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IMPART WP2 – FINAL RING TRIAL REPORT
SHORT SUMMARY

DETECTION OF CARBAPENEMASE-PRODUCING *ENTEROBACTERIACEAE*

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INTRODUCTION

Carbapenems are broad-spectrum beta-lactam antimicrobials, which are used as last-resort options for treatment of community-acquired and healthcare-associated infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Since carbapenem resistance results in resistance to nearly all beta-lactam antibiotics it narrows the therapeutic options dramatically. Carbapenems are prohibited in livestock in EU and are restricted to sporadic (off-label) use in companion animals. So far, carbapenemase-producing *Enterobacterales* (CPE) have been rarely detected in animals. Nevertheless, reports of CPE in livestock (pigs and broilers) on farms in Germany (1) underlined that livestock could constitute a potential source for the spread of CPE in the community. This was acknowledged by the European Food Safety Authority (EFSA) (2) and they advised to prioritize the monitoring of CPE in food-producing animals. With the enforcement of Commission Implementing Decision 2013/652 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (3), monitoring of ESBL/AmpC-producing *E. coli* became mandatory, but the monitoring of carbapenemase-producing (CP) *E. coli* was optional in the first years from 2014 through 2020. Screening for CP *E. coli* will be mandatory in the monitoring programmes from 2021. Simultaneously, the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) published protocols for isolation of ESBL/AmpC-producing and CP *E. coli* from caecal samples of slaughter animals and from meat. These protocols were mainly developed for the detection of ESBL/AmpC-producing *E. coli*. The sensitivity for detecting CP *E. coli* is unknown. As part of an early warning system a selective and sensitive culture method is needed to detect CPE in low concentrations in faecal samples of animals and in food.

Objectives

These were the aims for this ring trial:

1) To evaluate the performance of different selective agars described for the detection of CPE from samples of animal origin (meat and caecal samples) by performing a multicentre study, using the same enrichment protocol as described by the EURL-AR.
2) To isolate and perform correct species identification of the bacterial strains from each selective agar media
3) To perform correct genotyping of the presumptive CP strains.
4) To confirm phenotypic resistance to carbapenems.
5) Voluntary: To screen for genes causing carbapenemase production directly from the samples (overnight broth) by PCR.

STUDY DESIGIN

Participating laboratories

Twelve laboratories were involved in the final ring trial performed in September 2019: the organizer French Agency for Food, Environmental and Occupational Health & Safety (ANSES) Fougères Laboratory and the following eleven participants of the IMPART consortium; Animal and Plant Health Agency (APHA), ANSES Lyon Laboratory, Federal Institute for Risk Assessment (BfR), Technical University of Denmark (DTU), Istituto Zooprofilattico Sperimentale del Lazio e della Toscana (IZSLT),

Norwegian Veterinary Institute (NVI), National Veterinary Research Institute (PIWET/PULAWY), National Institute for Public Health and the Environment (RIVM), Statens Serum Institut (SSI), National Veterinary Institute (SVA) and Wageningen Bioveterinary Research (WBVR).

Method

A pre-ring trial between only three of the participating laboratories was performed in the beginning of the project to eliminate options to test among the eleven participants in the final ring trial. During the pre-ring trial an elimination of several selective culturing methods was performed. There was an agreement that pre-enrichment in buffered-peptone water (BPW) should be performed at 37°C. Two of the criteria in the pre-ring trial were to test all selective agar plates available in ALL European countries and to test both ready-to-use and in-house media as far as possible. The latter was a criteria because several of the participating laboratories in IMPART have experienced difficulty of getting media in time from manufacturers, and there is generally a three to four week delay in delivery from the day of ordering the media until it is delivered. Regarding the selective agars, seven different selective agar media were tested, two of which were tested as both ready-to-use and in-house, and those with the best performance were retested in this final ring trial.

Table 1. Overview of the selective agar plates available at the market for the WP2 pre-ring trial.

