-M 1	100	None	CTX	3+	3+	+	-	-	-
5	CTX-M 1	100	None	BZ/CTX	3+	3+	+	-	-	-
5	CTX-M 1	0	None	BPW	-	-	-	-	-	+
5	CTX-M 1	0	None	CTX	-	-	-	-	-	+
5	CTX-M 1	0	None	BZ/CTX	-	-	-	-	-	-
5	CTX-M 1	1000	All	BPW	3+	3+	+	-	-	+
5	CTX-M 1	1000	All	CTX	3+	3+	+	-	-	+
5	CTX-M 1	1000	All	BZ/CTX	3+	3+	+	-	-	+
5	CTX-M 1	1000	All	BPW	3+	3+	+	-	-	+
5	CTX-M 1	1000	All	CTX	3+	3+	+	-	-	+
5	CTX-M 1	1000	All	BZ/CTX	3+	3+	+	-	-	-

BPW – Buffered Peptone water; CTX – BPW + cefotaxime (CTX); BZ/CTX – BPW + CTX and benzo[b]thiophene-2-boronic acid (BZ).

Semi-quantitative counts after enrichment - 1 = 1 to 10 colonies; 2 = 11 to 100 colonies; 3 = > 100 colonies. Grey cells – False negatives by PCR

Tasks 1.2 and 1.3 – Table 3. Details of primers used in the LAMP assay for different ESBL groups

ESBL group	Primer type	Primer sequence details 5′ to 3′
CTX-M group 1	F3	AACTCTGCGGAATCTGAC
	B3	TTTCTGCCTTAGGTTGAGG
	FIP	CCCACAACCCAGGAAGCAAAAAGCTGGTGACATGGATGAA
	BIP	GTGGCTATGGCACCACCAAAAAGTAAGTGACCAGAATCAGC
	FLOOP	AGTCCAGCCTGAATGCTCG
	BLOOP	ACGATATCGCGGTGATCTGG
CTX-M group 2	F3	AAAGTGACGGCGTTTGCT
	B3	CTGTGCCCGCTGAGTTTC
	FIP	AATGGCGGTATTGAGCGTGGGAAAAACGCTCGTTGGGTGATGAG
	BIP	GTGATACCACCACGCCGCTCAAAAAGCTTTACCCAGCGTCAGAT
	FLOOP	GTTCTGTCCAGACGGAAGGT
	BLOOP	ATGGCGCAGACCCTGAA
CTX-M group 9	F3	CCAATGTGCAGTACCAGT
	B3	TATTCAGCGTAGGTTCAGTG
	FIP	CATTGTGCCGTTGACGTGTTCTTAATCAGCCTGTCGAGAT
	BIP	ACGCTGGCAGAACTGAGCCTGGGCAATCAATTTGTTCA
	FLOOP	TAGTTAACCAGATCGGCAGGC
	BLOOP	CGTTGCAGTACAGCGACAATAC
OXA 10 like	F3	GCATTAGCTAGTTCAATTACAGAA
	B3	GACACCAGTTTCTAGGCC
	FIP	GCTACTTTTACAAAGCACGAAAACAAAAATACGTCTTGGAACAAAGAGT
	BIP	TCCTGCGCTACCAATAACTTAGCAAAAATAATTGCGTTGGGGATCT
	FLOOP	GACGGCTTCGGCAGAGA
	BLOOP	TCGTGCATCAAAGGAATATCTTCC

F3, forward primer; B3, backward primer; FLOOP, forward LOOP primer; BLOOP, backward LOOP primer; FIP, forward internal primer; BIP, backward internal primer

Tasks 1.2 and 1.3 – Table 4. Specificity of LAMP assay for *bla*$_{CTX-M}$ groups 1, 2 and 9 and *bla*$_{OXA}$ 10 like ESBLs

Strain reference no.	ESBL Group	Gene sequence	LAMP primers specific for ESBL groups:-							
			Group 1 CTX		Group 2 CTX		Group 9 CTX		OXA 10 like	
			CT - value (Time)	MP	CT – value (Time)	MP	CT – value (Time)	MP	CT– Value (Time)	MP
12/C0322/02/10	CTX-M group 1	CTX 15	11.85	90.92	ND	84.83	58.69	76.15	ND	77.67
21/C0204/03/09	CTX-M group 1	CTX 15	11.91	90.88	ND	66.17	ND	75.7	ND	87.40
21/C0081/05/09	CTX-M group 1	CTX 15	14.95	90.88	ND	84.78	52.50	89.97	ND	77.15
LREC 61	CTX-M group 1	CTX 1	17.48	91.42	ND	84.89	ND	75.63	ND	77.13
B2329	CTX-M group 1	CTX 1	16.89	91.42	ND	85.40	ND	76.13	ND	76.10
LREC 217	CTX-M group 1	CTX 15	10.72	90.49	ND	66.70	ND	76.17	ND	77.13
B2319	CTX-M group 1	CTX 1	14.22	91.42	48.25	89.97	ND	75.58	ND	77.65
LREC 143	CTX-M group 1	CTX 3	10.67	90.92	ND	85.40	ND	76.10	ND	77.65
LREC 147	CTX-M group 2	CTX 2	ND	77.15	6.00	92.05	38.68	75.65	ND	77.13
B2326	CTX-M group 2	CTX 2	ND	77.65	6.93	92.03	ND	75.59	ND	77.67
LREC 149	CTX-M group 2	CTX 20	ND	77.10	10.00	92.00	ND	76.13	ND	77.13
B2323	CTX-M group 8	CTX 8	ND	77.10	ND	85.40	51.65	75.59	ND	77.13
LREC 94	CTX-M group 9	CTX 14	ND	77.13	ND	88.95	8.00	90.90	ND	77.13
LREC 144	CTX-M group 9	CTX 14B	ND	77.10	ND	84.89	14.86	90.96	ND	77.65
LREC 146	CTX-M group 26	CTX 40	54.88	89.95	ND	84.35	ND	75.63	ND	76.65
LREC 145	CTX-M group 26	CTX 26	ND	77.63	ND	85.40	ND	75.60	ND	76.60
B2306 (whole cell)	None	-	ND	77.63	ND	85.40	ND	75.61	ND	77.15
B2307 (cell lysate)	None	-	59.83	78.15	ND	91.03	48.27	76.15	ND	77.67
LREC 163	OXA-10 like	OXA 11	ND	89.45	50.75	93.53	ND	75.15	11.32	85.88
LREC 164	OXA-10 like	OXA-14	ND	77.15	36.83	84.85	ND	75.10	10.50	85.88
B2308	SHV/OXA/TEM	ND	53.26	77.63	17.93	84.90	57.98	76.15	38.60	85.88
B2309	TEM	ND	ND	77.15	ND	85.38	13.83	76.15	ND	77.15

The Ct value (in min) and MT (melting point, ºC) was used to indicate specificity. Grey highlight denotes positive results.

ND, not determined, was indicated for Ct- values where there was no product detected.

Tasks 1.2 and 1.3 – Table 5. Genes detected by the Check-Points CT102 array

Carbapenemases	**CTX-M ESBLs**	**TEM ESBLs vs. non-ESBL**[a]	**SHV ESBLs vs. non-ESBL**[a]	**AmpCs**	**Controls**
KPC NDM VIM IMP OXA-48	CTX-M-1 group CTX-M-2 group CTX-M-8 & -25 group CTX-M-9 group	TEM wt TEM E104K TEM R164S TEM R164H TEM G238S	SHV wt SHV G238S SHV G238A SHV E240K	-	DNA control Amplification control Hybridization control Negative control

Wt – wild type

a – E104K, glutamine to lysine change from wild type at amino acid 104 etc. The mutations give ESBL status to the TEM or SHV genes.

Tasks 1.2 and 1.3 – Table 6. Summary table for recovery of ESBL producers from spiked chicken samples on different agars after enrichment (all enrichment types included) and detection of ESBLs by LAMP assays

Spike cfu/gram chicken	Comp-etitors bacteria added (+) or not (-)	%[a] and numbers of spiked chicken samples yielding growth on agars			% and numbers of LAMP [b] positive results for ESBL groups as stated			
		CTX	BRILL	MacC+	CTX group 1	CTX group 2	CTX group 9	OXA-10 like
0	-	0%	0%	29.6%	0%	0%	0%	0%
		0 / 28	0 / 27	8 / 27	0 / 9	0 / 3	0 / 3	0 / 3
	+	0%	0%	30.0%	ND	ND	ND	ND
		0 / 10	0 / 8	3 / 10	ND	ND	ND	ND
10	-	100%	100%	90.0%	100%	100%	100%	100%
		30 / 30	30 / 30	27 / 30	9 / 9	3 / 3	3 / 3	3 / 3
	+	100%	86.7%	50.0%	77.8%	100%	100%	0%
		30 / 30	26 / 30	15 / 30	7 / 9 [c]	3 / 3	3 / 3	0 / 3
100	-	100%	100%	90.0%	100%	100%	100%	100%
		30 / 30	30 / 30	27 / 30	9 / 9	3 / 3	3 / 3	3 / 3
	+	100%	93.3%	46.7%	100%	100%	100%	33.3%
		29 / 29	28 / 30	14 / 30	9 / 9	3 / 3	3 / 3	1 / 3 [d]
1000	-	100%	100%	96.7%	100%	100%	100%	100%
		30 / 30	30 / 30	29 / 30	9 / 9	3 / 3	3 / 3	3 / 3
	+	100%	93.3%	56.7%	100%	100%	100%	100%
		30 / 30	28 / 30	17 / 30	9 / 9	3 / 3	3 / 3	3 / 3

ND – Not detected.
Competitor bacteria, where added, were added at ~ 10^6 cfu/gram
CTX, ChrOMagar CTX; BRILL, OXOID Brilliance ESBL agar; MacC +, MacConkey agar + 1 mg/L cefotaxime, RAM +, Rambach agar + CHROMagar CTX supplement.
a Percentage values represent sensitivity when they relate to the relative numbers of positive samples detected as such.
b Less samples were tested by the LAMP assay than by agar isolation as samples spiked with $bla_{\text{TEM-52}}$ or $bla_{\text{SHV-2}}$ and CTX *Salmonella* strains were not tested by LAMP assays.
c The chicken sample spiked with 10 cfu/gram group 1 CTX-M strain that was negative by LAMP when competitor strains were present was positive when the enrichment broth included both cefotaxime and the BZ compound.
d The chicken sample spiked with 100 cfu/gram group 1 OXA strain that was negative by LAMP when competitor strains were present was both positive when the enrichment broth included both cefotaxime and the BZ compound.

Tasks 1.2 and 1.3 – Table 7. Detection of *bla*CTX-M group 1, 2, and 9 from chicken by LAMP assays.

Spike Log cfu/g	Competebacteria	Enrichment type	CTX-M1 (Group 1)		CTX-M15 (Group 1)		CTX-M3 (Group 1)		CTX-M2 (Group 2)			CTX-M14 (Group 9)	
			1	2	1	2	1	2	1	2	3	1	2
0	NONE	BPW	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	NONE	BPW	16.69	17.35	16.93	13.55	16.95	9.8	<8	<8	8.73	32.19	34.58
2	NONE	BPW	15.75	14.94	14.06	11.51	14.97	10.31	<8	<8	8.4	25.89	22.71
3	NONE	BPW	14.95	35.67	14.9	14.24	14.12	8.66	<8	<8	8.36	21.21	21.9
1	ALL	BPW	NO CT	45.65	No Ct	23.32	No Ct	14.26	13.93	<8	10.93	16.99	14.94
2	ALL	BPW	NO CT	31.41	20.9	19.96	26.03	12.93	10.77	<8	11.02	17.72	16.47
3	ALL	BPW	20.93	29.03	20.57	13.54	19.9	12.62	10.26	<8	10.42	17.11	15.2
0	NONE	CTX+	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	NONE	CTX+	15.51	35.42	15.37	15.14	18.7	9.69	<8	<8	8.77	23.29	20.17
2	NONE	CTX+	14.91	14.11	15.63	11.26	14.91	9.96	8.06	<8	9.56	20.17	20.81
3	NONE	CTX+	14.93	16.12	14.66	9	15.33	9.35	10.8	<8	8.36	18.47	17.44
1	ALL	CTX+	20.63	18.2	26	16.16	19.69	11.79	10.5	<8	10.95	17.91	18.15
2	ALL	CTX+	20.75	18.66	21	16.73	15.35	10.39	10.34	<8	11.45	17.46	17.31
3	ALL	CTX+	18.82	15.84	17.46	11.59	17.81	11.43	9.72	<8	10.55	17.11	15.31
0	NONE	CTX	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	NONE	CTX	15.89	14.83	15.65	14.48	15.55	9.9	<8	<8	9.62	41	24.64
2	NONE	CTX	14.84	13.93	16.39	11.03	17.28	9.02	8.27	<8	9.72	21.75	22.29
3	NONE	CTX	14.84	16.7	15.98	11.88	14.37	8.19	8.13	<8	8.81	20.94	20.48
1	ALL	CTX	NO CT	14.57	24.52	19.49	26.54	12.89	11.7	<8	11.12	17.9	14.98
2	ALL	CTX	38.75	21.75	24.46	16.39	19.71	12.94	10.13	<8	10.84	17.44	14.92
3	ALL	CTX	20.01	19.06	17.61	13.34	18.99	11.84	10.93	<8	11.59	16.71	15.73

The type of enrichment and concentration of competitor organism, where appropriate, are given. 1 = fresh BPW; 2 = Boilates + glycerol; 3 = Frozen BPW + glycerol; each assay was performed 3 times

Tasks 1.2 and 1.3 – Table 8. Detection of *bla*CTX-M group 1, 2, and 9 from beef, pork, lamb and turkey by LAMP assays.

Spike Log cfu/g	Meat type	Compete bacteria	Enrichment type	CTX-M1 (Group 1)		CTX-M2 (Group 2)		CTX-M14 (Group 9)	
				1	2	1	2	1	2
0	Beef	Natural	BPW	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	Beef	Natural	BPW	10.18	9.33	8	<8	16.44	13.46
2	Beef	Natural	BPW	13.91	8.81	10.62	<8	16.49	13.46
0	Mince beef	Natural	BPW	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	Mince beef	Natural	BPW	18.98	12.99	<10	<8	19.86	14.85
2	Mince beef	Natural	BPW	14.53	9.99	10.33	<8	17.57	14.95
0	Mince lamb	Natural	BPW	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	Mince lamb	Natural	BPW	26.57	29.6	8.27	<8	21.46	19.5
2	Mince lamb	Natural	BPW	16.79	16.67	10.54	<8	24.55	21.95
0	Pork	Natural	BPW	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	Pork	Natural	BPW	11.08	12.01	10.89	<8	16.34	12.7
2	Pork	Natural	BPW	13.31	10.65	<10	<8	11.99	16.1
0	Turkey	Natural	BPW	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
1	Turkey	Natural	BPW	14.14	11.88	9.66	<8	23.64	19.78
2	Turkey	Natural	BPW	16.67	15.21	10.17	<8	17.75	16.83

1 = Frozen BPW + glycerol; 2 = Boilates + glycerol; the assay was performed in three replicates

Tasks 1.2 and 1.3 – Table 9. Direct detection of spiked ESBL strains from chicken, in the presence or absence of 10^6 CFU/g of competitor organism.

Log Cfu/gram of CTXM-2 Spike strain	Sample Spiked	Without Competitors at 10^6 cfu/gram		With Competitors at 10^6 cfu/gram	
		Time	Mp	Time	Mp
7	chicken	10.72	92.92	25.5	93.53
6	chicken	15.02	92.95	23.02	93.53
5	chicken	17.47	92.96	42.72	93.53
4	chicken	29	92.95	No Ct	86.3
3	chicken	No Ct	85.83	No Ct	86.3
NEG	chicken	No Ct	67.22		
7	PBS	18.48	93.46	12.76	93.03
6	PBS	22.78	93.49	11.33	93.44
5	PBS	9.15	91.96	53.29	93.46
4	PBS	14.62	91.99	No Ct	85.83
3	PBS	23.61	91.99	No Ct	85.83

The assay was performed in two replicates

Tasks 1.2 and 1.3 – Table 10. Comparisons of cefpodoxime disc method and results with chromogenic agars for isolation of ESBLs from chicken meat samples

Exp No	Spike	Spike Log_{10} cfu	Compete	Enrichment	CTX (ESBL)	Brill (ESBL)	Cefpodoxime disk results
2	CTX-M 1 - 245	1	All	BPW	2+	2+	-
2	CTX-M 1 - 245	1	All	CTX	2+	2+	+
2	CTX-M 1 - 245	1	All	BZ/CTX	2+	2+	+
2	CTX-M 1 - 245	1	None	BPW	3+	3+	+
2	CTX-M 1 - 245	1	None	CTX	3+	3+	+
2	CTX-M 1 - 245	1	None	BZ/CTX	3+	3+	+
2	None	0	None	BPW	-	-	-
2	None	0	None	CTX	-	-	-
2	None	0	None	BZ/CTX	-	-	-
4	CTX-M 14 -261	1	All	BPW	3+	2+	-
4	CTX-M 14 -261	1	All	CTX	3+	3+	-
4	CTX-M 14 -261	1	All	BZ/CTX	3+	3+	-
4	CTX-M 14 -261	1	None	BPW	3+	3+	+
4	CTX-M 14 -261	1	None	CTX	3+	3+	+
4	CTX-M 14 -261	1	None	BZ/CTX	3+	3+	+
4	CTX-M 14 -261	0	None	BPW	-	-	-
4	CTX-M 14 -261	0	None	CTX	-	-	-
4	CTX-M 14 -261	0	None	BZ/CTX	-	-	-
4	CTX-M 14 -261	1	All	None	-	-	-
4	CTX-M 14 -261	1	None	None	-	-	-
4	CTX-M 14 -261	0	None	None	-	-	-
6	CTX-M 15 -217	1	All	BPW	3+	3+	+
6	CTX-M 15 -217	1	All	CTX	3+	3+	+
6	CTX-M 15 -217	1	All	BZ/CTX	3+	3+	+
6	CTX-M 15 -217	1	None	BPW	3+	3+	+
6	CTX-M 15 -217	1	None	CTX	3+	3+	+
6	CTX-M 15 -217	1	None	BZ/CTX	3+	3+	+
6	CTX-M 15 -217	2	All	BPW	3+	3+	+
6	CTX-M 15 -217	2	All	CTX	3+	3+	+
6	CTX-M 15 -217	2	All	BZ/CTX	3+	3+	+
6	CTX-M 15 -217	2	None	BPW	3+	3+	+
6	CTX-M 15 -217	2	None	CTX	3+	3+	+
6	CTX-M 15 -217	2	None	BZ/CTX	3+	3+	+
6	CTX-M 15 -217	0	None	BPW	-	-	-
6	CTX-M 15 -217	0	None	CTX	-	-	-
6	CTX-M 15 -217	0	None	BZ/CTX	-	-	-
7	CTX-M 3-226	1	All	BPW	3+	3+	+
7	CTX-M 3-226	1	All	CTX	3+	3+	+
7	CTX-M 3-226	1	All	BZ/CTX	3+	3+	+
7	CTX-M 3-226	1	None	BPW	3+	3+	-
7	CTX-M 3-226	1	None	CTX	3+	3+	+
7	CTX-M 3-226	1	None	BZ/CTX	3+	3+	+
7	CTX-M 3-226	2	All	BPW	3+	3+	+
7	CTX-M 3-226	2	All	CTX	3+	3+	+

Exp No	Spike	Spike Log_{10} cfu	Compete	Enrichment	CTX (ESBL)	Brill (ESBL)	Cefpodoxime disk results
7	CTX-M 3-226	2	All	BZ/CTX	3+	3+	+
7	CTX-M 3-226	2	None	BPW	3+	3+	+
7	CTX-M 3-226	2	None	CTX	3+	3+	+
7	CTX-M 3-226	2	None	BZ/CTX	3+	3+	+
7	CTX-M 3-226	0	None	BPW	-	-	-
7	CTX-M 3-226	0	None	CTX	-	-	-
7	CTX-M 3-226	0	None	BZ/CTX	-	-	-

Grey highlight – Cefpodoxime discs method results does not agree with agar results.

Semi-quantitative counts after enrichment – 1+ = 1 to 1 0 colonies; 2+ = 11 to 100 colonies; 3+ = > 100 colonies.

BPW – Buffered Peptone water; CTX – BPW + cefotaxime (CTX); BZ/CTX – BPW + CTX and benzo[b]thiophene-2-boronic acid (BZ)

Tasks 1.2 and 1.3 – Table 11. Array results for spiked chicken samples spiked with CTX-M ESBL strains with and without competitor organisms.

APHA No	Sample details				Overall array result	Overall array CTX-M-group
	A	B	C	D		
19	1	1	Y	a	Negative	Negative
22	1	1	N	a	ESBL	CTX-M-1
31	-	0	N	a	ESBL	CTX-M-9
55	14	1	Y	a	Negative	Negative
58	14	1	N	a	ESBL	CTX-M-9
64	14	2	N	a	ESBL	CTX-M-9
65	14	2	N	b	ESBL	CTX-M-9
66	14	2	N	c	ESBL	CTX-M-9
70	14	3	Y	a	ESBL	CTX-M-1
71	14	3	Y	b	ESBL	CTX-M-1 and CTX-M-9
72	14	3	Y	c	ESBL	CTX-M-9
73	14	3	N	a	ESBL	CTX-M-9
74	14	3	N	b	ESBL	CTX-M-9
75	14	3	N	c	ESBL	CTX-M-9
83	1	1	Y	a	Negative	Negative
84	1	1	Y	b	ESBL	CTX-M-1
85	1	1	Y	c	Negative	Negative
86	1	1	N	a	ESBL	CTX-M-1
87	1	1	N	b	ESBL	CTX-M-1
88	1	1	N	c	ESBL	CTX-M-1
129	15	3	N	a	ESBL	CTX-M-1
157	3	3	N	a	ESBL	CTX-M-1

A – CTX-M type; B – spiked ESBL cfu/gram meat $\log_{10}$; C – competitors added Y/N; D – Enrichment method (a - BPW alone; b - BPW + cefotaxime; c - B - BPW + cefotaxime + ampC inhibitor).

Grey – where array has not detected presence of ESBLs

Tasks 1.2 and 1.3 – Table 12. Isolation of ESBL *Salmonella* on Rambach CTX agar from chicken samples spiked with ESBL *Salmonella*

Exp No	Spike ESBL strain	Spike Log cfu / gram	Compete	Enrichment	Isolation of ESBLs on different agars			
					CHROMagar CTX	ESBL Brilliance	MacConkey + 1 mg/L cefotaxime	Rambach CTX
16	S4330-11	0	All	BPW	-	-	-	-
15	S4327-11	0	All	BPW	-	-	-	-
15	S4327-11	0	All	BZ/CTX	-	-	2+	-
16	S4330-11	0	All	BZ/CTX	-	-	3+	-
16	S4330-11	0	All	CTX	-	-	-	-
15	S4327-11	0	All	CTX	-	-	-	-
16	S4330-11	0	All	None	-	-	-	-
15	S4327-11	0	All	None	-	-	-	-
15	S4327-11	0	None	BPW	-	-	ND	-
16	S4330-11	0	None	BPW	-	-	2+	-
16	S4330-11	0	None	BZ/CTX	-	-	-	-
16	S4330-11	0	None	CTX	-	-	-	-
16	S4330-11	0	None	None	-	-	-	-
16	S4330-11	1	All	BPW	3+	2+	-	2+
15	S4327-11	1	All	BPW	2+	2+	-	2+
15	S4327-11	1	All	BZ/CTX	2+	2+	3+	1+
16	S4330-11	1	All	BZ/CTX	3+	2+	3+	2+
15	S4327-11	1	All	CTX	1+	2+	-	2+
16	S4330-11	1	All	CTX	2+	2+	-	2+
15	S4327-11	1	All	None	-	-	-	-
16	S4330-11	1	All	None	-	-	-	1+
15	S4327-11	1	None	BPW	3+	3+	3+	2+
16	S4330-11	1	None	BPW	3+	3+	3+	3+
15	S4327-11	1	None	BZ/CTX	3+	3+	3+	3+
16	S4330-11	1	None	BZ/CTX	3+	3+	3+	3+
15	S4327-11	1	None	CTX	3+	3+	3+	3+
16	S4330-11	1	None	CTX	3+	3+	3+	3+
15	S4327-11	1	None	None	-	-	-	1+
16	S4330-11	1	None	None	-	-	-	-
15	S4327-11	2	All	BPW	2+	2+	-	2+
16	S4330-11	2	All	BPW	3+	2+	-	2+
15	S4327-11	2	All	BZ/CTX	2+	3+	3+	2+
16	S4330-11	2	All	BZ/CTX	2+	2+	2+	2+
16	S4330-11	2	All	CTX	2+	2+	-	2+
15	S4327-11	2	All	CTX	3+	3+	-	2+
16	S4330-11	2	All	None	-	1+	-	1+
15	S4327-11	2	All	None	1+	-	-	-
15	S4327-11	2	None	BPW	3+	3+	3+	3+
16	S4330-11	2	None	BPW	3+	3+	3+	3+
16	S4330-11	2	None	BZ/CTX	3+	3+	2+	3+
15	S4327-11	2	None	BZ/CTX	3+	2+	3+	3+

Exp No	Spike ESBL strain	Spike Log cfu / gram	Compete	Enrichment	Isolation of ESBLs on different agars			
					CHROMagar CTX	ESBL Brilliance	MacConkey + 1 mg/L cefotaxime	Rambach CTX
15	S4327-11	2	None	CTX	3+	3+	3+	2+
16	S4330-11	2	None	CTX	2+	2+	-	3+
16	S4330-11	2	None	None	-	-	-	1+
15	S4327-11	2	None	None	-	1+	1+	-
15	S4327-11	3	All	BPW	2+	3+	-	2+
16	S4330-11	3	All	BPW	3+	2+	-	2+
16	S4330-11	3	All	BZ/CTX	2+	2+	-	2+
15	S4327-11	3	All	BZ/CTX	3+	3+	3+	2+
16	S4330-11	3	All	CTX	2+	2+	-	3+
15	S4327-11	3	All	CTX	3+	2+	-	2+
16	S4330-11	3	All	None	1+	1+	-	2+
15	S4327-11	3	All	None	1+	1+	-	2+
15	S4327-11	3	None	BPW	3+	3+	3+	3+
16	S4330-11	3	None	BPW	3+	2+	2+	3+
15	S4327-11	3	None	BZ/CTX	3+	3+	3+	3+
16	S4330-11	3	None	BZ/CTX	3+	3+	3+	3+
15	S4327-11	3	None	CTX	3+	3+	3+	3+
16	S4330-11	3	None	CTX	3+	3+	2+	3+
15	S4327-11	3	None	None	1+	1+	1+	1+
16	S4330-11	3	None	None	1+	1+	1+	1+

BPW – Buffered Peptone water; CTX – BPW + cefotaxime (CTX); BZ/CTX – BPW + CTX and benzo[b]thiophene-2-boronic acid (BZ)

Semi-quantitative counts after enrichment – 1+ = 1 to 1 0 colonies; 2+ = 11 to 100 colonies; 3+ = > 100 colonies.

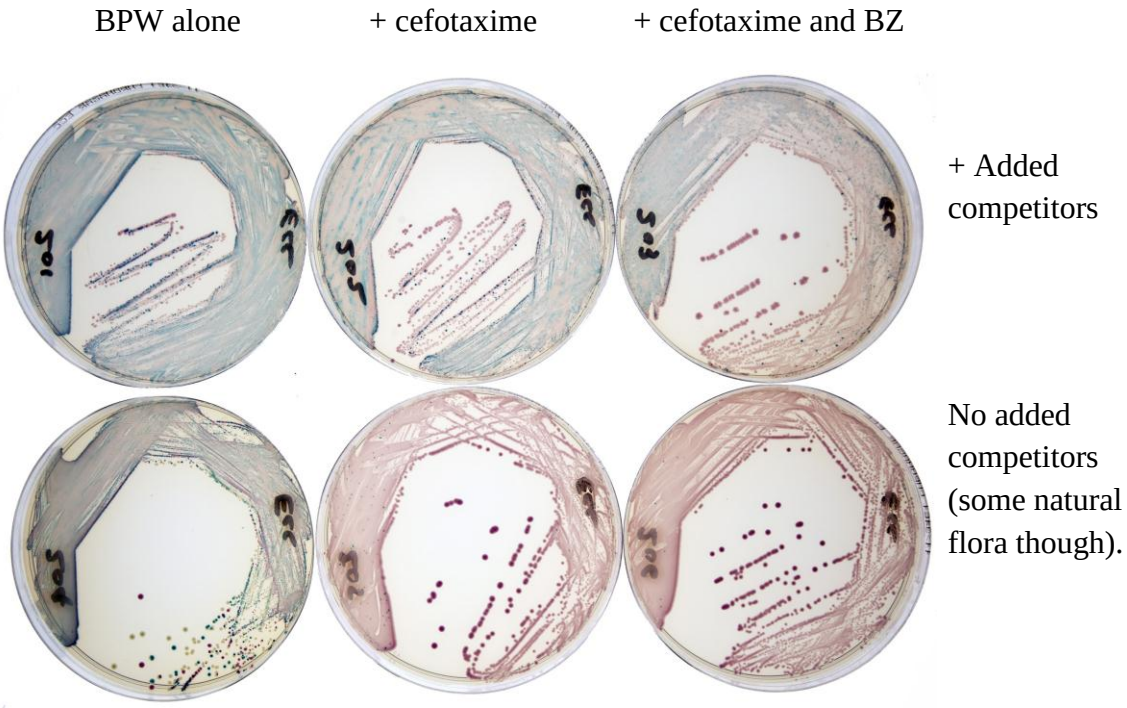
Grey – In some instances ESBLs were detected on Rambach agar from pre-enrichment BPW broths

Tasks 1.2 and 1.3 – Table 13. Isolation and detection of ESBLs from 15 retail chicken samples.

Exp No	Counter	Meat	Enrichment	Isolation of ESBLs on different agars			LAMP
				CHROMagar CTX	ESBL Brilliance	McConkey + 1 mg/L cefotaxime	CTX
13	330	Chicken	BPW	3+	3+	2+	+
13	331	Chicken	BPW	2+	3+	2+	+
13	332	Chicken	BPW	3+	3+	1+	+
13	333	Chicken	BPW	3+	3+	1+	+
13	334	Chicken	BPW	3+	3+	2+	+
13	335	Chicken	BPW	3+	3+	1+	+
13	336	Chicken	BPW	1+	2+	1+	-
13	337	Chicken	BPW	-	-	1+	-
13	338	Chicken	BPW	2+	3+	1+	+
13	339	Chicken	BPW	2+	3+	3+	+
13	340	Chicken	BPW	1+	2+	2+	-
13	341	Chicken	BPW	-	1+	1+	-
13	342	Chicken	BPW	3+	3+	1+	+
13	343	Chicken	BPW	2+	3+	1+	+
13	344	Chicken	BPW	3+	3+	1+	+

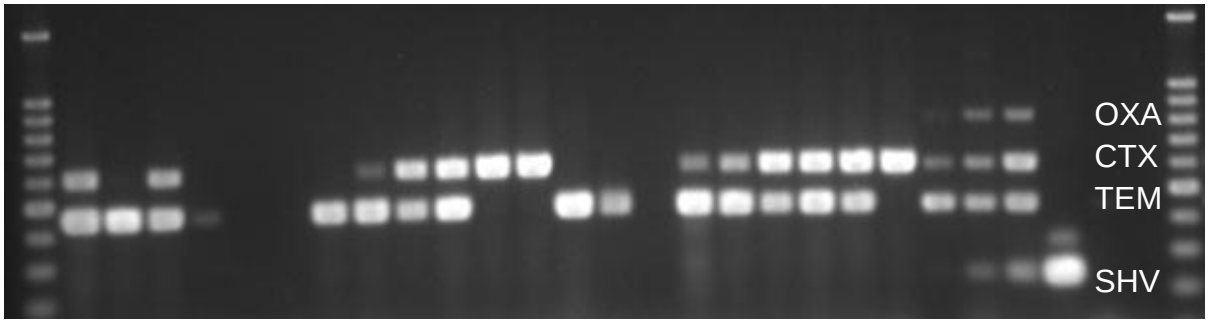
Grey – positive on at least one Chromogenic agar, but negative by LAMP assay

Tasks 1.2 and 1.3 - Figure 1. CHROMagar ECC plates from enriched chicken samples spiked with ~ 100 cfu/gram SHV ESBL *Klebsiella pneumoniae.*



Purple colonies, *Klebsiella pneumoniae* - SHV ESBL. White and blue/green colonies – non ESBLs.

Tasks 1.2 and 1.3 - Figure 2. CTX-M, OXA, SHV and TEM multiplex PCR for enriched broths from experiment 5 – CTX-M 14.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 Controls….

(a) Lanes 1 to 3, ~ 10 cfu CTX + competitors. 1, BPW, 2, + CTX, 3, CTX/BZ
(b) Lanes 4 to 6, ~ 10 cfu CTX + NO competitors. 1, BPW, 2, + CTX, 3, CTX/BZ
(c) Lanes 7 to 9, ~ 100 cfu CTX + competitors. 1, BPW, 2, + CTX, 3, CTX/BZ
(d) Lanes 10 to 12, ~ 100 cfu CTX + NO competitors. 1, BPW, 2, + CTX, 3, CTX/BZ
(e) Lanes 13 to 15, ~ Meat control, no added organisms.
(f) Lanes 16 to 18, ~ 1,000 cfu CTX + competitors. 1, BPW, 2, + CTX, 3, CTX/BZ
(g) Lanes 19 to 21, ~ 1,000 cfu CTX + NO competitors. 1, BPW, 2, + CTX, 3, CTX/BZ
(h) Lanes 21 +, PCR controls.

8. OBJECTIVE 01: Task 1.4

Assessment of the proportion of ESBL-producing bacteria in food. The total number of Enterobacteriaceae of a particular species, as indicated by the chromogenic media, will be counted to determine the proportion of resistant bacteria; to be carried out by plating food extracts on selective and non-selective media.[1]

Introduction

Most studies that focus on the isolation of ESBLs from meat,[13; 16; 42] or animal faeces[2; 16; 43] focus on the presence or absence of ESBLs, usually using some form of enrichment to ensure sensitive detection. However, such studies do not provide information of the numbers of ESBLs present. If a very sensitive enrichment technique is used, in theory, numbers of ESBL bacteria could be as low as ~ one ESBL per 25 grams of meat, which is the theoretical limit of detection possible using the recommended technique of this study (see Task 1.1). However, in the work of this project, the detection of ESBLs was validated down to ~ 10 ESBLs per gram of meat (see Tasks 1.2 and 1.3).

Whilst sensitive techniques are essential to provide details on the presence or absence of specific bacterial types within foodstuffs or animals, it can be useful to determine the numbers of bacteria present in foodstuffs and animals, in order to determine the risk in relation to, for example, infective dose of a particular bacteria. In recent studies at APHA Weybridge, counts of specific bacteria including ESBLs have been determined in animal faeces[1] and waste milk.[44] The purpose of task 1.4 was to determine if proportional counts of ESBL and non-ESBL Enterobacteriaceae could be determined in meat samples.

Materials and methods

Bacterial counts. Counts of bacteria in meat samples were performed as outlined for Tasks 1.2. and 1.3. Counts were performed from minced beef, minced lamb, pork, turkey and beef. Counts were not performed in this project from chicken meat, as the chicken samples that were used in this study were subjected to "ethanol sterilisation" as outlined in Tasks 1.2 and 1.3. However, later work for the FSA under Public Health project PR-R3-0212-21001 – "Defining reservoirs of ESBL-producing E. coli and the threat posed to personal, animal and public health in the UK" has provided counts for chicken meat.

[1] Performed at APHA Weybridge.

Counts were performed on five different agars (blood agar, CHROMagar ECC, CHROMagar CTX, MacConkey + cefotaxime and Rambach CTX), to give levels of different bacteria. On blood agar, most culturable aerobic bacteria, including Gram-positive bacteria such as *Enterococcus, Staphylococcus* and *Streptococcus* should grow, and as such, counts on this agar should be higher than counts on the other agars. All the other agars used are more selective for specific types of bacteria. CHROMagar ECC and MacConkey agar is mainly selective for *Enterobacteriaceae,* although members of the family *Pseudomonaceae* will also grow on these agars. The MacConkey agar contains 1 mg/L cefotaxime and is used in these counts as an agar comparable to that used in other studies (see Tasks 1.2 and 1.3) for isolation of ESBL type bacteria. However, MacConkey agar + 1 mg/L cefotaxime will not be as specific as the CHROMagar CTX and Rambach CTX agars, and will result in higher counts. The remaining two agars both contain the CTX supplement (see Tasks 1.2 and 1.3), which make them specific for mainly ESBLs. The CHROMagar CTX should allow all *Enterobacteriaceae* to grow, including *Salmonella* which will grow as white colonies on this agar. The Rambach agar is specific for ESBL *Salmonella,* as they will grow as pink / red colonies on this agar.

The counts of bacteria on the different meat types are shown in Table 1. On blood agar agar, the total counts were highest, as would be expected, with the highest count being from minced lamb, and corresponding to about a million bacteria per gram of meat. As Gram-positive bacteria grow on blood agar, but not generally on the other agars, the higher counts on blood agar suggest that many of these isolates on this agar are Gram-positive bacteria, although in general these were not identified by MALDI ToF as shown in Table 2.

The CHROMagar ECC showed up to ~ 1,000 cfu/gram of mainly *Enterobacteriaceae.* Many of these isolates were identified by MALDI ToF and were isolates such as *E. coli, Acinetobacter, Enterobacter cloacae, Hafnia alvei, Pseudomonas lundensis* and *Yersinia enterocolitica.*

None of the two agars shown to be specific for ESBLs gave rise to ESBLs, so proportional counts of ESBL and non-ESBL could not be determined for the meat samples examined.

Table 1 of Task 1.4. Counts of bacteria on different meats, on different types of agar

Meat type	Country of origin of meat	Counts (cfu/gram of meat on different agars)				
		Blood agar **Total aerobic bacteria**	**CA-ECC** **Mainly Entero-** **bacteriaceae**	**MacConkey +** **1 mg/L** **cefotaxime**	**CA-CTX** **All presumptive ESBLs**	**Rambach CTX** **Presumptive ESBL Salmonella**
M* beef	British	7.6×10^5	< 100	1.0×10^2	< 100	< 100
M* lamb	New Zealand	1.0×10^6	1.5×10^3	1.0×10^3	< 100	< 100
Pork	British	4.2×10^3	1.0×10^2	< 100	< 100	< 100
Turkey	British	< 100	1.0×10^2	< 100	< 100	< 100
Beef	Unknown	1.0×10^3	< 100	< 100	< 100	< 100

M*, minced; CA-ECC, CHROMagar ECC; CA-CTX, CHROMagar CTX.

Table 2 of Task 1.4. Major bacteria species from beef, lamb, pork and turkey samples as identified by MALDI-ToF

Meat type	Major bacteria species present as identified by MALDI-ToF
Minced beef	*E. coli, Acinetobacter* spp, *Acinetobacter genomospecies, Enterobacter cloacae*
Minced lamb	*E. coli, Aeromonas bestiarum, A. salmonicida, A.* spp., *Empedobacter brevis, Enterobacter cloacae, Hafnia alvei, Lactobacillus* spp., *Pseudomonas lundensis, Yersinia enterocolitica, Yersinia* spp.
Pork	*E. coli, Enterobacter kobei, Kocuria rhizophila, Moraxella* spp., *Proteus* spp., *Providencia* spp., *Serratia liquefaciens*
Turkey	*E. coli, Proteus mirabilis, Pseudomonas* spp, *Serratia liquefaciens*
Beef	*Enterobacter cloacae, Hafnia alvei, Micrococcus luteus, Yersinia* spp

Discussion

Whilst bacterial counts of only a few meat samples were determined, recent work for the FSA under Public Health project PR-R3-0212-21001 – "Defining reservoirs of ESBL-producing E. coli and the threat posed to personal, animal and public health in the UK"

suggests that these counts are representative of what would be seen if larger numbers of meat samples were tested. This work for the FSA has shown that most meat samples have ESBLs at below the detection level, which in recent work was ~ 55 cfu/gram. Thus, even if larger numbers of meat samples and chicken samples were included in the above counts, it is unlikely that many would have yielded proportional counts of ESBL and non-ESBL *Enterobacteriaceae*. In the previous work of Horton *et al.*,[6] proportional counts of ESBL and non-ESBL *Enterobacteriaceae* in chicken faecal / caecal samples were obtained, but obviously faecal / caecal will contain much higher counts of *Enterobacteriaceae* than meat samples. It is assumed that the main source of low counts of *Enterobacteriaceae* in meat samples is small amounts of contamination with faecal / caecal contents.

