A European multicenter evaluation study to investigate the performance on commercially available selective agar plates for the detection of carbapenemase producing *Enterobacteriaceae*

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ABSTRACT

The European Food Safety Authority (EFSA) advised to prioritize monitoring carbapenemase producing *Enterobacteriaceae* (CPE) in food producing animals. Therefore, this study evaluated the performance of different commercially available selective agars for the detection of CPE using spiked pig caecal and turkey meat samples and the proposed EFSA cultivation protocol. Eleven laboratories from nine countries received eight samples (four caecal and four meat samples). For each matrix, three samples contained approximately 100 CFU/g CPE, and one sample lacked CPE. After overnight enrichment in buffered peptone water, broths were spread upon *Brilliance*™ CRE Agar (1), CHROMID® CARBA (2), CHROMagar™ mSuperCARBA™ (3), Chromatic™ CRE (4), CHROMID® OXA-48 (5) and Chromatic™ OXA-48 (6). From plates with suspected growth, one to three colonies were selected for species identification, confirmation of carbapenem resistance and detection of carbapenemase encoding genes, by methods available at participating laboratories. Of the eleven participating laboratories, seven reported species identification, susceptibility tests and genotyping on isolates from all selective agar plates. Agars 2, 4 and 5 performed best, with 100% sensitivity. For agar 3, a sensitivity of 96% was recorded, while agar 1 and 6 performed with 75% and 43% sensitivity, respectively. More background flora was noticed for turkey meat samples than pig caecal samples. Based on this limited set of samples, most commercially available agars performed adequately. The results indicate, however, that OXA-48-like and non-OXA-48-like producers perform very differently, and one should consider which CPE strains are of interest to culture when choosing agar type.

1. Introduction

Carbapenemase producing *Enterobacteriaceae* (CPE) are emerging in the health care sector all over the world, including Europe, and are already endemic in some areas such as in Mediterranean areas. In the European Union (EU), although regulations can differ between member countries, carbapenem use is prohibited in livestock and restricted to exceptional cascade use in companion animals (European Commission, 2015). Nevertheless, occurrence of CPE has been reported in pig and broiler farms in Germany and meat products in Belgium (Borowiak et al., 2017; Fischer et al., 2013; Garcia-Graells et al., 2020; Guerra et al., 2014), and was recently reported in Italian fattening pigs (Diaconu et al., 2020). Furthermore, CPE has also been reported in livestock from non-European countries, where they sometimes appear to be more common (Hamza et al., 2016; Li et al., 2019; Mollenkopf et al., 2018; Tang et al., 2019; Wang et al., 2017).

The occurrence of CPE in livestock indicates that livestock could constitute a potential reservoir for CPE, and in China transfer of CPE has been described between humans and livestock (Li et al., 2019). In addition, the EFSA recognized in 2013 that presence of CPE in food producing animals could constitute a reservoir for humans, and they therefore advised to prioritize monitoring CPE in food producing animals (European Food Safety Authority, 2013). However, the monitoring of carbapenemase producing (CP) *Escherichia coli* using selective isolation remained optional until 2021 when it became mandatory for the national monitoring programs in Decision 2020/1729/EU (European Commission, 2020).

In an early warning system, a selective and sensitive culture method to detect CPE is needed and such a method should be capable of detecting low concentrations (<100 CFU/g) of CPE in faecal samples of animals and in food samples. The method should also at least be able to detect CPE that produce OXA-48 like enzymes (OXA-48 CPE) as well as CPE that produce other carbapenemases like NDM, IMP, VIM, and KPC (non-OXA-48 CPE). As both these CPE-groups are the most common in human clinical settings and are the ones that primarily have been detected in food or animals (Bonardi and Pitino, 2019).

Several studies have already been conducted on evaluating the performance of different CPE selective agar media (Cohen Stuart et al., 2013; Garcia-Fernandez et al., 2017; Girlich et al., 2013a; Girlich et al., 2013b; Girlich et al., 2019; Gottig et al., 2020). However, to our knowledge, no cross-country multicenter studies have been performed, nor a study including all commercially available selective agar plates on the market. To this end, a consortium called IMPART (IMproving Phenotypic Antimicrobial Resistance Testing) within the One Health European Joint Programme (https://onehealthejp.eu/) of both veterinary and public health laboratories was established in 2018 focusing on the development of sensitive screening assays for emerging resistances such as CPE. The aim was to evaluate the performance, sensitivity, and specificity, of different commercial selective agars available for the detection of CPE from spiked samples of animal origin, i.e. meat and caecal samples. This was carried out by performing a multicenter study, using the enrichment protocol used in the EU Decision 2013/652/EU as described by the EURL-AR.