NAME	PRODUCER	READY-TO-USE	IN-HOUSE
Brilliance™ CRE Agar	Oxoid	Yes	No
Brilliance™ ESBL/CRE, bi-plate*		Yes	No
CHROMID® CARBA Agar	bioMérieux	Yes	No
CHROMID® OXA-48 Agar		Yes	No
CHROMID® CARBA SMART Agar, bi-plate*		Yes	No
Chromatic™ CRE	Liofilchem	Yes	Yes
Chromatic™ OXA-48		Yes	No
Chromatic™ CRE/OXA-48, bi-plate*		Yes	No
CHROMagar™ mSuperCARBA™	CHROMagar	No	Yes
ChromArt CRE	BioLife	Yes	Yes
ChromArt OXA-48		Yes	Yes
HardyCHROM™ CRE Agar	HardyDiagnostics	Yes	Yes
Remel Spectra™ CRE	Thermo Fisher	Yes	No

*bi-plates not included in the pre- or final ring trial, also available as single plates

An overview of selective agar plates available on the market for detecting CPE is shown in Table 1. Selective agar plates which did not provide a full plate surface for isolates, like bi-plates, were ruled out. This is the reason why, none of the three bi-plates, *Brilliance*™ ESBL/CRE (Oxoid, Thermo Fisher Scientific, Basingstoke, Hampshire, United Kingdom), CHROMID® CARBA SMART Agar (bioMérieux, Marcy l'Étoile, France) or Chromatic™ CRE/OXA-48 (Liofilchem, Teramo, Italy) were selected for the pre- or final ring trial. The three selective agar plates Remel Spectra™ CRE agar (Thermo Fisher, Thermo Fisher Scientific, Waltham, Massachusetts, USA), ChromArt CRE and ChromArt OXA-48 (both Biolife, Milan, Italy) were left out of the final ring trial because they were not available in all European countries.

The performance of the ready-to-use versus the in-house made selective agars from Chromatic™ CRE (Liofilchem) did not give any significant difference in the pre-ring trial study. For the final ring trial it was decided to only use the ready-to-use agar. The CHROMagar™ mSuperCARBA™ (CHROMagar™, Paris, France) was only available as an in-house media. For the final ring trial, we had an agreement with Mast Group (MAST DIAGNOSTICS, Amiens, France) that they would produce these selective agar plates and this would eliminate possible differences resulting from in-house production errors. The following six selective agar plates were included in the final ring trial:

- Brilliance™ CRE Agar (Oxoid)
- CHROMID® CARBA Agar (bioMérieux)
- CHROMID® OXA-48 Agar (bioMérieux)
- Chromatic™ CRE (Liofilchem)
- Chromatic™ OXA-48 (Liofilchem)
- CHROMagar™ mSuperCARBA™ (CHROMagar)

Material

During the pre-ring trial, no significant differences in results were observed related to matrices, meat and caecal content, or the animal origin of samples, pig and turkey. For the final ring trial, meat from turkey and caecal content from pig were used for spiking with target strains.

Preparation of samples

The samples were prepared at ANSES Fougères Laboratory in France. In total eight samples, six spiked samples and two blank samples, were included in the final ring trial, see Table 3. These included three spiked and one blank meat sample from specific pathogen free (SPF) turkey raised in ANSES farm, and three spiked and one blank caecal sample from pigs. The pig caecal samples were collected at a French slaughterhouse in the framework of the antimicrobial resistance programme in France.

Non-contaminated caecal samples from pig and meat samples from turkey were frozen at -20°C until spiked. All matrices were checked to be negative for CPE using the EURL-AR protocol (https://www.eurl-ar.eu/protocols.aspx, accessed on June 2019) (6, 7).

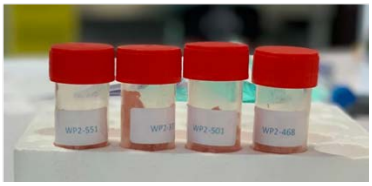
Strains were inoculated on blood agar and incubated overnight at 37°C. Prior to spiking, a 0.5 McFarland ($\approx 10^8$ CFU/ml) bacterial suspension was prepared in sodium chloride solution (0.9%) using fresh colonies. For each strain, the suspensions were diluted to obtain the target final concentration (100 CFU/g sample) in the pooled minced meat or caecal content samples. Contaminated samples were homogenized and aliquoted. The aliquots were stored at 4°C until shipping.

Table 3. Overview of the matrices and strains used to spike the samples in the final ring trial.