In view of the thick soup like nature of the meat homogenates, filtration of the homogenates using coarse filters to provide a less viscous liquid for bacterial filtration that would enable more sensitive counts to be performed, was not considered an option for several reasons. The very thick nature of the homogenate would make initial filtration difficult and as such this technique is not known to be a standard microbiological technique for foodstuffs, and it is unknown what proportion of bacteria would be retained by the initial filtration. Also, this would be a very time consuming process, and was not considered appropriate as the limit of detection is already 100 cfu/gram for this work, and ~ 50 cfu/gram for on-going work. It was considered very doubtful whether filtration would have improved these detection limits.

OBJECTIVE 01: Tasks 1.5

Evaluation of ESBL confirmation tests. The application of a variety of phenotypic tests (e.g. MAST test) will be considered and reviewed.[1]

Abstract

The methods of isolation and phenotypic characterisation of ESBL bacteria vary widely, although there are common themes that are reviewed in this document.

Use of specialised commercial ESBL chromogenic agars such as Brilliance ESBL agar (Oxoid) or CHROMagar CTX (CHROMagar) provide, according to published literature, greater sensitivity and specificity for the isolation of ESBLs compared to generic laboratory media such as MacConkey agar supplemented with a single antibiotic such as cefotaxime or ceftazidime. In view of the high degree of sensitivity and specificity of some of the chromogenic agars, growth on these agars can be considered as presumptive phenotypic identification of an ESBL. However, even the most sensitive and specific of agars can give rise to false positives, particularly from some isolates with certain $bla_{AMP\text{-}C}$ genes and carbapenamases. As such further phenotypic tests need to be performed to give a higher level of confidence that an isolate is an ESBL producer.

Most phenotypic confirmation tests for ESBLs depend on synergy between clavulanate and a cephalosporin antibiotic such as cefotaxime, ceftazidime or cefpodoxime, although other cephalosporin antibiotics can be used also. Various commercially available discs or strips are available and these are discussed in more detail below.

As different ESBL enzymes can have different affinities for different cephalosporin antibiotics sensitivity and specificity of disk may be improved if more than one disk set of cephalosporin antibiotics with and without clavulanate is used to predict ESBL phenotype. Even if three sets of discs are used, results may only be about 95 to 100% accurate. However, in one particular study results were improved if both ceftazidime and cefotaxime (both +/- clavulanate) discs were used, whilst the addition of cefpodoxime discs provided no additional benefits.

For unequivocal determination of ESBL status the ESBL gene needs to be detected and genetic methods are considered in the appendix of this document. This may be a relative simple exercise if the ESBL is a CTX-M as all CTX-M genes encode ESBL enzymes, but

[1] Review of literature performed at APHA, Weybridge with input from PHE.

can be much more complex if other ESBLs such as OXA, TEM or SHV are involved, as not all OXA, TEM and SHV genes are ESBLs.

Introduction

In this review, different isolation methods for ESBL-producing bacteria will be considered as well as phenotypic methods to confirm a bacterial isolate as an ESBL-producer, as isolation can be considered preliminary phenotypic confirmation of ESBL-producing bacteria.

At present there remains some controversy as to which β-lactamase enzymes should be classed as ESBLs.[45] Currently, the term ESBL refers to molecular class A, clavulanic acid inhibited enzymes with activity against extended spectrum β-lactam antibiotics[45]. Using this definition, high prevalence ESBLs would include mainly CTX-M's, but also TEM-ESBLs, SHV-ESBLs, VEB and PER enzymes and low prevalence ESBLs would include GES-1, -3, -7, -9, SFO-1, BES-1, BEL-1, TLA, IBC enzymes and CMT enzymes[45] and the genes for these enzymes tend to be mainly plasmid located in bacteria mainly of the family *Enterobacteriaceae*.

However, it has been proposed that plasmidic mediated AmpC enzymes, OXA enymes and carbapenemases are termed as ESBLs also.[45] Under the proposed new scheme, classical molecular class A ESBLs would termed $ESBL_A$, plasmidic mediated AmpC enzymes would be termed $ESBL_{M-C}$, OXA ESBLs would be termed $ESBL_{M-D}$ and carbapenemases would be termed $ESBL_{CARB}$ with various suffixes after "CARB" to denote different types of carbapenemases (e.g. $ESBL_{CARB-B}$ for the metallo carbapenemases such as IMP and VIM).

When considering isolation methods, phenotypic and genotypic tests for ESBLs, the main consideration in this section will be given to high prevalence molecular class A, clavulanic-acid inhibited enzymes with activity against extended spectrum cephalosporins (termed class $ESBL_A$ under the proposed new scheme, otherwise as ESBLs), although some mention will be made of the metallo-β-lactamases with respect to phenotypic tests. However, neither isolation media nor phenotypic tests will necessarily be highly specific for only molecular class A, clavulanic acid inhibited enzymes with activity against extended-spectrum antibiotics. For a completely specific result, genetic tests need to be performed on isolates.

Where the term ESBL is used in this section, it refers mainly to class $ESBL_A$ under the proposed new scheme, although some studies that are referenced also include OXA types as ESBLs.

Isolation methods for ESBLs

ESBLs can be found most commonly in *Enterobacteriaceae (*e.g. *E.coli, Klebsiella* and *Enterobacter* species) and rarely in non-fermenters such as *Pseudomonas,*[46] and as such, any media for isolation of ESBLs should be favourable to these types of organisms.

In a clinical setting, the failure to detect ESBL-mediated resistance has led to treatment failure (Ambrose, 2006)[47; 48] and contributed to the uncontrolled spread of ESBL-producing organisms.[49] Conversely, laboratory-based detection of patients infected or colonized by ESBL-producing organisms by surveillance cultures has proven useful to control and terminate nosocomial outbreaks.[50; 51; 52] As such, rapid isolation and identification of ESBLs is important in a clinical setting. However, there is also a need to monitor the prevalence of ESBLs in food animals and their products. In recent years there have been studies looking for CTX-M ESBLs in cattle, poultry and swine in France,[12] in cattle in Hong Kong,[53] in chickens, ducks, pigs and partridges in China,[11] in horses in the Netherlands,[54] in cattle in the UK (Liebana *et al*., 2006) and in pets and wild animals in Portugal.[7; 8] Thus, there is a need for media to rapidly and sensitively isolate ESBLs from other bacterial flora and presumptively identify them from both a clinical and surveillance viewpoint.

Methods for isolating ESBLs and presumptively identifying them tend to be as diverse as the different types of ESBLs themselves. Different strategies have evolved in different laboratories and there are different types of commercial media. In 2003 the National Committee for Clinical Laboratory standards[55] recommended to test for presumptive ESBLs by testing for growth in Mueller Hinton broth containing any two of following:- 4 mg/L cefpodoxime; 1 mg/L ceftazidime; 1 mg/L aztreonam; 1 mg/L cefotaxime or 1 mg/L ceftriaxone. Similarly, in the UK, for *Enterobacteriaceae* from hospital infections, resistance to cefotaxime and ceftazidime or resistance to cefpodoxime is used as a primary screen for possible ESBLs whilst for *Enterobacteriaceae* from community patients, resistance to cefpodoxime is considered a good first line indicator of ESBL production. Addition of some of the above antibiotics therefore tends to form the basis of most selective media for isolation of presumptive ESBL producers.

Various selective laboratory media have been proposed to assess the carriage of ESBL producers in stools. Examples of such media include Drigalski agar supplemented with cefotaxime,[56] MacConkey agar supplemented with ceftazidime,[57] and nutrient agar supplemented with ceftazidime, vancomycin, and amphotericin B.[50] In other studies aiming to isolate bacteria carrying ESBL genes from a mixed bacterial flora, cefotaxime has been added to selective agar at 1 mg/L[7] or 2 mg/L[53; 58; 59] (Stürenburg *et al*., 2005); ceftazidime has also been used[39] Stürenburg *et al*., 2005).

Commercial agars for isolation and presumptive identification of ESBLs include Oxoid ESBL Brilliance agar [60], Chromagar ESBL-Bx – biomerieux,[39] BLSE agar (AES France)[61] and CHROMagar CTX (CHROMagar, France) aimed to isolated mainly CTX-M ESBLs.[18]

In the above study[60] comparing ESBL Brilliance agar (OX; Oxoid, Basingstoke, United Kingdom), with ChromID ESBL agar (ESBL-Bx; bioMérieux, Marcy l'Etoile, France) and MacConkey agar with a ceftazidime disk (MCC), the sensitivities of MCC, ESBL-Bx, and OX were 74.6, 94.9, and 94.9%, respectively. The specificities of MCC, ESBL-Bx, and OX by specimens reached 94.9, 95.5, and 95.7%, respectively, when only coloured colonies were considered on the two selective chromogenic media. The confirmation of ESBL-producing isolates in this study was performed by combined double disks (30 μg ceftazidime and 30 μg cefotaxime with and without 10 μg clavulanic acid) according to CLSI guidelines and the challenge set of organisms tested for the ability to grow on the two chromogenic media comprised 200 isolates, including 156 *Enterobacteriaceae* and 44 non-fermenting Gram-negative bacteria. The high negative predictive value (99.3%) found for OX suggests that this medium may constitute an excellent screening tool for the rapid exclusion of patients not carrying ESBL producers. As such this agar could be considered to presumptively identify ESBL bacteria.

In the study with Chromagar ESBL-Bx,[39] a total of 644 clinical samples, including 561 stool, 63 lower respiratory tract (sputum, bronchial, or endotracheal aspirates), and 20 miscellaneous samples (wound swabs or ear-nose-throat specimens), were analysed for the isolation of ESBLs, comparing Chromagar ESBL-Bx to MacConkey agar supplemented with 2 mg/L ceftazidime - MCKC.[39] The sensitivities were 97.7 and 84.1% for ESBL-Bx and MCKC but on either one of the two media, natural AmpC-hyperproducing derepressed *Enterobacter* spp. (n = 25) and *Citrobacter* spp. (n = 14) were the most common false positives as well as non-ESBL-producing *Klebsiella oxytoca* (n = 18) on ESBL-Bx and *Morganella morganii* (n = 10) on MCKC.

In another study comprising a similar number of clinical human samples, Chromagar ESBL-Bx (biomerieux) was also compared to BLSE (AES, France) agar for the isolation of ESBLs.[61] BLSE agar consists of a bi-plate made of two selective media (Drigalski and MacConkey agar supplemented with 1.5 mg/L and 2 mg/L cefotaxime and ceftazidime respectively). Again, Chromagar ESBL-Bx gave better results than those obtained using BLSE agar for isolation of ESBLs.

CHROMagar CTX is designed for the specific isolation of CTX-M type ESBLs,[18] and this agar contains an inhibitor of AmpC strains. Whilst this agar has not been evaluated with human clinical specimens, it has been evaluated for the ability to allow growth of CTX-M positive strains whilst inhibiting non-CTX-M strains compared to agar with 1, 2, 4 and 8 mg/L of either cefotaxime or ceftazidime and to Chromagar ESBL-Bx (biomerieux) for a panel of 150 *Enterobacteriaceae* (Table1). Additionally, CHROMagar CTX was also compared to Chromagar ESBL-Bx (biomerieux) and agars containing cefotaxime for the isolation of CTX-M positive strains from a total of 342 farm animal faecal samples including some samples spiked with different levels of CTX-M strains with and without AmpC strains (Table 2). CHROMagar CTX was found to be superior to other agars tested with 100% sensitivity and 64.2% specificity for growth of the CTX-M strains in the panel and 90.1% of colonies from animal faeces plated on CHROMagar CTX were CTX-M strains. CHROMagar CTX inhibited more AmpC strains (Table 1) than Chromagar ESBL-Bx (biomerieux) but also inhibited some of the non-CTX-M ESBLs and as such, its use would be limited to isolation of CTX-M ESBLs rather than all ESBLs. As such this agar also could be considered to presumptively identify mainly CTX-M ESBL bacteria.

Summary for ESBL isolation methods

It remains unlikely that a standardized method for isolation and presumptive identification of ESBL producers will be adopted worldwide in the near future, but both in-house and commercial agars exist that help the clinician or researcher to isolate presumptive ESBLs producers from a background of other flora, and such agars are essential for the sensitive isolation of ESBLs. The commercial Chromogenic agars in general give higher sensitivity and specificity than laboratory media. Based on the above studies, Oxoid ESBL Brilliance agar and CHROMagar CTX (CHROMagar) give the best sensitivity and specificity.

Phenotypic methods of identification of ESBLs

It has been recommended and generally accepted that ESBL isolates (even when MICs are in the susceptible range) should be reported as resistant to all penicillins (except temocillin), all cephalosporins (except cefoxitin) and aztreonam.[46; 62] There were various reasons for this, the major one being because of increased mortality of patients infected with ESBL strains when treated with cephalosporins, even when MICs were in the susceptible range.[46; 62] In view of this, susceptibility testing methods including CLSI, BSAC, CA-SFM, and SRGA used to recommend the use of ESBL screening and confirmation tests on a routine basis, (Tundridge, 2007). However, EUCAST and CLSI have now lowered cephalosporin breakpoints and

recommend that isolates are reported as found based on MICs (Dr Neil Woodford, personal communication). Additionally, EUCAST and CLSI no longer recommend ESBL tests for patients, and if done, confirmatory tests are for infection control and epidemiological purposes only (Dr Neil Woodford, personal communication).

Reports suggest that the rapid detection and identification of ESBLs in a clinical setting not only improves the outcome of treatment,[47; 48] but also has proven useful to control and terminate nosocomial outbreaks.[50; 51; 52]

Most phenotypic confirmation tests for ESBLs depend on synergy between clavulanate and a cephalosporin antibiotic.[46] However, Turnidge[63] highlights some problems with phenotypic tests for ESBLs that are worth mentioning before we consider different phenotypic tests for ESBLs:-

1. The problem of defining an adequate number of substrates to ensure sufficiently sensitive screening. Ideally, one should include a minimum of 4, namely cefpodoxime, ceftazidime, ceftriaxone or cefotaxime, and aztreonam, and at concentrations that often differ from those used for susceptibility breakpoints.

2. The lack of reliable phenotypic methods to detect ESBLs in species with inducible AmpC β-lactamases. Some of these species have been shown to be important reservoirs for ESBLs, and resistance to extended-spectrum cephalosporins cannot solely be attributed to stable de-repression of AmpC.

3. The failure of current methods to provide advice on the interpretation of a positive screening test but a negative confirmation test, especially if the isolates are "susceptible" to extended-spectrum cephalosporins using method-recommended breakpoints. Such strains have been shown to harbour OXA enzymes, inhibitor-resistant TEM enzymes, or particularly plasmid-borne AmpC enzymes with significant frequency. Thus there is no current phenotypic or genotypic test that can be practically and effectively applied in the routine laboratory with sufficient sensitivity to detect the emerging range of transmissible enzymes.

The Health Protection Agency[46] in the UK do recommend that all *Enterobacteriaceae* should be tested first line against an indicator cephalosporin (cefotaxime and ceftazidime or cefpodoxime) or if direct sensitivities are performed on clinical specimens they should include suitable cephalosporins to detect possible ESBLs for all clinical specimens likely to

harbour ESBLs. Once an isolate is identified as a possible ESBL producer, confirmatory tests can be done.

Confirmatory tests for ESBLs include:-

(1). Double disk tests. Discs containing cefotaxime or ceftazidime (30 µg) or cefpodoxime (10 µg) are placed either side of a disk with co-amoxclav (20 + 10 µg) or c. 25-30 mm from it. ESBL production is inferred when the zone of *either* cephalosporin is expanded by the presence of clavulanate. This method is not recommended by the HPA.[46]

(2). Combination disk method (such as MAST disks). The method compares zones of inhibition for a disk with a cephalosporin alone, compared to the same cephalosporin with clavulanate. Interpretation criteria vary with discs from different companies, but increased zone diameters for discs with clavulanate imply the strain is an ESBL. Commercially available discs include cefotaxime, cefepime, ceftazidime, cefipirome and cefpodoxime +/- clavulanate.

(3). E-test ESBL strips. Work on the same principle as the combination disk method. These commercially available strips have a cephalosporin gradient at one end and a cephalosporin / clavulanate gradient at the other end. E-test strips are also available for detection of metallo-β-lactamases.

(4). **MICs.** A ≥ 3 fold reduction in the MICs of cefotaxime or ceftazidime in the presence of clavulanic acid is considered indicative that the strain could be an ESBL.[55]

(5). **Automated systems such as Vitek and Phoenix.** These systems incorporate ESBL detection systems or strategies based on synergy between cephalosporin antibiotics and clavulanate.

(6). **The Cica-Beta test for ESBL detection.** Cica-Beta test strips are available commercially to detect ESBLs, metallo-ESBLs and ampC strains. The test works on the basis that narrow spectrum β-lactamases can not break down the compound HMRZ-86 which is dropped onto the test strip. ESBLs, metallo-ESBLs and ampC strains are distinguished from each other by the presence of different inhibitors on the different strips.

Whilst there are other phenotypic tests for ESBLs and metallo-ESBLs, the above list comprised the main types of phenotypic tests for ESBLs and other tests tend to be variations of the above.

The HPA point out some pitfalls to the above phenotypic ESBL tests:-[46]

1. ESBLs are harder to detect in *Enterobacteriaceae* with inducible AmpC enzymes such as *Enterobacter* species. For ESBL tests on *Enterobacter* species it is best to use an AmpC-stable cephalosporin such as cefepime or cefpirome in the clavulanate synergy tests.

2. About 10-20% of *Klebsiella oxytoca* strains hyper-produce their class A "K1" chromosomal β-lactamase. This may give positive clavulanate synergy with cefotaxime or cefepime, but not with ceftazidime.

3. The above phenotypic tests for ESBLs were not developed for *Acinetobacter* species, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* and should not be used for these isolates.

There are numerous papers that compare the different types of phenotypic ESBL tests, and it is beyond the scope of this report to go into great detail, but some studies are briefly discussed below.

Combination discs and E-test.[64] A total of 100 epidemiologically distinct strains including ESBL and non-ESBL de-repressed class 1 β-lactamase-producing strains and reference strains, were used. The ß-lactamases were characterized using a combination of PCR to detect bla_{TEM}, bla_{SHV}, bla_{BIL} and bla_{PER}, iso-electric focusing, substrate profiles and in some cases DNA sequencing. Combination discs with cefotaxime +/- clavulanate and ceftazidime +/- clavulanate (MDD) were compared to the E-test for identifying non-CTX-M ESBL producers.[64] The sensitivity of the MDD test for detecting ESBLs using discs containing ceftazidime was 86%. When using cefotaxime, the sensitivity was 65.5%. The sensitivity was increased to 93% if the results obtained using both agents are taken into consideration**.**

From the data, it appears that the use of both cefotaxime and ceftazidime MAST discs is important as ESBLs can be missed if just a single disc is used.

Combination discs and the Cica-β-Test.[65] A total of 123 epidemiologically distinct strains (mainly *E. coli*) were characterised genotypically (tested for *bla*CTX-M *bla*OXA, *bla*SHV, *bla*TEM and AmpC genes) and phenotypically (cefepime, cefotaxime, cefpodoxime and ceftazidime MAST ESBL ID discs, the Cica-Beta test and MICs (Randall *et al*., 2008). The panel comprised ~ 54 and ~ 32% of strains positive for *bla*CTX-M and AmpC genes respectively. The sensitivity and specificity of MAST ESβL ID discs for confirming the *bla*CTX-M strains as ESBL strains ranged from 93.9 to 100% for the each set of discs but the sensitivity and specificity could be increased to between 98.5 and 100% by taking results from two sets of discs. The Cica-β-Test test showed a 77.3 and 90.9% sensitivity and specificity for confirming the *bla*CTX-M strains as ESBL strains.

Livermore *et al*.[65] found that the Cica-β-Test correctly identified 85, 77 and 72% of strains tested as ESBLs, metallo-β-lactamase and AmpC β-lactamase after 24 hours.

From this data is also appears that at least two sets of combination discs should be used to increase accurate detection of CTX-M ESBLs. The Cica test did not perform as well as the discs.

E-test strips with cefepime.[66] This study focused on a panel of 54 mainly *Enterobacter* strains which were confirmed as ESBL and comprised strains with *bla*CTX-M *bla*OXA, *bla*SHV, *bla*TEM genes. Cefotaxime-clavulanate and ceftazidime-clavulanate E-test strips were compared with cefepime-clavulanate E-test strips. With this panel of strains, Cefotaxime-clavulanate and ceftazidime-clavulanate E-test strips were 83 and 74% sensitive respectively at detecting strains as ESBLs whilst cefepime-clavulanate E-test strips were 98% sensitive. However, the cefepime-clavulanate E-test strips gave false positives of 4/6 *Klebsiella oxytoca* strains tested.

E-tests strips (or combination discs) containing cefepime-clavulanate should be a valuable addition to the different phenotypic tests for ESBLs, particularly when testing Enterobacter species, but false positives may occur with Klebsiella oxytoca strains.

Detection of metallo-β-lactamases.[67; 68] Discs with imipenem and 750 µg EDTA were effective at detecting metallo-β-lactamases in *Pseudomonas* spp. and *Acinetobacter* spp. (Yong *et al*., 2002).

Several E-test (AB BIODISK, Solna, Sweden) gradient formats were developed for detection of metallo-β-lactamases based on the reduction of imipenem (IP) or ceftazidime (TZ) MICs in the presence of EDTA or 2-mercaptopropionic acid (MPA).[67] The E-test metallo-β-lactamase (E-test MBL) strips consisted of a double-sided seven-dilution range of IP or TZ (4 to 256 μg/ml) and IP or TZ (1 to 64 μg/ml) overlaid with a constant concentration of EDTA or MPA. The prototype strips were evaluated with 138 challenge strains. EDTA was found to be a better inhibitor of metallo-β-lactamases, especially for anaerobes. IP was better than TZ. Mueller-Hinton agar was the preferred medium, particularly when compared to Isosensitest agar, which frequently produced falsely low MICs for IP. E-test IP plus IP-EDTA with Mueller-Hinton agar had a sensitivity of 94% (79 of 84) and specificity of 95% (124 of 130). The E-test MBL strip appears to be an acceptable diagnostic reagent to detect metallo-β-lactamase phenotypes in the clinical microbiology laboratory.

Strips or discs containing imipenem and EDTA appear to have a high sensitivity and specificity for identification of strains with metallo-β-lactamases and are now available commercially.

Comparisons of cefotaxime, ceftazidime and cefpodoxime combination discs.[69] In a study of 438 clinical isolates of cefatoxime resistant *Enterobacteriaceae*, the cefotaxime +/- clavulanate discs performed best for detection of strains as ESBLs, but results were improved if both ceftazidime and cefotaxime (both +/- clavulanate) discs were used, whilst the addition of cefpodoxime discs provided no additional benefits.

Cefotaxime and ceftazidime discs used together are a good combination for detection of ESBLs in Enterobacteriaceae.

Automated methods. Automated systems include the BD Phoenix (BD Diagnostics), the Vitek System (bioMerieux and the MicroScan WalkAway expert system (Dade Behring). These systems can perform automated identification and antibiotic resistance profiling of bacterial isolates. For ESBL identification, the automated systems work on the basis of synergy between clavulanate and suitable cephalosporin antibiotics.

Vitek automated system:- In a study of 157 well characterised strains, the sensitivity and specificity of the Vitek ESBL test was found to be 99.5 and 100% respectively compared to

98.1 and 99.4% for double disk tests and the Vitek system was found to be capable of detecting hyper-production of AmpC.[70]

In a study comparing the the Vitek 2 system to E-test strips, the Vitek 2 system correctly identified all *E. coli*, *Klebsiella* and *Salmonella* reference ESBLs.[71] In a more recent study the Vitek 2 system (Spanu *et al*., 2006) was compared to genetic methods for identification of strains as ESBLs for a panel of 1,129 clinically relevant *Enterobacteriaceae.* ESBL production was correctly identified in 306 / 312 ESBL-producing organism isolates (sensitivity of 98.1%; positive predictive value 99.3%).

Phoenix and MicroScan WalkAway automated systems: The BD Phoenix system was evaluated for its ability to identify ESBL-producing strains compared to NCCLS confirmatory results for 194 clinical isolates and showed 100% sensitivity and 95% specificity.[72] In a more recent study the Phoenix system was compared to the Micro Scan WalkAway system, and the Micro Scan WalkAway system was more accurate for ESBL detection.[73] In another study of 494 clinical isolates, the Phoenix BD system was shown to have 98% sensitivity and 98.7% specificity for ESBL identification.[74]

Automated systems in general give good results for identifying ESBL isolates as such, although one author has commented that automated results for all Klebsiella pneumoniae, K. oxytoca and E. coli isolates confirmed as ESBLs should be confirmed by an alternative method prior to release of results.[73]

Summary of Phenotypic methods of identification of ESBLs

Although there are standardised international methods recommended for phenotypic detection of ESBLs, a variety of methods are used in different laboratories, and none of the phenotypic tests will give 100% sensitivity or specificity for all *Enterobacteriaceae* in all instances. BSAC and CLSI guidelines recommend that cefotaxime and ceftazidime antibiotics +/- clavulanate are used (e.g. MICs, suitable discs or E-test strips) together and published data also suggests these are a good combination. Discs or strips containing cefepime +/- clavulanate should be a valuable addition to the different phenotypic tests for ESBLs when testing for *Enterobacter* species.

Automated methods can correctly assign ESBL status in c. ≥ 95% of strains. Results reported for the Cica-β-Test showed this test to be less accurate for assigning ESBL status than results obtain using two sets of combination discs or E-test strips.

To get an absolutely definitive ESBL identification requires genetic analysis, but if two or more suitable sets of combination discs or E-test strips are used, or an automated system such as Vitek or BD Phoenix, then it is likely that phenotypic ESBL identification accuracy will be c. $\geq$ 95%.

Task 1.5 - Table 1. Ability of different strains types to grow on different agars.[18]

Characteristic	No. strains tested	No. of strains growing on agar									
		CHROMagar ECC + CTX mg/L				CHROMagar ECC + CAZ mg/L				Biomerieux ESBL	CHROMagar CTX
		1	2	4	8	1	2	4	8		
All ESBLs [a]	101	95	89	84	77	91	86	86	84	99	91
CTX-M ESBLs	70	70	67	67	63	63	58	58	58	70	70
OXA ESBLs	2	2	2	2	2	2	2	2	2	2	2
PER ESBLs	1	1	1	1	1	1	1	1	1	1	1
SHV ESBLs	7	5	5	1	0	5	5	5	5	7	5
TEM ESBLs	5	4	3	2	1	5	5	5	5	5	3
VEB-1 ESBLs	1	1	1	1	1	1	1	1	1	1	1
Unknown ESBLs [b]	15	12	10	10	9	14	14	14	12	13	9
ESBLs CTX-M -	31	25	22	17	14	28	28	28	26	29	21
All AmpC	37	34	32	30	23	33	33	31	31	28	5
Chromosomal AmpC	6	5	3	3	3	5	5	4	4	2	1
MOXM group	1	0	0	0	0	0	0	0	0	0	0
CITM group	25	25	25	24	17	24	24	23	23	23	4
DHAM group	3	2	2	2	2	2	2	2	2	1	0
ACCM group	1	1	1	1	1	1	1	1	1	1	0
FOXM group	1	1	1	0	0	1	1	1	1	1	0
Klebsiella oxytoca K1	1	1	0	0	0	0	0	0	0	1	0
All non-ESBL [c]	49	39	41	34	24	39	38	34	33	38	8

a All ESBLs, all strains positive with at least one pair of MAST ESBL discs.

b Unknown ESBLs, phenotypic ESBL but genetic mechanism for ESBL status not confirmed.

c Negative for ESBLs by all MAST ESBL discs.[18]

Task 1.5 - Table 2. Recovery of CTX-M positive colonies from 48 bovine faecal [a] mixtures spiked with ~ 10^1 to 10^4 cfu/g CTX-M positive strains with and without ~ 10^4 cfu/g AmpC positive strains.[18]

Agar / **Spike level**	**% of cultures that were CTX-M positive[b] for spike levels** With / without presence of AmpC strains			
	10^1	**10^2**	**10^3**	**10^4**
CHROMagar EEC (CA)	**0** / 0	**17** / 0	**0** / 0	**20** / 17
CA + 2 mg/L CTX	**0** / 80	**0** / 17	**50** / 100	**50** / 100
CA + 8 mg/L CTX	**17** / 100	**0** / 100	**17** / 100	**50** / 100
bioMerieux ESBL	**0** / 67	**0** / 50	**33** / 83	**83** / 100
CHROMagar CTX	**67** / 83	**67** / 100	**67** / 100	**67** / 100

CTX - cefotaxime.

a Faecal samples were checked to be free of CTX-M and AmpC strains prior to spiking using standard protocols.[75]

b A total of 240 colonies was isolated from the agars and tested by PCR for the presence of $bla_{\text{CTX-M}}$

9. OBJECTIVE 02: Tasks 2.1

Evaluation of procedures of bacterial speciation. Two methods (API strips and MALDI-ToF) will be compared for a panel of approximately 100 ESBL *Enterobacteriaceae*. Both options will be included in the screening protocol and their relative benefits and limitations described including cost.[1]

Introduction

In a recent extensive review of MALDI-ToF to identify clinical bacteria, it was recommended as "a rapid, precise, and cost-effective method for identification of intact bacteria, compared to conventional phenotypic techniques or molecular biology"[11]. Additionally, MALDI-ToF was recently approved by the FDA as a method to identify clinical isolates of bacteria. Whilst the percentage of isolates that are correctly identified in comparison to a gold standard (usually 16S rDNA sequencing or type strain status) vary to some extend between studies, and the nature of the isolates examined, in two studies of clinical isolates 99.1% of 680[12] and 95.2% of 1116[17] isolates were correctly identified.

At APHA Weybridge, we have been using MALDI-ToF in comparison to identification by other methods (such as 16S rDNA sequencing, PCR, serotyping, biochemical tests, selective media and API) as a method to identify bacteria for several years. For this objective, we provide results for ~ 100 isolates of *Enterobacteriaceae*, comparing results of identification by MALDI-ToF and other methods as appropriate, including API.

Materials and methods

Table 1 shows the results of 100 bacteria identifications by MALDI-ToF and other methods. Isolates identified included *Enterobacter* species (n=2), *Escherichia coli* (n=56), *Klebsiella* species (n=15), *Salmonella* enterica species (n=15), *Serratia* species (n=5) and *Yersinia* species (n=7).

MALDI-ToF was unable to identify *Salmonella* enterica to the serotype level but correctly identified all *Salmonella*. For all other isolates, identification to the species level agreed, except for one isolate of isolate of *Yersinia*, which was identified by API

[1] Performed at APHA Weybridge

20E as *Yersinia frederiksenii* but was identified by MALDI-ToF as *Yersinia enterocolitica.* Without a third method, it is not possible to comment on which is these two results are correct.

Discussion

MALDI-ToF was recently approved by the FDA as a method to identify clinical isolates of bacteria and there are literally hundreds of peer reviewed papers to support its as an accurate, quick and cost effective method to identify bacteria, as recently reviewed[114]. It is now used as the main method of identifying bacteria in many hospitals.

In this small study, results of identification by MALDI-ToF were shown to agree to the genera level for 100% of isolates tested and to the species level for 99% of the isolates tested. Whilst not all the isolates in this study were confirmed as ESBLs, the presence of a particular antibiotic resistance type has not been reported to influence bacterial identification by MALDI-ToF, as far as we are aware.

Compared to API, MALDI-ToF is much quicker and easier to perform, and results in an automated printed output of the results. Cost for matrix per test is ~ £0.05, and the only other reagent cost for identification is the calibration standard at about £1.30 per week (for as many tests that can be done in that week).

With respect to cost and speed of identification, in a previous study, a total of 824 bacterial isolates were assessed for identification by MALDI-ToF.[115] On average, identification by MALDI-ToF was 1.45 days earlier than previously used biochemical methods and estimated savings on labour and reagent by use of MALDI-ToF (including instrument maintenance) were 56.9% within 12 months.[115] The authors concluded that this saving could be applied to other moderate to high volume laboratories.[115]

In conclusion, we would recommend MALDI-ToF as a rapid, reliable and cost effective method to identify bacteria, over and above tests such as API. However, approximately 4,000 bacterial isolates identified per year are required by MALDI ToF, so that savings in reagents and staff time can offset servicing costs.

For small laboratories that cannot afford to purchase or access a MALDI-ToF machine then suitable selective media, suitable biochemical tests and API 20E are all suitable methods to identify *Enterobacteriaceae.*

It is not always appropriate or the best option to consider API as the first method of identification for *Enterobacteriaceae* if MALDI-ToF is not available as a method of identification. Results in this small study (Task 2.1) show that suitable CHROMagar ECC or CHROMagar CTX was a reliable way to identify *E. coli* for those who are

experienced in its use and simple biochemical tests, such as oxidase and indole, can give added confidence that blue / green colonies from such agars are *E. coli*, thus saving time and costs over performing API tests. However, for non *E. coli* and non *Salmonella*, for the small laboratory without access to a MALDI-ToF machine, then use API 20E strips is an appropriate method to identify *Enterobacteriaceae.*

Task 2.1 - Table 1. Comparison of identification of *Enterobacteriaceae* by MALDI-ToF to identification by other appropriate methods (including API)

Reference Number	Genera by non MALDI method	Species by non MALDI method	ESBL status if known	Method of non MALDI identification	Genera by MALDI-ToF	Genera by MALDI-ToF	Agreement between MALDI-ToF and non MALDI methods
NCTC10006	Enterobacter	*aerogenes*	Not known	API 20E	Enterobacter	*aerogenes*	Yes
14/M54/09/12	Enterobacter	*cloacae*	Not known	API 20E	Enterobacter	*cloacae*	Yes
191	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
620	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
494	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
955	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
186	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
951	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
804	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
966	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
942	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
166	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
944	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
170	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
171	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
173	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
212	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
805	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
6-P16-P	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-DC3-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C16-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C13-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes

Reference Number	Genera by non MALDI method	Species by non MALDI method	ESBL status if known	Method of non MALDI identification	Genera by MALDI-ToF	Genera by MALDI-ToF	Agreement between MALDI-ToF and non MALDI methods
1-C8-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C6-W	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C4-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P22-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-DC26-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C26-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-LY47-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-E6-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-LY33-W	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-LY33-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-LY8-W	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-LY1-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-HY50-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C3-W	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P13-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-IND1-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P20-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P14-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P18-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P17-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P16-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C21-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P14-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-C25-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes

Reference Number	Genera by non MALDI method	Species by non MALDI method	ESBL status if known	Method of non MALDI identification	Genera by MALDI-ToF	Genera by MALDI-ToF	Agreement between MALDI-ToF and non MALDI methods
1-P10-B	Escherichia	*coli*	ESBL	CHROMagar	Escherichia	*coli*	Yes
1-P8-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-P6-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-P5-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-P4-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-C28-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-HY38-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-P15-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-IND10-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-IND24-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-IND23-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
1-IND21-B	*Escherichia*	*coli*	ESBL	CHROMagar	*Escherichia*	*coli*	Yes
44	*Klebsiella*	*oxytoca*	Not known	API 20E	*Klebsiella*	*oxytoca*	Yes
30	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
125	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
134	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
137	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
154	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
167	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
194	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
B0252/07/12	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
M320/08/12	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
M40/09/12	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
M116/09/12	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes

Reference Number	Genera by non MALDI method	Species by non MALDI method	ESBL status if known	Method of non MALDI identification	Genera by MALDI-ToF	Genera by MALDI-ToF	Agreement between MALDI-ToF and non MALDI methods
P302/09/12	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
P0237/12/12(N4)	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
P0237/12(T2)	*Klebsiella*	*pneumoniae*	Not known	API 20E	*Klebsiella*	*pneumoniae*	Yes
S0074-98	*Salmonella*	Derby	Not known	Serotyping	*Salmonella*	species	Genera
NCTC 12709	*Salmonella*	Dublin	Not known	Serotyping	*Salmonella*	species	Genera
S5472-03	*Salmonella*	Enteritidis	Not known	Serotyping	*Salmonella*	species	Genera
S08274/02	*Salmonella*	Enteritidis	Not known	Serotyping	*Salmonella*	species	Genera
S6106-91	*Salmonella*	Hadar	Not known	Serotyping	*Salmonella*	species	Genera
S4606-91	*Salmonella*	Kedougou	Not known	Serotyping	*Salmonella*	species	Genera
S3095-06	*Salmonella*	Mbandaka	Not known	Serotyping	*Salmonella*	species	Genera
S3095-06	*Salmonella*	Mbandaka	Not known	Serotyping	*Salmonella*	species	Genera
S4806-91	*Salmonella*	Montevideo	Not known	Serotyping	*Salmonella*	species	Genera
S4806-91	*Salmonella*	Montevideo	Not known	Serotyping	*Salmonella*	species	Genera
S4430-91	*Salmonella*	Newport	Not known	Serotyping	*Salmonella*	species	Genera
S4430-91	*Salmonella*	Newport	Not known	Serotyping	*Salmonella*	species	Genera
NCTC 7832	*Salmonella*	Nottingham	Not known	Serotyping	*Salmonella*	species	Genera
NCTC 4840	*Salmonella*	Poona	Not known	Serotyping	*Salmonella*	species	Genera
NCTC 5776971	*Salmonella*	Pullorum	Not known	Serotyping	*Salmonella*	species	Genera
24	*Serratia*	*liquefaciens*	Not known	API 20E	*Serratia*	*liquefaciens*	Yes
26	*Serratia*	*liquefaciens*	Not known	API 20E	*Serratia*	*liquefaciens*	Yes
117	*Serratia*	*liquefaciens*	Not known	API 20E	*Serratia*	*liquefaciens*	Yes
57	*Serratia*	*marcescens*	Not known	API 20E	*Serratia*	*marcescens*	Yes
78	*Serratia*	*marcescens*	Not known	API 20E	*Serratia*	*marcescens*	Yes
3	*Yersinia*	*enterocolitica*	Not known	API 20E	*Yersinia*	*enterocolitica*	Yes

Reference Number	Genera by non MALDI method	Species by non MALDI method	ESBL status if known	Method of non MALDI identification	Genera by MALDI-ToF	Genera by MALDI-ToF	Agreement between MALDI-ToF and non MALDI methods
110	*Yersinia*	*enterocolitica*	Not known	API 20E	*Yersinia*	*enterocolitica*	Yes
130	*Yersinia*	*enterocolitica*	Not known	API 20E	*Yersinia*	*enterocolitica*	Yes
15/P146/9/12	*Yersinia*	*enterocolitica*	Not known	API 20E	*Yersinia*	*enterocolitica*	Yes
171	*Yersinia*	*frederiksenii*	Not known	API 20E	*Yersinia*	*enterocolitica*	Genera
193	*Yersinia*	*frederiksenii*	Not known	API 20E	*Yersinia*	*frederiksenii*	Yes
101	*Yersinia*	*pseudotuberculosis*	Not known	API 20E	*Yersinia*	*pseudotuberculosis*	Yes

Biochemical identifications include, as appropriate, API strips or appropriate diagnostic media such as CHROMagar ECC or CHROMagar CTX for identification of *E. coli* (blue/green colonies).

Grey highlight – Agreement on identification is the Genera only for this one isolate.

10. OBJECTIVE 02: Tasks 2.2

Review of genetic screening approaches.[1] A comparison of genetic screening approaches will be carried out by building on the data from the SafeFoodEra project (B14016/B14017). The costs and applicability of micro-array and other molecular screening approaches will be reviewed in terms of cost, speed, ease of use and transferability.

Introduction

The genetic characterisation and comparison of isolates from food and humans is necessary to establish their epidemiology for risk analysis purposes. Micro-array technology has been used for the genetic screening of bacterial host and plasmid antimicrobial resistance, virulence and O and H antigen genes. This enables identification of those isolates with similar or identical genetic profiles for more detailed investigation. The SafeFoodEra project has array data on some 800 ESBL isolates from humans, food and food producing animals. The utility of micro-arrays and other molecular screening technologies such as replicon typing, Multi-Locus Sequence Typing (MLST), pulsed-field gel electrophoresis (PFGE) will be reviewed for ESBL screening. This objective will discuss the above methodologies and their role in characterisation of bacteria, and in particular ESBL *E. coli.*

Discussion

APHA Weybridge has historically used micro-arrays, replicon typing, MLST and PFGE for analysing strains of interest, including ESBL *Enterobacteriaceae.* Micro-arrays are used to determine the presence of genes (such as antibiotic resistance genes and virulence genes) in isolates, replicon typing is used to determine the plasmid types carried by isolate, MLST is used to determine the clonal type of an isolate and PFGE is generally used to determine a more precise genetic fingerprint of an isolate (e.g. sub types of clonal types).