Sample	Matrix	Animal origin	Species	Carbapenemase gene
M-1	Meat	Turkey	BLANK	-
M-2	Meat	Turkey	*K. pneumoniae* ATCC BAA-1705	bla_{KPC-2}
M-3	Meat	Turkey	*E. coli* 16874	bla_{OXA-48}
M-4	Meat	Turkey	*S.* Kentucky 2014LSAL00827	bla_{NDM-1}
C-1	Caecal	Pig	BLANK	-
C-2	Caecal	Pig	*E. coli* NCTC 13476	bla_{IMP}
C-3	Caecal	Pig	*K. pneumoniae* NCTC 13442	bla_{OXA-48}
C-4	Caecal	Pig	*E. coli* R1180	bla_{VIM-1}

Shipping of samples

The samples were prepared at ANSES Fougères Laboratory (see illustration photo) on Friday September 6 2019 and shipped to the eleven participants on Monday September 9 2019 in compliance with UN3373 regulations at 4°C. The laboratories received the samples with a unique code for each sample. Analysis started immediately upon arrival of the samples.



Prior to the sample shipment the ring trial protocol was distributed per email. The six selective agar plates were shipped with the samples.

Protocol

The protocol is summarized in the work flow visualized in Figure 1. The first enrichment step is identical to the one recommended by the EURL-AR for the isolation of CP *E. coli* from caeca and meat samples, Version 6 (accessed on June 2019) (6, 7).

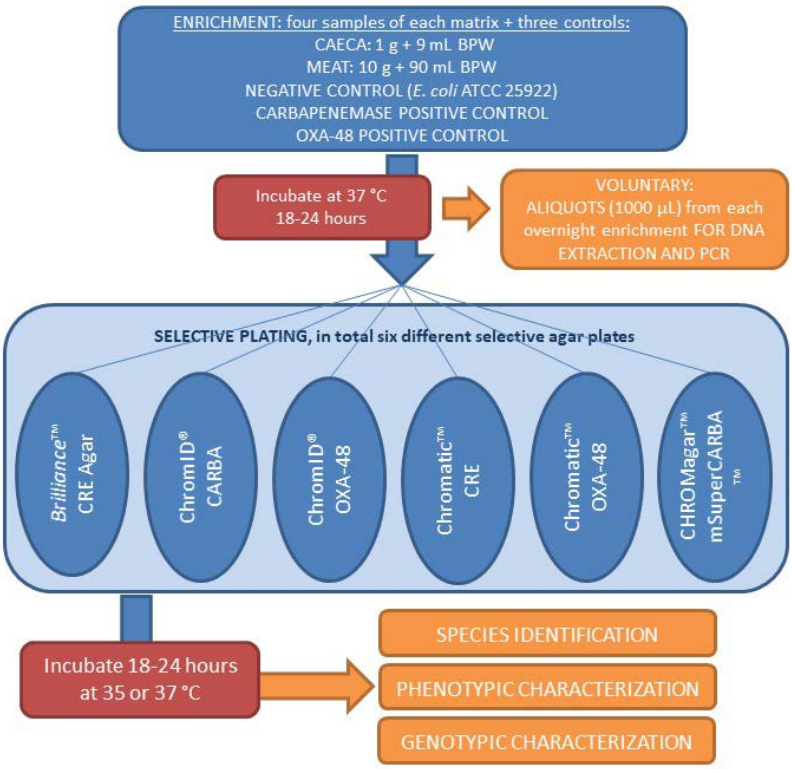


Figure 1. Schematic outline of the ring trial.

Control strains

E. coli ATCC 25922 was used as a susceptible quality control strain to validate the performance of each selective agar medium. To validate the selective agar performance on detecting carbapenem resistant bacteria, at least two control strains were included; one carrying a non-OXA-48 genotype and one carrying a bla_{OXA-48} genotype. Nine of the eleven laboratories used the two control strains distributed by the EURL-AR in 2014: *E. coli* TZ 3638 (bla_{GES-5}) and *E. coli* 16874 (bla_{OXA-48}). One lab used an internal bla_{KPC-3} positive *E. coli* 40336 and a bla_{OXA-48} positive *K. pneumoniae*, and one lab used a bla_{VIM-1} positive *E. coli* TZ 116 and the *E. coli* 16874 (bla_{OXA-48}) from the EURL-AR.

Detection steps

Direct PCR screening

As a voluntary step, direct PCR screening was performed by seven out of eleven laboratories on DNA extracts from the overnight enrichment broth.

Selective agar screening

Table 4 gives a description of which geno- /phenotypes each plate should detect, but there are some exceptions as some CP strains have low carbapenemase activity, like strains carrying bla_{VIM} or bla_{IMP} genes. Both CHROMID® CARBA and CHROMagar™ mSuperCARBA™ report that strains with low carbapenemase activity might not be detected on their agar plates, but this is not reported by the manufacturers of the Brilliance™ CRE Agar nor Chromatic™ CRE. Plates should be read according to the plate reading scheme in Table 5, which is based on the morphology described in the product

information by the manufacturers of the different selective agar plates. There was no information from any of the manufacturers regarding any colour on their respective media for colonies of *Salmonella* spp.