Replicon typing, MLST and PFGE can all be used as methods to determine if isolates from animals or food are similar to isolates from humans with respect to plasmid type (replicon type) or isolate type (MLST and PFGE), or for example, if isolates are similar between farms, different species of animals etc. For example, the CTX-M 15

[1] This brief review was performed at APHA Weybridge.

E. coli clonal MLST 131 belonging to serotype O25b:H4 is widely recognized as a human pandemic strain.[116] By definition, the MLST type 131 is homogeneous with respect to housekeeping gene sequence across the 7 MLST loci; however, diversity of PFGE profiles has provided insights into the ecology of ST131.[117] The presence of ST131 isolates with similar PFGE profiles in widely dispersed locales and of isolates with quite different profiles in the same locale has suggested rapid and ongoing global dissemination of ST131.[117] As such, the combined uses of MLST and PFGE as molecular typing methods have the ability to provide a "genetic fingerprint" of this and other strains, and this can be coupled with details of the serotype, plasmid type and CTX-M or other ESBL sequence type as well as the presence of antibiotic resistance and virulence genes as determined by arrays to further "fingerprint" isolates and also provide details of their characteristics such as antibiotic resistance.

In a recent study at APHA ESBL gene sequencing, arrays, and replicon typing were used to characterise CTX-M *E. coli* isolates from cattle, chickens and turkeys in Great Britain.[118] The main CTX-M enzymes identified in *E. coli* from cattle, chicken and turkeys were 14 and 15, 1 and 15, and 1 and 14 respectively. The plasmid replicon type I1-γ was most common and seen in 23%, 95% and 50% of the isolates tested from cattle, chickens and turkeys respectively, whilst types F, FIA, FIB and K were common to isolates from cattle and turkeys only. Thirty-five different antibiotic resistance (Figure 1) genes and thirty-nine different fitness and virulence genes were detected by micro-array. Fisher exact test and hierarchical clustering of the antibiotic resistance and virulence gene results showed some genes were more commonly associated with isolates from chickens or cattle.

In another study at APHA,[119] the presence of virulence genes (Figure 2) in isolates of CTX-M *E. coli* from diseased chickens was compared to isolates from healthy chickens and from urinary tract infections in people. The results supported previous findings that CTX-M *E. coli* strains in chickens were generally different from those causing disease in humans.

At APHA ESBL gene sequencing, replicon typing, MLST, serotyping and PFGE (Figure 3) have also been used to characterize ESBL isolates of *E. coli* from healthy chickens and turkeys,[2] and from waste milk.[44] In both of these studies, these molecular tools were useful to show both similarities of some isolates, and distinctiveness of other isolates, and overall that none of the isolates from chickens,

turkeys or waste milk were the human pandemic O25:H4-ST131 CTX-M-15 clone.[120; 121]

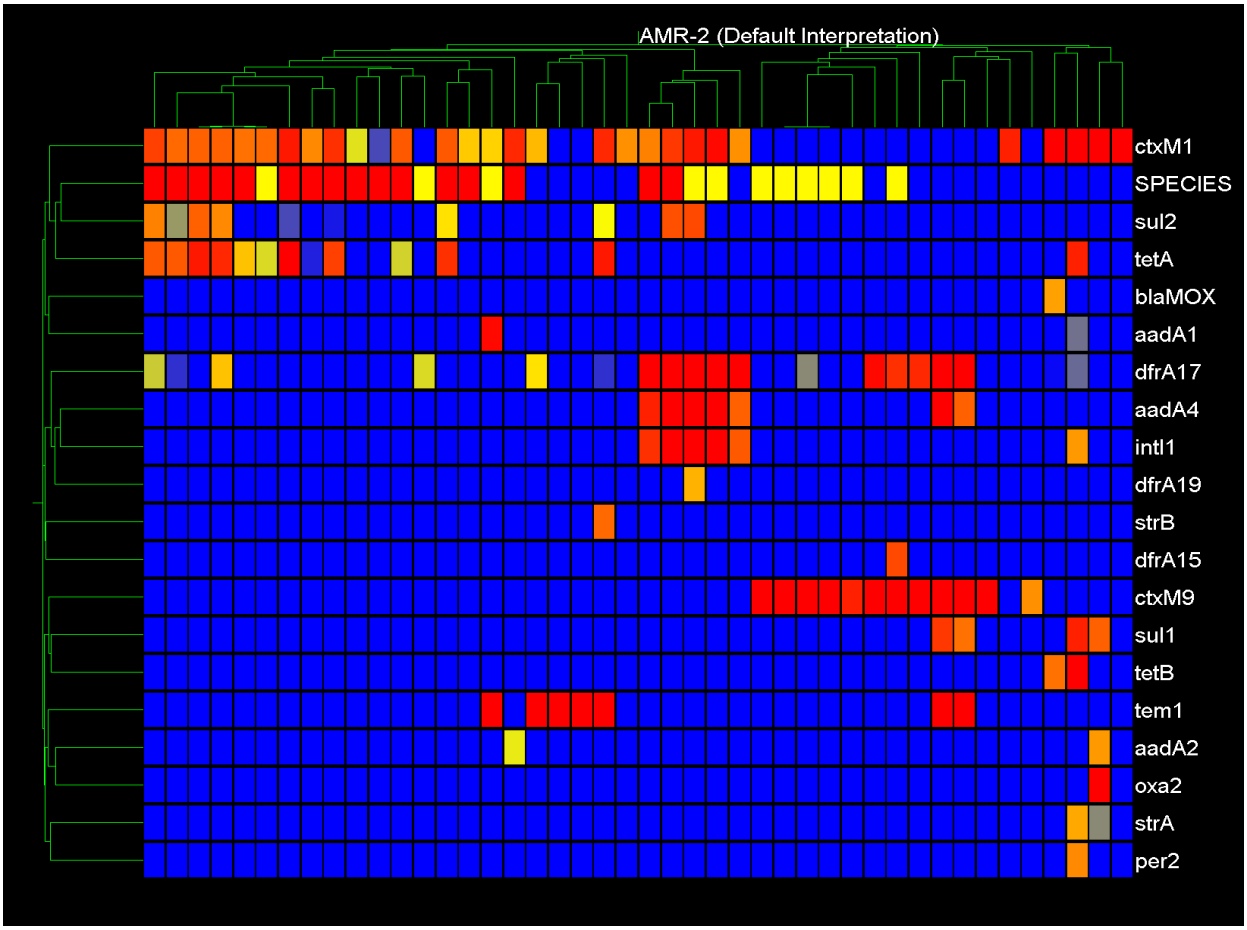
However, whilst these methods are useful, arrays, MLST, PFGE and replicon typing are all labour intensive methods, and therefore expensive. They are also time-consuming with respect to turn around time of the tests.

In recent years, the cost of whole genome sequencing of bacterial isolates has reduced considerably, and can now be done for around £50 to £200 per isolate, excluding time to prepare DNA and subsequent analysis of the data. In November 2009, "Complete Genomics" published a peer-reviewed paper in *Science* demonstrating its ability to sequence a complete human genome for $1,700. If true, this would mean the cost of full genome sequencing of human cell has come down exponentially within just a single year from around $100,000 to $50,000 to $1,700. Whilst bacteria were then cheaper to sequence than human DNA, these figures to give some idea of how much the costs have fallen.

Whole genome sequencing has the potential to provide details of MLST, plasmid type, ESBL gene type, other antibiotic resistance genes, virulence and fitness genes in a single test, once suitable pipelines are developed for analysis.

In a recent study, whole genome sequencing was found to provide superior resolution over classical typing methods and did not support the previously proposed occurrence of frequent clonal transmission of ESBL-positive *E. coli* from chickens to humans.[122] This work demonstrated 4216 - 4470 single nucleotide polymorphisms (SNPs) between human & chicken/meat strains but only a maximum 6 SNPs in German *E. coli* outbreak isolates.[122] Thus it seems likely, as costs for whole genome sequencing fall, and improved pipelines for analysis are developed, that whole genome sequencing may soon replace methods such as arrays, MSLT, PFGE and replicon analysis as ways of analysing strains, both to reduce costs, and provide a superior level of analysis.

Figure 1. Antibiotic resistance gene profiles for all cattle, chicken and turkey transconjugant isolates.[123]



Genes were clustered using GeneSpring® 7 software (Silicon Genetics) and Spearman correlation.

Colour scheme - Blue, gene negative or ambiguous; blue green to dark yellow, result ambiguous; yellow to orange, gene positive; red, gene strongly positive. In the

SPECIES row red represents chicken isolates, yellow turkey isolates and blue cattle isolates.

Figure 2. Hierarchial clustering demonstrating the distance between similar genes and isolates (GeneSpring software).[119]

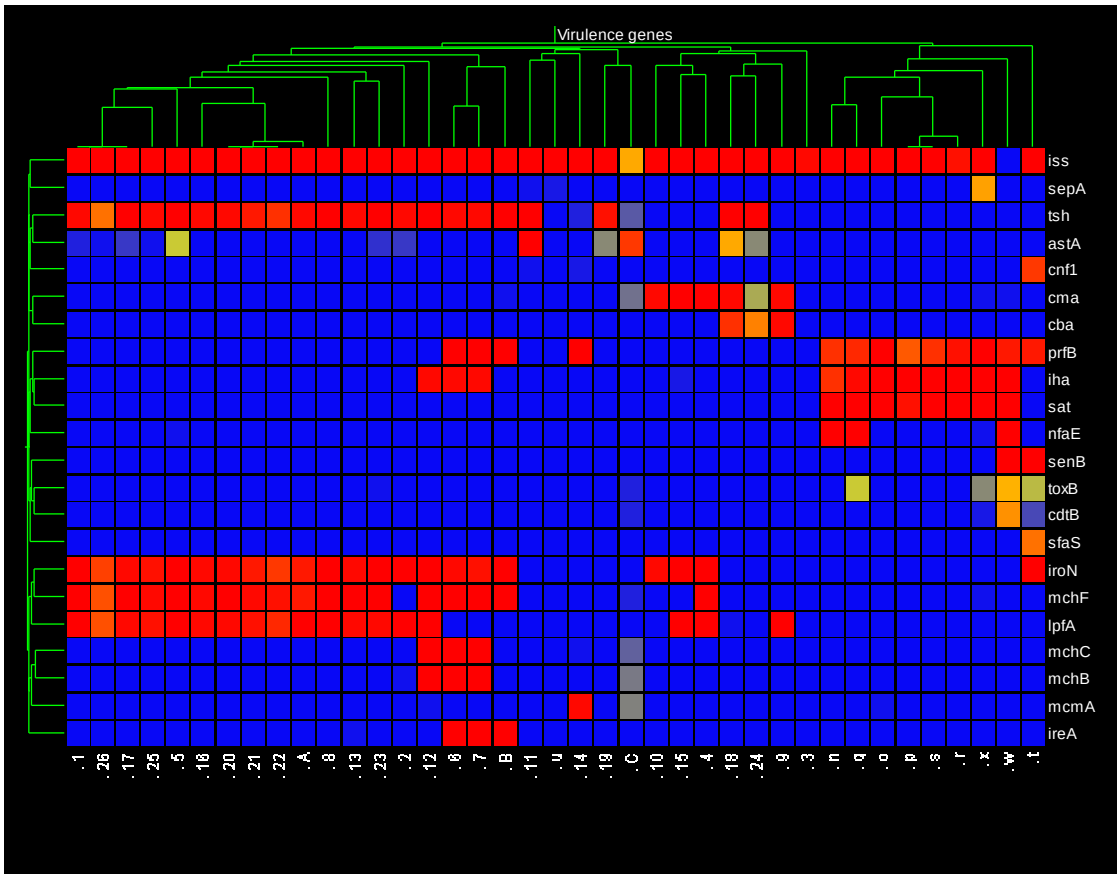
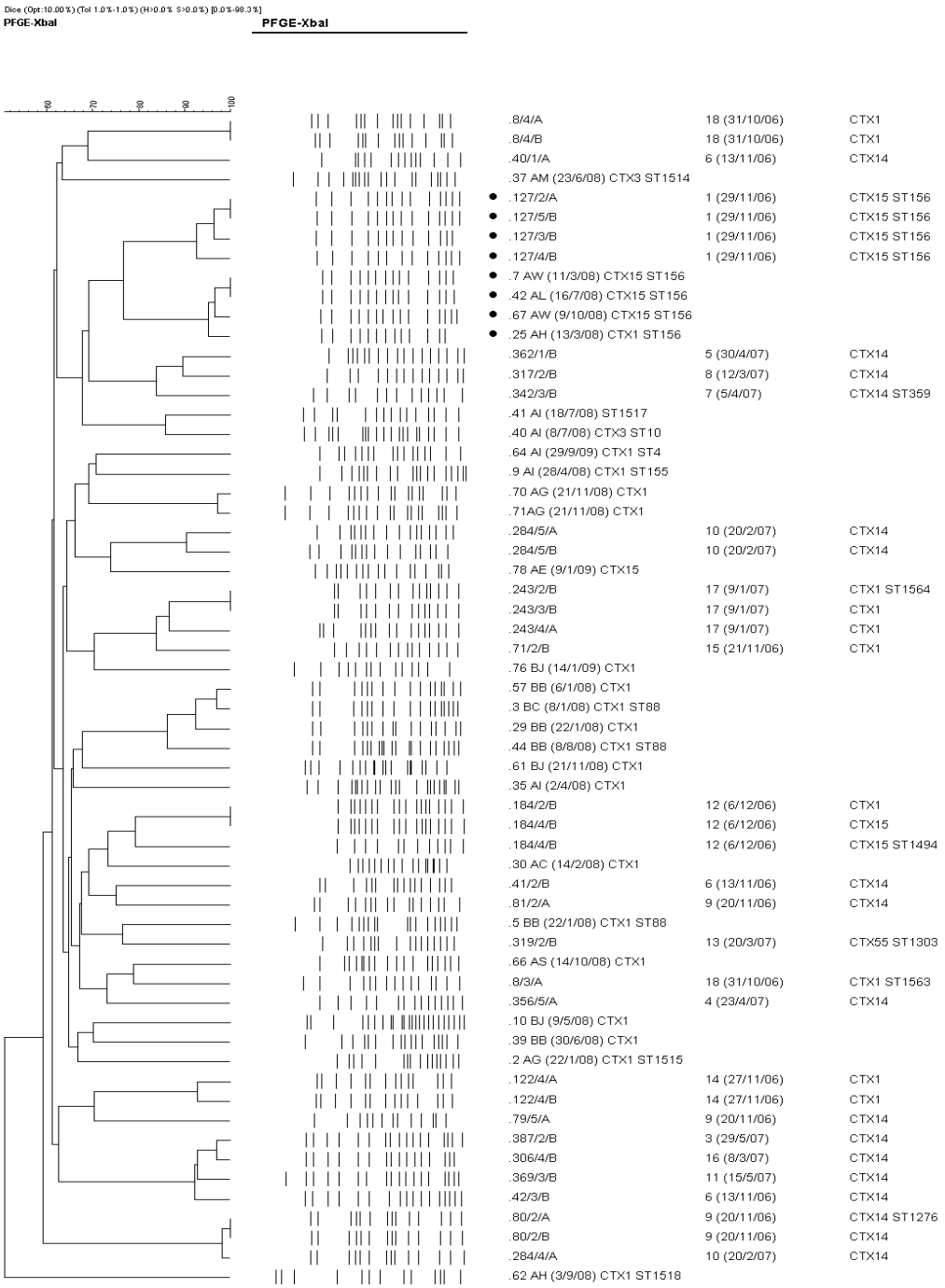


Figure 3. Dendrogram of PFGE types for combined chicken and turkey CTX-M-positive *E. coli* isolates.[2]



Key to details:- Turkey isolates have strain details further right. For both turkey and chicken isolates details are - symbol for MLST type 156, isolate number, farm or abattoir code, date of isolation, CTX-M sequence type, MLST (where available).

11. OBJECTIVE 03: Tasks 3.1

Method selection and industry seminar.[1] Protocol options for the screening method for ESBL detection in food will be discussed and the best method(s) selected. A seminar will be held to disseminate information to industry and other interested parties on findings of the method development stage and to gain feedback from industry.

Introduction

A seminar entitled "*Antimicrobial Resistance: The Detection of Extended Spectrum Beta Lactamases (ESBLs) of Enterobacteriaceae in Food*" was held at Leatherhead Food Research on the 16th May 2012 to disseminate current understanding on the relevance of ESBL-resistance to the UK food industry and consumers with particular emphasis on the development of new reliable methods of detection for use in surveillance and enforcement. The findings of the FSA funded project FS241023: 'Evaluation and recommendation of a screening protocol for the detection of Extended Spectrum Beta Lactamases of Enterobacteriaceae in food' were reviewed to support a recommendation for the most suitable method in terms of analytical capability and costs.

The following information on the seminar is provided in Appendix IV

Programme
Publicity material for industry seminar
Delegate list
Comparative costs of analytical methods

Presentations: pdf files of the presentations have been sent to the FSA

[1] Leatherhead Food Research organised the industry seminar.

Discussion

The consensus of the seminar delegates was that a combination of phenotypic and genotypic methods would produce the most reliable and useful results. The phenotypic method using standard cultural isolation of presumptive ESBLs was favoured by industry and contract testing laboratories, on the basis that the science and costs were understood and accepted by industry. The LAMP method was generally considered to be the more robust molecular approach in terms of giving meaningful results, with the added potential of allowing the future development of cost effective rapid tests and automated equipment for on-site or near-site testing applications.

Conclusions

Recommended method for industry (text prepared by APHA and submitted to the FSA in August 2012). Based on results, our recommendation would be to use the standard protocol of the food industry initially (homogenize 25 grams of meat and then enrich in BPW without additives for ~ 24 hours at 37°C) then plate BPW to CHROMagar CTX and also use a crude DNA extract from the same BPW for LAMP assays to detect bla_{OXA} and $bla_{\text{CTX-M}}$ groups 1, 2 and 9. This will give a phenotypic and genotypic result from a single sample, which should both endorse each other providing added robustness to the test.

The rationale behind this protocol is as follows:

It was felt that, culture is needed to catch ESBL types not covered by PCR or LAMP assays. Culture also is quick with respect to operator time, cheap, and needs little training to perform.

However, genetic tests are needed to confirm culture results and the LAMP assay has advantages over multiplex PCR in that it can give the CTX-M group and it is more sensitive. At present in the UK, CTX-M ESBLs are probably the main ones associated with food.

The disadvantages of the LAMP assay compared to the multiplex PCR are that it does not detect SHV or TEM genes. However, not all TEM and SHV genes are ESBLs, so as such a multiplex PCR (for CTX, OXA, SHV and TEM genes) positive result from enrichment broth for SHV or TEM genes is not 100% confirmation of an ESBLs, as the PCR signal may come from non-ESBL SHV or TEM genes.

With the above in mind, the following test strategy is recommended:-

1. Standard enrichment – 25 gram meat to 225 ml BPW broth, then overnight incubation at 37°C
2. Plate 10 ml of enriched BPW broth to CHROMagar CTX and incubate for 24 to 48 hours at 37°C.
3. Prepare a crude DNA extract from BPW and test in LAMP assay for CTX-M groups 1, 2 and 9 and ESBL OXAs.
4. If CHROMagar CTX plate shows growth and LAMP assay is positive, record as ESBL. LAMP assay will give indication of ESBL type. It is hoped this will capture most ESBLs in meat.
5. If CHROMagar CTX plate is negative, but LAMP assay is positive, record as genotypically positive but phenotypically negative. Investigate further.
   If CHROMagar CTX plate is positive, but LAMP assay is negative, test isolates for presence of CTX, OXA, SHV and TEM genes using multiplex PCR. If the PCR is positive, record as ESBL with type as indicated by the PCR. If the PCR is negative, record results as "ambiguous - needs further work" (resources allowing). This step is not part of the validation work to be done in this project, but would be needed to confirm ESBL status if LAMP results were negative.

Addendum from APHA dated 3-3-2014

This is the first time, to our knowledge, that an ESBL selective agar has been validated for isolation of ESBLs from food samples. The LAMP assays are newly developed and have recently been published as such.[41] The combination of a phenotypic chromogenic isolation agar and LAMP assays, to not only isolate presumptive ESBLs, but to also perform some initial genetic characterisation of ESBLs in food samples, is entirely novel as a method to recommend to the food industry.

12. OBJECTIVE 03: Tasks 3.2

In-house single laboratory validation and field application of ESBL screening procedure.[1] A protocol (draft SOP) for the screening for ESBLs will be produced and trialled on a range of food products in a range of different formats and performance of the method(s) will be assessed in a formal in-house validation exercise using a protocol based on BS EN ISO 16140:2003. The method(s) will then be demonstrated in a test validation exercise,[2] comparing levels of ESBLs found in a range of farm and retail samples to samples prepared in the laboratory.

Introduction

A protocol (draft SOP) produced by APHA (**Appendix I**) for the screening for ESBLs was produced and trialled on a range of food products (including chicken, turkey, pork and beef) in a range of different formats (including ready-to-eat meals). Performance of the method(s) was assessed in an in-house validation exercise using a protocol based on BS EN ISO 16140:2003. The method(s) were then evaluatedin a further test validation exercise, by determining the number of positive ESBLs detected in a set of 300 poultry samples by both the phenotypic (agar) and LAMP methods (as outlined in the SOP and which had been agreed as suitable techniques to use) (see Task 3.1).

Methods

Selective agar. Media preparation, QC and preparation of overnight enrichment broths were carried out in accordance with the SOP 'Isolation of presumptive Extended Spectrum Beta Lactamase (ESBL) bacteria from meats; ref BAC0280; APHA' – **See Appendix I.**

[1] This part of the work was performed in part by Leatherhead Food Research and in part by APHA. The method to use for this part of the study and for industry in general was agreed on following discussions between APHA, PHE and LFR. There were also some limited discussions about the methodology to use for industry at the seminar (Task 3.1).

[2] For the test validation exercise in order to analyse a sufficient number of samples to generate relevant test performance statistics, a total of 300 samples were used to validate the detection methodology.

LAMP assays. Setting up of the assays, QC and preparation of sample boilates were carried out in accordance with the SOP 'The detection of CTX-M group 1, group 2, group 9 and OXA ESBLs in meat samples using Loop-Mediated Isothermal Amplification (LAMP); ref BAC0282; APHA' – **See Appendix II.**

Results

Selective agar media QC

Task 3.2 - Table 1. Control organisms for CHROMagar CTX

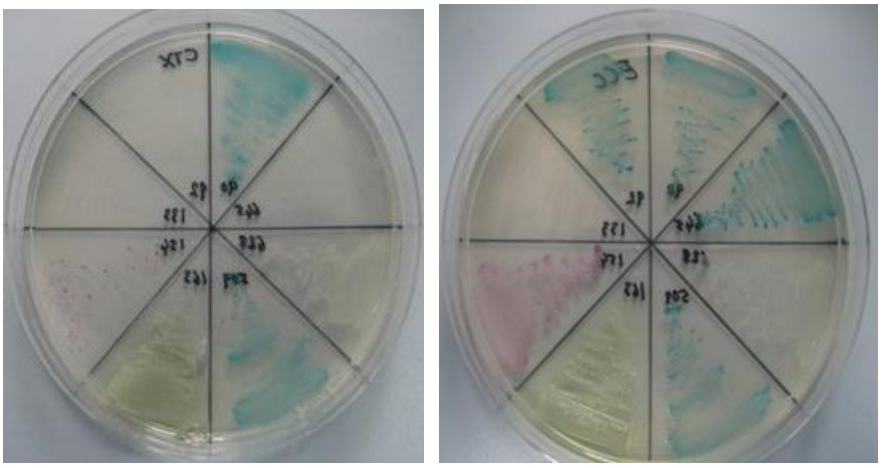
Ref no.	**Bacteria**	**ESBL type**	**Growth**	**Colour**
LREC90	*E.coli*	CTX M14	Yes	Blue/Green
LREC92	*E.coli*	-	No	Blue/Green
LREC133	*Salmonella*	-(AmpC)	No	Cream
LREC154	*K.pneumoniae*	SHV 5	Yes	Mauve
LREC163	*Ps.aeruginosa*	OXA 11	Yes	Cream
LREC509	*E.coli*	TEM 52	Yes	Blue/Green
LREC645	*E.coli*	-(AmpC)	No	Blue/Green
S628	*Salmonella*	CTX	Yes	Cream

Task 3.2 - Table 2. Control organisms for CHROMagar ECC

Ref no.	**Bacteria**	**ESBL type**	**Growth**	**Colour**
LREC90	*E.coli*	CTX M14	Yes	Blue/Green
LREC92	*E.coli*	-	Yes	Blue/Green
LREC133	*Salmonella*	-(AmpC)	No	Cream
LREC154	*K.pneumoniae*	SHV 5	Yes	Mauve
LREC163	*Ps.aeruginosa*	OXA 11	Yes	Cream
LREC509	*E.coli*	TEM 52	Yes	Blue/Green
LREC645	*E.coli*	-(AmpC)	Yes	Blue/Green
S628	*Salmonella*	CTX	Yes	Cream

All media used in the study was assessed for the growth and colony appearance of the control organisms and approved as QC compliant.

Task 3.2 - Figure 1. Examples of control strains on CHROMagar CTX and CHROMagar ECC



CHROMagar CTX CHROMagar ECC

In-house validation - selectivity. Presumptive ESBL negative strains (30 non target strains) were sourced from LFR's culture collection and used to assess the selectivity of the CHROMagar CTX selective agar (Table 3). On the CHROMagar ECC non-selective base media, 28 out of the 30 showed positive growth. Two strains of *Morganella morganii* showed no growth. On the CHROMagar CTX selective agar, 30 out of 30 showed no growth, indicating they were all presumptive negative for ESBL. All 30 presumptive ESBL negative samples were also classified as negative for CTX M-1, CTX M-2, CTX M-9 and OXA when screened in the LAMP assay.

Presumptive ESBL positive strains were supplied by APHA (Table 4). On the CHROMagar ECC non-selective base media, 17 out of the 17 showed positive growth. On the CHROMagar CTX selective agar, 15 out of 17 showed positive growth and were classified as presumptive ESBL positive. Samples 7 and 8 produced colouring of the agar but no colony formation was observed, even under the microscope.

Task 3.2 - Table 3. Presumptive ESBL negative sample screen

Sample No.	Strain	BPW	CHROMagar CTX		CHROMagar ECC	
		Turbidity	Growth	Colour	Growth	Colour
1	*Salmonella* Arizonae	G	NG	No	G	Green
2	*Salmonella* spp	G	NG	No	G	Cream
3	*Salmonella* Enteritidis	G	NG	No	G	Cream
4	*Salmonella* Infantis	G	NG	No	G	Cream
5	Salmonella Enteritidis PT30	G	NG	No	G	Cream
6	*Salmonella* Typhimurium	G	NG	No	G	Cream
7	*Salmonella* Typhimurium	G	NG	No	G	Cream
8	*Salmonella* Typhimurium	G	NG	No	G	Cream
9	*Salmonella* Newington	G	NG	No	G	Green
10	*Salmonella* Oranienburg	G	NG	No	G	Green
11	*Escherichia coli*	G	NG	No	G	Green
12	*Escherichia coli K12*	G	NG	No	G	Green
13	*Escherichia coli AB1157*	G	NG	No	G	Green
14	*Escherichia coli 1*	G	NG	No	G	Green
15	*Escherichia coli O157:K88a*	G	NG	No	G	Green
16	*Escherichia coli*	G	NG	No	G	Green
17	*Escherichia coli*	G	NG	No	G	Pink
18	*Escherichia coli*	G	NG	No	G	Green
19	*Escherichia coli*	G	NG	No	G	Green
20	*Enterobacter cloacae*	G	NG	No	G	Green
21	*Enterobacter aerogenes*	G	NG	No	G	Pink
22	*Enterobacter sakazakii*	G	NG	No	G	Pink
23	*Klebsiella pneumoniae*	G	NG	No	G	Pink
24	*Morganella morganii*	G	NG	No	NG	NG
25	*Yersinia enterocolica*	G	NG	No	G	Cream
26	*Shigella dysenteriae*	G	NG	No	G	Cream
27	*Citrobacter freundii*	G	NG	No	G	Mauve
28	*Enterobacter aerogenes*	G	NG	No	G	Mauve
29	*Morganella morgani*	G	NG	No	NG	No Growth
30	*Citrobacter freundii*	G	NG	No	G	Mauve
Controls						
31	S628	G	G	Cream	G	Cream
32	90	G	G	Green	G	Green
33	154	G	G	Mauve	G	Mauve
34	BPW	NG	NG	NG	NG	NG

Key: G growth; NG no growth

Task 3.2 - Table 4. Presumptive ESBL positive sample screen

S.no	Ref.no	Group	BPW	Chromagar CTX		Chromagar ECC	
			Turbidity	Growth	colour	Growth	colour
1	90	9	G	G	Green	G	Green
2	94	9	G	G	Green	G	Green
3	147	2	G	G	Cream	G	Cream
4	149	2	G	G	Green	G	Green
5	164	OXA	G	G	Cream	G	Cream
6	217	1	G	G	Green	G	Green
7	200	2	G	NG	Pink	G	Pink
8	201	2	G	NG	Pink	G	Pink
9	212	1	G	G	Green	G	Green
10	220	1	G	G	Green	G	Green
11	243	9	G	G	Green	G	Green
12	513	1	G	G	Green	G	Green
13	519	1	G	G	Green	G	Green
14	526	9	G	G	Green	G	Green
15	529	9	G	G	Green	G	Green
16	B307466	9, OXA	G	G	Green	G	Green
17	B307467	1	G	G	Green	G	Green

LAMP assay control strains 147, 149, 164, B307466 and B307467, plus S628 *Salmonella* and LREC 163 *Pseudomonas aeruginosa*, were screened in the LAMP assay (Table 5). All 7 strains were classified correctly according to their assigned ESBL group.

Task 3.2 - Table 5. Presumptive ESBL positive sample screen by LAMP assay

Sample	Expected	Positive	Negative
S628	CTX-M1	CTX-M1	CTX-M2, CTX-M9, OXA
LREC163	OXA-11	OXA	CTX-M1, CTX-M2, CTX-M9
147	CTX-M2	CTX-M2	CTX-M1, CTX-M9, OXA
149	CTX-M2	CTX-M2	CTX-M1, CTX-M9, OXA
164	OXA-14	OXA	CTX-M1, CTX-M2, CTX-M9
B307466	CTX-M9	CTX-M9	CTX-M1, CTX-M2, OXA
B307467	CTX-M1	CTX-M1	CTX-M2, CTX-M9, OXA

Limit of detection (LOD). ESBL positive control strains were enriched overnight in BPW and enumerated on CHROMagar CTX to yield $>10^8$ cfu/ml. Two chicken broths, confirmed ESBL negative on CHROMagar CTX, were enumerated on CHROMagar ECC to confirm the background TVC (Broth 1- $2.6x10^6$ cfu/ml and

Broth 2- 1.1x10^6 cfu/ml). The chicken broths were spiked with the control strains at ~ 1, 10 and 100 cfu/ml, enumerated on CHROMagar CTX (Table 6) and a boilate prepared for analysis by LAMP assay (Table 7).

Task 3.2 - Table 6. LOD determination on CHROMagar CTX

ESBL		**Chicken broth 1 (background count on ECC – 2.6x10^6 cfu/ml)**			**Chicken broth 2 (background count on ECC – 1.1x10^6 cfu/ml)**		
Ref	Inoculum cfu/ml	10^2 cfu/ml	10^1 cfu/ml	10^0 cfu/ml	10^2 cfu/ml	10^1 cfu/ml	10^0 cfu/ml
CTX-M1 S628	1.50x10^8	96	9	1	99	14	7
CTX-M2 147	1.95x10^8	89	12	4	112	15	3
CTX-M9 90	2.63x10^8	107	15	3	97	11	2
OXA 163	1.90x10^8	117	6	1	103	14	3

Task 3.2 - Table 7. LOD determination by LAMP

Lamp assay	**S**	**Ct**	**MP**	**Classification**	**Range (cfu/ml)**	**LOD (cfu/ml)**
CTX-M 1	10^0	20.57	90.5	+	1-7	≥1
	10^1	19.75	90.5	+	9-14	
	10^2	19.35	90.5	+	96-99	
CTX-M2	10^0	19.54	91.5	+	3-4	≥3
	10^1	18.89	91.5	+	12-15	
	10^2	18.61	91.5	+	89-112	
CTX-M9	10^0	24.66	90.5	+	2-3	≥2
	10^1	22.13	90.5	+	11-15	
	10^2	21.58	90.5	+	97-107	
OXA	10^0	17.38	85.0	+	1-3	≥1
	10^1	15.45	85.0	+	6-14	
	10^2	13.34	85.0	+	103-117	
CTX-M1	Ctrl	16.84	90.5	+	NA	NA
CTX-M2	Ctrl	10.89	91.5	+	NA	NA
CTX-M9	Ctrl	18.29	90.0	+	NA	NA
OXA	Ctrl	14.97	85.5	+	NA	NA

The limit of detection using CHROMagar CTX selective agar was determined as CTX-M 1 ≥1 cfu/ml, CTX-M 2 ≥3 cfu/ml, CTX-M 9 ≥2 cfu/ml, OXA ≥1 cfu/ml, with a competing background flora of >10^6 cfu/ml. All boilates prepared from these samples were classified as positive in the LAMP assay, thus indicating an equal LOD capability.

Precision. Assay precision was determined by the analysis of three sets of triplicate samples (n=9) in each assay (Table 8). Both the within (intra) and between (inter) assay precision was calculated as ≤10 % coefficient of variation (CV).

Task 3.2 - Table 8. Precision of LAMP assays

Lamp assay	Reps	mean	SD	% CV	
				Intra	Inter
CTX-M1	14.30	13.87	0.44	3.2	10.0
	13.90				
	13.43				
	14.01	14.44	0.37	2.6	
	14.60				
	14.70				
	17.11	16.84	0.26	1.5	
	16.80				
	16.60				
CTX-M2	10.74	10.44	0.65	6.2	2.2
	9.70				
	10.89				
	11.01	10.84	0.15	1.4	
	10.77				
	10.73				
	10.83	10.86	0.83	7.6	
	11.70				
	10.04				
CTX-M9	15.98	16.02	0.04	<0.1	7.7
	16.03				
	16.06				
	16.88	16.09	0.69	4.3	
	15.61				
	15.79				
	18.18	18.29	0.11	0.6	
	18.40				
	18.29				
OXA	13.71	13.77	0.60	4.4	4.2
	14.40				
	13.20				
	14.71	14.25	0.42	2.9	
	14.14				
	13.90				
	14.89	14.97	1.39	9.3	
	16.40				
	13.62				

CV – coefficient of variation

Recovery of ESBLs from food matrices. ESBL positive control strains were grown overnight in BPW and enumerated on CHROMagar CTX to yield >10^8 cfu/ml. A total of 6 food matrices comprising 4 raw meats and 2 ready-to-eat products were confirmed as ESBL negative on CHROMagar CTX and enumerated on CHROMagar ECC to confirm the background mainly *Enterobacteriaceae* count. The samples were spiked with the control ESBL strains at ~ 10^3 cfu/ml, enumerated on CHROMagar CTX and a boilate prepared for analysis by LAMP assay.

The results for recovery of ESBLs from using agar are shown in Table 9, whilst the detection by LAMP assays are shown in Table 10.

Task 3.2 - Table 9. Recovery of ESBLs from food matrices by CHROMagar CTX

Sample		**Chicken (diced breast)**	**Turkey (diced breast)**	**Pork (fillet)**	**Beef (diced steak)**	**Fried chicken mini fillets**	**Ham sandwich**
		Background count (aerobic TVC) cfu/ml					
ESBL		$2.6x10^6$	$5.7x10^6$	$3.2x10^5$	$2.6x10^6$	$4.0x10^1$	$2.1x10^2$
Ref	Inoculum cfu/ml	**Recovery of ESBL cfu/ml (% recovery based on initial spike level)**					
CTX-M1 S628	$1.78x10^3$	$1.21x10^3$ (68)	$1.32x10^3$ (74)	$1.44x10^3$ (81)	$9.43x10^2$ (53)	$9.26x10^2$ (52)	$1.28x10^3$ (72)
CTX-M2 147	$1.34x10^3$	$8.44x10^2$ (63)	$7.64x10^2$ (57)	$1.02x10^3$ (76)	$7.91x10^2$ (59)	$6.83x10^2$ (51)	$8.58x10^2$ (64)
CTX-M9 90	$2.29x10^3$	$1.63x10^3$ (71)	$1.42x10^3$ (87)	$1.90x10^3$ (83)	$1.37x10^3$ (60)	$1.35x10^3$ (59)	$1.67x10^3$ (73)
OXA 163	$1.59x10^3$	$1.35x10^3$ (85)	$1.19x10^3$ (75)	$1.40x10^3$ (88)	$9.22x10^2$ (58)	$8.37x10^2$ (52)	$1.03x10^3$ (65)

TCV- total viable count

Task 3.2 - Table 10. Detection of ESBLs from food matrices by LAMP assay

Lamp assay		**Chicken**	**Turkey**	**Pork**	**Beef**	**Fried chicken**	**Ham sandwich**
CTX-M 1	Ct	19.24	19.65	19.43	20.63	20.11	19.41
	MP	90.5	90.5	90.5	90.5	90.5	90.5
	Id	+	+	+	+	+	+
CTX-M2	Ct	18.54	18.64	19.87	21.97	19.18	19.81
	MP	91.5	91.5	91.5	91.5	91.5	91.5
	Id	+	+	+	+	+	+
CTX-M9	Ct	21.34	21.15	21.26	23.12	21.36	20.04
	MP	90.5	90.5	90.5	90.5	90.5	90.5
	Id	+	+	+	+	+	+
OXA	Ct	13.38	13.28	13.66	14.67	13.51	14.07
	MP	85.0	85.0	85.0	85.0	85.0	85.0
	Id	+	+	+	+	+	+

The ESBL positive control strains were detected in all the food matrices with recoveries in the range of 51-88%. Boilates prepared from the same samples were similarly classified as positive for ESBL in the qualitative LAMP assay.

Test validation study. The aim of the study was to analyse a sufficient number of poultry samples (neck flap and caecal contents, n=300) to establish standard test performance parameters, provide statistical confidence of the robustness of procedures for ESBL detection, as well as robust data on background levels and a comparison between the phenotypic and genotypic methods.

Abattoir samples (neck flap and caecal samples) were sourced over a 4 month period (October 2014 to February 2015) from an existing ongoing FSA study (FS241051). Abattoir samples were stratified by month, randomised and weighted by abattoir throughput. In addition to these 300 abbattoir samples, 30 randomly selected retail chicken meat samples (collected over a ~ 2 to 3 week period) were also tested to provide proof of principle for different sample types. The retail samples were tested by Leatherhead foods, and the abattoir samples at APHA.

All samples (both abattoir and retail) were screened for presumptive ESBL bacteria by enriching overnight in BPW and then plating on CHROMagar CTX. To detect the presence of ESBL bacteria by LAMP, boilates were made from the enriched overnight BPW and the presence of CTX-M group 1, CTX-M group 2, CTX-M group 9 and OXA detected by the aforementioned LAMP assays. In addition, for the 300 abbattoir samples, suspect colonies from CHROMagar CTX were tested by multiplex PCR for CTX, OXA, SHV and TEM genes.[33] PCR was able to confirm or refute if suspect ESBL colonies on CHROMagar CTX were positive for ESBL genes, since white colonies on this agar may be, for example, *E. coli*, *Pseudomonas* or *Salmonenella enterica.*

For the 300 abbattoir samples, sensitivity and specificity was calculated as well as positive and negative predictive values, by comparing results from agar alone or PCR for CTX-M genes, to the results by the LAMP CTX-M assays. Predictive values give the probabilities of abnormality for test results. For the 30 retail samples, sensitivity and specificity only was calculated, comparing agar results to results by CTX LAMP assay.

For the 300 abbattoir samples, since the study was based on randomly selected samples of caecum and neck flap (FS241051), the test validation results are considered to be applicable for these sample types in future studies. This is a considerable additional benefit to the study, compared to that set out in the initial proposal because in a non-randomised sampling strategy, positive predictive value would not necessarily have general application to the population.

Establishing proof of principle for thirty retail samples.