Table 4. Description of which genotypes included that should be detected on the different selective agar plates.

NAME	Carbapenemase genes, non-OXA-48 (NDM, VIM, KPC, IMP)	Oxacillinase gene (OXA-48)
Brilliance™ CRE Agar	YES	YES
CHROMID® CARBA Agar	YES[#]	NO
CHROMID® OXA-48 Agar	NO	YES
Chromatic™ CRE	YES	YES
Chromatic™ OXA-48	NO	YES
CHROMagar™ mSuperCARBA™	YES[#]	YES

[#]Reported issues of not detecting strains showing low level of carbapenemase-producing activity like strains carrying bla_{VIM} and bla_{IMP}.

Table 5. Description of the morphology of possible bacterial species on the selective agar plates. The grey fields is translated by the authors to more easily comprehend the table.

Species	*Brilliance*™ CRE Agar	CHROMID® CARBA and OXA-48	Chromatic™ CRE and OXA-48	CHROMagar™ mSuperCARBA™
Escherichia coli	1–2 mm, pale pink	Pink to burgundy	Red	Dark pink to reddish
CPE coliforms (other than *E. coli)*	Unknown/blue (*Klebsilla* sp.)	Spontaneous bluish-green to bluish-grey coloration	Blue-violet/blue-green/blue with red halo	Metallic blue
CPE *Pseudomonas*	Unknown	Unknown	Unknown	Translucent, +/- natural pigment cream to green
Acinetobacter	1–2 mm, colourless to cream	Unknown	Unknown	Cream
CR / OXA-48 non-*Enterobacterales*	Unknown	Unknown	White to natural pigmented	Colourless, natural pigmentation

Species identification

Species identification and confirmation should be performed on at least one colony from each selective agar plate where growth was detected. A maximum of **three colonies** should be selected from each selective agar plate, but species identification was only needed to be performed on one of them. If this colony was negative, the other two colonies should be tested. Species identification should be performed with the method normally used in each lab (MALDI-TOF MS, API20E and PCR).

Phenotypic identification

Susceptibility testing should be performed by either broth microdilution or disk diffusion method on isolates of either *E. coli, K. pneumoniae* or *Salmonella* from each positive sample by reporting meropenem as the mandatory agent.

Genotypic identification

Genotypic identification should be performed on all presumptive pure cultured CPE isolates by either PCR or real-time PCR to detect the following genotypes: bla_{IMP}, bla_{NDM}, bla_{KPC}, bla_{VIM} and bla_{OXA-48}. Methods used should be according to the protocol mentioned in Appendix 1 of the final ring trial or by using an in-house method.

Reporting the results

The reporting scheme for the ring trial was prepared by the NVI and sent to the participants as an excel file. One sheet in the excel file was available to report all information regarding the general testing, one sheet each per sample (in total eight), and one sheet for quality control strain (in total three strains).

SUMMARY OF RESULTS

All eleven participants of the multicentre study delievered their result in the excel spread sheet distributed together with the protocol. They all received a final report containing the results from their own laboratory and what was expected to be detected in the samples distributed. This was not a ring trial checking the performance of each lab. The multicentre study was conducted to test the protocol used at most national reference laboratory for antimicrobial resistance in the harmonized monitoring of CPE in caecal and meat samples of animal origin, using a variety of commercially available selective agar plates.

- All agars (**Brilliance™ CRE Agar**, **CHROMagar™ mSuperCARBA™**, **CHROMID® CARBA**, **Chromatic™ CRE and CHROMID® OXA-48**), except the **Chromatic OXA-48**, performed well in the trial to detect CPE strains.
- **Brilliance™ CRE Agar** did not detect the control strains, which might lead to unreliable results.
- **Direct PCR protocol** on DNA extracted from an enrichment of the sample works well for meat samples, but should be improved for caecal samples.
- The prescribed method is invalid to detect the bla_{VIM-1} positive strains included in this trial.
- To include the detection of bla_{VIM-1} positive strains, a selective enrichment step (including meropenem and cefotaxime) or culturing on an in-house made agar (MacConkey agar including meropenem and cefotaxime) should be added or validated for the detection of other CPE as well.

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