For the 30 retail samples, data was analysed in two ways:-

(i) Assuming culture as a gold standard (i.e. so calculating the relative sensitivity of LAMP to culture).

(ii) Using Bayesian approaches without assuming a gold standard. However because there was only one population in the sample, it was necessary to make additional assumptions and therefore a specificity of culture of 100% was assumed.

These results are shown in Table 11 for retail samples. The LAMP assay was 95% specific and 60% sensitive compared to culture, assuming culture as the gold standard.

Task 3.2 - Table 11. Summary of the sensitivity and specificity estimates (with 95% confidence intervals) from the analysis of 30 random retail meat samples tested in parallel by LAMP for CTX-M group 1, 2, and 9, OXA and culture.

Parameter	Assuming culture gold standard	Non gold standard approach
Culture specificity	100% assumed	100% assumed
Culture sensitivity	100% assumed	83% (45%, 99%)
LAMP specificity	95% (75%, 99.8%)	94% (76%, 1%)
LAMP sensitivity	60% (26%, 88%)	54% (26%, 81%)
ESBL Prevalence	33% (17%, 53%)	39% (22%, 68%)

All these samples were negative for OXA

300 abattoir samples

Data for the 300 abattoir samples was analysed using Bayesian approaches without assuming a gold standard. A 3-test model assuming conditional independence between tests was employed, adjusted to account for the PCR test being only applied to culture positives. Non-informative priors for all parameters (ESBL prevalence and sensitivity/specificity of each test) were used. Convergence was tested by using several different starting values and applying the Gelman-Rubin method as implemented in WinBUGS. A total of 5,000 iterations of the model were used for estimation of posterior densities, with a burn-in of 5,000.

The model was applied to neck and caecal sample data separately to see whether there were any apparent differences in test performance by sample type, and also to the data when combined (assuming sensitivity and specificity values did not differ between sample types). Positive predictive values (the probability that a true positive sample would test positive) and negative predictive values (probability that a true negative sample would test negative) were also calculated for each test.

For the 300 abatoir neck flap and caecal contents samples, the summary results are shown in tables 12 (samples positive by different tests), 13 (sensitivity and specificity) and 14 (positive and negative predictive values). A table of individual results is given in Appendix IV.

Task 3.2 – Table 12. Summary of the total number of abattoir samples (n = 300) positive for ESBL-phenotype by culture and ESBL-genotype for CTX-M genes by PCR and CTX-M group 1, 2 and 9 by LAMP assay.

Sample	Diagnostic test		
	Culture[a]	PCR[b]	CTX-M LAMP
Caeca	36/150 (24%)	33/149 (22%)	40/150 (26%)
Neck Flap	94/150 (63%)	60/147 (41%)	99/150 (62%)
Total	**130/300 (43%)**	**93/296 (31%)**	**139/300 (46%)**

a - Positive on agar included all colony colours. For agar results to be comparable to the LAMP results, all colonies on CHROMagar CTX including white colonies were scored as positive. Such white colonies can often be *Pseudomonas species*, which can be intrinsically resistant, but some white colonies can be *E. coli*, or other *Enterobacteriaceae* that can be ESBL-producers

b – Only one presumptive ESBL-colony was tested by PCR from agar, but LAMP assay and culture on agar assessed the entire enriched bacterial population.

Task 3.2 - Table 13. Bayesian estimates of sensitivity - Se and specificity - Sp (plus 95% confidence intervals) of culture and LAMP (CTX-M group 1, 2, and 9) to detect ESBLs in abattoir samples (n = 300) from (i) analysing the neck and caecal sample data separately and (ii) analysing the data together.

Parameter	Sample type		
	Neck sample	Caecal Sample	Caeca/neck data combined
Culture Se	93.2 (83.7, 99.6)	86.3 (66.6, 99.1)	91.7 (81.3, 99.6)
Culture Sp	91.8 (80.2, 99.5)	97.9 (91.8, 99.9)	94.8 (89.4, 99.8)
CTX-M LAMP Se	96.8 (88.6, 99.9)	88.4 (72.7, 99.2)	97.1 (88.1, 99.9)
CTX-M LAMP Sp	89 (73.8, 99.4)	95.2 (87.9, 99.7)	93.4 (85.5, 99.6)
ESBL Prevalence	**62.3 (51.7, 72)**	**26.1 (17.9, 35.9)**	**43.5 (36.2, 51.3)**

Task 3.2 - Table 14. Percentage positive and negative predictive values for culture and CTX-M LAMP applied to abattoir samples, stratified by sample type (caeca, neck and caeca/neck data combined).

	Positive predictive value		Negative predictive value	
	Culture	CTX-M LAMP	Culture	CTX-M LAMP
Caeca	93.5	86.6	95.3	95.9
Neck	95.0	93.5	89.1	94.4
Caeca/Neck combined	**93.1**	**91.5**	**93.7**	**97.6**

Of the 300 poultry abattoir samples (150 neck flaps and 150 caecal contents) used in the test validation exercise, 130 or 43% of both sample types were positive for presumptive ESBLs on agar. This included 94 or 63% neck flap and 36 or 24% cecal samples (Table 12). The “true” prevalance was estimated to be 43.5%, 62.3% and 26.1% for all samples, neck flap samples and caecal contents, respectively (Table 13).

CTX-M LAMP had the highest number of samples positive (Table 12), followed by culture and PCR having the fewest number positive. This resulted in high estimates of sensitivity for LAMP, with CTX-M LAMP being the most sensitive test and PCR being the least sensitive (Table 13). However, there were a number of samples that were positive for culture and/or PCR, that were negative for CTX-M LAMP (n=20), leading to an estimate of CTX-M LAMP specificity of 93.4% overall (Table 13). Similarly, culture was also found to have imperfect specificity (Table 13). It was

found that there was very little power in the data to estimate PCR specificity (this is the probability that a true negative sample would test negative by PCR, and since PCR was only applied to selected colonies on agar plates from positive samples, there were no true negative samples to which PCR was applied). Therefore PCR sensitivity and specificity was not reported in this study, and the positive predictive and negative predictive values were not calculated for PCR.

Positive predictive and negative predictive values were over 90% overall for both culture and CTX-M LAMP, with culture having a higher positive predictive value than CTX-M LAMP but a lower negative predictive value (Table 14).

It is important to note that the agar and LAMP results are comparable, as both test the enrichment broth, but the LAMP and the PCR results are less comparable, since the PCR tests only one randomly chosen colony from CHROMagar CTX plates for a number of ESBL genes, and not the entire enriched bacteria population i.e. all colonies present on the antibiotic selective plate. Similarly, a limitation of LAMP is that only the CXT-M and OXA specific genes are being detected, while culture is looking at any ESBL harbouring bacteria. However, the fact that the results from culture and LAMP CTX-M assays are highly concordant suggests that the majority of ESBL positive bacteria present on plates were harbouring CTX-M group 1, 2 or 9 genes. In fact the majority of CTX-M sequence types, as determined by both LAMP assay and PCR of individual colonies, were CTX-M group 1 (results not shown).

In contrast, comparison of samples for presence of OXA-10 like ESBL genes by LAMP with CHROMagar CTX was poor. The specificity was ~82% and sensitivity ~29%, using agar as the gold standard.

Discussion

The selective agar and LAMP methods developed by APHA were successfully transferred to LFR and validated against standard analytical performance criteria. The method of selectivity (accurate reporting of positive and negative samples), limit of detection (detection of <10 target organisms with a background flora of $>10^6$ cfu/ml), precision (inter and intra assay precision <10% coefficient of variation) and recovery from different matrices, were all acceptable.

Based on a test validation exercise of 300 abattoir neck flap and caecal samples from chicken, 43%, 63% and 24% of all samples, neck flap, and caecal samples, respectively, were found to be positive for presumptive ESBLs by agar. The "true" prevalance was estimated to be 43.5%, 62.3% and 26.1% for all samples, neck flap samples and caecal contents respectively, so these results were very close to those observed from the agar results.

For all 300 samples, the culture and CTX-M LAMP sensitivity was 91.7 and 97.1% respectively, while the specificity was 94.8% and 93.4%, respectively. The culture and CTX-M LAMP positive predictive values were 93.1 and 91.5% respectively, while the negative predictive values were 93.7 and 97.6%, respectively. The sensitivity for caecal samples for both culture and CTX-M LAMP assays was slightly lower than that for neck samples; wheras the converse was true for the specificity.

For the 30 retail meat samples examined to demonstrate proof of principle (where LAMP results were only compared to results from agar), the specificity was similar to that seen for the 300 abattoir samples, but the sensitivity was lower. The reasons for this are not apparent, but it could be that if the sample size for these retail meats was larger, the results may have been more comparable to those observed for the abattoir samples.

The SOP for this work recommends using both agar and LAMP results together to detect the presence of ESBL-producing bacteria. If both results are used in combination, whilst there would be a small overall reduction in sensitivity compared to using results from agar or LAMP tests alone, there would be increased specificity, but also the CTX-M group is simultaneously determined for CTX-M positive samples by LAMP, and a culture is isolated for future work if required. However, it will be important in future to determine by sequencing the identity of all LAMP positive / agar negative samples to see if they were "true" negatives or had actually been "missed" by culture on CHROMagar CTX agar plates.

We noted poor sensitivity of the LAMP OXA-10 like ESBL results compared to results from CHROMagar CTX. It is not clear why sensitivity in this instance was poor, and this will require further work. Two explanations are possible, firstly that the agar inhibits some of these OXA-10 like ESBLs, or that the LAMP OXA also detects some non-ESBL OXA genes. On the first count, in other work the agar has been compared to other commercial ESBL agars with comparable results, and on the

second count OXA-10 and its derivatives are all ESBLs and mainly present in *Pseudomonas*, and include OXA-10, -11, -13, -14, -15, -16, -17, -19 and -28. As the "OXA-10 like" LAMP assay was designed to detect this group, it should be specific for ESBLs, and the agar should allow such ESBL to grow. Sequencing of a PCR product using the LAMP external primers in future will help identify the gene detected in LAMP OXA positive, culture negative samples and determine whether these result from non-specific amplification by the LAMP OXA primers or underscoring by the CHROMagar CTX-M plates. Since most ESBLs in poultry in the UK are currently CTX-M type ESBLs, these results do not impinge on the reliability of the combined results from agar and LAMP for the practical detection of those ESBLs which are currently most frequent in poultry.

In combination (e.g. agar and LAMP), the test is capable of both isolating ESBLs (for further work if needed) and rapidly detecting major ESBLs gene types and classifying them as OXA ESBLs, or specific groups of CTX-M ESBLs.

In view of the culture and CTX-M LAMP sensitivity of 91.7 and 97.1% respectively, specificity of 94.8% and 93.4%, respectively, positive predictive values of 93.1 and 91.5% respectively, and negative predictive values of 93.7 and 97.6%, respectively, the combined tests used should provide reliable and accurate determination of ESBL phenotype coupled with CTX-M genotype in poultry samples.

13. OBJECTIVE 03: Tasks 3.3

Ring Trial[1]

Introduction

The ESBL screening method was further evaluated in a small ring trial to determine the transferability, robustness and analytical performance of the method. The trial was performed in strict accordance with the SOPs 'Isolation of presumptive Extended Spectrum Beta Lactamase (ESBL) bacteria from meats; ref BAC0280; APHA' and 'The detection of CTX-M group 1, group 2, group 9 and OXA ESBLs in meat samples using Loop-Mediated Isothermal Amplification (LAMP); ref BAC0282; APHA' – See appendices I and II for SOPs.

Method

A total of 10 blind samples (samples 1-6 chicken/turkey; samples 7-10 poultry boilates) were screened by Leatherhead Food Research and two independent operators for the isolation of presumptive positive ESBLs on CHROMagar CTX and confirmation by LAMP assay for CTX-M group 1, CTX-M group 2, CTX-M group 9 and OXA, as shown in Table 13.

Results

The results of the trial are shown in Table 1. All 10 samples were correctly identified on both agar and by LAMP assay.

The results of the ring trial showed good agreement between operators with all samples giving the same classification. Following limited practice runs, no problems were reported in following the SOP and executing the work.

[1] The ring trial was organised by Leatherhead Food Research

Task 3.3 - Table 1. The combined results for isolation on CHROMagar CTX and identification by LAMP assay.

Sample	Operator	Agar EEC	Agar CTX	Lamp CTX1 Ct/MP	Lamp CTX2 Ct/MP	Lamp CTX9 Ct/MP	Lamp OXA Ct/MP	ID
1	1	G	G	37.39 88	55.84 90	17.18 90	NA 87	CTX 9
	2	G	G	NA 76.5	30.70 90	17.22 90	NA 76.5	CTX 9
	3	G	G	45.09 85.5	19.37 89	17.29 89.5	NA 86.5	CTX 9
2	1	G/W	G/W	NA 76.5	42.44 89.5	NA 83	6.24 94.5	-
	2	G/W	G/W	48.72 88.5	NA 90	NA 83	NA 94.5	-
	3	G/W	G/W	32.07 86.5	37.38 90	58.56 89.5	NA 90	-
3	1	G/M	M	NA 76.5	39.24 90.5	NA 83	NA 94.5	-
	2	G/M	M	NA 76.5	NA 90	NA 83	NA 90	-
	3	G/M	M	NA 76	44.03 89.5	NA 83	NA 94.5	-
4	1	G/M/W	G/W	NA 76.5	32.19 89	NA 83	13.08 85.5	OXA
	2	G/M/W	G/W	28.42 87.5	46.80 89.5	NA 83	12.97 85.5	OXA
	3	G/M/W	G/W	36.58 88	48.62 89.5	NA 82.5	13.22 85.5	OXA
5	1	G	G	NA 89.5	NA 90	NA 82.5	NA 77	-
	2	G	G	28.87 87.5	57.47 89.5	NA 82.5	NA 91.5	-
	3	G	G	36.38 87.5	29.32 89.5	NA 82.5	NA 91.5	-
6	1	G/M/W	G/M	42 88	32.78 89.5	NA 82.5	NA 85.5	-
	2	G/M/W	G/M	50.03 88.5	36.30 89.5	NA 82.5	NA 94.5	-
	3	G/M/W	G/M	27.54 88.5	33.13 90	NA 8.25	NA 81	-
7	1	ND	ND	16.22 90	-	-	-	CTX 1
	2	ND	ND	16.81 90	-	-	-	CTX 1
	3	ND	ND	17.22 90	-	-	-	CTX 1
8	1	ND	ND	-	12.83 92	-	-	CTX 2
	2	ND	ND	-	13.75 92	-	-	CTX 2
	3	ND	ND	-	12.96 92	-	-	CTX 2
9	1	ND	ND	-	-	19.17 90.5	-	CTX 9
	2	ND	ND	-	-	18.45 90.5	-	CTX 9
	3	ND	ND	-	-	18.03 90.5	-	CTX 9
10	1	ND	ND	-	-	-	17.41 85	OXA
	2	ND	ND	-	-	-	16.99 85	OXA
	3	ND	ND	-	-	-	17.98 85	OXA

Dual values in cells for LAMP results are Ct (top) and MP (below).
ND, not determined as samples 7 to 10 were DNA boilates, so no cultural work could be carried out. Samples 1-6 were enriched chicken samples, and samples 1 and 4 were spiked with ESBL strains as detected. G green, M mauve, W white, Ct value, MP melt point

Discussion

The selective agar and LAMP methods developed by APHA were successfully transferred to LFR and were shown to be transferable and robust when examined in the small ring-trial. There were some samples that gave growth on the CHROMagar CTX, but yielded negative results by the LAMP assays. These results would have warranted further investigation to determine if the LAMP results were false negatives, or the agar results were false positives. In the test validation exercise (Task 3.2), test validation parameters were similar with the culture and CTX-M LAMP sensitivity was 91.7 and 97.1% respectively, while the specificity was 94.8% and 93.4%, respectively. The culture and CTX-M LAMP positive predictive values were 93.1 and 91.5% respectively, while the negative predictive values were 93.7 and 97.6%, respectively

For the purposes of this ring trial, all operators managed to learn the technique and got identical results. This small ring trial also highlights the usefulness of having both a phenotypic (agar) and genotypic tests (LAMP assays) combined to ensure maximum and robust isolation and detection of ESBLs.

14. Final discussion and conclusions

Each of the 10 task sections of this report has its own discussion. As such, the final discussion given here provides a summary of overall conclusions.

- The project was successful in developing, evaluating, trialling and then recommending to industry a suitable screening method for isolating, detecting and partially characterising ESBLs in Enterobacteriaceae in foods.
- Standard industry methods for isolation of ESBLs from meat / foodstuffs with enrichment and MacConkey agar + antibiotic[15] were compared with (i) Enrichment + / - additives followed by isolation with chromogenic agars or (ii) Enrichment + / - additives followed by isolation using the cefpodoxime disk method[13] or (iii) Enrichment + / - additives followed by detection using ESBL micro-arrays or (iv) Enrichment + / - additives followed by detection using the newly developed LAMP assays for rapid detection of $bla_{\text{CTX-M}}$ groups 1, 2 and 9 and $bla_{\text{OXA-10-like}}$ ESBLs from meat.
- None of the different food matrices (diced and minced beef, chicken, pork, lamb, turkey or ready meals) presented any problems for the isolation and detection of ESBLs. Since the method first involved an enrichment stage through broth, it was considered that the methodology would be suitable for all meat and food types, unless they contained substances that would be inhibitory to the growth of bacteria in the enrichment broth.
- The chromogenic agars showed improved sensitivity and specificity for detection of ESBLs compared with MacConkey agar supplemented with cefotaxime, the cefpodoxime disc method or ESBL microarray. Chromogenic agars were capable of detecting 10 cfu/ESBLs per gram of meat or less, even in the presence of high numbers of competitor bacteria.
- The LAMP assays showed similar sensitivity and specificity to the chromogenic agars and worked well for all the different meat matrices tested. They were very quick and relatively easy to perform, giving results more quickly than microarrays and were also less expensive than microarrays.
- There was agreement amongst key staffs at APHA, PHE and LFR with respect to an overall method to recommend to industry, and to recommend for a pilot field trial at LFR.

- The agreed selective agar and LAMP methods developed by APHA were successfully transferred to Leatherhead Food Research with suitable SOPs and validated against standard analytical performance criteria. The method selectivity (accurate reporting of positive and negative samples), limit of detection (detection of ≤ 10 target organisms with a background flora of ≥ 10^6 cfu/ml) and precision (inter and intra assay precision <10% CV) were all acceptable.
- In a test avaulation study performed on 300 abattoir samples, the culture and CTX-M LAMP sensitivity was 91.7 and 97.1% respectively, while the specificity was 94.8% and 93.4%, respectively. The culture and CTX-M LAMP positive predictive values were 93.1 and 91.5% respectively, while the negative predictive values were 93.7 and 97.6%, respectively
- The SOP for this work recommends using both agar and LAMP results together to detect the presence of ESBL-producing bacteria. If both results are used in combination, whilst there would be a small overall reduction in sensitivity compared to using results from agar or LAMP tests alone, there would be increased specificity, but also the CTX-M group is simultaneously determined for CTX-M positive samples by LAMP, and a culture is isolated for future work if required.
- The method was shown to be transferable and robust when examined by ring-trial.

Overall the recommended methods have the potential to both isolate and rapidly identify ESBLs from meats and other foodstuffs. Whilst the use of a chromogenic agar will increase the costs slightly compared to use of MacConkey agar supplemented withantibiotics, the improved sensitivity and specificity will lead to added confidence in results and could be more cost-effective if further confirmatory work is not required, particularly when results are combined with those from LAMP assays.

To our knowledge, this is the first time that LAMP assays have been developed and used for detection of ESBLs from foods, and the dual agar and LAMP approach is also, to our knowledge, a novel and robust approach to screening for and partially characterising ESBLs from food. The combined use of the LAMP assays have the added benefit of being able to confirm agar results, and chactacterise ESBLs down to ESBL family type, and even CTX-M group type. If a novel ESBL or an ESBL other

than those detected by the LAMP assays is present, then the agar should allow such strains to grow.

The extra LAMP assays for *bla*$_{\text{CMY-2,}}$ *bla*$_{\text{NDM-1,}}$ *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{VIM}}$ genes were designed and initially validated over and above the contractural requirements. As such these add value to the project and help future proof the work should there be an urgentneed to test food products for these important antibiotic resistance genes.

15. Contractor recommendations for further work

The Department of Health has funded a project to assess the public health relevance of non-human reservoirs of ESBL-producing *E. coli*. This will include sampling meat from five UK regions to give nationally-relevant information. The FSA have added to the award to allow vegetables and fruits to be assessed, and to allow ESBL producers to be counted in food samples.

Future work could involve:-

1. Technology transfer of all the developed LAMP assays to Public Health England.
2. Bi-annual monitoring of meats, slurry and vegetables for ESBLs and carbapenamase bacteria – jointly by PHE and APHA.
3. The advantage of the LAMP assay is the rapid result which can be provided; further development could entail matching the types of enzyme which can be detected by this technique to those found to be of greatest importance from this current research on retail meat.

16. Acknowledgments

We are grateful to the Foods Standard Agency (FSA) for funding this work under project code FS241023.

CHROMagar CTX was developed at the APHA as reported previously.[18]

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18. Appendix I – APHA internal SOP of methodology for isolation of presumptive Extended Spectrum Beta Lactamase (ESBL) bacteria from meats

AHVLA Animal Health and Veterinary Laboratories Agency

STANDARD OPERATING PROCEDURE

Title: **Isolation of presumptive Extended Spectrum Beta Lactamase (ESBL) bacteria from meats**

SOP Reference:	**BAC 0280**	*Author:*	**Dr L. P. Randall**
Edition:	**[CAPS IN BOLD]**	*Implementation date:*	[DD.MM.YYYY]

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Contents of SOP BAC 280

INTRODUCTION

Purpose/Scope of this SOP

To isolate presumptive Extended Spectrum Beta Lactamase (ESBL) bacteria from meat samples by enrichment followed by plating on selective agar

Background information

Bacteria with resistance to ESBL antibiotics have been shown to be widespread in farm animals in the UK[1; 2; 3; 4; 5] and other countries.[6; 7; 8; 9; 10; 11; 12] As such these bacteria may pose a threat to human health via ingestion of contaminated meat products.[13]

Several studies to date have isolated ESBLs from meat. These include a study where CTX-M 1, 2 and 14 *E. coli* were isolated from UK and imported chicken breast fillets, [13] a study in Portugal where ESBLs were isolated from 60% of uncooked chicken carcasses[15] and a study in the Netherlands where 94% of chicken samples contained at least one *E. coli* isolate with an ESBL phenotype with ESBL genes CTX-M 1, 2, 14, SHV-2, 12 and TEM-20 in the *E. coli*.[16]

Several different methods for isolation of ESBLs from meat have been used. In a UK study meat samples were enriched in Buffered Peptone water (BPW) before plating on Cystine Lactose Electrolyte Deficient (CLED) agar + 8 mg/L ciprofloxacin then looking for growth inside the zone of inhibition caused by a cefpodoxime disc.[13] In another study, meat samples were again enriched in BPW then plated on MacConkey (MacC) agar with and without cefotaxime (CTX) or ceftazidime (CAZ) at 1 mg/L,[15] whilst another study first of all enriched meat samples in peptone water, then in MacC CTX broth and finally on MacC CTX agar.[17] Finally, another study used first enrichment in BPW before plating to BCIG agar and looking for growth inside a CTX disk.[14] All of these studies did however have a common stage of enrichment in a peptone broth of some sort, usually BPW.

The method used in this SOP is from validated work that was done at the Animal and Plant Health Agency (Weybridge) and is based on the industry standard method of enrichment in BPW, but involves an isolation step using a chromogenic ESBL agar, CHROMagar CTX.

SAFETY

See current version of APHA Health and Safety Policy, relevant Safety Unit Codes of Practice on intraVet and appropriate Risk Assessments for this procedure.

Staff who carry out this work must be appropriately trained to work with ACDP hazard group 2 organisms, and must have read, understood and abide by the requirement set out in the risk assessments as below as a minimum. Staff must also be trained in the used of a class I or II safety cabinet.

All work must be carried out in a laboratory designated for ACDP hazard group II organisms with reference to appropriate risk assessments for the orgaisation performing the work.

MATERIALS

Documentation and software

There are two associated forms with this SOP. One is for recording the results from growth on CHROMagar CTX and Rambach CTX (if used) and the other is for recording details of media.

Form BA835:- Isolation of presumptive Extended Spectrum Resistance bacteria from meat samples.

Form BA141:- Pages for media QC book.

Chemicals and reagents

Ethanol – 100%

Ethanol – 70% with distilled water

Media

Buffered Peptone water: 225 ml volumes in suitable container made according to SOP BPU1157 or according to manufacturer's instructions (Van Waters Rogers international [VWR] cat no.1.07228.0500). This media has a one year shelf life once made up and when stored at 2°C to 8°C.

CHROMagar ECC: CHROMagar ECC is made according to SOP BPU BPU 1363 or according to manufacturers instructions (Cat. No. EF320, CHROMagar, France). These plates have a two month shelf life once made up and when stored at 2°C to 8°C.
CHROMagar CTX: CHROMagar CTX supplement (Cat No CX062, CHROMagar, France) is added to CHROMagar ECC agar base (Cat. No. EF322, CHROMagar, France) according to SOP BPU1773 or according to

manufacturers instructions to make CHROMagar CTX. These plates have a one month shelf life once made up and when stored at 2°C to 8°C.
Rambach CTX agar: The above supplement (Cat No CX062, CHROMagar, France) can be added to Rambach agar (Van Waters Rogers international [VWR] cat no. 1.0750.0001) to make Rambach CTX agar (SOP BPU 1818) which will give a specific pink coloured colonies for isolation of Salmonella. These plates have a one month shelf life once made up and when stored at 2°C to 8°C.
Luria Bertani (LB) broth: 3 ml volumes in bijouxs made according to SOP BPU0343 or according to manufacturer's instructions (Van Waters Rogers international [VWR] cat no. 1.10285.500). This media has a one year shelf life once made up and when stored at 2°C to 8°C.
Dorset egg slopes: ~ 3 ml volumes in bijouxs (SOP BPU0014). This media has a one year shelf life once made up and when stored at 2°C to 8°C.

Cryobank beads: Suitable beads for long term frozen storage of bacteria, for example MAST cryobank bacterial storage system, catalogue number CRYO/Y. Use according to manufacturers' instructions.

Micro-organisms

Control organisms for CHROMagar CTX

Ref. No.	Bacteria	ESBL type	Expected growth	Expected colour
LREC 90	*E. coli*	CTX-M 14	Yes	Blue / green
LREC 92	*E. coli*	-	No	Blue / green
LREC 133	*Salmonella*	- (AmpC)	No	White / cream
LREC 154	*K. pneumoniae*	SHV-5	Yes	Mauve
LREC 163	*Ps. aeruginosa*	OXA-11	Yes	Cream/brown
LREC 509	*E. coli*	TEM-52	Yes	Blue / green
LREC 645	*E. col*	- (AmpC)	No	Blue / green
S628	*Salmonella*	CTX	Yes	White / cream

Control organisms for Rambach agar CTX

Ref. No.	Bacteria	ESBL type	Expected growth	Expected colour
LREC 90	*E. coli*	CTX-M 14	Yes	Blue / green
LREC 92	*E. coli*	-	No	Blue / green
LREC 133	*Salmonella*	- (AmpC)	No	Pink to red
LREC 154	*K. pneumoniae*	SHV-5	Yes	Blue / green
LREC 163	*Ps. aeruginosa*	OXA-11	Yes	Light pink
LREC 509	*E. coli*	TEM-52	Yes	Blue / green
LREC 645	*E. col*	- (AmpC)	No	Blue / green
S628	*Salmonella*	CTX	Yes	Pink to red

See SOP appendix A for photo of how control strains grow on CHROMagar ECC, CHROMagar CTX, Rambach agar and Rambach CTX agar.

Equipment

Suitable stomacher capable of stomaching ~ 50 to 100 ml volumes

Suitable sterile stomacher bags capable of holding ~ 50 to 100 ml volumes

Scissors and forceps that can be sterilised by autoclaving

PROCEDURE/METHOD

Test Reliability

The test has been validated with chicken, beef, lamb, pork and turkey samples spiked with different ESBL-producing strains at different levels, with and without competitor strains. The results with respect to sensitivity and specificity of CHROMagar CTX for isolation of ESBLs compared with other agars is shown in Table1.

It should be emphasised that this gives a “presumptive ESBL” that will need confirming by other tests (such as PCR, LAMP assay or ESBL disks) not covered by this SOP. However, in practice about 90% to 100% of isolates of the correct colour and morphology that grow on this agar have been found to be ESBLs to date (2012).

It should also be emphasised that each batch of agar must be tested and pass with controls, must be correctly stored, and must be used within the shelf life.

Appendix I - Table 1 – Recovery of ESBLs from spiked chicken samples on different agars after enrichment – grey is agar used in this SOP.

Spike cfu/gram chicken	Competitors added (+) or not (-)	% and numbers of spiked chicken samples yielding growth of presumptive spike organism on agars				Comment
		CTX	BRILL	MacC+	RAM+	
0	+ and -	6%	4%	22%	0%	False positives
		3 / 50	2 / 50	11 / 50	0 / 14	False positives
10	-	100%	100%	93.3%	100%	True positives
		30 / 30	30 / 30	28 / 30	6 / 6	True positives
	+	100%	76.7%	40.0%	100%	True positives
		30 / 30	23 / 30	12 / 30	6 / 6	True positives
100	-	100%	100%	90.0%	100%	True positives
		30 / 30	30 / 30	27 / 30	6 / 6	True positives
	+	100%	83.3%	40.0%	100%	True positives
		29 / 29	25 / 30	12 / 30	6 / 6	True positives
1000	-	100%	100%	100%	100%	True positives
		30 / 30	30 / 30	30 / 30	6 / 6	True positives
	+	100%	83.3%	60.0%	100%	True positives
		30 / 30	25 / 30	18 / 30	6 / 6	True positives

CTX, CHROMagar CTX; BRILL, OXOID Brilliance ESBL agar ; MacC +, MacConkey agar + 1 mg/L cefotaxime, RAM +, Rambach agar + CHROMagar CTX supplement.

QC of selective media

All agar plates used must be "dried" prior to use. Plates should be dried open but face down in a laminar flow cabinet for 30 minutes or open but face down in a "clean" 37°C incubator. Once dried plates can be stored at 2 to 8°C within their shelf-life until needed.

Control strains as outline in 3.4 should be stored at -70°C on cryogenic beads according to the manufacturer's instructions.

Prepare broth stocks of control strains from bead stocks by using sterile forceps or a sterile blunt ended needle to remove one bead to 3 mls of sterile LB-G broth. Incubate overnight at 37°C and check purity of isolates in broth by plating to CHROMagar ECC without CTX-M supplement.

If isolates are pure, prepare Dorset egg slope cultures from the broths and store these are room temperature with a one year shelf life.

The above LB-G broths with control strains can be stored for 2 months at 2 to 8°C. After two months discard the LB-G broths and make new LB-G broths from the Dorset egg slopes, but again checking for purity by plating the new broths to CHROMagar ECC.

Dilute LB-G control strains 1:10 in sterile saline and streak 1 µl of these dilutions to at least one CHROMagar CTX or Rambach agar CTX plate (e.g. divide plate into eight segments) for each batch of agar used. Incubate the plate for 24 to 48 hours at 37°C. Normally results can be recorded at 24 hours, but if colonies are very small or some isolates have not grown that should grow, incubate for a further 24 hours before recording results

Record results for each batch of agar tested with the final result on form BA141.

If media fails QC test, re-test with QC isolates freshly prepared from bead stocks. If media still fails discard the batch unless other suitable QC strains show media to be suitable.

After one year, prepare fresh Dorset egg slopes of control strains from bead stocks checking purity as outlined above and give these Dorset egg cultures a one year shelf life at room temperature. Use these Dorset egg cultures for preparing LB-G broth cultures for regular QC testing of media for the year. This ensures bead stocks are only used once a year.

Weighing out and stomaching of the meat sample and incubation

Allocate each meat sample to be tested a sample ID number and enter details on form BA835 in a registered file for specific project. Label appropriate numbers of 225 ml sterile BPW's with sample ID numbers

Work in a room with a class I or II safety cabinet and turn the cabinet on. Put on gloves and spray with 70% ethanol and allow the gloves to air dry. Place a sterile stomacher bag on a top pan balance and open the top of the stomacher bag to receive meat samples. Tare the balance so reading is zero.

Using sterile scissors cut off pieces of meat (if needed) in small pieces of about 3 to 5 grams into the stomacher bag on the balance until 25 grams (+/- 0.5 grams) has been weighed into the stomacher bag. If the meat is minced or already in small pieces, use sterile forceps to transfer to the stomacher until 25 grams (+/- 0.5 grams).

From relevant marked BPW pour ~ 50 mls of sterile BPW into the stomacher bag. Place the stomacher bag + meat sample + BPW into the stomacher and stomach for 4 minutes at 260 beats per minute until most of the meat forms a homogenous suspension. As the sample is being stomached, place the lid back

on the top of the remaining BPW and place into the safety cabinet.

Pour the meat / BPW homogenate back into the original BPW container (which should be inside a safety cabinet) that is marked with the sample ID.

Use of newly sterilised scissors and forceps for each meat sample processed are essential to avoid cross contamination. This can be achieved by either having suitable numbers of packs of sterile scissors and forceps for all meat samples ready (this is the preferred option), or by sterilising them between meats with ethanol and flaming.

If sterilising scissors and forceps between meat samples with ethanol and flaming, work with ~ 4 to 5 sets of scissors and forceps and leave all these soaking vertically in 100% ethanol when not in use with the top 1 to 2 inches of the scissors or forceps out of the ethanol for safe handling. From the ethanol take a pair of scissors or forceps that have not been used for the longest time of all sets (e.g. the set that has most time in the ethanol, and not the set last used) and pass through a Bunsen burner flame. Allow ethanol to burn off and the scissors or forceps are now ready to use.

If sterilising scissors or forceps using 100% ethanol, after use wipe them clean before placing in 100% ethanol, so that the ethanol does not become mixed with meat tissue and juices as far as possible. Each set of scissors or forceps should have at least 10 minutes soaking in 100% ethanol before use.

Once all the meats have been processed, spray the BPW containers with 70% ethanol and after 15 minutes remove from the safety cabinet. Close and turn off the safety cabinet in line with procedures for the safety cabinet.

Place the BPWs + meat in a 37°C aerobic incubator and incubate for 16 to 24 hours

Plating out from BPW to agar and recording growth

All agar plates used must be "dried" prior to use. Plates should be dried open but face down in a laminar flow cabinet for 30 minutes or open but face down in a "clean" 37°C incubator. Once dried plates can be stored at 2 to 8°C within their shelf-life until needed.

Label plates with sample IDs to correspond to sample IDs entered on form BAC835.

After 16 to 24 hours incubation remove meat samples in BPW from 37°C incubator and using a 10µl sterile loop, plate from BPWs to appropriately labelled plate.

Incubate plate at aerobically at 37°C for 24 to 48 hours. In most cases, growth is visible by 24 hours, but if colonies are very small at 24 hours, or if there is no growth on the plate, it should be incubated for a further 24 hours so that colonies can clearly be seen or if no growth after 48 hours, the result is taken to be negative

Record results of growth using form BAC835.

Storage of isolates for further characterisation

For short term storage of up to 2 weeks, isolates can be stored on the original isolation plate.

For longer term storage either store isolates on a Dorset egg slope or ideally on cryogenic beads.

Prior to longer term storage, take a single colony from original isolation plate and re-plate to the same agar as originally used. This should provide a pure culture suitable for storage and further characterisation. Do not store direct from original isolation media, as the culture may be mixed with other unseen bacteria.

Disposal of used media

All used media containing live bacteria should be disposed of safely, e.g. by incineration or as appropriate after killing all bacteria with a suitable autoclave program.

RESULTS for SOP

Results are only valid if the media used is both in date and has passed QC checks

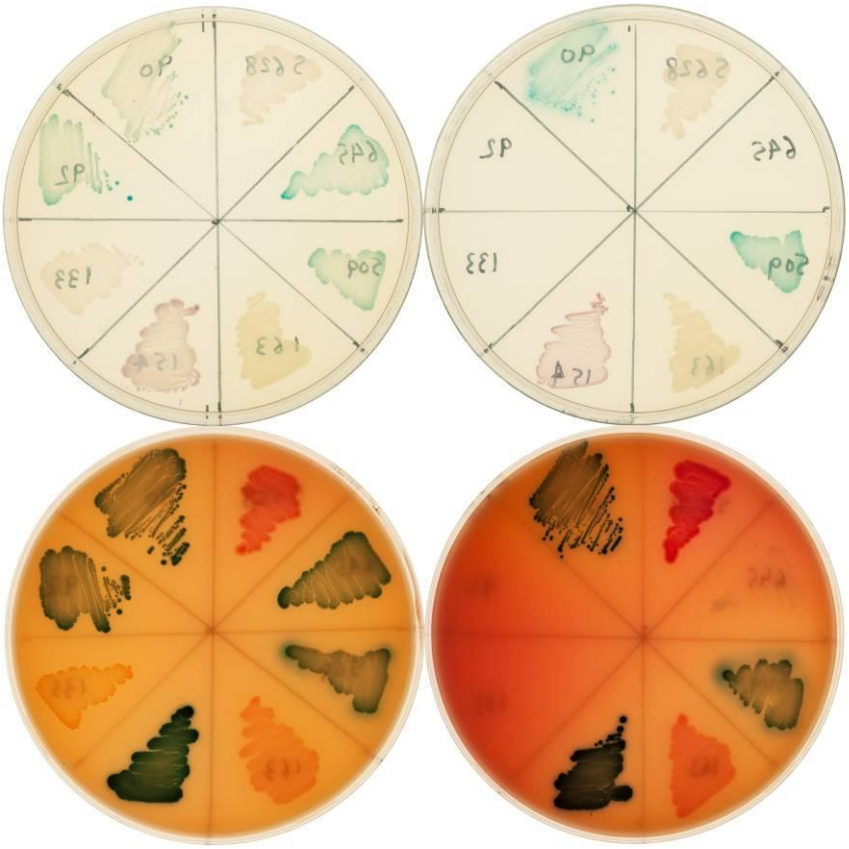
Typical colony colours for *E. coli* (blue / green) and non-*E. coli Enterobacteriaceae* (mauve) can be seen in SOP appendix B. Most presumptive ESBLs will normally be either blue / green or mauve. However, some presumptive ESBLs can be white / cream (non-fermenters such as *Pseudomonas* and *Salmonella*). Rarely *E. coli* are glucuronidase-negative and these also grow with white / cream colonies. The most common false positives are non-ESBL *Pseudomonas.*

Results are "presumptive ESBL" only as further genetic tests are needed to confirm the presence of a specific ESBL gene. However, if the colonies are blue / green or mauve, in most cases such isolates are ESBLs. If colonies are white / cream, the isolates may be an ESBL but can also be a false positive such as non-ESBL *Pseudomonas.*

Appendix A of appendix I

Appendix I - Figure 1 – Control strains on CHROMagar ECC, CHROMagar CTX, Rambach agar and Rambach CTX agar

No Supplement With CHROMagar CTX supplement



No Supplement With CHROMagar CTX supplement

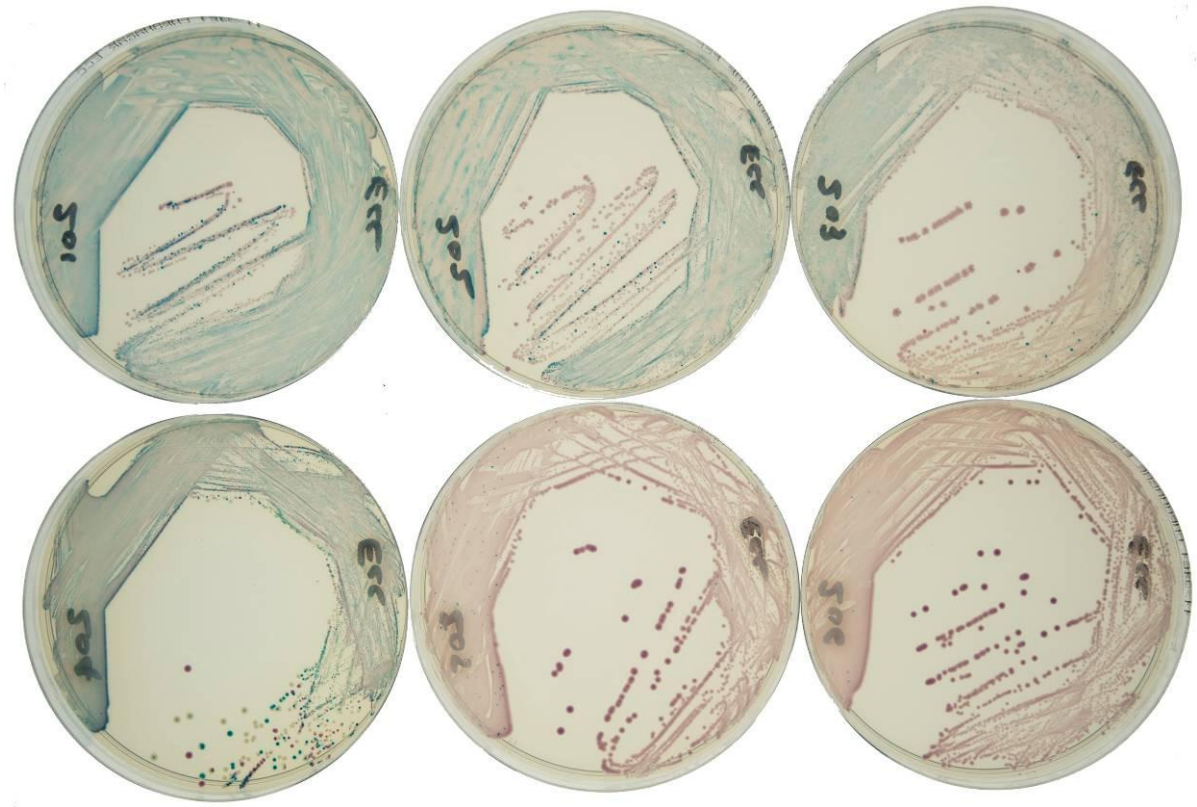
Top – CHROMagar ECC: +/- CTX supplement

Bottom - Rambach agar: +/- CTX supplement

Strains from top left going anticlockwise – LREC 90, 92, 133, 154, 163, 509, 645 and S 628.

Appendix B of appendix I

Appendix I - Figure 2 – *E. coli* (blue / green) and non *E. coli* but *Enterobacteriaceae* such as *Klebsiella*, *Citrobacter*, *Enterobacter* (mauve) colonies growing on CHROMagar ECC to give an example of colony colours.



Non-fermenters such as *Pseudomonas* and *Salmonella* grow as white / cream colonies (not shown) as do glucuronidase-negative *E. coli* (not shown) although these are not common.

The most common false positives are some *Pseudomonas*, although in some case *Pseudomonas* can carry ESBL genes.

19. Appendix II – APHA SOP for detection of CTX-M group 1, 2, 9 and OXA ESBLs in meat samples using a Loop-Mediated Amplification (LAMP) assay.

AHVLA Animal Health and Veterinary Laboratories Agency

STANDARD OPERATING PROCEDURE

Title: **The detection of CTX-M group 1, group 2, group 9 and OXA ESBLs in meat samples using a Loop-Mediated Isothermal Amplification (LAMP) assay.**

SOP Reference:	**[CAPS IN BOLD]**	*Author:*	**JENNIFER CORK**
Edition:	**Edition 1**	*Implementation date:*	[DD.MM.YYYY]

Authorisation	
Approver Name:	

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Contents

1. INTRODUCTION

1.1. Purpose/Scope of this SOP

1.1.1. Extended spectrum beta lactamase (ESBL) enzymes are capable of breaking down certain antibiotics called cephalosporins and ESBL-producing bacteria can be found in food animals and the environment. This SOP describes a method to test DNA isolated from raw meat samples for CTX-M group 1, 2 and 9 and Oxa 10-type ESBLs using a Loop-Mediated Isothermal Amplification (LAMP) assay.

1.1.2. Staff must undergo suitable training before carrying out the procedures detailed in this SOP.

1.2 Background information

1.2.1 Generally ESBLs provide resistance to ß-lactam antibiotics commonly used in hospitals including penicillins, cephalosporins and monobactams. Increases in the number of ESBL-producing bacteria have been observed in both healthcare and community settings as well as among animals in recent years suggesting that food and the environment could be sources of these resistant bacteria.

1.2.2. The CTX-M ESBLs are so called after their ability to hydrolyse the third generation (extended spectrum) cephalosporin cefotaxime. Other ESBLs typically have greater activity against other extended spectrum beta lactam antibiotics such as ceftazidime.

1.2.3. Samples undergo an initial processing and enrichment process to produce a homogenised meat sample in buffered peptone water. (See SOP BAC 2080). Boilates of these samples are then prepared to lyse the cells and release the deoxyribonucleic acid (DNA), which is used as template for the four LAMP assays, CTX-M group 1, 2 and 9 and OXA.

1.2.4 Loop-mediated Isothermal Amplification, LAMP, is a rapid amplification used to amplify small regions of DNA. In LAMP, the target sequence is amplified at a constant temperature of 65 °C using four sets of primers and a polymerase with high strand displacement activity in addition to a replication activity.

1.2.5 Four different primers, forward and reverse internal primers and forward and reverse external primers designed to recognize six distinct regions on the target gene are used to detect and amplify the target ESBL gene. Inclusion of a further two primers, the loop primers, increases the speed of amplification process.

1.2.6 Samples are incubated with a mastermix containing the primers, DNA Polymerase, dNTPs and EvaGreen®. EvaGreen® dye is a DNA binding dye that emits fluorescence when bound to DNA but is inactive in the absence of DNA. In samples containing the target gene amplification of the DNA occurs during

incubation and corresponding levels of fluorescence are recorded.

1.2.7 Melt Curve analysis of the amplified product is performed by heating a reaction-mixture that contains double-stranded DNA sequences and measuring dissociation against temperature. The melting point is the temperature at which 50% of the DNA has denatured. As the energy required to break the base-base hydrogen bonding between two strands of DNA is dependent on their length, GC content and their complementarity, melt point analysis can be used to help check the specificity of the amplification reaction.

2.0 SAFETY

2.1 See current version of APHA Health and Safety Policy, relevant Safety Unit Codes of Practice on intraVet and appropriate Risk Assessments for this procedure.

2.2 ⚠ Areas within this procedure which refer to Safety Critical activities are denoted in the paragraph number column with the ⚠ sign to highlight these areas to users.

2.3 Staff who carry out this work must be appropriately trained to work with

ACDP hazard group 2 organisms, and must have read, understood and

abide by the requirement set out in the appropriate risk assessments.

3.0 MATERIALS

3.1 Documentation and software

3.1.1 MX3000P Real-time PCR system setup and users guide -Stratagene

3.1.2 MX3000P system software installation CD - Stratagene

3.1.3 SOP BAC 2080

3.2 Chemicals and reagents

3.2.1	Nuclease-free water	Ambion – catalogue number 9937 or equivalent
3.2.2	LAMP Isothermal Mastermix	Optigene – IS0-001

Oligonucleotide primers are ordered from Sigma Genosys and are ordered desalted

3.2.3 CTX-M group 1 F3 oligonucleotide primer : AACTCTGCGGAATCTGAC

C. fetus for. forward oligonucleotide primer: GCT AAG GGT GAG GTT GAT GGG

3.2.4 CTX-M group 1 B3 oligonucleotide primer :

TTTCTGCCTTAGGTTGAGG

3.2.5 CTX-M group1 FIP oligonucleotide primer :

CCCACAACCCAGGAAGCAAAAAGCTGGTGACATGGATGAA

3.2.6 CTX-M group 1 BIP oligonucleotide primer :

GTGGCTATGGCACCACCAAAAAGTAAGTGACCAGAATCAGC

3.2.7 CTX-M group 1 FLOOP oligonucleotide primer :

AGTCCAGCCTGAATGCTCG

3.2.8 CTX-M group 1 BLOOP oligonucleotide primer :

ACGATATCGCGGTGATCTGG

3.2.9 CTX-M group 2 F3 oligonucleotide primer :

AAAGTGACGGCGTTTGCT

3.2.10 CTX-M group 2 B3 oligonucleotide primer :

CTGTGCCCGCTGAGTTTC

3.2.11 CTX-M group 2 FIP oligonucleotide primer :

AATGGCGGTATTGAGCGTGGGAAAAACGCTCGTTGGGTGATGAG

3.2.12 CTX-M group 2 BIP oligonucleotide primer :

GTGATACCACCACGCCGCTCAAAAAGCTTTACCCAGCGTCAGAT

3.2.13 CTX-M group 2 FLOOP oligonucleotide primer :

GTTCTGTCCAGACGGAAGGT

3.2.14 CTX-M group 2 BLOOP oligonucleotide primer :

ATGGCGCAGACCCTGAA

3.2.15 CTX-M group 9 F3 oligonucleotide primer:

CCAATGTGCAGTACCAGT

3.2.16 CTX-M group 9 B3 oligonucleotide primer :

TATTCAGCGTAGGTTCAGTG

3.2.17 CTX-M group 9 FIP oligonucleotide primer :

CATTGTGCCGTTGACGTGTTCTTAATCAGCCTGTCGAGAT

3.2.18 CTX-M group 9 BIP oligonucleotide primer:

ACGCTGGCAGAACTGAGCCTGGGCAATCAATTTGTTCA

3.2.19 CTX-M group 9 FLOOP oligonucleotide primer :

TAGTTAACCAGATCGGCAGGC

3.2.20 CTX-M group 9 BLOOP oligonucleotide primer :

CGTTGCAGTACAGCGACAATAC

3.2.21 OXA 19 F3 oligonucleotide primer :

GCATTAGCTAGTTCAATTACAGAA

3.2.22 OXA 19 B3 oligonucleotide primer :

GACACCAGTTTCTAGGCC

3.2.23 OXA 19 FIP oligonucleotide primer:

GCTACTTTTACAAAGCACGAAAACAAAAATACGTCTTGGAACAAAGA
GT

3.2.24 OXA 19 BIP oligonucleotide primer:
TCCTGCGCTACCAATAACTTAGCAAAAATAATTGCGTTGGGGATCT

3.2.25 OXA 19 FLOOP oligonucleotide primer:

GACGGCTTCGGCAGAGA

3.2.26 OXA 19 BLOOP oligonucleotide primer:

TCGTGCATCAAAGGAATATCTTCC

3.2.27 CTX-M group 1 strain grown overnight as positive control and boiled. Boilate of 20 000 cfu/ml of CTX-M3 LREC143.

3.2.28 CTX-M group 2 strain grown overnight as positive control and boiled. Boilate of 233 000 cfu/ml of CTX-M2 LREC147.

3.2.29 CTX-M group 9 strain grown overnight as positive control and boiled. Boilate of 3 000 000 cfu/ml of CTX-M14B LREC144

3.2.30 OXA strain grown overnight as positive control and boiled. Boilate of 233 000 cfu/ml of OXA 11 LREC 163B

3.3 Media

3.3.1 LB-G Broth

3.3.2 LB-G Agar plates

3.4 Animals/Micro-organisms/Cells

3.4.1 OXA 11 strain LREC 163 in *Ps. Aeruginosa* stored in glycerol or on beads at -80°C.

3.4.2 CTX-M14B strain LREC 144 in *E. Coli* stored in glycerol or on beads at -80°C.

3.4.3 CTXM-2 strain LREC 147 in *E. Coli* stored in glycerol or on beads at -80°C.

3.4.4 CTX-M1 strain LREC 143 in *E. Coli* stored in glycerol or on beads at -80°C.

3.5 Equipment

3.5.1	Mx3000P Real-Time PCR system and dedicated computer	Stratagene
3.5.2	Thermo-Fast 96 well plate Non-skirted Natural	Abgene – catalogue number AB-0600
3.5.3	Thermo-strips and Ultra Clear Cap Strips Natural	Abgene – catalogue number AB-1183

3.5.4	Ultra clear cap strips Natural	Abgene – catalogue number AB-0866
3.5.5	Range of nuclease-free barrier pipette tips from 1µl to 1000µl	Continental Lab Products or equivalent
3.5.6	Selection of pipettes	
3.5.7	2ml DNase free safelock microcentifuge tubes	Eppendorf catalogue number 0030 121.597 or similar
3.5.8	Hot block capable of heating 2ml tubes tubes to 95°C	
3.5.9	37°C +/- 1°C incubator	
3.5.10	Shaker capable of shaking cultures at 200rpm at 37°C.	
3.5.11	Centrifuge capable of spinning microcentrifuge tubes at 6000g	

4.0 PROCEDURE/METHOD

4.1 Test Reliability

4.1.1 These assays have been validated with chicken, beef, lamb, pork and turkey samples spiked with different ESBL-producing strains at different levels, with and without competitor strains.

4.1.2 Each new batch of reagents should be checked as fit for purpose and must be stored correctly and used within the shelf life.

4.1.3 The appropriate validated standard positive must be included on every assay and must perform within expected parameters. All assays should include a NTC or Negative well.

4.2 Preparation of standard positive controls

Standard controls used for each assay are prepared as below.

4.2.1 Prepare broth stocks of control strains from glycerol stocks or bead stocks by using a sterile 10µl loop to transfer approx 10ul of glycerol stock or by using sterile forceps or a sterile blunt ended needle to transfer one bead into 7 mls of sterile LB-G broth. Incubate overnight at 37□C shaking at 200rpm.

4.2.2 Prepare 10 fold dilutions of overnight culture from 10^{-1} to 10^{-8} in Nuclease-free water. Prepare a minimum of 600ul of each dilution.

4.2.3 Spot 3 x 20µl spots of each dilution onto LB-G agar plates, keeping each spot separate, 4 different dilutions (12 spots) can be tested on one agar plate by dividing plate into quarters. Record which dilutions are placed where. Incubate overnight at 37°C

4.2.4 Prepare boilates of each dilution (see 4.3). This should be done immediately after 4.2.2 and 4.2.3 to prevent any further growth of the bacteria. Store at -18°C or lower.

4.2.5 After overnight incubation of culture plate, where possible, count the number of colonies in each spot. Using the mean number of colonies for each dilution calculate the colony forming units per ml, CFU/ml, for each dilution using the formula No of colonies x (1000/20).

4.2.5 Select the boilate that has the correct CFU/ml for the positive control being prepared, see 3.2.27 – 3.2.30. If none of the boilates are the correct CFU/ml a dilution can be made using Nuclease-free water. Test the sample on the LAMP assay alongside the current batch of positive control. The new batch must be comparable to the current batch and perform within the set criteria for the control see results section.

4.2.6 Store standard control at -18°C or lower.

4.3 Preparation of boilates from homogenised samples.

4.31 Boilates of overnight enrichment broths, the latter prepared according to SOP BAC2080, are produced for use in the LAMP assays. Homogenates can be stored at -18°C or lower prior to preparing boilate.

4.3.2 To prepare sample boilate place 500µl of overnight enrichment broths into a microcentrifuge tube.

4.3.3 Centrifuge sample at 8000rpm (~6000xg) for 10 minutes to pellet bacterial cells.

4.3.4 Carefully remove and discard supernatant.

4.3.5 Resuspend pellet in 500µl of Nuclease free water.

4.3.6 Place sample in heat block @95°C for 20 mins.

4.3.7 Spin samples to remove debris, transfer supernatant to fresh tube. Samples can now be used as template for LAMP assay.

4.3.8 Boliates should be stored frozen at -18°C or lower.

4.4 Preparation of oligonucleotide primers

4.4.1 All four assays have the same cycling conditions and can be run in different wells on the same 96-well plate if required.

4.4.2. Lyophilised primers and probes and LAMP mastermixes should be prepared in a designated clean room or area.

4.4.3 Lyophilised primers and probes should be made up to a stock concentration of 100µM in nuclease free water and stored at -18^{o}C or lower for upto 3 years.

4.4.4 Prepare a 50µM working dilution of the Internal primers, IPS, FIP and BIP. e.g. 50µl of 100µM stock of FIP + 50µl of 100µM stock of BIP.

4.4.5 Prepare a 5µM working dilution of the external primers, EPS, F3 and B3. e.g. 5µl of 100µM stock of F3 + 5µl of 100µM Stock of B3 + 90µl of nuclease free water.

4.4.6 Prepare a 25µM working dilution of the LOOP, FLOOP and BLOOP. E.g. 25µl of 100µM stock of FLOOP + 25µl of 100µM Stock of BLOOP + 50µl of nuclease free water.

4.4.7 Working stocks of primers can be stored at -18°C or lower for 1 year.

4.5 Lamp assay

4.5.1. Prepare a plan showing the position of the samples on the PCR plate, Each sample should be tested in triplicate. Include a positive control on each plate. Additionally each plate must include at least one no template control (NTC) well. All four assays are performed using the same protocol and can be performed on the same plate if required. Selection of the appropriate primers determine the assay performed.

4.5.2. Calculate the volume of reagents required to prepare sufficient Master mix for all samples and controls to be tested using the following recipe:

Mastermix per well

15µl Isothermal Mastermix

6µl Nuclease free water

1µl IPS working stock (50µM)

1µl EPS working stock (5µM)

1µl Loop working stock (25µM)

4.5.3. Selecting the primers appropriate for the assay to be performed thaw the reagents, at room temperature in designated clean room / area.

4.5.4 Thaw sample and appropriate controls at room temperature.

4.5.5 While the samples and reagents are thawing, set up the Mx3000P machine to allow time for the bulb to warm up. When setting up the machine select experiment / project type Eva green with dissociation curve.

Test samples should be selected as 'unknown', positive controls as 'positive', and negative controls as 'NTC'. Data should be collected for the EVAgreen channel. The following cycling conditions should be set up:

Segment	Temp	Time in seconds	No of cycles	Data collection
1	65°C	60	60	End of each cycle
2	95°C	30	1	All (continuous)

4.5.6 When all the reagents are thawed, make up the assay mastermix as previously calculated.

4.5.7 Aliquot 24µl of mastermix into the required no wells of a 96 well plate or 8 well strip. Cover plate and transfer sample handling area away from designated clean room / area.

4.5.8 Add 1µl of sample or control to each well following plate plan. Cap the tubes securely using the cap strips. Load the plate in the Mx3000P machine and run the programme.

5.0 RESULTS

5.1.1 Before viewing the results, go to the options menu, and open the analysis term settings, baseline correction option. The adaptive baseline box should be checked, but the Mx algorithm box below should not be checked

5.1.2 For each well tested two results are obtained the Ct value and the Melt point.

5.1.3 On the analysis selection / set up page select the wells to be analysed.

5.1.4. The Ct Value can be viewed by selecting plate sample values on the analysis page this gives the results in plate format. The Ct value is the point at which the fluorescence increases above the threshold and indicates a positive result.

5.1.5 For the assay to be valid the standard controls must fall with in the expected range:

Expected Ct ranges for the standard positive controls are:

CTX-M1 assay standard positive = 18-24

CTX-M2 assay standard positive = 18-24

CTX-M9 assay standard positive = 21-27

OXA assay standard positive = 18 – 24.

All assays NTC and standard negatives must be No Ct.

5.1.6. Check the Amplification plots of all the samples. An example of an amplification plot for positive samples can be seen in Appendix A.

The baseline for the assay is automatically set by the MX machine during the first 6-10 cycles. Samples that react in under ten minutes can appear to be negative by examination of the Ct value as they have reacted before the baseline is established an example of this can be seen in appendix B. For these samples the Ct value will have to be manually established from the graph.

5.1.7 The dissociation curve can be seen by selecting dissociation curve on the analysis page. The peak of the curve shows the melt point for that well. Appendix three shows some typical melt curves for positive samples.

Negative samples give low peaks at a much lower temperature than that seen with the positive sample, this would be combined with a No Ct result.

Non-specific results are seen when a positive Ct value is recorded but the melt point is outside the expected range for that assay. Appendix D shows the melt curves for a selection of positive and negative samples including a non specific melt curve.

5.1.8 The Ct Values and Melt points for the samples can also be examined by selecting

the text report on the analysis page.

5.1.9 A well is scored positive if the Ct value is <30 and the melt point is in the expected range for the target gene.

Melt point ranges:

CTX-M group 1	90.0 – 92.0°C
CTX-M group 2	91.0 – 93.0°
CTX-M group 9	90.0 - 92.0°C
OXA	85.0 – 86.0°C

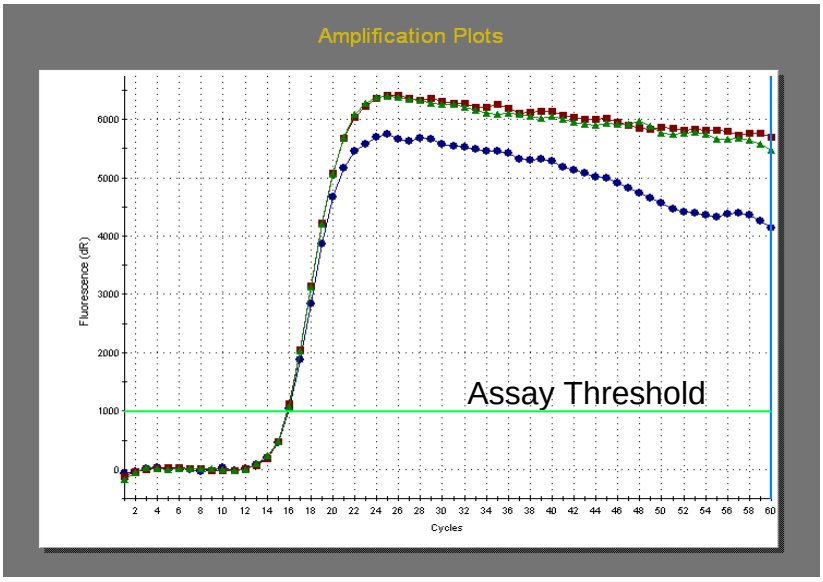
5.1.10 Samples with 3/3 wells positive are considered to be positive.

Samples with 1/3 or 2/3 wells positive are inconclusive and should be repeated. If the same results are obtained the sample should be considered positive.

Samples with 0/3 wells positive (all No Ct) are negative.

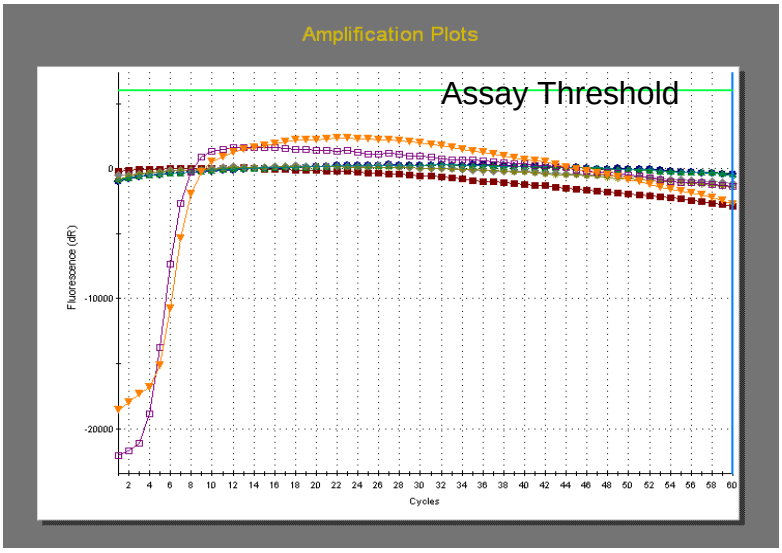
Appendix A of appendix II

Appendix II – Figure 1. Example of amplification plots obtained with a positive sample known to harbour a relevant ESBL gene.



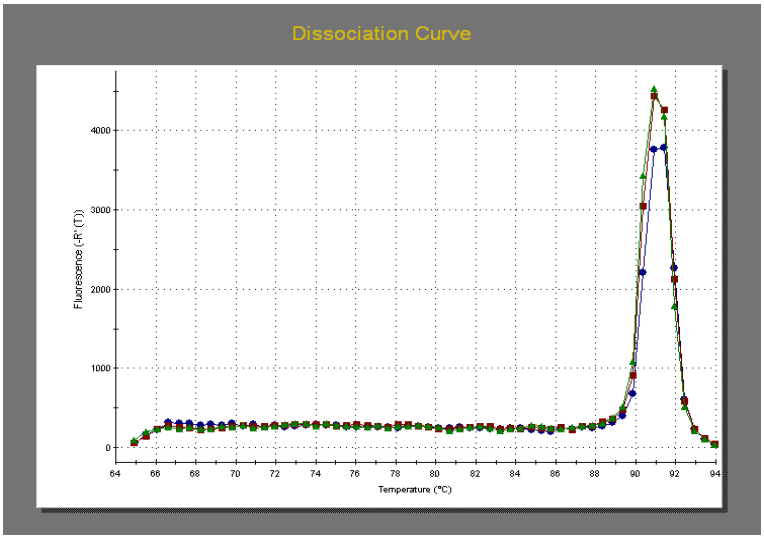
Appendix B of appendix II

Appendix II – Figure 2. Example of amplification plots obtained with a positive sample known to harbour a relevant ESBL gene where the positive samples have reacted before the baseline has been established, these positives samples would give a No Ct result.



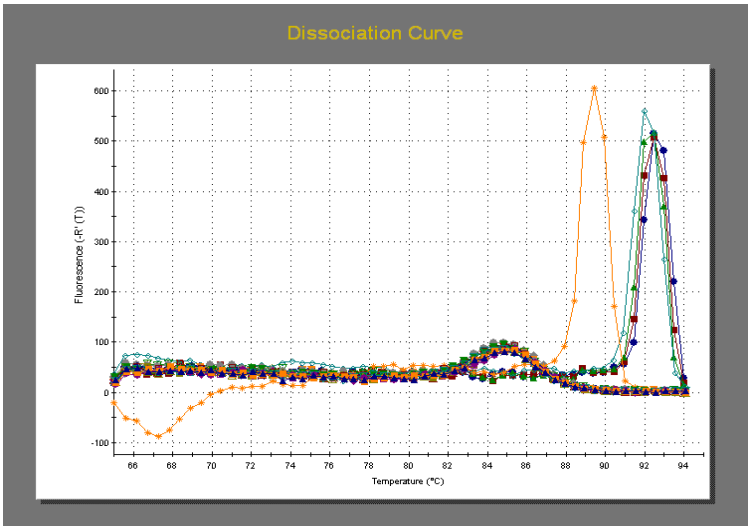
Appendix C of appendix II

Appendix II – Figure 3. Example of melt curves seen with positive samples known to harbour relevant ESBL genes.



Appendix D of appendix II

Appendix II – Figure 4. Example of melt curves for a selection of positive (known to harbour a relevant ESBL gene) and a negative sample (known to be free of relevant ESBL gene) including a non-specific melt curve.



20. **Appendix III** – Raw data tables for all experiments in which chicken meat samples were spiked with ESBL bacteria for method appraisal and validation

Appendix III - Table 1. Chicken meat samples spiked with different ESBLs strains, with different types of enrichment and with and without competitor bacteria, results on agars and by multiplex PCR

Broth ID	Spike cfu ESBL/gram	Added competitors	Exp No	Spike organism used	Enrichment method used	Presumptive ESBLs on different agars				Cefpodoxime disk result	Results with multiplex PCR			
						CTX (ESBL)	Brill	McC (ESBL)	RAM (SA)		CTX	OXA	SHV	TEM
55	1	All	4	CTX-M 14 -261	BPW	3+	2+	2+		-	+	-	-	+
56	1	All	4	CTX-M 14 -261	CTX	3+	3+	3+		-	+	-	-	+
57	1	All	4	CTX-M 14 -261	BZ/CTX	3+	3+	3+		-	+	-	-	+
58	1	None	4	CTX-M 14 -261	BPW	3+	3+	3+		+	+	-	-	-
59	1	None	4	CTX-M 14 -261	CTX	3+	3+	3+		+	+	-	-	-
60	1	None	4	CTX-M 14 -261	BZ/CTX	3+	3+	3+		+	+	-	-	-
61	2	All	4	CTX-M 14 -261	BPW	3+	3+	3+			+	-	-	+
62	2	All	4	CTX-M 14 -261	CTX	3+	3+	3+			+	-	-	+
63	2	All	4	CTX-M 14 -261	BZ/CTX	3+	3+	3+			+	-	-	+
64	2	None	4	CTX-M 14 -261	BPW	3+	3+	3+			+	-	-	-
65	2	None	4	CTX-M 14 -261	CTX	3+	3+	3+			+	-	-	-
66	2	None	4	CTX-M 14 -261	BZ/CTX	3+	3+	3+			+	-	-	-
67	0	None	4	CTX-M 14 -261	BPW	-	-	-		-	-	-	-	+
68	0	None	4	CTX-M 14 -261	CTX	-	-	-		-	-	-	-	-
69	0	None	4	CTX-M 14 -261	BZ/CTX	-	-	-		-	-	-	-	-
70	3	All	4	CTX-M 14 -261	BPW	3+	3+	3+			+	-	-	-
71	3	All	4	CTX-M 14 -261	CTX	3+	3+	3+			+	-	-	+
72	3	All	4	CTX-M 14 -261	BZ/CTX	3+	3+	3+			+	-	-	+
73	3	None	4	CTX-M 14 -261	BPW	3+	3+	3+			+	-	-	-

74	3	None	4	CTX-M 14 -261	CTX	3+	3+	3+			+	-	-	-
75	3	None	4	CTX-M 14 -261	BZ/CTX	3+	3+	3+			+	-	-	-
76	1	All	4	CTX-M 14 -261	None	-	-	-		-				
77	1	None	4	CTX-M 14 -261	None	-	-	-		-				
78	2	All	4	CTX-M 14 -261	None	-	-	-						
79	2	None	4	CTX-M 14 -261	None	-	1+	-						
80	0	None	4	CTX-M 14 -261	None	-	-	-		-				
81	3	All	4	CTX-M 14 -261	None	-	1+	1+						
82	3	None	4	CTX-M 14 -261	None	1+	-	1+						
83	1	All	5	CTX-M 1 - 245	BPW	3+	2+	-			+	-	-	+
84	1	All	5	CTX-M 1 - 245	CTX	1+	1+	-			-	-	-	+
85	1	All	5	CTX-M 1 - 245	BZ/CTX	3+	3+	3+			+	-	-	+
86	1	None	5	CTX-M 1 - 245	BPW	3+	3+	3+			-	-	-	-
87	1	None	5	CTX-M 1 - 245	CTX	3+	3+	3+			-	-	-	-
88	1	None	5	CTX-M 1 - 245	BZ/CTX	3+	3+	3+			-	-	-	-
89	2	All	5	CTX-M 1 - 245	BPW	3+	3+	-			-	-	-	+
90	2	All	5	CTX-M 1 - 245	CTX	3+	3+	-			+	-	-	+
91	2	All	5	CTX-M 1 - 245	BZ/CTX	3+	3+	-			+	-	-	+
92	2	None	5	CTX-M 1 - 245	BPW	3+	3+	3+			+	-	-	+
93	2	None	5	CTX-M 1 - 245	CTX	3+	3+	3+			+	-	-	-
94	2	None	5	CTX-M 1 - 245	BZ/CTX	3+	3+	3+			+	-	-	-
95	0	None	5	CTX-M 1 - 245	BPW	-	-	3+			-	-	-	+
96	0	None	5	CTX-M 1 - 245	CTX	-	-	3+			-	-	-	+
97	0	None	5	CTX-M 1 - 245	BZ/CTX	-	-	-			-	-	-	-
98	3	All	5	CTX-M 1 - 245	BPW	3+	3+	3+			+	-	-	+
99	3	All	5	CTX-M 1 - 245	CTX	3+	3+	3+			+	-	-	+
100	3	All	5	CTX-M 1 - 245	BZ/CTX	3+	3+	3+			+	-	-	+
101	3	None	5	CTX-M 1 - 245	BPW	3+	3+	3+			+	-	-	+
102	3	None	5	CTX-M 1 - 245	CTX	3+	3+	3+			+	-	-	+
103	3	None	5	CTX-M 1 - 245	BZ/CTX	3+	3+	3+			+	-	-	-
104	1	All	5	CTX-M 1 - 245	None	-	-	-						
105	1	None	5	CTX-M 1 - 245	None	-	-	-						
106	2	All	5	CTX-M 1 - 245	None	-	-	-						
107	2	None	5	CTX-M 1 - 245	None	-	-	-						

108	0	None	5	CTX-M 1 - 245	None	-	-	-						
109	3	All	5	CTX-M 1 - 245	None	-	-	-						
110	3	None	5	CTX-M 1 - 245	None	-	-	-						
111	1	All	6	CTX-M 15 -217	BPW	3+	3+	-		+	+	-	-	+
112	1	All	6	CTX-M 15 -217	CTX	3+	3+	-		+	+	-	-	+
113	1	All	6	CTX-M 15 -217	BZ/CTX	3+	3+	-		+	+	-	-	+
114	1	None	6	CTX-M 15 -217	BPW	3+	3+	3+		+	+	-	-	+
115	1	None	6	CTX-M 15 -217	CTX	3+	3+	3+		+	+	-	-	+
116	1	None	6	CTX-M 15 -217	BZ/CTX	3+	3+	3+		+	+	-	-	+
117	2	All	6	CTX-M 15 -217	BPW	3+	3+	-		+	+	-	-	+
118	2	All	6	CTX-M 15 -217	CTX	3+	3+	-		+	+	-	-	+
119	2	All	6	CTX-M 15 -217	BZ/CTX	3+	3+	2+		+	+	-	-	+
120	2	None	6	CTX-M 15 -217	BPW	3+	3+	3+		+	+	-	-	+
121	2	None	6	CTX-M 15 -217	CTX	3+	3+	3+		+	+	-	-	+
122	2	None	6	CTX-M 15 -217	BZ/CTX	3+	3+	3+		+	+	-	-	+
123	0	None	6	CTX-M 15 -217	BPW	-	-	-		-	-	-	-	+
124	0	None	6	CTX-M 15 -217	CTX	-	-	-		-	-	-	-	-
125	0	None	6	CTX-M 15 -217	BZ/CTX	-	-	-		-	-	-	-	-
126	3	All	6	CTX-M 15 -217	BPW	3+	3+	-			+	-	-	+
127	3	All	6	CTX-M 15 -217	CTX	3+	3+	-			+	-	-	+
128	3	All	6	CTX-M 15 -217	BZ/CTX	3+	3+	3+			+	-	-	+
129	3	None	6	CTX-M 15 -217	BPW	3+	3+	3+			+	-	-	+
130	3	None	6	CTX-M 15 -217	CTX	3+	3+	3+			+	-	-	+
131	3	None	6	CTX-M 15 -217	BZ/CTX	3+	3+	3+			+	-	-	+
132	1	All	6	CTX-M 15 -217	None	-	-	-						
133	1	None	6	CTX-M 15 -217	None	-	-	-						
134	2	All	6	CTX-M 15 -217	None	-	-	-						
135	2	None	6	CTX-M 15 -217	None	-	-	-						
136	0	None	6	CTX-M 15 -217	None	-	-	-						
137	3	All	6	CTX-M 15 -217	None	-	1+	1+						
138	3	None	6	CTX-M 15 -217	None	1+	1+	1+						
139	1	All	7	CTX-M 3-226	BPW	3+	3+	1+		+	+	-	-	+
140	1	All	7	CTX-M 3-226	CTX	3+	3+	1+		+	+	-	-	+
141	1	All	7	CTX-M 3-226	BZ/CTX	3+	3+	1+		+	+	-	-	+

142	1	None	7	CTX-M 3-226	BPW	3+	3+	3+		-	+	-	-	+
143	1	None	7	CTX-M 3-226	CTX	3+	3+	3+		+	+	-	-	+
144	1	None	7	CTX-M 3-226	BZ/CTX	3+	3+	3+		+	+	-	-	+
145	2	All	7	CTX-M 3-226	BPW	3+	3+	-		+	+	-	-	+
146	2	All	7	CTX-M 3-226	CTX	3+	3+	3+		+	+	-	-	+
147	2	All	7	CTX-M 3-226	BZ/CTX	3+	3+	3+		+	+	-	-	+
148	2	None	7	CTX-M 3-226	BPW	3+	3+	3+		+	+	-	-	+
149	2	None	7	CTX-M 3-226	CTX	3+	3+	3+		+	+	-	-	-
150	2	None	7	CTX-M 3-226	BZ/CTX	3+	3+	3+		+	+	-	-	-
151	0	None	7	CTX-M 3-226	BPW	-	-	1+		-	-	-	-	+
152	0	None	7	CTX-M 3-226	CTX	-	-	2+		-	-	-	-	+
153	0	None	7	CTX-M 3-226	BZ/CTX	-	-	-		-	-	-	-	-
154	3	All	7	CTX-M 3-226	BPW	3+	3+	3+			+	-	-	+
155	3	All	7	CTX-M 3-226	CTX	3+	3+	3+			+	-	-	+
156	3	All	7	CTX-M 3-226	BZ/CTX	3+	3+	3+			+	-	-	+
157	3	None	7	CTX-M 3-226	BPW	3+	3+	3+			+	-	-	+
158	3	None	7	CTX-M 3-226	CTX	3+	3+	3+			+	-	-	+
159	3	None	7	CTX-M 3-226	BZ/CTX	3+	3+	3+						
160	1	All	7	CTX-M 3-226	None	1+	-	-						
161	1	None	7	CTX-M 3-226	None	-	-	-						
162	2	All	7	CTX-M 3-226	None	1+	-	1+						
163	2	None	7	CTX-M 3-226	None	-	-	-						
164	0	None	7	CTX-M 3-226	None	-	-	-						
165	3	All	7	CTX-M 3-226	None	1+	1+	2+						
166	3	None	7	CTX-M 3-226	None	1+	1+	1+						
167	1	All	8	OXA-11-163	BPW	1+	-	-			-	+	-	+
168	1	All	8	OXA-11-163	CTX	1+	-	-			-	-	-	+
169	1	All	8	OXA-11-163	BZ/CTX	3+	2+	3+			-	+	-	+
170	1	None	8	OXA-11-163	BPW	2+	2+	-			-	-	-	-
171	1	None	8	OXA-11-163	CTX	3+	3+	-			-	-	-	-
172	1	None	8	OXA-11-163	BZ/CTX	3+	3+	3+			+	+	+	+
173	2	All	8	OXA-11-163	BPW	2+	2+	-			-	+	-	+
174	2	All	8	OXA-11-163	CTX	3+	2+	3+			-	-	-	+
175	2	All	8	OXA-11-163	BZ/CTX	3+	3+	3+			-	+	-	+

176	2	None	8	OXA-11-163	BPW	3+	3+	-			-	-	-	+
177	2	None	8	OXA-11-163	CTX	3+	3+	3+			-	-	-	+
178	2	None	8	OXA-11-163	BZ/CTX	3+	3+	-			+	-	-	+
179	0	None	8	OXA-11-163	BPW	-	-	1+			-	-	-	+
180	0	None	8	OXA-11-163	CTX	-	-	-			-	-	-	+
181	0	None	8	OXA-11-163	BZ/CTX	-	-	-			-	-	-	-
182	3	All	8	OXA-11-163	BPW	3+	3+	3+			-	+	-	+
183	3	All	8	OXA-11-163	CTX	3+	3+	-			-	-	-	+
184	3	All	8	OXA-11-163	BZ/CTX	3+	3+	3+			-	-	-	+
185	3	None	8	OXA-11-163	BPW	3+	3+	3+			-	-	-	-
186	3	None	8	OXA-11-163	CTX	3+	3+	3+			-	-	-	-
187	3	None	8	OXA-11-163	BZ/CTX	3+	3+	3+			-	-	-	-
188	1	All	8	OXA-11-163	None	-	-	-						
189	1	None	8	OXA-11-163	None	-	-	-						
190	2	All	8	OXA-11-163	None	1+	-	-						
191	2	None	8	OXA-11-163	None	1+	-	-						
192	0	None	8	OXA-11-163	None	-	-	-						
193	3	All	8	OXA-11-163	None	1+	-	-						
194	3	None	8	OXA-11-163	None	1+	1+	-						
227	1	All	10	CTX-M 2	BPW	3+	1+	-			+	+		+
228	1	All	10	CTX-M 2	CTX	3+	-	3+			+	-	-	+
229	1	All	10	CTX-M 2	BZ/CTX	3+	-	3+			+	+	-	+
230	1	None	10	CTX-M 2	BPW	3+	3+	3+			+	-	-	+
231	1	None	10	CTX-M 2	CTX	3+	3+	3+			+	-	-	+
232	1	None	10	CTX-M 2	BZ/CTX	3+	3+	3+			+	-	-	+
233	2	All	10	CTX-M 2	BPW	3+	-	-			+	+	-	+
234	2	All	10	CTX-M 2	CTX	3+	-	3+			+	-	-	+
235	2	All	10	CTX-M 2	BZ/CTX	ND	1+	3+			+	?	-	+
236	2	None	10	CTX-M 2	BPW	3+	3+	3+			+	-	-	+
237	2	None	10	CTX-M 2	CTX	3+	3+	2+			+	-	-	+
238	2	None	10	CTX-M 2	BZ/CTX	3+	3+	3+			?	-	-	+
239	3	All	10	CTX-M 2	BPW	3+	-	3+			?	+	-	+
240	3	All	10	CTX-M 2	CTX	3+	1+	-			+	-	-	+
241	3	All	10	CTX-M 2	BZ/CTX	3+	-	3+			+	-	-	+

242	3	None	10	CTX-M 2	BPW	3+	3+	3+			+	-	-	+
243	3	None	10	CTX-M 2	CTX	3+	3+	3+			+	-	-	+
244	3	None	10	CTX-M 2	BZ/CTX	3+	3+	3+			+	-	-	+
245	0	None	10	CTX-M 2	BPW	-	-	-			-	-	-	+
246	0	None	10	CTX-M 2	CTX	-	-	-			-	-	-	-
247	0	None	10	CTX-M 2	BZ/CTX	-	-	-			-	-	-	-
248	1	All	10	CTX-M 2	None	-	-	-						
249	1	None	10	CTX-M 2	None	-	+	?						
250	2	All	10	CTX-M 2	None	1+	-	-						
251	2	None	10	CTX-M 2	None	-	-	-						
252	3	All	10	CTX-M 2	None	1+	-	-						
253	3	None	10	CTX-M 2	None	1+	1+	1+						
254	0	None	10	CTX-M 2	None	-	-	-						
255	1	All	11	TEM 52	BPW	2+	1+	-			+	-	-	+
256	1	All	11	TEM 52	CTX	3+	3+	-			+	-	-	+
257	1	All	11	TEM 52	BZ/CTX	3+	3+	-			+	-	-	+
258	1	None	11	TEM 52	BPW	3+	3+	3+			-	-	-	+
259	1	None	11	TEM 52	CTX	3+	3+	3+			-	-	-	+
260	1	None	11	TEM 52	BZ/CTX	3+	3+	3+			-	-	-	+
261	2	All	11	TEM 52	BPW	3+	3+	-			+	-	-	+
262	2	All	11	TEM 52	CTX	3+	3+	-			+	-	-	+
263	2	All	11	TEM 52	BZ/CTX	3+	3+	-			+	-	-	+
264	2	None	11	TEM 52	BPW	3+	3+	3+			-	-	-	+
265	2	None	11	TEM 52	CTX	3+	3+	3+			-	-	-	+
266	2	None	11	TEM 52	BZ/CTX	3+	3+	3+			-	-	-	+
267	3	All	11	TEM 52	BPW	3+	3+	-			+	-	-	+
268	3	All	11	TEM 52	CTX	3+	3+	-			+	-	-	+
269	3	All	11	TEM 52	BZ/CTX	3+	3+	-			+	-	-	+
270	3	None	11	TEM 52	BPW	3+	3+	3+			-	-	-	+
271	3	None	11	TEM 52	CTX	3+	3+	3+			-	-	-	+
272	3	None	11	TEM 52	BZ/CTX	3+	3+	3+			-	-	-	+
273	0	None	11	TEM 52	BPW	-	-	-			-	-	-	+
274	0	None	11	TEM 52	CTX	-	-	2+			-	-	-	+
275	0	None	11	TEM 52	BZ/CTX	-	-	3+			-	-	-	+

276	1	All	11	TEM 52	None	-	-	-						
277	1	None	11	TEM 52	None	-	-	-						
278	2	All	11	TEM 52	None	-	-	-						
279	2	None	11	TEM 52	None	-	-	-						
280	3	All	11	TEM 52	None	-	-	-						
281	3	None	11	TEM 52	None	-	1+	-						
282	0	None	11	TEM 52	None	-	-	-						
370	1	All	15	S4327-11	BPW	2+	2+	-	2+		+	+	-	+
371	1	All	15	S4327-11	CTX	1+	2+	-	2+		+	-	-	+
372	1	All	15	S4327-11	BZ/CTX	2+	2+	3+	1+		+	+	-	+
373	1	None	15	S4327-11	BPW	3+	3+	3+	2+		+	-	-	+
374	1	None	15	S4327-11	CTX	3+	3+	3+	3+		+	-	-	+
375	1	None	15	S4327-11	BZ/CTX	3+	3+	3+	3+		+	-	-	+
376	2	All	15	S4327-11	BPW	2+	2+	-	2+		+	+	-	+
377	2	All	15	S4327-11	CTX	3+	3+	-	2+		+	-	-	+
378	2	All	15	S4327-11	BZ/CTX	2+	3+	3+	2+		+	+	-	+
379	2	None	15	S4327-11	BPW	3+	3+	3+	3+		+	-	-	+
380	2	None	15	S4327-11	CTX	3+	3+	3+	2+		+	-	-	+
381	2	None	15	S4327-11	BZ/CTX	3+	2+	3+	3+		+	-	-	+
382	3	All	15	S4327-11	BPW	2+	3+	-	2+		+	+	-	+
383	3	All	15	S4327-11	CTX	3+	2+	-	2+		+	-	-	+
384	3	All	15	S4327-11	BZ/CTX	3+	3+	3+	2+		+	+	-	+
385	3	None	15	S4327-11	BPW	3+	3+	3+	3+		+	-	-	+
386	3	None	15	S4327-11	CTX	3+	3+	3+	3+		+	-	-	+
387	3	None	15	S4327-11	BZ/CTX	3+	3+	3+	3+		+	-	-	+
388	0	All	15	S4327-11	BPW	-	-	-	-		+	+	-	+
389	0	All	15	S4327-11	CTX	-	-	-	-		-	-	-	+
390	0	All	15	S4327-11	BZ/CTX	-	-	2+	-		+	+	-	+
391	0	All	15	S4327-11	None	-	-	-	-					
392	0	All	15	S4327-11	None	?	?	?	?					
393	1	All	15	S4327-11	None	-	-	-	-					
394	1	None	15	S4327-11	None	-	-	-	1+					
395	2	All	15	S4327-11	None	1+	-	-	-					
396	2	None	15	S4327-11	None	-	1+	1+	-					

397	3	All	15	S4327-11	None	1+	1+	-	2+					
398	3	None	15	S4327-11	None	1+	1+	1+	1+					
399	0	None	15	S4327-11	BPW	-	-	ND	-					
400	1	All	16	S4330-11	BPW	3+	2+	-	2+		+	+	-	+
401	1	All	16	S4330-11	CTX	2+	2+	-	2+		+	-	-	+
402	1	All	16	S4330-11	BZ/CTX	3+	2+	3+	2+		+	+	-	+
403	1	None	16	S4330-11	BPW	3+	3+	3+	3+		+	-	-	+
404	1	None	16	S4330-11	CTX	3+	3+	3+	3+		+	-	-	-
405	1	None	16	S4330-11	BZ/CTX	3+	3+	3+	3+		+	-	-	-
406	2	All	16	S4330-11	BPW	3+	2+	-	2+		+	+	-	+
407	2	All	16	S4330-11	CTX	2+	2+	-	2+		+	-	-	+
408	2	All	16	S4330-11	BZ/CTX	2+	2+	2+	2+		+	+	-	+
409	2	None	16	S4330-11	BPW	3+	3+	3+	3+		+	-	-	+
410	2	None	16	S4330-11	CTX	2+	2+	-	3+		+	-	-	-
411	2	None	16	S4330-11	BZ/CTX	3+	3+	2+	3+		+	-	-	-
412	3	All	16	S4330-11	BPW	3+	2+	-	2+		+	+	-	+
413	3	All	16	S4330-11	CTX	2+	2+	-	3+		+	-	-	+
414	3	All	16	S4330-11	BZ/CTX	2+	2+	-	2+		+	-	-	+
415	3	None	16	S4330-11	BPW	3+	2+	2+	3+		+	-	-	+
416	3	None	16	S4330-11	CTX	3+	3+	2+	3+		+	-	-	-
417	3	None	16	S4330-11	BZ/CTX	3+	3+	3+	3+		+	-	-	-
418	0	None	16	S4330-11	BPW	-	-	2+	-		-	-	-	+
419	0	None	16	S4330-11	CTX	-	-	-	-		-	-	-	-
420	0	None	16	S4330-11	BZ/CTX	-	-	-	-		-	-	-	-
421	0	None	16	S4330-11	None	-	-	-	-		?	?	?	?
422	0	All	16	S4330-11	None	-	-	-	-					
423	1	None	16	S4330-11	None	-	-	-	-					
424	1	All	16	S4330-11	None	-	-	-	1+					
425	2	None	16	S4330-11	None	-	-	-	1+					
426	2	All	16	S4330-11	None	-	1+	-	1+					
427	3	None	16	S4330-11	None	1+	1+	1+	1+					
428	3	All	16	S4330-11	None	1+	1+	-	2+					
429	0	All	16	S4330-11	BPW	-	-	-	-		-	+	-	+
430	0	All	16	S4330-11	CTX	-	-	-	-		-	-	-	+

431	0	All	16	S4330-11	BZ/CTX	-	-	3+	-		-	+	-	+
432	1	All	17	SHV-2 LREC151	BPW	3+	3+	1+			-	+	+	+
433	1	All	17	SHV-2 LREC151	CTX	3+	3+	1+			-	-	+	+
434	1	All	17	SHV-2 LREC151	BZ/CTX	3+	3+	2+			+	-	+	+
435	1	None	17	SHV-2 LREC151	BPW	3+	3+	1+			-	-	+	+
436	1	None	17	SHV-2 LREC151	CTX	2+	2+	-			+	-	+	+
437	1	None	17	SHV-2 LREC151	BZ/CTX	3+	3+	1+			+	-	+	+
438	2	All	17	SHV-2 LREC151	BPW	3+	3+	1+			-	+	+	+
439	2	All	17	SHV-2 LREC151	CTX	3+	3+	-			-	-	+	+
440	2	All	17	SHV-2 LREC151	BZ/CTX	3+	3+	3+			+	-	+	+
441	2	None	17	SHV-2 LREC151	BPW	3+	3+	1+			+	-	+	+
442	2	None	17	SHV-2 LREC151	CTX	3+	3+	1+			+	-	+	+
443	2	None	17	SHV-2 LREC151	BZ/CTX	3+	3+	1+			-	-	+	+
444	3	All	17	SHV-2 LREC151	BPW	3+	3+	-			-	+	+	+
445	3	All	17	SHV-2 LREC151	CTX	3+	3+	1+			-	-	+	+
446	3	All	17	SHV-2 LREC151	BZ/CTX	3+	3+	3+			-	-	+	+
447	3	None	17	SHV-2 LREC151	BPW	3+	3+	-			-	-	+	+
448	3	None	17	SHV-2 LREC151	CTX	3+	3+	3+			-	-	+	+
449	3	None	17	SHV-2 LREC151	BZ/CTX	3+	3+	1+			-	-	+	-
450	0	All	17	SHV-2 LREC151	BPW	-	-	3+			-	+	-	+
451	0	All	17	SHV-2 LREC151	CTX	-	?	-			-	-	-	+
452	0	All	17	SHV-2 LREC151	BZ/CTX	-	?	-			-	-	-	+
453	0	None	17	SHV-2 LREC151	BPW	-	?	-			+	-	-	+
454	0	None	17	SHV-2 LREC151	CTX	-	-	-			-	-	-	-
455	0	None	17	SHV-2 LREC151	BZ/CTX	-	-	-			-	-	-	-
456	0	All	17	SHV-2 LREC151	None	-	-	1+						
457	0	None	17	SHV-2 LREC151	None	-	-	-						
458	1	All	17	SHV-2 LREC151	None	-	-	1+						
459	1	None	17	SHV-2 LREC151	None	-	-	-						
460	2	All	17	SHV-2 LREC151	None	-	1+	1+						
461	2	None	17	SHV-2 LREC151	None	-	-	-						
462	3	All	17	SHV-2 LREC151	None	1+	1+	1+						
463	3	None	17	SHV-2 LREC151	None	-	1+	1+						

BPW – Buffered Peptone water; CTX – BPW + cefotaxime (CTX); BZ/CTX – BPW + CTX and benzo[b]thiophene-2-boronic acid (BZ)1 = 1 to 1 0 colonies; 2 = 11 to 100 colonies; 3 = > 100 colonies.

Appendix III – Table 2. Non chicken meat samples spiked with different ESBLs strains, results on agars and by multiplex PCR

Broth ID	Meat	Spike cfu ESBL / gram	Added competitors	Spike organism used	Enrichment method used	Presumptive ESBLs on different agars			Results with multi-plex PCR			
						CTX (ESBL)	Brill (ESBL)	McC (ESBL)	CTX-PCR	OXA-PCR	SHV-PCR	TEM-PCR
283	Beef	0	Natural	None	BPW	-	-	-	-	-	-	+
284	Beef	1	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
285	Beef	2	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
286	Beef	1	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
287	Beef	2	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
288	Beef	1	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
289	Beef	2	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
290	Mince beef	0	Natural	None	BPW	-	1+	-	-	-	-	+
291	Mince beef	1	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
292	Mince beef	2	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
293	Mince beef	1	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
294	Mince beef	2	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
295	Mince beef	1	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
296	Mince beef	2	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
297	Mince lamb	0	Natural	None	BPW	-	-	-	-	-	-	+
298	Mince lamb	1	Natural	CTX-M 1 - 245	BPW	2+	3+	1+	+	-	-	+
299	Mince lamb	2	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
300	Mince lamb	1	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
301	Mince lamb	2	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
302	Mince lamb	1	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
303	Mince lamb	2	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
304	Pork	0	Natural	None	BPW	-	-	-	-	-	-	+
305	Pork	1	Natural	CTX-M 1 - 245	BPW	2+	3+	3+	+	-	-	+

306	Pork	2	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
308	Pork	1	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
309	Pork	2	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
310	Pork	1	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
311	Pork	2	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
312	Turkey	0	Natural	None	BPW	-	-	-	-	-	-	+
313	Turkey	1	Natural	CTX-M 1 - 245	BPW	3+	3+	3+	+	-	-	+
314	Turkey	2	Natural	CTX-M 1 - 245	BPW	3+	3+	2+	+	-	-	+
315	Turkey	1	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
316	Turkey	2	Natural	CTX-M 2	BPW	3+	3+	3+	+	-	-	+
317	Turkey	1	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+
318	Turkey	2	Natural	CTX-M 14 -261	BPW	3+	3+	3+	+	-	-	+

BPW – Buffered Peptone water; CTX – BPW + cefotaxime (CTX); BZ/CTX – BPW + CTX and benzo[b]thiophene-2-boronic acid (BZ)

1 = 1 to 1 0 colonies; 2 = 11 to 100 colonies; 3 = > 100 colonies.

21. **Appendix IV –** Details of industrial seminar help at Leatherhead Foods research

Appendix IV - Objective 3. Task 3.1 – Material for industry seminar

Programme for industry seminar

Antimicrobial Resistance: The Detection of Extended Spectrum Beta Lactamases (ESBLs) of Enterobacteriaceae in Food

Wednesday 16th May 2012

Leatherhead Food Research, Leatherhead, Surrey

Lecture Hall 1

10.30 **Welcome** - John Haines, LFR

10.45 **ESBLs in farm animals and food** - Christopher Teale, APHA

11.15 **ESBLs – the medical perspective** - Neil Woodford, HPA

11.45 **ESBLs - relevance to the UK food industry and consumer** - Paul Cook, FSA

12.15 Lunch

12.45 **New screening protocols for the detection of ESBLs in foods**

12.45 **Phenotypic methods** - Luke Randall, APHA

13.15 **Genotypic methods** - Muna Anjum, APHA

13.45 **Gene array** - Daniele Meunier, HPA

14.15 **Open discussion on relevance of ESBL to UK industry and consumer, including requirements for new screening methods** - Jeremy Hall, Bernard Matthews

15.00 Close

Publicity material for industry seminar

Antimicrobial Resistance – The Detection of Extended Spectrum Beta Lactamases (ESBLs) of *Enterobacteriaceae* in Food

16 May 2012

Mini Description

Antibiotic-resistant bacteria in intensively-reared farm animals have the potential to spread to humans. Learn its relevance to the UK food industry and consumers, and how current research will enable improved surveillance and enforcement strategies.

Description

Antibiotic-resistant bacteria in intensively-reared farm animals have the potential to spread to humans. Attend this seminar and learn more about current understanding of antimicrobial resistance in farm animals and the impact on the food chain, hear about current work to develop new methods for detection of ESBL resistance, and get an opportunity to input your views and help steer technology development.

It has been reported that ESBL resistance is now present in more than 30% of dairy farms and an estimated 50% of poultry companies in Britain. There is an increasing awareness and demand for tighter national and international controls on the use of antibiotics classified by the WHO as critically important in human medicine. Without action, there is the potential for the loss of antimicrobial therapy in animal welfare, and subsequently, human medicine.

Leatherhead's new seminar, brought to you in partnership with the Veterinary Laboratories Agency, Health Protection Agency and Food Standards Agency, will disseminate current understanding on the relevance of ESBL-resistance to the UK food industry and consumers, with particular emphasis on the development of new reliable methods of detection for use in surveillance and enforcement.

The seminar is free of charge to attend.

Who should attend?

- National enforcement agencies
- Public Health Authorities
- Food testing laboratories
- Abattoir operators
- Importers/exporters of livestock, meat and poultry
- Food manufacturers, distributors and suppliers of meat and poultry products
- Trade associations with links to animal farming

From this course you will

- Learn more about the current understanding of antimicrobial resistance in farm animals and its impact on the food chain
- Gain an insight into the medical perspective
- Hear about current work to develop new methods for detection of ESBL resistance
- Get an opportunity to input your views and help steer technology development

Delegate list for industry seminar

Name	Company
Evangelia Komitopoulou	Leatherhead Food Research
John Haines	Leatherhead Food Research
Christopher Teale	Animal and Plant Health agency (APHA)
Nick Coldham	Animal and Plant Health agency (APHA)
Luke Randall	Animal and Plant Health agency (APHA)
Muna Anjum	Animal and Plant Health agency (APHA)
Neil Woodford	Health Protection Agency
Daniele Meunier	Health Protection Agency
Jeremy Hall	Bernard Matthews
Paul Cook	Food Standards Agency
Robert H Davies	Animal and Plant Health agency (APHA)
Felicity Clifton-Hadley	Animal and Plant Health agency (APHA)
Peter Silley	MB Consult Limited
Andrew Fox	HPA Microbiology Services Preston
Kristian Hoel	ANIMALIA
Alessandra Carattoli	Istituto Superiore di Sanità (Italy)
Fraser J Gormley	Aviagen Ltd
Liam Herbert	Aviagen Ltd
Catherine Cockcroft	Exova
Graham Hannant	Invicta Food Group
Alison Cox	Diversey - part of Sealed Air
Clare Bridges	SVA Ltd
Robert Morley	Health Protection Agency
Chris Hudson	Newtec Laboratories Limited
Deborah Charles	Public Health Wales - Health Protection
Pat Higgins	IFF
Ka Yi Chan	Smiths Detection Diagnostics
Karen Sims	Waitrose
Yvonne Stedman	Sainsbury's
Roberto Marcello La Ragione	University of Surrey
Richard Cooper	EBVC
Nicole Batey	Veterinary Medicines Directorate
Athanasios Dolias	FUDCO
André Steentjes	Veterinair Centrum Someren (Veterinary Practice and Laboratory)
Dr Manal AbuOun	Animal and Plant Health agency
Matthew Stokes	Animal and Plant Health agency
Laura Holding	HPA
Kirsti Sandberg	ANIMALIA
Kara Thomas	FSA

Comparative costs of analytical methods

Summary of method time / cost per sample

Method	Time (h)		Costs (£)	
	TTR	**Staff hours**	**Equipment**	**Per test**
Phenotypic	48	1 sample-10 min 10 samples-2 h	2,798 stomacher	3.80
LAMP	4	1 sample-1 h 10 samples-2 h	19,500 RTPCR	4.00
ARRAY	9	5	8,395 array reader, thermocycler,mixer	84.00

Selection based on:

- Analytical performance
- TTR is from provision of an extract that has been enriched in BPW overnight.
- Cost
- Ease of use

22. **Appendix V –** Test Validation Study - Presence of ESBL-producing bacteria in 300 abattoir chicken neck flaps and caecal contents by agar, CTX-M PCR and CTX-M LAMP

Sample No.	Source	CHROMagar CTX	PCR CTX	LAMP CTX all
31	Caecal - C	-	ND	-
32	Caecal - C	+	+	+
33	Caecal - C	+	+	+
34	Caecal - C	+	+	+
35	Neck - F	+	+	+
36	Caecal - C	+	+	+
37	Neck - F	+	-	+
38	Caecal - C	-	ND	-
39	Neck - F	-	ND	-
40	Caecal - C	+	+	+
41	Neck - F	+	+	+
42	Caecal - C	-	ND	-
43	Neck - F	+	+	+
44	Caecal - C	-	ND	-
45	Neck - F	+	+	-
46	Caecal - C	-	ND	-
47	Neck - F	-	ND	-
48	Caecal - C	-	ND	-
49	Neck - F	-	ND	-
50	Caecal - C	-	ND	+
51	Neck - F	+	+	+
54	Neck - F	-	ND	-
55	Neck - F	-	ND	-
56	Neck - F	-	ND	-
57	Caecal - C	+	+	-
58	Neck - F	+	-	+
59	Caecal - C	-	ND	-
60	Neck - F	+	+	-
61	Caecal - C	-	ND	-
62	Neck - F	+	+	+
63	Caecal - C	-	ND	-
64	Neck - F	-	ND	-
65	Caecal - C	-	ND	-
66	Neck - F	+	-	+
67	Caecal - C	-	ND	-
68	Neck - F	+	+	+
69	Caecal - C	+	+	+
70	Neck - F	+	-	+
71	Caecal - C	-	ND	-
72	Neck - F	+	+	+
73	Caecal - C	-	ND	-
74	Neck - F	+	-	+
75	Caecal - C	-	ND	-
76	Neck - F	+	+	+
77	Caecal - C	+	+	+
78	Neck - F	+	-	+
79	Caecal - C	-	ND	-

80	Neck - F	+	+	+
81	Caecal - C	+	+	+
82	Neck - F	+	+	+
83	Caecal - C	+	+	+
84	Neck - F	+	+	+
85	Caecal - C	+	-	+
86	Neck - F	+	+	+
89	Caecal - C	-	ND	-
90	Neck - F	-	ND	-
91	Caecal - C	+	+	+
92	Neck - F	+	-	+
93	Caecal - C	-	ND	-
94	Neck - F	-	ND	-
95	Caecal - C	-	ND	-
96	Neck - F	+	-	+
97	Caecal - C	-	ND	-
98	Neck - F	-	ND	+
99	Caecal - C	-	ND	-
100	Neck - F	-	ND	-
102	Neck - F	+	+	+
105	Caecal - C	-	ND	-
106	Neck - F	-	ND	-
107	Caecal - C	-	ND	-
108	Neck - F	-	ND	-
109	Caecal - C	-	ND	-
110	Neck - F	-	ND	-
111	Caecal - C	-	ND	-
112	Neck - F	-	ND	-
113	Caecal - C	-	ND	-
114	Neck - F	-	ND	-
115	Caecal - C	-	ND	-
116	Neck - F	+	-	+
117	Caecal - C	-	ND	-
118	Neck - F	-	ND	-
119	Caecal - C	-	ND	-
120	Neck - F	+	-	+
121	Caecal - C	-	ND	-
122	Neck - F	-	ND	+
124	Neck - F	-	ND	+
125	Caecal - C	-	ND	-
126	Neck - F	+	+	+
127	Caecal - C	-	ND	-
128	Neck - F	-	ND	-
129	Caecal - C	-	ND	-
130	Neck - F	+	+	+
131	Caecal - C	+	+	+
132	Neck - F	+	+	+
133	Caecal - C	-	ND	-
134	Neck - F	-	ND	-
135	Caecal - C	-	ND	-
136	Neck - F	-	ND	-
137	Caecal - C	-	ND	-
138	Neck - F	-	ND	-
139	Caecal - C	-	ND	-
140	Neck - F	-	ND	-

141	Caecal - C	-	ND	-
142	Neck - F	-	ND	-
143	Caecal - C	+	+	+
144	Neck - F	+	+	+
145	Caecal - C	+	+	+
146	Neck - F	+	+	+
147	Caecal - C	-	ND	-
148	Neck - F	-	ND	+
149	Caecal - C	+	+	+
150	Neck - F	+	+	+
155	Caecal - C	-	ND	-
156	Neck - F	+	-	+
157	Caecal - C	-	ND	-
158	Neck - F	-	ND	-
159	Caecal - C	+	+	+
160	Neck - F	-	ND	-
161	Caecal - C	-	ND	-
162	Neck - F	+	+	+
163	Caecal - C	-	ND	-
164	Neck - F	+	-	+
165	Caecal - C	+	+	+
166	Neck - F	+	+	+
167	Caecal - C	-	ND	+
168	Neck - F	+	-	+
169	Caecal - C	+	+	+
170	Neck - F	+	+	+
171	Caecal - C	+	+	-
172	Neck - F	+	+	+
173	Caecal - C	-	ND	-
174	Neck - F	+	-	+
175	Caecal - C	-	ND	-
176	Neck - F	+	+	-
177	Caecal - C	-	ND	-
178	Neck - F	+	-	+
179	Caecal - C	-	ND	-
180	Neck - F	-	ND	-
181	Caecal - C	-	ND	-
182	Neck - F	+	-	+
183	Caecal - C	-	ND	-
184	Neck - F	+	-	+
185	Caecal - C	+	-	+
186	Neck - F	+	+	+
187	Caecal - C	-	ND	-
188	Neck - F	+	+	+
189	Caecal - C	+	+	+
190	Neck - F	+	-	+
193	Caecal - C	-	ND	-
194	Neck - F	+	+	+
195	Caecal - C	-	ND	-
196	Neck - F	-	ND	-
197	Caecal - C	+	+	+
198	Neck - F	+	+	+
199	Caecal - C	+	+	+
200	Neck - F	+	+	+
201	Caecal - C	+	+	+

202	Neck - F	+	-	+
203	Caecal - C	-	ND	-
204	Neck - F	+	-	+
205	Caecal - C	-	ND	-
206	Neck - F	+	-	+
207	Caecal - C	-	ND	-
208	Neck - F	+	-	+
209	Caecal - C	-	ND	-
210	Neck - F	+	+	+
211	Caecal - C	-	ND	-
212	Neck - F	-	ND	-
213	Caecal - C	-	ND	-
214	Neck - F	+	+	+
215	Caecal - C	-	ND	-
216	Neck - F	-	ND	-
217	Caecal - C	-	ND	-
218	Neck - F	+	-	+
219	Caecal - C	-	ND	+
220	Neck - F	-	ND	-
221	Caecal - C	-	ND	-
222	Neck - F	+	-	+
223	Caecal - C	-	ND	
224	Neck - F	+	+	+
225	Caecal - C	-	ND	-
226	Neck - F	+	+	+
227	Caecal - C	+	ND	+
228	Neck - F	+	-	+
229	Caecal - C	-	ND	-
230	Neck - F	+	+	+
231	Caecal - C	-	ND	+
232	Neck - F	+	+	+
233	Caecal - C	-	ND	-
234	Neck - F	-	ND	-
235	Caecal - C	+	+	-
236	Neck - F	+	+	-
237	Caecal - C	-	ND	-
238	Neck - F	-	ND	-
239	Caecal - C	-	ND	-
240	Neck - F	+	+	+
241	Caecal - C	-	ND	-
242	Neck - F	+	ND	+
243	Caecal - C	-	ND	-
244	Neck - F	+	-	+
245	Caecal - C	-	ND	-
246	Neck - F	+	+	+
247	Caecal - C	-	ND	-
248	Neck - F	-	ND	-
249	Caecal - C	-	ND	-
250	Neck - F	-	ND	+
251	Caecal - C	-	ND	+
252	Neck - F	+	+	+
253	Caecal - C	-	ND	+
254	Neck - F	+	+	-
255	Caecal - C	-	ND	-
256	Neck - F	+	+	+

257	Caecal - C	-	ND	-
258	Neck - F	+	ND	+
259	Caecal - C	-	ND	-
260	Neck - F	+	+	+
261	Caecal - C	-	ND	+
262	Neck - F	-	ND	-
263	Caecal - C	-	ND	-
264	Neck - F	-	ND	+
265	Caecal - C	-	ND	+
266	Neck - F	+	+	+
267	Caecal - C	-	ND	-
268	Neck - F	+	-	+
269	Caecal - C	-	ND	-
270	Neck - F	+	+	+
271	Caecal - C	-	ND	-
272	Neck - F	-	ND	-
273	Caecal - C	-	ND	-
274	Neck - F	-	ND	+
275	Caecal - C	-	ND	-
276	Neck - F	-	ND	-
277	Caecal - C	-	ND	-
278	Neck - F	-	ND	-
279	Caecal - C	-	ND	+
280	Neck - F	-	ND	-
281	Caecal - C	-	ND	-
282	Neck - F	+	+	+
283	Caecal - C	+	+	+
284	Neck - F	+	+	+
285	Caecal - C	-	ND	-
286	Neck - F	+	+	+
287	Neck - F	+	ND	+
288	Neck - F	+	-	+
289	Caecal - C	+	+	-
290	Neck - F	-	ND	-
291	Caecal - C	+	+	+
292	Neck - F	+	-	+
293	Caecal - C	-	ND	-
294	Neck - F	-	ND	-
295	Caecal - C	-	ND	-
296	Neck - F	-	ND	-
297	Caecal - C	+	+	-
298	Neck - F	-	ND	-
299	Caecal - C	-	ND	-
300	Neck - F	-	ND	+
301	Caecal - C	-	ND	-
302	Neck - F	-	ND	-
303	Caecal - C	-	ND	-
304	Neck - F	-	ND	-
305	Caecal - C	-	ND	-
306	Neck - F	-	ND	+
307	Caecal - C	-	ND	-
308	Neck - F	-	ND	+
309	Caecal - C	-	ND	-
310	Neck - F	-	ND	+
311	Caecal - C	-	ND	-

312	Neck - F	+	+	+
313	Caecal - C	+	+	+
314	Neck - F	+	+	+
315	Caecal - C	-	ND	-
316	Neck - F	+	+	+
317	Caecal - C	+	+	+
318	Neck - F	+	ND	+
319	Caecal - C	-	ND	-
320	Neck - F	+	+	-
321	Caecal - C	+	+	+
322	Neck - F	+	ND	+
323	Caecal - C	+	+	+
324	Neck - F	+	+	+
325	Caecal - C	-	ND	-
326	Neck - F	-	ND	-
327	Caecal - C	-	ND	-
328	Neck - F	+	+	+
329	Caecal - C	+	+	+
330	Neck - F	+	+	+
331	Caecal - C	-	ND	-
332	Neck - F	+	+	+
333	Caecal - C	-	ND	-
334	Neck - F	+	+	+
335	Caecal - C	-	ND	-
336	Neck - F	-	ND	-
337	Caecal - C	-	ND	-
338	Neck - F	+	+	+
339	Caecal - C	-	ND	-
340	Neck - F	+	+	+
341	Caecal - C	-	ND	-
342	Neck - F	-	ND	-
343	Caecal - C	-	ND	-
344	Neck - F	-	ND	-

23. **Appendix VI-** Development and initial validation of a loop-mediated isothermal amplification (LAMP) assay for the detection of blaOXA-48

Summary

- A LAMP assay to detect the carbapenemase *bla*$_{OXA-48}$ was developed.
- Positive results were obtained within 16 minutes.
- The assay had 100% sensitivity and 97% specificity for a panel of 44 reference strains (one false positive result was obtained).
- The assay had an analytical sensitivity of 750 gene copies.
- Further validation of the assay with field samples will be required.

Aim

Develop a LAMP assay for the detection of blaOXA-48, a carbapenem-hydrolysing class D β-lactamase reported in enterobacterial species.

The blaOXA48 gene family

To date 11 variants of blaOXA-48 have been described, summarised in Table 1. The chromosome-encoded blaOXA-54 of *Shewanella oneidensis* has been proposed as the origin of blaOXA-48 but these genes have only 84% nucleotide identity 24, and therefore blaOXA-54 has not been included in Table 1.

Appendix VI - Table 1. *bla*$_{OXA-48}$ variants.

Gene	Acc	Position	Author	References
bla$_{OXA-48}$	AY236073	2188..2985	Poirel et al. 2004	Antimicrob. Agents Chemother. 48 (1), 15-22 (2004)
bla$_{OXA-48b}$	JX644945	3780..4577	Zong, Z	PLoS ONE 7 (10), E48280 (2012)
bla$_{OXA-162}$	GU197550	1..798	Unpublished	Direct Submission
bla$_{OXA-163}$	HQ700343	1..786	Poirel et al. 2011	Antimicrob. Agents Chemother. 55 (6), 2546-2551 (2011)
bla$_{OXA-181}$	HM992946	1..798	Castanheira et al.	Antimicrob Agents Chemother. 55(3):1274-8 (2011)
bla$_{OXA-199}$	JN704570	4039..4836	Zong, Z.	PLoS ONE 7 (10), E48280 (2012)
bla$_{OXA-204}$	JQ809466	5375..6172	Potron et al. 2013	Antimicrob. Agents Chemother. 57 (1), 633-636 (2013)
bla$_{OXA-232}$	JX423831	2677..3474	Potron et al. 2013	Int. J. Antimicrob. Agents 41 (4), 325-329 (2013)
bla$_{OXA-244}$	JX438000	1..798	Oteo et al. 2013	J. Antimicrob. Chemother. 68 (2), 317-321 (2013)
bla$_{OXA-245}$	JX438001	1..798	Oteo et al. 2013	J. Antimicrob. Chemother. 68 (2), 317-321 (2013)
bla$_{OXA-247}$	JX893517	1..786	Gomez et al. 2013	Clin. Microbiol. Infect. 19 (5), E233-E235 (2013)

Design of LAMP assay primers

The nucleotide sequence (coding region) of each gene listed in Table 1 was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) into EditSeq (DNAStar). The sequences were aligned in MegAlign (DNAStar) using ClustalV and the consensus sequence saved as a FASTA file.

The consensus FASTA file was used as input into Primer Explorer 4 (Eiken Chemical Company Limited, Japan) for LAMP primer design (http://primerexplorer.jp/elamp4.0.0/index.html). Many LAMP primer sets were designed. Three sets were selected based on the criteria set out in the Primer Explorer 4 manual (A Guide to LAMP primer designing No.1, No.2, and No.3; http://primerexplorer.jp/e/v4_manual/index.html). Loop primers were then designed for each LAMP primer set using Primer Explorer 4, and two pairs of Loop primers selected for each set based on criteria in the Primer Explorer 4 manual. The final selection of primers is given in Table 2.

Appendix VI - Table 2. LAMP primer sets selected for detection of *bla*$_{\text{OXA-48}}$ group genes.

LAMP Set	Primer Name	Primer Sequence (5'-3')
Set 1	OXA48-Set1-F3	GCGTGGTTAAGGATGAACA
	OXA48-Set1-B3	CCTCATTACCATAATCGAAAGC
	OXA48-Set1-FIP	TGATTAGATTATGATCGCGATTCCA-CCAAGTCTTTAAGTGGGATG
	OXA48-Set1-BIP	CCGCGATGAAATATTCAGTTGTG-ATGTAGCATCTTGCTCATACG
	OXA48-Set1-LF1	GCGATATCGCGCGTCTGT
	OXA48-Set1-LB1	ATCAAGAATTTGCCCGCCAAATTG
	OXA48-Set1-LF2	GGCGATATCGCGCGTCTGT
	OXA48-Set1-LB2	GAATTTGCCCGCCAAATTGG
Set 3	OXA48-Set3-F3	TCGATTATGGTAATGAGGACAT
	OXA48-Set3-B3	GCCCGAATAATATAGTCGCC
	OXA48-Set3-FIP	TTCTTAAAAAGCTGATTTGCTCCGT-CGGGCAATGTAGACAGTT
	OXA48-Set3-BIP	AGCTGTATCACAATAAGTTACACGT-CAGCATGGCTTGTTTGAC
	OXA48-Set3-LF1	CCGAAATTCGAATACCACCGTCGA
	OXA48-Set3-LB1	GGAGCGCAGCCAGCGTATT
	OXA48-Set3-LF2	GCCGAAATTCGAATACCACCG
	OXA48-Set3-LB2	GAGCGCAGCCAGCGTAT
Set 4	OXA48-Set4-F3	AATAGCTTGATCGCCCTC
	OXA48-Set4-B3	CCATAATCGAAAGCATGTAGC
	OXA48-Set4-FIP	GATTCCAAGTGGCGATATCGC-GGCGTGGTTAAGGATGAAC
	OXA48-Set4-BIP	TAATCACCGCGATGAAATATTCAGT-CTTGCTCATACGTGCCTC
	OXA48-Set4-LF1	TCTGTCCATCCCACTTAAAGACTT
	OXA48-Set4-LB1	AAGAATTTGCCCGCCAAATTG
	OXA48-Set4-LF2	GTCTGTCCATCCCACTTAAAGACT
	OXA48-Set4-LB2	GAATTTGCCCGCCAAATTGG

Bacterial strains

The bacterial strains used in this work were taken from collections held at APHA and are listed in Table 3. The panel included 11 *bla*$_{OXA-48}$ positive isolates. To test for specificity of the assays, the panel also included 25 isolates with other *bla*$_{OXA}$ genes: *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-7}$, *bla*$_{OXA-9}$, *bla*$_{OXA-23}$, *bla*$_{OXA-40}$, *bla*$_{OXA-51,}$ and/or *bla*$_{OXA-58}$. The remaining isolates had other β-lactamase genes. DNA was prepared from these strains by DNA lysis, as described previously. [34]

Appendix VI - Table 3. Bacterial strains used for LAMP assay development

Strain	Species	β-lactamase genes (selected only)			
74560385	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
90640804	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
NCTC 13302	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
NCTC 13304	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
NCTC 13305	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
NCTC 13420	*Acinetobacter baumannii*	bla_{OXA-}			
NCTC 13421	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
NCTC 13424	*Acinetobacter baumannii*	bla_{OXA-}	bla_{OXA-}		
CIT	*Citrobacter freundii*	bla_{CMY}			
84520159	*Citrobacter freundii*	bla_{CMY}	bla_{NDM}		
85120312	*Enterobacter* spp.	bla_{CMY}	bla_{NDM}	bla_{OXA-}	
84560680	*Escherichia coli*	bla_{CMY}	bla_{NDM}	bla_{OXA-}	bla_{OXA-}
90220853	*Escherichia coli*	bla_{CMY}	bla_{NDM}	bla_{OXA-}	
74560386	*Klebsiella pneumoniae*	bla_{OXA-}	bla_{OXA-}		
80820413	*Klebsiella pneumoniae*	bla_{OXA-}			
81320179	*Klebsiella pneumoniae*	bla_{OXA-}			
82240675	*Klebsiella pneumoniae*	bla_{OXA-}			
84080146	*Klebsiella pneumoniae*	bla_{OXA-}			
84140235	*Klebsiella pneumoniae*	bla_{OXA-}	bla_{OXA-}		
84220315	*Klebsiella pneumoniae*	bla_{OXA-}	bla_{OXA-}		
84340096	*Klebsiella pneumoniae*	bla_{NDM}	bla_{OXA-}		
85240324	*Klebsiella pneumoniae*	bla_{OXA-}			
85240325	*Klebsiella pneumoniae*	bla_{OXA-}			
NCTC 13443	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM-}	bla_{OXA-}	bla_{OXA-}
90480355	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM}	bla_{OXA-}	bla_{OXA-}
90480398	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM}	bla_{OXA-}	
90540829	*Klebsiella pneumoniae*	bla_{OXA-}			
90640544	*Klebsiella pneumoniae*	bla_{OXA-}	bla_{OXA-}		
91220314	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM}	bla_{OXA-}	
91340362	*Klebsiella pneumoniae*	bla_{NDM}	bla_{OXA-}		
91340363	*Klebsiella pneumoniae*	bla_{NDM}	bla_{OXA-}	bla_{OXA-}	
91340364	*Klebsiella pneumoniae*	bla_{NDM}	bla_{OXA-}		
NCTC 13439	*Klebsiella pneumoniae*	bla_{VIM-}			
NCTC 13440	*Klebsiella pneumoniae*	bla_{VIM-}			
NCTC 13442	*Klebsiella pneumoniae*	bla_{OXA-}			
92000514	*Klebsiella pneumoniae*	bla_{CMY}	bla_{OXA-}	bla_{OXA-}	
91000848	*Klebsiella* spp.	bla_{CMY}	bla_{NDM}		
NCTC 13437	*Pseudomonas aeruginosa*	bla_{VIM-}	bla_{OXA-}		
VIM	*Pseudomonas aeruginosa*	bla_{VIM-}	bla_{OXA-}		
90700129	*Pseudomonas fluorescens /*	bla_{VIM-}			
B2317 (197511)	*Salmonella* spp.	bla_{CMY}			
B2308 (E111592)	*Escherichia coli*	bla_{OXA-}			
B2314	*Escherichia coli*	bla_{OXA-}			
B2318 (C134)	*Escherichia coli*	bla_{OXA-}			

LAMP Assay Method

The LAMP reactions were run essentially as described in SOP BAC0282, with minor modifications (such as use of different primers). All LAMP assays were set up and run as shown in Tables 4 and 5. Reactions were run on the Mx3000P (Stratagene) and data analysed using MxPro software (Mx3000P v4.10 Build 389, Schema 85; Stratagene). Samples returning a Ct value <30 were considered positive.

Appendix VI - Table 4. Reaction set-up for a LAMP assay

Reagent	**Stock Conc^{n}**	**Volume for single Reaction (µl)**
ISO-001 Mastermix (Optigene)	As supplied	15
External Primers (F3 + B3)	5 µM	1
Internal Primers (FIP + BIP)	50 µM	1
Loop Primers (LF + LB)	25 µM	1
Molecular Water	As supplied	6
Template	Not applicable	1

Appendix VI - Table 5. LAMP reaction and read conditions.

Temp	Time	# cycles	READ
65°C	1MIN	X60	1 END read
95°	1MIN	X 1	ALL Read

Preliminary Assessment of LAMP Assays

A total of six LAMP assays were assessed in the first instance: OXA48 Set1 Loop1, OXA48 Set1 Loop2, OXA48 Set3 Loop1, OXA48 Set3 Loop2, OXA48 Set4 Loop1, and OXA48 Set4 Loop2.

Each assay was tested with a 1:250 dilution (in water) from three bacterial strains in triplicate: NCTC 13302 (*bla*$_{\text{OXA-40}}$ and *bla*$_{\text{OXA-51}}$), NCTC 13443 (*bla*$_{\text{OXA-1}}$ and *bla*$_{\text{OXA-9}}$), and NCTC 13442 (*bla*$_{\text{OXA-48}}$). All assays were positive for NCTC 13442, the

bla$_{OXA-48}$ reference strain, and negative for the isolates with other *bla*$_{OXA}$ genes (Table 6).

Two assays were selected for additional testing: OXA48 Set1 Loop2 and OXA48 Set4 Loop1. Each was tested further using 1:100 dilutions (in water) of the same three bacterial strains in triplicate. Both assays were positive for NCTC 13442 (the *bla*$_{OXA-48}$ reference strain) and negative for the isolates with other *bla*$_{OXA}$ genes (Table 6). These two assays were therefore selected and subjected to sensitivity and specificity testing.

Appendix VI - Table 6. Results of initial assessment of six *bla*$_{OXA-48}$ LAMP assays. (Note: Ct values > 30 are negative).

LAMP Assay	Dilution	NCTC 13302 (*bla*$_{OXA-40}$ and *bla*$_{OXA-51}$)	NCTC 13443 (*bla*$_{OXA-1}$ and *bla*$_{OXA-9}$)	NCTC 13442 (*bla*$_{OXA-48}$)
OXA48 Set1 Loop1	1:250	53.84	No Ct	5.39
OXA48 Set1 Loop2	1:250	43.31	No Ct	5.31
	1:100	No Ct	50.31	5.52
OXA48 Set3 Loop1	1:250	No Ct	No Ct	10.54
OXA48 Set3 Loop2	1:250	No Ct	No Ct	9.80
OXA48 Set4 Loop1	1:250	No Ct	No Ct	7.65
	1:100	No Ct	50.31	5.52
OXA48 Set4 Loop2	1:250	No Ct	No Ct	7.59

Analytical Sensitivity

The *bla*$_{oxa-48}$ gene was amplified from the reference strain NCTC 13442 using the primers in Table 7. The PCR product was gel purified, recovered, and the yield quantitated using the Nanodrop. The number of copies of the gene was determined using the calculator at the URI Genomics & Sequencing Center, (http://cels.uri.edu/gsc/cndna.html).

Appendix VI - Table 7. PCR primers to amplify the *bla*$_{oxa-48}$ gene.

Primer Name	Sequence (5'-3')	Tm (ºC)	Amplicon size	Reference
OXA48_GENE_F	TGTATTAGCCTTATCGGCTGTG	59.3	777 bp	This study
OXA48_GENE_R	TCCTGTTTGAGCACTTCTTTTG	59.5		

The PCR product was used to test the analytical sensitivity of the LAMP assay. Analytical sensitivity was tested in three separate experiments, each with a freshly prepared 10-fold dilution series. Each dilution was tested in triplicate, and the results are summarised in Tables 8 and 9.

OXA48 Set1 Loop2 had an analytical sensitivity of 750 gene copies.

OXA48 Set4 Loop1 had an analytical sensitivity of 500 gene copies.

Appendix VI - Table 8. Analytical sensitivity for LAMP assay OXA48 Set1 Loop2

Number of	OXA48_LAMP_Expt6 (14/08/13)		OXA48_LAMP_Expt7 (20/08/13)		OXA48_LAMP_Expt8 (20/08/13)	
3×10^{7}	3/3 replicates positive	Ct 5.27 (average)	not tested	not tested	not tested	not tested
3×10^{6}	3/3 replicates positive	Ct 5.87 (average)	not tested	not tested	not tested	not tested
3×10^{5}	3/3 replicates positive	Ct 6.50 (average)	3/3 replicates positive	Ct 7.07 (average)	not tested	not tested
3×10^{4}	3/3 replicates positive	Ct 7.33 (average)	3/3 replicates positive	Ct 7.77 (average)	not tested	not tested
3×10^{3}	3/3 replicates positive	Ct 8.84 (average)	3/3 replicates positive	Ct 9.13 (average)	3/3 replicates positive	Ct 8.73 (average)
1.5×10^{3}	3/3 replicates positive	Ct 9.63 (average)	3/3 replicates positive	Ct 10.29 (average)	3/3 replicates positive	Ct 9.98 (average)
1×10^{3}	not tested	not tested	3/3 replicates positive	Ct 9.39 (average)	3/3 replicates positive	Ct 14.06 (average)
7.5×10^{2}	not tested	not tested	3/3 replicates positive	Ct 10.70 (average)	3/3 replicates positive	Ct 10.97 (average)
5×10^{2}	not tested	not tested	2/3 replicates positive	Ct 13.89 / 10.05	3/3 replicates positive	Ct 12.80 (average)
3×10^{2}	2/3 replicates positive	Ct 8.97 / 13.77	2/3 replicates positive	Ct 10.79 / 15.29	2/3 replicates positive	Ct 11.40 / 13.27
3×10^{1}	0/3 replicates positive	No Ct	1/3 replicates positive	Ct 11.86	0/3 replicates positive	No Ct
3×10^{0}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
3×10^{-1}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
NTC	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct

Appendix VI - Table 9. Analytical sensitivity for LAMP assay OXA48 Set4 Loop1

Number	OXA48_LAMP_Expt6 (14/08/13)		OXA48_LAMP_Expt7 (20/08/13)		OXA48_LAMP_Expt8 (20/08/13)	
3×10^{7}	3/3 replicates positive	Ct 7.87 (average)	not tested	not tested	not tested	not tested
3×10^{6}	3/3 replicates positive	Ct 8.85 (average)	not tested	not tested	not tested	not tested
3×10^{5}	3/3 replicates positive	Ct 10.43 (average)	3/3 replicates positive	Ct 9.80 (average)	not tested	not tested
3×10^{4}	3/3 replicates positive	Ct 11.45 (average)	3/3 replicates positive	Ct 11.17 (average)	not tested	not tested
3×10^{3}	3/3 replicates positive	Ct 13.54 (average)	3/3 replicates positive	Ct 12.96 (average)	3/3 replicates positive	Ct 13.85 (average)
1.5×10^{3}	3/3 replicates positive	Ct 14.76 (average)	3/3 replicates positive	Ct 13.46 (average)	3/3 replicates positive	Ct 14.55 (average)
1×10^{3}	not tested	not tested	3/3 replicates positive	Ct 14.42 (average)	3/3 replicates positive	Ct 14.72 (average)
7.5×10^{2}	not tested	not tested	3/3 replicates positive	Ct 15.33 (average)	3/3 replicates positive	Ct 15.14 (average)
5×10^{2}	not tested	not tested	3/3 replicates positive	Ct 15.02 (average)	3/3 replicates positive	Ct 15.44 (average)
3×10^{2}	2/3 replicates positive	Ct 16.65 / 17.00	3/3 replicates positive	Ct 16.56 (average)	3/3 replicates positive	Ct 15.70 (average)
3×10^{1}	1/3 replicates positive	Ct 17.22	1/3 replicates positive	Ct 15.25	0/3 replicates positive	No Ct
3×10^{0}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
3×10^{-1}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
NTC	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct

Analytical Specificity

The DNA lysate of every strain listed in Table 3 was diluted 1:1000 in water and used to test the analytical specificity of both LAMP assays. Results are summarised in Table 10. No false negative results were obtained, as all isolates containing *bla*$_{\text{oxa-48}}$ were positive (with Ct values < 11). A single false positive result was obtained: isolate # 84340096 (*Klebsiella pneumoniae*; *bla*$_{\text{OXA-9}}$ positive) (Table 10).

Further testing of this isolate demonstrated that it was PCR negative for *bla*$_{\text{oxa-48}}$, using the PCR primers in Table 7 and two published *bla*$_{\text{oxa-48}}$ PCRs [124; 125]. The isolate was demonstrated to be PCR positive for *bla*$_{\text{oxa-9}}$, as expected, using published primers [35]. A new DNA lysate of isolate # 84340096 was prepared and fresh dilutions of both lysates tested by LAMP. However, intermittent LAMP positive results were still obtained with this isolate. Therefore Table 10 lists isolate # 84340096 as a false positive result. Assay OXA48 Set1 Loop2 gave the least number of false positive results (data not shown).

It is possible that the *bla*$_{\text{oxa-9}}$ gene in isolate # 84340096 has sufficient nucleotide identity with the LAMP primers used to give a false positive result. The gene can be sequenced to establish this.

It is noteworthy that four other isolates with *bla*$_{\text{oxa-9}}$ were included in the panel and none gave false positive results (Table 10).

Appendix VI - Table 10. Analytical sensitivity of *bla*$_{\text{OXA-48}}$ gene LAMP assays.

Strain	Species	Plate Well	Plate Well	OXA48 Set1	OXA48 Set4	Gene			
74560385	*Acinetobacter baumannii*	A1	A7	No Ct	No Ct	*bla*$_{\text{OXA-51}}$	*bla*$_{\text{OXA-58}}$		
90640804	*Acinetobacter baumannii*	B1	B7	No Ct	No Ct	*bla*$_{\text{OXA-40}}$	*bla*$_{\text{OXA-51}}$		
NCTC 13302	*Acinetobacter baumannii*	C1	C7	57.56	No Ct	*bla*$_{\text{OXA-40}}$	*bla*$_{\text{OXA-51}}$		
NCTC 13304	*Acinetobacter baumannii*	D1	D7	No Ct	No Ct	*bla*$_{\text{OXA-23}}$	*bla*$_{\text{OXA-51}}$		
NCTC 13305	*Acinetobacter baumannii*	E1	E7	No Ct	No Ct	*bla*$_{\text{OXA-51}}$	*bla*$_{\text{OXA-58}}$		
NCTC 13420	*Acinetobacter baumannii*	F1	F7	No Ct	No Ct	*bla*$_{\text{OXA-51}}$			
NCTC 13421	*Acinetobacter baumannii*	G1	G7	No Ct	No Ct	*bla*$_{\text{OXA-23}}$	*bla*$_{\text{OXA-51}}$		
NCTC 13424	*Acinetobacter baumannii*	H1	H7	54.53	No Ct	*bla*$_{\text{OXA-23}}$	*bla*$_{\text{OXA-51}}$		
CIT	*Citrobacter freundii*	A2	A8	33.00	No Ct	*bla*$_{\text{CMY}}$			
84520159	*Citrobacter freundii*	B2	B8	32.84	No Ct	*bla*$_{\text{CMY}}$	*bla*$_{\text{NDM}}$		
85120312	*Enterobacter* spp.	C2	C8	No Ct	No Ct	*bla*$_{\text{CMY}}$	*bla*$_{\text{NDM}}$	*bla*$_{\text{OXA-1}}$	
84560680	*Escherichia coli*	D2	D8	No Ct	No Ct	*bla*$_{\text{CMY}}$	*bla*$_{\text{NDM}}$	*bla*$_{\text{OXA-1}}$	*bla*$_{\text{OXA-2}}$
90220853	*Escherichia coli*	E2	E8	No Ct	No Ct	*bla*$_{\text{CMY}}$	*bla*$_{\text{NDM}}$	*bla*$_{\text{OXA-2}}$	
74560386	*Klebsiella pneumoniae*	F2	F8	5.09	8.51	*bla*$_{\text{OXA-48}}$	*bla*$_{\text{OXA-1}}$		
80820413	*Klebsiella pneumoniae*	G2	G8	5.23	9.76	*bla*$_{\text{OXA-48}}$			
81320179	*Klebsiella pneumoniae*	H2	H8	4.70	9.14	*bla*$_{\text{OXA-48}}$			
82240675	*Klebsiella pneumoniae*	A3	A9	5.33	10.41	*bla*$_{\text{OXA-48}}$			
84080146	*Klebsiella pneumoniae*	B3	B9	No Ct	No Ct	*bla*$_{\text{OXA-1}}$			
84140235	*Klebsiella pneumoniae*	C3	C9	5.16	9.66	*bla*$_{\text{OXA-48}}$	*bla*$_{\text{OXA-1}}$		
84220315	*Klebsiella pneumoniae*	D3	D9	5.30	9.87	*bla*$_{\text{OXA-48}}$	*bla*$_{\text{OXA-1}}$		
84340096	*Klebsiella pneumoniae*	E3	E9	False Positive	False Positive	*bla*$_{\text{NDM}}$	*bla*$_{\text{OXA-9}}$		
85240324	*Klebsiella pneumoniae*	F3	F9	5.46	10.00	*bla*$_{\text{OXA-48}}$			
85240325	*Klebsiella pneumoniae*	G3	G9	No Ct	No Ct	*bla*$_{\text{OXA-1}}$			

NCTC 13443	*Klebsiella pneumoniae*	H3	H9	No Ct	No Ct	bla_{CMY}	bla_{NDM-1}	bla_{OXA-1}	bla_{OXA-9}
90480355	*Klebsiella pneumoniae*	A4	A10	No Ct	No Ct	bla_{CMY}	bla_{NDM}	bla_{OXA-1}	bla_{OXA-9}
90480398	*Klebsiella pneumoniae*	B4	B10	No Ct	No Ct	bla_{CMY}	bla_{NDM}	bla_{OXA-9}	
90540829	*Klebsiella pneumoniae*	C4	C10	5.18	9.70	bla_{OXA-48}			
90640544	*Klebsiella pneumoniae*	D4	D10	5.23	9.33	bla_{OXA-48}	bla_{OXA-1}		
91220314	*Klebsiella pneumoniae*	E4	E10	No Ct	No Ct	bla_{CMY}	bla_{NDM}	bla_{OXA-1}	
91340362	*Klebsiella pneumoniae*	F4	F10	51.01	No Ct	bla_{NDM}	bla_{OXA-1}		
91340363	*Klebsiella pneumoniae*	G4	G10	No Ct	No Ct	bla_{NDM}	bla_{OXA-1}	bla_{OXA-7}	
91340364	*Klebsiella pneumoniae*	H4	H10	No Ct	No Ct	bla_{NDM}	bla_{OXA-1}		
NCTC 13439	*Klebsiella pneumoniae*	A5	A11	No Ct	No Ct	bla_{VIM-1}			
NCTC 13440	*Klebsiella pneumoniae*	B5	B11	No Ct	No Ct	bla_{VIM-1}			
NCTC 13442	*Klebsiella pneumoniae*	C5	C11	5.22	9.78	bla_{OXA-48}			
92000514	*Klebsiella pneumoniae*	D5	D11	4.78	9.56	bla_{OXA-48}	bla_{CMY}	bla_{OXA-1}	
91000848	*Klebsiella* spp.	E5	E11	No Ct	No Ct	bla_{CMY}	bla_{NDM}		
NCTC13437	*Pseudomonas aeruginosa*	F5	F11	40.48	No Ct	bla_{VIM-10}	bla_{OXA-7}		
VIM	*Pseudomonas aeruginosa*	G5	G11	No Ct	No Ct	bla_{VIM-1}	bla_{OXA-2}		
90700129	*Pseudomonas fluorescens / putida*	H5	H11	No Ct	No Ct	$bla_{VIM-2gr}$			
B2317 (197511)	*Salmonella* spp.	A6	A12	58.79	No Ct	bla_{CMY}			
B2308 (E111592)	*Escherichia coli*	B6	B12	No Ct	No Ct	bla_{OXA-9}			
B2314 (J53pSKL54)	*Escherichia coli*	C6	C12	No Ct	No Ct	bla_{OXA-7}			
B2318 (C134)	*Escherichia coli*	D6	D12	46.89	No Ct	bla_{OXA-1}			

Conclusion

OXA48 Set1 Loop2 can be used as the *bla*$_{\text{OXA-48}}$ LAMP assay, and test performance is summarised in Table 11. The false positive reaction with one isolate containing *bla*$_{\text{oxa-9}}$ is noted.

Appendix VI - Table 11. Summary of test OXA48 Set1 Loop2 test performance

Index	**Estimate**	**Lower 95% CI**	**Upper 95% CI**
Sensitivity	100.00%	71.51%	Cannot be determined
Specificity	96.97%	84.24%	99.92%

24. Appendix VII – Development and initial validation of a loop-mediated isothermal amplification (LAMP) assay for the detection of *bla*$_{VIM}$

Summary

- A LAMP assay to detect the carbapenemase *bla*$_{VIM}$ was developed.
- The assay had 100% sensitivity and specificity using a panel of 44 reference strains.
- The assay had an analytical sensitivity of 750 – 1,000 gene copies.
- Positive results were obtained within 18 minutes.
- Further validation of the assay with field samples will be required.

Aim

Develop a LAMP assay for the detection of *bla*$_{VIM}$, a carbapenem-hydrolysing class B β-lactamase reported in Enterobacteriaceae and *Pseudomonas aeruginosa*.

The bla$_{VIM}$ genes

To date 35 variants of *bla*$_{VIM}$ have been described, summarised in Table 1. These are listed in the database curated by the Lahey clinic, last accessed 25/06/2013 (http://www.lahey.org/Studies/other.asp#table1). For 16 variants the nucleotide sequence was available but there was no associated publication, and these are indicated as direct submissions in Table 1. Four *bla*$_{VIM}$ variants have been assigned, but no sequence was available.

There are three groups of *bla*$_{VIM}$ genes: *bla*$_{VIM-1}$, *bla*$_{VIM-2}$, and *bla*$_{VIM-7}$ [126]. *bla*$_{VIM-12}$ can be considered a 'hybrid' as the 5' region has greatest nucleotide identity to *bla*$_{VIM-1}$ while the 3' region has greatest nucleotide identity to *bla*$_{VIM-2}$ [127].

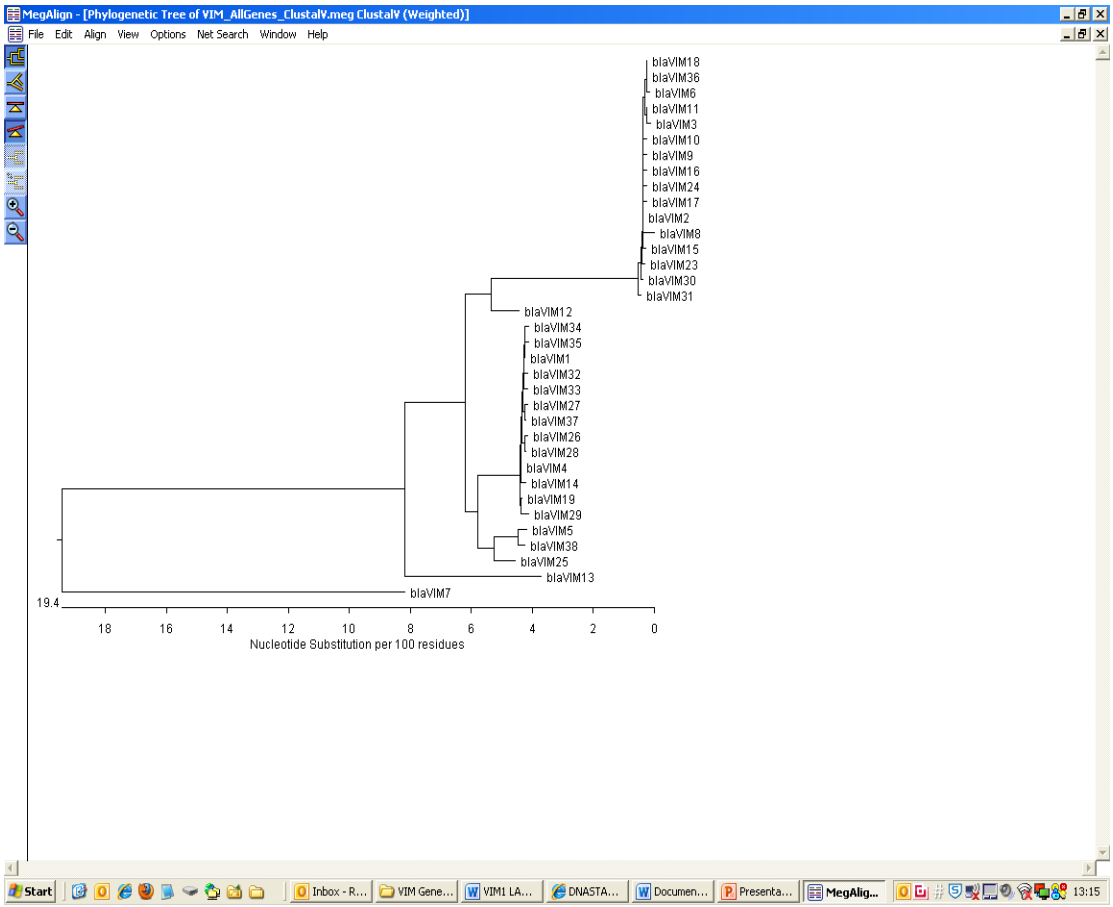
Appendix VII - Table 1. *bla*$_{VIM}$ variants.

***bla*$_{VIM}$ variant**	**Nucleotide Accession Number**	**Reference**
bla$_{VIM-1}$	Y18050	AAC 43: 1584, 1999
bla$_{VIM-2}$	AF191564	AAC 44: 891, 2000
bla$_{VIM-3}$	AF300454	AAC 45: 2224, 2001
bla$_{VIM-4}$	AY135661	AAC 46: 4026, 2002
bla$_{VIM-5}$	AY144612	JAC 54:282, 2004
bla$_{VIM-6}$	AY165025	AAC 48:2334, 2004
bla$_{VIM-7}$	AJ536835	AAC48:329, 2004
bla$_{VIM-8}$	AY524987	JCM 42:5094-5101, 2004
bla$_{VIM-9}$	AY524988	Direct submission
bla$_{VIM-10}$	AY524989	Direct submission
bla$_{VIM-11}$	AY605049	AAC 49:474-5, 2005.
bla$_{VIM-12}$	DQ143913	AAC 49:5153-5156, 2005
bla$_{VIM-13}$	DQ365886	AAC 52:3589-3596, 2008
bla$_{VIM-14}$	AY635904	Direct submission
bla$_{VIM-15}$	EU419745	AAC 52:2977-2979, 2008
bla$_{VIM-16}$	EU419746	AAC 52:2977-2979, 2008
bla$_{VIM-17}$	EU118148	AAC 53:1325-1330, 2009
bla$_{VIM-18}$	AM778091	AAC 53:1225-1227, 2009
bla$_{VIM-19}$	FJ822963	AAC 54:471-476, 2010
bla$_{VIM-20}$	Assigned	
bla$_{VIM-21}$	Assigned	
bla$_{VIM-22}$	Assigned	
bla$_{VIM-23}$	GQ242167	Direct submission
bla$_{VIM-24}$	HM855205	AAC 55:2428-2430, 2011
bla$_{VIM-25}$	HM750249	Direct submission
bla$_{VIM-26}$	FR748153	Direct submission
bla$_{VIM-27}$	HQ858608	AAC 55:3570-3572, 2011
bla$_{VIM-28}$	JF900599	Direct submission
bla$_{VIM-29}$	JX311308	Direct submission
bla$_{VIM-30}$	JN129451	Direct submission
bla$_{VIM-31}$	JN982330	AAC 56:3283-3287, 2012
bla$_{VIM-32}$	JN676230	Direct submission
bla$_{VIM-33}$	JX258134	Direct submission
bla$_{VIM-34}$	JX013656	Direct submission
bla$_{VIM-35}$	JX982634	Direct submission
bla$_{VIM-36}$	JX982635	Direct submission
bla$_{VIM-37}$	JX982636	Direct submission
bla$_{VIM-38}$	KC469971	Direct submission
bla$_{VIM-39}$	Assigned	

Alignment of *bla*$_{VIM}$ nucleotide sequences

The nucleotide sequence (coding region) of each gene listed in Table 1 was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). Nucleotide sequences were aligned using ClustalV in MegAlign (DNAStar Inc, Madison, WI, USA). Figure 1 shows that the *bla*$_{VIM}$ genes fell into the expected three groups, and identified the members of each group.

Appendix VII - Figure 1. Phylogenic tree of *bla*$_{VIM}$ nucleotide sequences (MegAlign using Clustal V).



Design of LAMP assay primers

A LAMP assay for *bla*$_{VIM-2}$ had been published in 2006.[128] This assay was not tested in this work for several reasons:

- the article is in Japanese and could nottherefore be assessed critically without translation.
- the assay was developed using a single *bla*$_{VIM-2}$ gene sequence from *Achromobacter xylosoxidans* (Accession number AY204637) and hence may not detect other variants.

- AY204637 is not the reference *bla*$_{VIM-2}$ gene sequence (*c.f.* Table 1).
- No one has cited the paper in any publications.

The nucleotide sequence diversity between all *bla*$_{VIM}$ genes was large, and the consensus sequence generated from these was not suitable for LAMP assay design (as there were too many SNPs). Therefore the *bla*$_{VIM-1}$ group genes were selected as the target for LAMP assay design. A FASTA file containing all 16 *bla*$_{VIM-1}$ group nucleotide sequences and *bla*$_{VIM-12}$ was made, used as input for alignment by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the alignment saved.

LAMP design was done with Primer Explorer 4 (Eiken Chemical Company Limited, Japan) (http://primerexplorer.jp/elamp4.0.0/index.html), using the ClustalW alignment file as input. To generate primers able detect all *bla*$_{VIM-1}$ genes, the 'common' design option was selected. Many LAMP primer sets were designed by Primer Explorer 4, although most fell into the same small region of the gene and had minor differences between them.

Three sets were selected based on the least number of SNPs and the criteria set out in the Primer Explorer 4 manual (A Guide to LAMP primer designing No.1, No.2, and No.3; http://primerexplorer.jp/e/v4_manual/index.html).

Loop primers were then designed for each LAMP primer set using Primer Explorer 4, and two pairs of Loop primers selected for each set based on criteria in the Primer Explorer 4 manual. The LAMP and loop primers selected are presented in Table 2. Note that Set 1 and Set 2 used the same Primer F3, Primer BIP, and loop primers. Given the high degree of similarity between Set 1 and Set 2, only primers for Set 1 and Set 3 were tested.

Appendix VII - Table 2. LAMP and loop primer sets selected for detection of bla_{VIM-1} group genes.

LAMP	Primer Name	Primer Sequence (5'-3')
Set 1	VIM1-Set1-F3	TGACCGCGTCTGTCATGG
	VIM1-Set1-B3	ACCCCACGCTGTATCAATCA
	VIM1-Set1-FIP	GGACCTCTCCGACCGGAATTTC-
	VIM1-Set1-BIP	AACGCAGTCGTTTGATGGCGC-
	VIM1-Set1-LF1	GTCGGATACTCACCACTCGG
	VIM1-Set1-	TCTACCCGTCCAATGGTCTC
	VIM1-Set1-LF2	TGTCGGATACTCACCACTCGG
	VIM1-Set1-	GGTCTACCCGTCCAATGGTCT
Set 2	VIM1-Set1-F3	as VIM1-Set1-F3
	VIM1-Set2-B3	CGCTGTGTTTTTCGCACC
	VIM1-Set2-FIP	CGGACCTCTCCGACCGGAATTT-
	VIM1-Set1-BIP	as VIM1-Set1-BIP
	VIM1-Set1-LF1	as VIM1-Set1-LF1
	VIM1-Set1-	as VIM1-Set1-LB1
	VIM1-Set1-LF2	as VIM1-Set1-LF2
	VIM1-Set1-	as VIM1-Set1-LB2
Set 3	VIM1-Set3-F3	CGTGATGGTGATGAGTTGCT
	VIM1-Set3-B3	GCGTGGGAATCTCGTTCC
	VIM1-Set3-FIP	CACGCGTTACGGGAAGTCCAAT-GATACAGCGTGGGGTGC
	VIM1-Set3-BIP	CACTTTCATGACGACCGCGTCG-CGTGTCGACGGTGATGC
	VIM1-Set3-LF1	CGAGAAGTGCCGCTGTGT
	VIM1-Set3-	GCGGCGTTGATGTCCTTCGG
	VIM1-Set3-LF2	GCGAGAAGTGCCGCTGT
	VIM1-Set3-	CGGCGTTGATGTCCTTCGG

Bacterial strains

The bacterial strains used in this work were taken from collections held at APHA and are listed in Table 3. The panel included three isolates positive for a bla_{VIM-1} family gene and two isolates with a bla_{VIM-2} family gene. The remaining isolates had other β-lactamase genes. DNA was prepared from these strains by DNA lysis as described previously [129].

Appendix VII - Table 3. Bacterial strains used for LAMP assay development

Strain	Species	β-lactamase genes (selected only)			
74560385	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
90640804	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
NCTC 13302	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
NCTC 13304	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
NCTC 13305	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
NCTC 13420	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$			
NCTC 13421	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
NCTC 13424	*Acinetobacter baumannii*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-}}$		
CIT	*Citrobacter freundii*	bla_{CMY}			
84520159	*Citrobacter freundii*	bla_{CMY}	bla_{NDM}		
85120312	*Enterobacter* spp.	bla_{CMY}	bla_{NDM}	$bla_{\text{OXA-1}}$	
84560680	*Escherichia coli*	bla_{CMY}	bla_{NDM}	$bla_{\text{OXA-1}}$	$bla_{\text{OXA-2}}$
90220853	*Escherichia coli*	bla_{CMY}	bla_{NDM}	$bla_{\text{OXA-2}}$	
74560386	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-1}}$		
80820413	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$			
81320179	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$			
82240675	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$			
84080146	*Klebsiella pneumoniae*	$bla_{\text{OXA-1}}$			
84140235	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-1}}$		
84220315	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-1}}$		
84340096	*Klebsiella pneumoniae*	bla_{NDM}	$bla_{\text{OXA-9}}$		
85240324	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$			
85240325	*Klebsiella pneumoniae*	$bla_{\text{OXA-1}}$			
NCTC 13443	*Klebsiella pneumoniae*	bla_{CMY}	$bla_{\text{NDM-1}}$	$bla_{\text{OXA-1}}$	$bla_{\text{OXA-9}}$
90480355	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM}	$bla_{\text{OXA-1}}$	$bla_{\text{OXA-9}}$
90480398	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM}	$bla_{\text{OXA-9}}$	
90540829	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$			
90640544	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$	$bla_{\text{OXA-1}}$		
91220314	*Klebsiella pneumoniae*	bla_{CMY}	bla_{NDM}	$bla_{\text{OXA-1}}$	
91340362	*Klebsiella pneumoniae*	bla_{NDM}	$bla_{\text{OXA-1}}$		
91340363	*Klebsiella pneumoniae*	bla_{NDM}	$bla_{\text{OXA-1}}$	$bla_{\text{OXA-7}}$	
91340364	*Klebsiella pneumoniae*	bla_{NDM}	$bla_{\text{OXA-1}}$		
NCTC 13439	*Klebsiella pneumoniae*	$bla_{\text{VIM-1}}$			
NCTC 13440	*Klebsiella pneumoniae*	$bla_{\text{VIM-1}}$			
NCTC 13442	*Klebsiella pneumoniae*	$bla_{\text{OXA-}}$			
92000514	*Klebsiella pneumoniae*	bla_{CMY}	$bla_{\text{OXA-}}$	$bla_{\text{OXA-1}}$	
91000848	*Klebsiella* spp.	bla_{CMY}	bla_{NDM}		
NCTC 13437	*Pseudomonas aeruginosa*	$bla_{\text{VIM-10}}$	$bla_{\text{OXA-7}}$		
VIM	*Pseudomonas aeruginosa*	$bla_{\text{VIM-1}}$	$bla_{\text{OXA-2}}$		
90700129	*Pseudomonas fluorescens / putida*	$bla_{\text{VIM-}}$			
B2317 (197511)	*Salmonella* spp.	bla_{CMY}			

B2308 (E111592)	*Escherichia coli*	bla_{OXA-9}
B2314 (J53pSKL54)	*Escherichia coli*	bla_{OXA-7}
B2318 (C134)	*Escherichia coli*	bla_{OXA-1}

LAMP Assay Method

The LAMP reactions were run essentially as described in SOP BAC0282, with minor modifications (such as use of different primers). All LAMP assays were set up and run as shown in Tables 4 and 5. Reactions were run on the Mx3000P (Stratagene) and data analysed using MxPro software (Mx3000P v4.10 Build 389, Schema 85; Stratagene). Samples returning a Ct value <30 were considered positive.

Appendix VII - Table 4. Reaction set-up for a LAMP assay

Reagent	**Stock Conc^{n}**	**Volume for single Reaction (µl)**
ISO-001 Mastermix (Optigene)	As supplied	15
External Primers (F3 + B3)	5 µM	1
Internal Primers (FIP + BIP)	50 µM	1
Loop Primers (LF + LB)	25 µM	1
Molecular Water	As supplied	6
Template	Not applicable	1

Appendix VII - Table 5. LAMP reaction and read conditions.

Temp	Time	# cycles	READ
65°C	1MIN	X60	1 END read
95°	1MIN	X 1	ALL Read

Preliminary Assessment of LAMP Assays

A total of four LAMP assays were assessed in the first instance: VIM1 Set1 Loop1, VIM1 Set1 Loop2, VIM1 Set3 Loop1, and VIM1 Set3 Loop2.

Each assay was tested with 1:250 dilutions in water of lysates from three bacterial strains: NCTC 13439 (bla_{VIM-1}), NCTC 13437 (bla_{VIM-10}), and VIM (bla_{VIM-1}). Note that bla_{VIM-10} is a member of the bla_{VIM-2} group (Figure 1). Each assay was tested in two repeats and each lysate was tested in triplicate on each occasion, results are summarised in Table 6.

Appendix VII - Table 6. Results of initial assessment of four *bla*$_{VIM-1}$ group LAMP assays.

LAMP Assay	Repeat	NCTC 13439	NCTC 13437	VIM (*bla*$_{VIM-1}$)
VIM1 Set1 Loop1	1	9.61	17.39	13.62
	2	10.23	26.48	14.09
VIM1 Set1 Loop2	1	9.46	19.68	13.21
	2	10.19	31.3	14.51
VIM1 Set3 Loop1	1	7.77	8.58	9.99
	2	8.01	9.63	11.04
VIM1 Set3 Loop2	1	7.65	8.40	9.69
	2	8.15	9.56	11.56

Two assays were selected for further work:

VIM1 Set1 Loop2 as it gave higher Ct values for *bla*$_{VIM-2}$ group genes.

VIM1 Set3 Loop1 as it detected both *bla*$_{VIM-1}$ group and *bla*$_{VIM-2}$ group genes within 12 minutes.

Analytical Sensitivity

The *bla*$_{VIM-1}$ gene was amplified from the reference strain NCTC 13439 using the primers in Table 7. The PCR product was gel purified, recovered, and the yield quantitated using the Nanodrop. The number of copies of the gene was determined using the calculator at the URI Genomics & Sequencing Center, (http://cels.uri.edu/gsc/cndna.html).

Appendix VII - Table 7. PCR primers to amplify the *bla*$_{VIM-1}$ gene.

Primer Name	Sequence (5'-3')	Tm (ºC)	Amplicon size	Reference
VIM1_GENE_F	TTATGGAGCAGCAACGATGT	56.4	920	[130]
VIM1_GENE_R	CAAAAGTCCCGCTCCAACGA	60.5		

The PCR product was used to test the analytical sensitivity of the LAMP assay. Analytical sensitivity was tested in three separate experiments, each with a freshly prepared 10-fold dilution series. Each dilution was tested in triplicate, and the results are summarised in Tables 8 and 9.

VIM1 Set1 Loop2 had an analytical sensitivity of 2,000 gene copies.

VIM1 Set3 Loop1 had an analytical sensitivity of 750 – 1,000 gene copies.

Appendix VII - Table 8. Analytical sensitivity for LAMP assay VIM1 Set1 Loop2

# of	VIM1_LAMP_Expt3 (08/08/13)		VIM1_LAMP_Expt4 (14/08/13)		VIM1_LAMP_Expt4 (22/08/13)	
3×10^{8}	not tested	not tested	3/3 replicates positive	Ct 7.75 (average)	not tested	not tested
3×10^{7}	3/3 replicates positive	Ct 8.51 (average)	not tested	not tested	not tested	not tested
3×10^{6}	3/3 replicates positive	Ct 9.91 (average)	not tested	not tested	not tested	not tested
3×10^{5}	3/3 replicates positive	Ct 11.39 (average)	3/3 replicates positive	Ct 11.48 (average)	not tested	not tested
3×10^{4}	3/3 replicates positive	Ct 12.82 (average)	3/3 replicates positive	Ct 12.86 (average)	not tested	not tested
3×10^{3}	3/3 replicates positive	Ct 15.26 (average)	3/3 replicates positive	Ct 16.55 (average)	3/3 replicates positive	Ct 14.71 (average)
2×10^{3}	not tested	not tested	3/3 replicates positive	Ct 15.42 (average)	3/3 replicates positive	Ct 14.89 (average)
1×10^{3}	not tested	not tested	3/3 replicates positive	Ct 16.98 (average)	2/3 replicates positive	Ct 14.83 / 16.90
7.5×10^{2}	not tested	not tested	not tested	not tested	1/3 replicates positive	Ct 16.95
5×10^{2}	not tested	not tested	1/3 replicates positive	Ct 28.22	1/3 replicates positive	Ct 29.15
3×10^{2}	1/3 replicates positive	Ct 17.75	1/3 replicates positive	Ct 15.75	0/3 replicates positive	No Ct
3×10^{1}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
3×10^{0}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
3×10^{-1}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
NTC	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct

Appendix VII - **Table 9.** Analytical sensitivity for LAMP assay VIM1 Set3 Loop1

# of copies	VIM1_LAMP_Expt3 (08/08/13)		VIM1_LAMP_Expt4 (14/08/13)		VIM1_LAMP_Expt4 (22/08/13)	
3×10^8	not tested	not tested	3/3 replicates positive	Ct 7.56 (average)	not tested	not tested
3×10^7	3/3 replicates positive	Ct 8.73 (average)	not tested	not tested	not tested	not tested
3×10^6	3/3 replicates positive	Ct 9.40 (average)	not tested	not tested	not tested	not tested
3×10^5	3/3 replicates positive	Ct 10.29 (average)	3/3 replicates positive	Ct 10.48 (average)	not tested	not tested
3×10^4	3/3 replicates positive	Ct 12.02 (average)	3/3 replicates positive	Ct 11.74 (average)	not tested	not tested
3×10^3	3/3 replicates positive	Ct 14.70 (average)	3/3 replicates positive	Ct 13.77 (average)	3/3 replicates positive	Ct 15.33 (average)
2×10^3	not tested	not tested	3/3 replicates positive	Ct 13.64 (average)	3/3 replicates positive	Ct 15.70 (average)
1×10^3	not tested	not tested	3/3 replicates positive	Ct 17.16 (average)	3/3 replicates positive	Ct 15.98 (average)
7.5×10^2	not tested	not tested	not tested	not tested	3/3 replicates positive	Ct 16.87 (average)
5×10^2	not tested	not tested	2/3 replicates positive	Ct 15.11 / 19.20	2/3 replicates positive	Ct 14.86 / 27.17
3×10^2	2/3 replicates positive	Ct 18.64 / 28.95	2/3 replicates positive	Ct 15.51 / 16.03	2/3 replicates positive	Ct 16.76 / 23.45
3×10^1	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	1/3 replicates positive	Ct 21.18
3×10^0	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
3×10^{-1}	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct
NTC	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct	0/3 replicates positive	No Ct

Analytical Specificity

The DNA lysate of every strain listed in Table 3 was diluted 1:1000 in water and used to test the analytical specificity of both LAMP assays.

VIM1 Set1 Loop2 was positive for the three *bla*$_{\text{VIM-1}}$ group isolates, negative for one *bla*$_{\text{VIM-2}}$ group isolate and was positive with a high Ct value for the other *bla*$_{\text{VIM-2}}$ group isolate (Ct 26.58), Table 10. Additionally three samples were positive in the absence of a *bla*$_{\text{VIM}}$ gene, Table 10, however, the Tm of the amplification product was incorrect (at ~71º C rather than the required ~90ºC) and therefore these samples are negative.

VIM1 Set3 Loop1 was positive for all five *bla*$_{\text{VIM}}$ samples by Ct 12, and no false positive results were obtained, Table 10.

Appendix VII - Table 10. Analytical sensitivity of *bla*$_{VIM-1}$ gene LAMP assays. (Samples indicated by an asterisk and red fill returned Ct values but had an incorrect product Tm values, and are therefore negative).

Strain	Species	VIM1 Set1	VIM1 Set3	β-lactamase genes (selected only)			
74560385	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-51}$	*bla*$_{OXA-58}$		
90640804	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-40}$	*bla*$_{OXA-51}$		
NCTC 13302	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-40}$	*bla*$_{OXA-51}$		
NCTC 13304	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-23}$	*bla*$_{OXA-51}$		
NCTC 13305	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-51}$	*bla*$_{OXA-58}$		
NCTC 13420	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-51}$			
NCTC 13421	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-23}$	*bla*$_{OXA-51}$		
NCTC 13424	*Acinetobacter baumannii*	No Ct	No Ct	*bla*$_{OXA-23}$	*bla*$_{OXA-51}$		
CIT	*Citrobacter freundii*	No Ct	No Ct	*bla*$_{CMY}$			
84520159	*Citrobacter freundii*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$		
85120312	*Enterobacter* spp.	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$	*bla*$_{OXA-1}$	
84560680	*Escherichia coli*	23.50 *	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$	*bla*$_{OXA-1}$	*bla*$_{OXA-2}$
90220853	*Escherichia coli*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$	*bla*$_{OXA-2}$	
74560386	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$	*bla*$_{OXA-1}$		
80820413	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$			
81320179	*Klebsiella pneumoniae*	20.84 *	No Ct	*bla*$_{OXA-48}$			
82240675	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$			
84080146	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-1}$			
84140235	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$	*bla*$_{OXA-1}$		
84220315	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$	*bla*$_{OXA-1}$		
84340096	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{NDM}$	*bla*$_{OXA-9}$		
85240324	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$			

85240325	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-1}$			
NCTC 13443	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM-1}$	*bla*$_{OXA-1}$	*bla*$_{OXA-9}$
90480355	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$	*bla*$_{OXA-1}$	*bla*$_{OXA-9}$
90480398	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$	*bla*$_{OXA-9}$	
90540829	*Klebsiella pneumoniae*	9.55 *	No Ct	*bla*$_{OXA-48}$			
90640544	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$	*bla*$_{OXA-1}$		
91220314	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$	*bla*$_{OXA-1}$	
91340362	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{NDM}$	*bla*$_{OXA-1}$		
91340363	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{NDM}$	*bla*$_{OXA-1}$	*bla*$_{OXA-7}$	
91340364	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{NDM}$	*bla*$_{OXA-1}$		
NCTC 13439	*Klebsiella pneumoniae*	9.59	7.77	*bla*$_{VIM-1}$			
NCTC 13440	*Klebsiella pneumoniae*	9.05	7.45	*bla*$_{VIM-1}$			
NCTC 13442	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{OXA-48}$			
92000514	*Klebsiella pneumoniae*	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{OXA-48}$	*bla*$_{OXA-1}$	
91000848	*Klebsiella* spp.	No Ct	No Ct	*bla*$_{CMY}$	*bla*$_{NDM}$		
NCTC 13437	*Pseudomonas aeruginosa*	No Ct	9.49	*bla*$_{VIM-10}$	*bla*$_{OXA-7}$		
VIM	*Pseudomonas aeruginosa*	14.78	11.02	*bla*$_{VIM-1}$	*bla*$_{OXA-2}$		
90700129	*Pseudomonas fluorescens / putida*	26.58	9.01	*bla*$_{VIM-2gr}$			
B2317 (197511)	*Salmonella* spp.	No Ct	No Ct	*bla*$_{CMY}$			
B2308 (E111592)	*Escherichia coli*	No Ct	No Ct	*bla*$_{OXA-9}$			
B2314 (J53pSKL54)	*Escherichia coli*	No Ct	No Ct	*bla*$_{OXA-7}$			
B2318 (C134)	*Escherichia coli*	No Ct	No Ct	*bla*$_{OXA-1}$			
NTC	Water	No Ct	No Ct				

Conclusion

VIM1 Set3 Loop1 is recommended for use as the *bla*$_{VIM}$ LAMP assay, and test performance is summarised in Table 11.

Appendix VII - Table 11. Summary of test VIM1 Set3 Loop1 test performance

Index	**Estimate**	**Lower 95% CI**	**Upper 95% CI**
Sensitivity	100.00%	47.82%	cannot be calculated
Specificity	100.00%	90.97%	cannot be calculated

25. Appendix VIII - Development and initial validation of a loop-mediated isothermal amplification (LAMP) assay for the detection of *bla*$_{CMY-2}$

Summary of results

- Three sets of LAMP primers were designed based on a conserved region of *bla*$_{CMY-2}$ which was identical in 35 other *bla*$_{CMY-2}$-like genes.
- Each set was tested with known *bla*$_{CMY}$ positive DNA and only two sets worked.
- The 2 functional sets were tested further to assess sensitivity, and set5 was found to be most sensitive, able to detect 500 copies/µl of the target gene.
- The specificity of the set5 primers was also good. It was able to detect all known CMY positive isolates, with the exception of three isolates which were negative in the LAMP assay and also by PCR. The plasmid containing CMY may have been lost during storage.
- All CMY negative isolates were also negative in the LAMP assay with set5 primers.

Background

The *bla*$_{CMY}$ gene encodes an AmpC-type enzyme which is able hydrolyse both extended spectrum cephalosporins but also cephamycins such as cefoxitin. Of particular concern in veterinary medicine is *bla*$_{CMY-2}$ as this gene has been particularly associated with *Salmonella* Newport strains, which are of concern particularly in the US. The *bla*$_{CMY-2}$ gene was first described in 1996 in a *Klebsiella* infection and the gene originated from a chromosomally encoded AmpC gene of *Citrobacter freundii*. The *bla*$_{CMY}$ gene was mobilised on to a plasmid allowing for its spread and proliferation. The genes are often carried on large multi-resistant plasmids [131]. There are now 108 CMY genes described (http://www.lahey.org/Studies/other.asp).

Primer design

Of the 108 CMY genes assigned in the Lahey clinic database only 85 have entries in the GenBank database. All these sequences were received and an alignment performed. This demonstrated that there were distinct CMY types based on sequence homology and it was not possible to identify a conserved region in all genes for LAMP primer design. Therefore a sub-set of these genes were selected which were most similar to *bla*$_{CMY-2}$. In total, 36 sequences were identified for which a conserved region could be selected for primer design, this 267 base-pair (bp) region was inputted into the Eiken software (http://loopamp.eiken.co.jp/e/lamp/) to allow the design of the external (EPS) and internal primer sets (IPS). The loop primers were then designed for each set of EPS and IPS. Five primer sets (EPS and IPS) were selected initially, however it was only possible to design loop primers for 3 of these sets. Two pair's of loop primers were selected for each EPS/IPS set. The selected primers are listed in Table 1.

Primer selection

All primer sets were tested with a *bla*$_{CMY}$ positive isolate to identify the best primer set. Dilutions were prepared (1:10, 1:100, 1:1000) to compare each primer pair (Table 2.). The Cycle threshold (Ct) values were determined and used to compare each primer set. For primer set 2 no amplification was detected even at the higher DNA concentrations. Amplification was observed for primer set 3 with the lowest Ct values obtained with loop set 2 (Table 2.). These ranged from 11.53-18.47 across the DNA dilution range. Set 5 primers gave the lowest Ct values (Table 2.), but there was very little difference between the Ct values for each loop primer set. Therefore loop primer set 1 was selected for further analysis. Figure 1 shows the amplification and melting curves for the *bla*$_{CMY}$ set5 primers.

Determining assay sensitivity

The sensitivity of the CMY LAMP assay was determined using a CMY PCR product. The entire gene was amplified using primers designed previously [132] from isolate B2317. The gene was purified, quantified and the number of gene copies estimated using a web-based program (http://cels.uri.edu/gsc/cndna.html) which can calculate number of copies of a template from fragment size and concentration. Once the

number of copies was known a dilution series was prepared between $1.4x10^{10}$ and $1x10^{-1}$ gene copies/µl (Table 3.). Primer sets 3 (LOOP 2) and 5 (LOOP 1) were then tested using the dilution series to determine the minimum number of template copies each primer set could detect. The first test demonstrated that CMY set 5 was able to detect the gene at a level of $1x10^{2}$ copies/µl. However, this was not consistent and it was found that not all three replicates were positive. The primers behaved most consistently (i.e. all three replicates were positive) were at a concentration of $5x10^{2}$ copies/µl. While for primer set 3 the detection limit was much higher at $1x10^{4}$ copies/µl. Therefore this primer set was not tested further. The sensitivity testing of primer set 5 was repeated and was found to be reproducible (Table 3.).

Testing the specificity of CMY primer Set 5 loop set 1.

The CMY set 5 primers with loop set 1 were tested with a panel of 41 genomic DNA preparations, which included 12 *bla*$_{CMY}$ positive isolates (as determined by resistance gene array) and isolates carrying other antimicrobial resistance genes. It was found that 9/12 *bla*$_{CMY}$ positive isolates tested were positive in the LAMP assay i.e. had a Ct value of ≤12 and the resulting product had a melting point between 88.4 and 89.4◦C (Tables 4.). However, the three which were negative by LAMP but positive by array were tested using a *bla*$_{CMY}$ specific PCR (CIT, AmpC group)[133] and were negative. Therefore it appears that the CMY gene has been lost in there three strains and therefore the LAMP assay detects all 9 confirmed positive isolates. All *bla*$_{CMY}$ negative isolates were also negative by LAMP assay.

Appendix VIII - Table 1. Primers designed and tested during this study

Primer	Function	Sequence
Set2 F3	EPS	TCTGCTGCTGACAGCCTC
Set2 B3	EPS	AAACAGCGTTTGCTGCGT
Set2 FIP	IPS	CGGGGTGATGGTGCGATTAACGTTTCTCCACATTTGCTGCCG
Set2 BIP	IPS	GGTATGGCCGTTGCCGTTATCTCTGGGTGGTTATTGGCGATA
Set2 LF1	Loop F	TCGGCAATCTGTTGTTCTGTTTTTG
Set2 LB1	Loop B	ACCAGGGAAAACCCTATTATTTCAC
Set2 LF2	Loop F	ATCGGCAATCTGTTGTTCTGTT
Set2 LB2	Loop B	AAAACCCTCTTCTTTCACCTGGGG
Set3 F3	EPS	GCCGCAAAAACAGAACAACA
Set3 B3	EPS	ACACGCCGTTAAACGTCTT
Set3 FIP	IPS	CCATACCCGGAATAGCCTGCTCTGCCGATATCGTTAATCGCA
Set3 BIP	IPS	CTGGGGTAAAGCCGATATCGCCGATCCTAGCTCAAACAGCGT
Set3 LF1	Loop F	GCATCAACGGGGTGATGG
Set3 LB1	Loop B	AATTAACCACCCAGTCACGCAG
Set3 LF2	Loop F	CTGCATCAACGGGGTGATG
Set3 LB2	Loop B	CACCCAGTCACGCAGCA
Set5 F3	EPS	TGCCGATATCGTTAATCGCA
Set5 B3	EPS	ACACGCCGTTAAACGTCTT
Set5 FIP	IPS	CCTGGTAGATAACGGCAACGGCCATCACCCCGTTGATGCA
Set5 BIP	IPS	CTGGGGTAAAGCCGATATCGCCGATCCTAGCTCAAACAGCGT
Set5 LF1	Loop F	CATACCCGGAATAGCCTGCT
Set5 LB1	Loop B	TAACCACCCAGTCACGCA
Set5 LF2	Loop F	CATACCCGGAATAGCCTGCTCC
Set5 LB2	Loop B	AATAACCACCCAGTCACGCA

Appendix VIII - Table 2. Testing of each primer set to identify the best set for further testing. Each value represents the average of three replicate wells and the values highlighted in blue and the primers selected for further analysis**.**

Strain ID	Set 2				Set 3				Set 5			
	Loop 1		Loop2		Loop 1		Loop2		Loop 1		Loop2	
	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm	Ct	Tm
HPA 16 neat	43.19	89.95	38.01	89.42	17.07	88.42	12.34	88.95	7.47	88.95	7.25	88.49
HPA16 1:10	46.23	89.42	42.62	89.42	15.65	88.42	13.19	88.95	7.63	88.45	7.44	88.46
HPA16 1:100	37.82	80.2	49.42	88.9	21.83	88.42	11.53	88.92	8.74	88.42	8.38	88.46
HPA16 1:1000	No Ct	79.71	No Ct	79.7	22.1	88.42	18.71	88.42	10.1	88.44	9.91	88.46

Appendix VIII - Table 3. Testing of CMY primer sets 3 and 5 with a CMY PCR amplicon to determine test sensitivity. CMY5 primers were tested in duplicate and CMY3 primers tested only once as following the first test CMY5 primers were found to have better sensitivity. (**indicates when not all three replicates were positive in an assay and shaded boxes indicate when 1 or more of the replicates was positive for CMY in the LAMP assay).

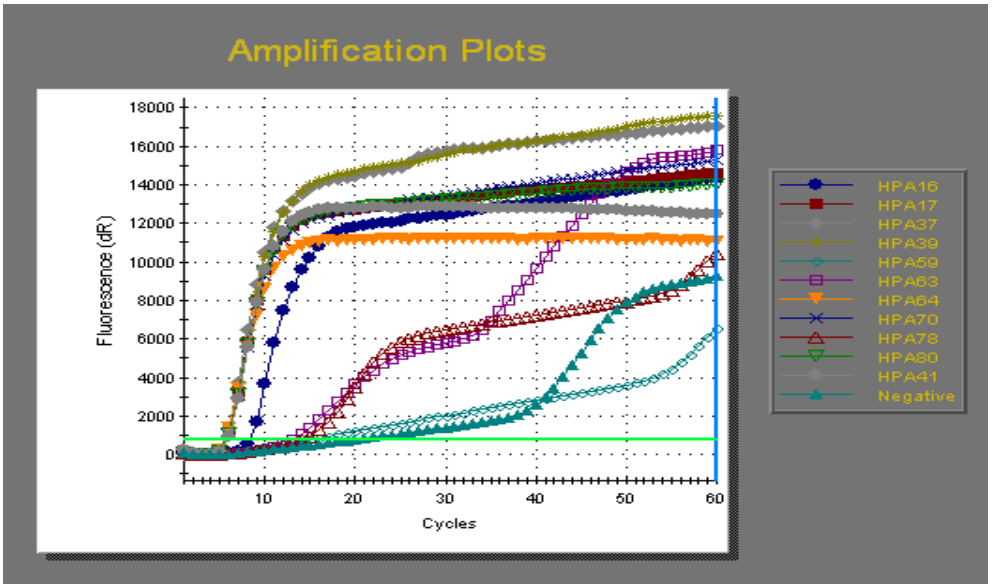
No. of copies / µl	CMY Primer set 5 (loop1)		CMY Primer set 5 (loop1) Repeat run		CMY primer set 3 (loop2)	
	Ct	Tm	Ct	Tm	Ct	Tm
1.42E+10	5.07	88.86	4.34	88.75	10.45	88.88
1.00E+10	5.03	88.9	4.43	88.78	10.97	88.9
1.00E+08	6.11	88.9	5.52	88.8	14.8	88.9
1.00E+06	7.8	88.92	6.87	88.78	18.68	88.9
1.00E+04	10.27	88.95	8.57	88.8	23.4	88.95
1.00E+03	13.33	88.95	10.22	88.83	29.84**	88.96
1.00E+02	14.95**	88.96	10.59	88.83	No Ct	81.83
1.00E+01	16.5	91	13.82	90.38	No Ct	82.35
1.00E+00	22.55	90.95	10.41	90.8	No Ct	82.25
1.00E-01	18.13	90.95	12.9	90.28	No Ct	81.76
5.00E+02	13.53	88.9	11.03	89.3	28**	88.9
2.50E+02	15.69**	88.9	10.59**	88.78	42.99**	88.9

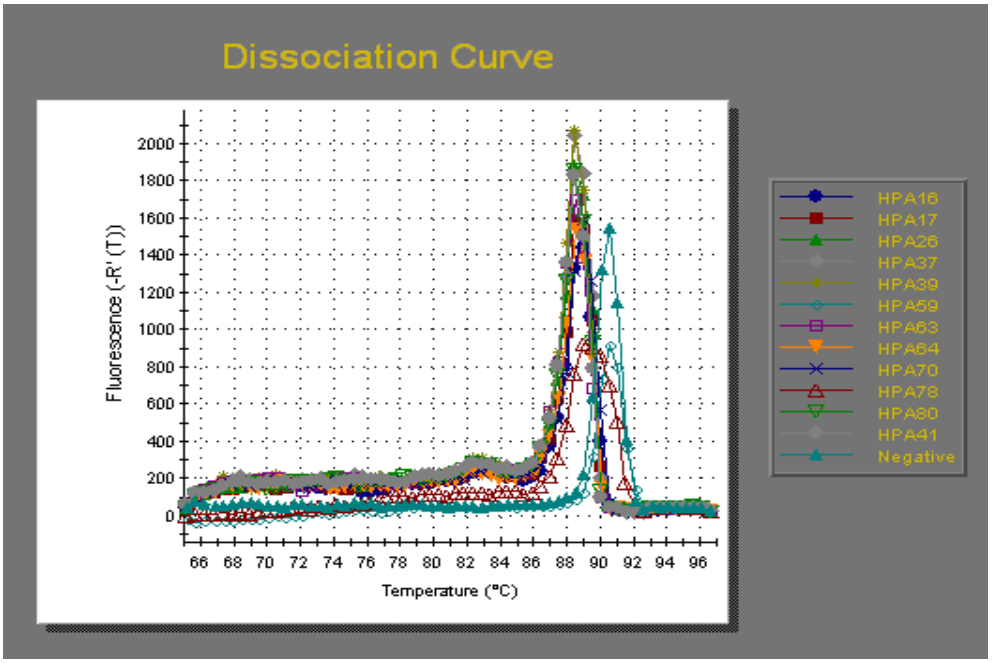
Appendix VIII - Table 4. Testing of CMY primer set 5 to assess specificity. Ct and Tm values are averages of triplicate wells and those highlighted in blue are results for *bla*$_{CMY}$ positive isolates and those with a red border were negative by LAMP assay and *bla*$_{CMY}$ PCR.

HPA #	Strain Ref	Species	OXA types		Other Beta-lactamase genes			Average of three replicates	
			1	2	1	2	3	Ct	Tm (◦C)
5	74560385	*Acinetobacter baumannii*	Oxa51	Oxa58				36.48	90.92
6	90640804	*Acinetobacter baumannii*	Oxa40	Oxa51				29.92	91.46
7	NCTC13302	*Acinetobacter baumannii*	Oxa40	Oxa51				34.22	90.92
8	NCTC13304	*Acinetobacter baumannii*	Oxa23	Oxa51				31.57	90.96
9	NCTC13305	*Acinetobacter baumannii*	Oxa51	Oxa58				30.71	90.45
10	NCTC13420	*Acinetobacter baumannii*	Oxa51					32	90.49
11	NCTC13421	*Acinetobacter baumannii*	Oxa23	Oxa51				36.57	90.99
12	NCTC13424	*Acinetobacter baumannii*	Oxa23	Oxa51				32.18	90.47
16	CIT	*Citrobacter freundii*			*bla*$_{CMY}$			10.32	88.85
17	84520159	*Citrobacter freundii*			*bla*$_{CMY}$	NDM		7.32	89.38
26	85120312	*Enterobacter* sp			*bla*$_{CMY}$	NDM		6.79	88.92
37	84560680	*Escherichia coli*			*bla*$_{CMY}$	NDM		7	88.9
39	90220853	*Escherichia coli*			*bla*$_{CMY}$	NDM		7.5	88.9
46	74560386	*Klebsiella pneumoniae*	Oxa48					29.25	91
47	80820413	*Klebsiella pneumoniae*	Oxa48					31.49	90.45
48	81320179	*Klebsiella pneumoniae*	Oxa48					36.86	91
49	82240675	*Klebsiella pneumoniae*	Oxa48					14.6	91.45
50	84080146	*Klebsiella pneumoniae*	Oxa48					36.64	91.45
51	84140235	*Klebsiella pneumoniae*	Oxa48					26.56	90.92
52	84220315	*Klebsiella pneumoniae*	Oxa48					30.36	90.45
53	84340096	*Klebsiella pneumoniae*				NDM		30.82	90.96
56	85240324	*Klebsiella pneumoniae*	Oxa48					30.06	90.46
57	85240325	*Klebsiella pneumoniae*	Oxa48					32.56	91

59	90300671 = NCTC 13443	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM1		34.45	91.53
63	90480355	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM		32.89	90.95
64	90480398	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM		7.02	88.88
65	90540829	*Klebsiella pneumoniae*	Oxa48					31.68	90.42
66	90640544	*Klebsiella pneumoniae*	Oxa48					27.91	90.96
70	91220314	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM		7.22	88.9
71	91340362	*Klebsiella pneumoniae*				NDM		16.61	88.9
72	91340363	*Klebsiella pneumoniae*				NDM		36.07	91
73	91340364	*Klebsiella pneumoniae*				NDM		50	90.4
75	NCTC13439	*Klebsiella pneumoniae*					VIM1	40.48	90.44
76	NCTC13440	*Klebsiella pneumoniae*					VIM1	52.21	90.44
77	81040548 = NCTC 13442	*Klebsiella pneumoniae*	Oxa48					45.04	90.47
78	92000514	*Klebsiella pneumoniae*		Oxa48	*bla*$_{CMY}$			50.37	89.95
80	91000848	*Klebsiella* sp.			*bla*$_{CMY}$	NDM		8.12	88.45
93	NCTC13437	*Pseudomonas aeruginosa*					VIM10	48.08	90.47
95	VIM	*Pseudomonas aeruginosa*					VIM1	47.27	90.03
96	90700129	*Pseudomonas fluorescens /*					VIM?	50.12	90.91
N/A	B2317	*Salmonella* spp.			*bla*$_{CMY}$			8.31	88.4

Appendix VIII - Figure 1. Amplification and dissociation curves for twelve bla_{CMY} positive isolates tested during this study.





26. Appendix IX - Development and initial validation of a loop-mediated isothermal amplification (LAMP) assay for the detection of New Delhi Metallo-ß-lactamase-1 (*bla*$_{NDM-1}$)

Summary of results

- Two published primer sets were tested for sensitivity and specificity.
- Sensitivity testing demonstrated that the Qi primer sets could reliably detect the gene at a level of 1,000 copies/µl, while the level of detection for the Liu primers was 10-fold higher.
- Specificity testing demonstrated that the Liu and Qi primers detected in the NDM gene in 9/12 of the isolates determined as positive by array. However, the three isolates negative by LAMP assay were also PCR negative for the NDM gene.
- All isolates negative by array for the NDM gene were also negative in both NDM LAMP assays.

Background

New Delhi Metallo-ß-lactamase-1 (*bla*$_{NDM-1}$) is a newly emerging problem. It was first reported in 2009 [134] and is of particular concern as it is able to confer resistance to the carbapenems in addition to most other beta-lactam antibiotics. It is most commonly associated with *Klebsiella pneumoniae* and *Escherichia coli* but has been detected in other bacterial species. There are currently ten *bla*$_{NDM}$ genes deposited in the Lahey clinic repository for gene numbering (http://www.lahey.org/Studies/other.asp#table1).

Current available methods

Two LAMP assays have been published for NDM-1 detection both in 2012 [21; 22]. Both methods were designed using the NDM-1 sequence (FN396876.1) as the template. In addition, Qi *et al*. (2012) also used the NDM-1 sequences AB571289.1, HM853678.1 and HQ171206.1. Each primer set is designed to detect a different region of the NDM-1 gene therefore both primer sets were tested at the APHA to determine their sensitivity and specificity. Sequence alignments of all available NDM sequences demonstrated that the sequence amplified by the Qi primers was completely conserved among all NDM genes. While for the Liu primer set there are

single nucleotide changes in NDM-7 (JX262694) and NDM-8 (AB744718) in the FIP primer sequence.

METHODS

LAMP assay method

Genomic DNA was isolated from a panel of 41 isolates. LAMP assays were performed as described here; each 25µl reaction consisted 15µl of LAMP assay master-mix, 6µl of water, and 5pMol of the EPS, 20pMol of Loops and 40pMol of IPS. One microlitre of genomic DNA was added either neat or diluted 1:1000. The cycling run consisted of 60 cycles of 65◦C for 1 min with reads taken at the end of each cycle to monitor amplification and 1 cycle of 95◦C for 1 min with continuous reading taken to determine melting temperature (Tm).

DNA preparation for sensitivity testing

For sensitivity testing a PCR product of the NDM-1 gene was prepared. PCR was performed using the primers (NDM-1-F(132) and NDM-1-R(794)) described in Table 1, which were designed for this purpose. Each reaction consisted of; 1X PCR buffer (Promega), 3mM MgCl2, 200uM dNTP of each, 20pMol of each primer, and 1.25U GoTaq DNA polymerase. PCR cycling conditions consisted of; one cycle of 95◦C for 5min, followed by 30 cycles of 95◦C 30sec, 60◦C 30sec, and 72◦C 45sec, followed by one cycle of 72◦C for 7min. The expected product has a size of 663bp and the presence of the correct band was confirmed by visualisation of the product on a 2% agarose gel. PCR product was cleaned using the Promega Wizard PCR clean-up kit prior to use. The DNA concentration was determined using the Nanodrop and this value along with product size (663bp) was used to calculate the number of copies. The website; http://cels.uri.edu/gsc/cndna.html was used for this calculation. A dilution series was prepared for the 663bp NDM-1 PCR product from a starting concentration of $3.81x10^{11}$ copies/µl to $1x10^{-1}$ copies/µl.

RESULTS

Sensitivity testing

To test the sensitivity of each assay a PCR product was prepared of the NDM-1 gene. A 663bp region from base 132 to 794 was prepared using HPA 37 as the template. The PCR product was cleaned, the concentration determined and this was used to estimate the number of copies of NDM-1. A ten-fold dilution series was then prepared between the initial concentration of $3.8x10^{11}$ copies/µl and $1x10^{-1}$ copies/µl. Each dilution was then tested with both primer sets to estimate the sensitivity of each assay (Table 2.). Using the Qi primers, a sensitivity of 500 copies/µl was achieved, although only 2/3 replicates were positive and the Ct values (for positive replicates) at this DNA concentration were between 22.43 and 24.1 (Table 3, Figure 1.). The assay was repeated on several occasions and it was possible to detect as few as 10 copies/µl but this was not consistent between runs. Therefore the results suggest that the sensitivity of the assay is between 500 and 1000 copies/µl. The Liu primer set was also tested in the same manner. It was very difficult to determine the sensitivity of this assay as in all reactions, even in the absence of DNA; the Tm of the product was identical. However, the Ct values suggest that the sensitivity may be 10,000 copies/ µl, as this is where a large change in Ct values in observed (Table 2.).

Specificity testing

Both sets of primers were tested for there ability to specifically detect NDM in a panel of 41 isolates. In total, 13 NDM positive isolates were included in this panel (Table 4.) and the Qi and Liu primers were both able to detect 10/13 of these isolates. All NDM negative isolates (n= 28) were also negative in both NDM LAMP assays. For the Qi primer set the lowest cycle threshold (Ct) value among NDM negative isolates was 30.26, while for the Liu primer set this value was 19.55. It was also found that for the Liu primer set the product generated from the negative isolates had a similar Tm to those from NDM positive isolates. The Liu primers were quicker in generating a signal with Ct values ranging from 7.9-9.64 in NDM positive isolates. For the Qi primers the range of Ct values was 10.85-13.89 (Table 4.). Three isolates previously determined to be NDM-1 positive were negative in both LAMP assays. A PCR was performed with the primers NDM-1-F(132) and NDM-1-R(794) to determine if the

isolates were still positive. All three isolates were negative by PCR suggesting a loss of the plasmid carrying the gene and confirming the results of the LAMP assay.

Appendix IX - Table 1. Published primer sets tested during this study and primers designed for amplification of NDM-1.

Primer	Sequence	Author
CJXJ1F3	GCATAAGTCGCAATCCCCG	Liu *et al.* (2012)
CJXJ1B3	GGTTTGATCGTCAGGGATGG	
CJXJ1FIP	CTGGCGGTGGTGACTCACGTTTTGCATGCAGCGCGTCCA	
CJXJ1BIP	CGCGACCGGCAGGTTGATCTTTTGGTCGATACCGCCTGGAC	
CJXJ1LF1	GCATCAGGACAAGATGGGC	
CJXJ1LB1	TCCAGTTGAGGATCTGGGC	
F3	GGCCACACCAGTGACAAT	Qi *et al.* (2012)
B3	GCGGAATGGTCATCACGAT	
FIP	ACTTGGCCTTGCTGTCCTTGATGTTGGGATCGACGGCAC	
BIP	CGCTCGGCAATCTCGGTGATGCTGGCCTTGGGGAACGC	
LF1	GCTGTAGCGAAAACCACCG	
LB1	ACACTGAGCACTACGCCG	
NDM-1-F(132)	ACGGTTTGGCGATCTGGTTTTC	This study
NDM-1-R(794)	ATGCGGGCCGTATGAGTGATTG	

Appendix IX - Table 2. Results of testing to determine the sensitivity of the published NDM-1 LAMP assay, the test was performed using a PCR amplicon of the NDM-1 gene.

Number of copies of PCR product	Qi primers		Qi primers (repeat)		Qi primers (repeat 2)		Liu primers	
	Ct	Tm	Ct	Tm	Ct	Tm	Results Ct	Results Tm
$3.8x10^{11}$	5	93.58			4.53	93.47	5.06	92.96
$1x10^{11}$	5.11	93.08			4.68	93.47	5.18	93
$1x10^{10}$	6.41	93.08			5.3	92.97	5.77	93
$1x10^{9}$	8.44	93.09			6.73	93.03	6.78	93.05
$1x10^{8}$	9.87	93.1	11.85	93.55	7.87	93.03	8.04	93.03
$1x10^{7}$	11.43	93.13	13.23	93.55	8.58	93.05	9.81	93.05
$1x10^{6}$	12.71	93.13	15.01	93.59	10	93.05	11.88	93.08
$1x10^{5}$	14.63	93.65	17.61	93.6	10.95	93.08	15.1	93.08
$1x10^{4}$	17.86	93.58	20.8	93.08	12.22	93.47	17.95	92.96
$1x10^{3}$	25.42	93.08	50.45	91.6	13.85	93.47	22.81	93
$7.5\ x10^{2}$			27.35**	93.59				
$5x10^{2}$	23.92**	93.13	58.31	91.03	13.75	93.05	27.64	93.08
$2.5x10^{2}$	47.78	91.1	No Ct	94.58	14.86	93.58	27.06	93.58
$1x10^{2}$	57.07	91.03	56.41	91.6	16.29	93	26.92	93.03
$1x10^{1}$	58.7	91.05	58.79	92.1	16.43	93.03	27.76	93.05
$1x10^{0}$	54.59	91.08			20.45	93.03	27.79	93.03
$1x10\text{-}^{1}$	49.98	91.08			39.23	91.01	27.75	93.03

Appendix IX - Table 3. *Values for replicates at 10000, 1000, 500 and 250 copies/ul using Qi primer set.*

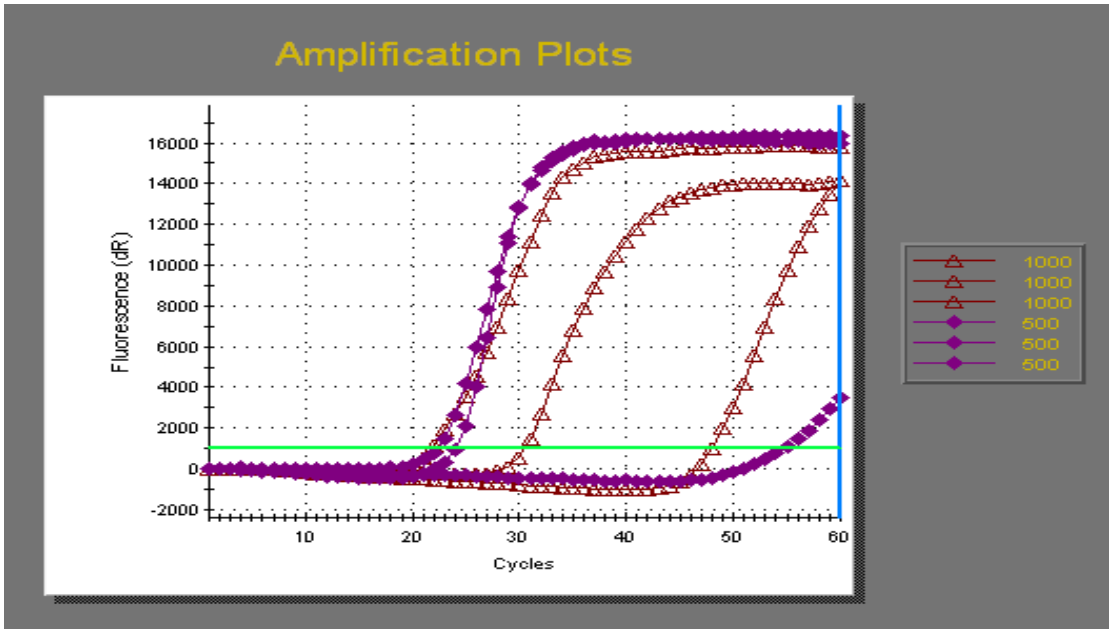
Number of copies/ul	*Replicate 1*		*Replicate 2*		*Replicate 3*	
	Ct	*Tm*	*Ct*	*Tm*	*Ct*	*Tm*
10,000	*18.11*	*93.58*	*17.08*	*93.58*	*18.92*	*93.58*
1000	*21.81*	*93.08*	*30.64*	*93.58*	*48*	*93.08*
500	*24.1*	*93.13*	*22.43*	*93.13*	*54.89*	*91.09*
250	*46.48*	*91.1*	*47.41*	*91.1*	*52.75*	*91.6*

Appendix IX - Table 4. Results of specificity testing with both published NDM-1 primer sets. Results highlighted in blue are those known NDM positive isolates tested in this study. Those boxed in red are where LAMP assay was negative. Ct and Tm values are the average of three replicates.

HPA #	Strain	Species	OXA Types		Other Beta-lactamase genes			Qi Primer set		Liu Primer set	
			1	2	1	2	3	Ct	Tm	Ct	Tm
5	74560385	*Acinetobacter baumannii*	Oxa51	Oxa58				49	91.45	26.74	93.56
6	90640804	*Acinetobacter baumannii*	Oxa40	Oxa51				53.01	91.46	26.73	93.59
7	NCTC13302	*Acinetobacter baumannii*	Oxa40	Oxa51				56.87	90.96	27.45	93.61
8	NCTC13304	*Acinetobacter baumannii*	Oxa23	Oxa51				51.32	91.5	26.56	93.61
9	NCTC13305	*Acinetobacter baumannii*	Oxa51	Oxa58				32.92	93.05	27.24	93.09
10	NCTC13420	*Acinetobacter baumannii*	Oxa51					54.85	91.03	25.56	93.13
11	NCTC13421	*Acinetobacter baumannii*	Oxa23	Oxa51				55.84	91.03	25.2	93.13
12	NCTC13424	*Acinetobacter baumannii*	Oxa23	Oxa51				45.52	91.55	22.1	93.67
16	CIT	*Citrobacter freundii*			bla_{CMY}			38.39	91.45	26.2	93.59
17	84520159	*Citrobacter freundii*			bla_{CMY}	NDM		11.72	93	9.64	93.06
26	85120312	*Enterobacter* sp			bla_{CMY}	NDM		10.91	93	8.66	92.58
37	84560680	*Escherichia coli*			bla_{CMY}	NDM		11.32	93.03	8.72	92.58
39	90220853	*Escherichia coli*			bla_{CMY}	NDM		11.48	93.05	9.05	92.59
46	74560386	*Klebsiella pneumoniae*	Oxa48					47.86	91.53	27.27	93.13
47	80820413	*Klebsiella pneumoniae*	Oxa48					52.64	91.53	19.55	93.15
48	81320179	*Klebsiella pneumoniae*	Oxa48					30.26	91.03	26.25	93.67
49	82240675	*Klebsiella pneumoniae*	Oxa48					54.76	90.95	26.12	93.59
50	84080146	*Klebsiella pneumoniae*	Oxa48					52.03	90.95	24.46	93.06
51	84140235	*Klebsiella pneumoniae*	Oxa48					51.83	92.47	26.41	93.1
52	84220315	*Klebsiella pneumoniae*	Oxa48					52.62	91.5	23.16	93.1
53	84340096	*Klebsiella pneumoniae*				NDM		No Ct	93.56	26.27	93.1
56	85240324	*Klebsiella pneumoniae*	Oxa48					45.89	91.53	21.31	93.13

57	85240325	*Klebsiella pneumoniae*	Oxa48					45.99	92.04	22.95	93.15
59	90300671 =	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM1		34.9	91.55	26.8	93.15
63	90480355	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM?		49.88	90.95	27.01	93.59
64	90480398	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM		11.52	93.53	8.87	93.06
65	90540829	*Klebsiella pneumoniae*	Oxa48					42.89	92.5	22.73	93.1
66	90640544	*Klebsiella pneumoniae*	Oxa48					54.26	90.46	25.66	93.1
70	91220314	*Klebsiella pneumoniae*			*bla*$_{CMY}$	NDM		11.39	93.05	9.15	92.6
71	91340362	*Klebsiella pneumoniae*				NDM		10.85	93.03	9.05	92.63
72	91340363	*Klebsiella pneumoniae*				NDM		11.35	93.09	9.61	92.65
73	91340364	*Klebsiella pneumoniae*				NDM		13.09	93.46	7.9	93.49
75	NCTC13439	*Klebsiella pneumoniae*					VIM1	47.61	66.17	21.52	93.49
76	NCTC13440	*Klebsiella pneumoniae*					VIM1	46.71	92.47	23.47	93.5
77	81040548 =	*Klebsiella pneumoniae*	Oxa48					44.38	91.47	21.17	93.58
78	92000514	*Klebsiella pneumoniae*		Oxa48	*bla*$_{CMY}$			57.26	91.97	22.91	93.05
80	91000848	*Klebsiella* sp.			*bla*$_{CMY}$	NDM		13.89	93.55	8.3	93.03
93	NCTC13437	*Pseudomonas aeruginosa*					VIM10	40.41	90.53	24.78	93.54
95	VIM	*Pseudomonas aeruginosa*					VIM1	46.67	93.06	23.61	93.55
96	90700129	*Pseudomonas fluorescens*					VIM	47.68	67.24	23.24	93.49
N/A	B2317	*Salmonella* spp.			*bla*$_{CMY}$			58.63	91.44	23.18	93.49

Appendix IX - Figure 1. Graph showing the differential amplification profiles obtained with 500 and 1000 copies/µl of NDM-1 PCR product using the Qi primers.



27. Underpinning evidence

No more evidence to present.