2. Material and methods

2.1. Participants

In total, twelve institutes (eleven participants and one organizer) from nine countries participated in the multicenter evaluation study conducted in 2019, see Table 1.

Before starting the multicenter evaluation study, all participants were asked to fill in a questionnaire on the methods used in their institute to detect CPE in samples (see Supplementary file 1).

Prior to the execution of the multicenter evaluation study, a pre-test was conducted in 2018 that involved four of the laboratories (Anses Fougères Laboratory, NVI, RIVM and WBVR). The purpose of this pre-test was to evaluate growth conditions and available selective agar plates in order to narrow down test conditions for the eleven participants in the main multicenter evaluation study (see Supplementary file 2 and for the report see https://zenodo.org/record/3676437#.YW1r-xpBxPY). In summary the pre-test showed that: i) incubating selective agar plates at 44 °C compared to 35–37 °C resulted in false negative samples, ii) ready-to-order plates gave the same results as in-house made selective agar plates from the same manufacturer, and iii) there was no interference by the matrix i.e. caecal and meat, to detect the spiked isolate.

2.2. Matrices and strains

Meat from specific pathogen free (SPF) turkey and caecal samples from pigs collected at a French slaughterhouse were used. Matrices were checked to be negative for CPE using the protocol from the EURL-AR (accessed on June 2019) (Hasman et al., 2018a; Hasman et al., 2018b). Verified CPE free samples were frozen at -20 °C until spiked. In total eight samples, six spiked samples and two blank samples were prepared (Table 2).

Strains used for spiking are listed in Table 2, and inclusion criteria for the strains were: i) strains should be well defined (reference strains), ii) strains should carry a CPE gene, and iii) included strains should express different minimum inhibitory concentration (MIC) values to the carbapenems meropenem, ertapenem and imipenem. Antimicrobial susceptibility towards beta-lactam antibiotics including different carbapenems was determined by the organizer using the broth microdilution method with the Sensititre™ TREK EUVSEC2 panel (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Strains were grown on blood agar and incubated overnight at 37 °C. From the overnight cultures, a 0.5 McFarland ($\approx10^8$ CFU/mL) bacterial suspension for each strain was prepared in saline (0.9% NaCl). Suspensions were then diluted to obtain the target final concentration (100 CFU/g sample) in the pooled minced meat or caecal content samples. Spiked samples were homogenized in a mixer for meat and with a stirrer for caecal content for approximately 5 min and aliquoted on a Friday. All aliquots were stored at 4 °C until shipping the following Monday at 4 °C in accordance with UN3373 regulation. For each sample, homogeneity was tested on ten aliquots randomly selected from the positive test samples and on three aliquots randomly selected from the negative test samples. The stability of each sample was tested on three aliquots randomly chosen among positive test samples. Stability was assessed by comparing the data from the day of shipment (data obtained in the homogeneity study) and from one day after reception and analysis of the samples by the participating laboratories. All laboratories were instructed to start analyses immediately

Table 1
Overview of participating institutes.

No.	Participant lab	Country
1	Anses, Fougères Laboratory	France (organizer)
2	Norwegian Veterinary Institute (NVI)	Norway
3	Anses, Lyon Laboratory	France
4	Statens Serums Institut (SSI)	Denmark
5	National Veterinary Institute (SVA)	Sweden
6	Technical University of Denmark (DTU)	Denmark
7	German Federal Institute for Risk Assessment (BfR)	Germany
8	National Institute for Public Health and the Environment (RIVM)	The Netherlands
9	Animal and Plant Health Agency (APHA)	United Kingdom
10	Państwowy Instytut Weterynaryjny (PIWET)	Poland
11	Istituto Zooprofilattico Sperimentale del Lazio e della Toscana *M. Aleandri* (IZSLT)	Italy
12	Wageningen Bioveterinary Research (WBVR)	The Netherlands

upon arrival of the samples.

2.3. Selective CPE agar plates

To be included in the multicenter evaluation study, the commercial selective agar plates had to fulfill the following criteria: i) plates should be available in all countries, ii) they should be available as ready-to-use plates, iii) no bi/dual plates or similar should be included. One exception to the criteria was done for the CHROMagar™ mSuperCARBA™ which is only available as an in-house medium. To avoid any preparation biases of the CHROMagar™ mSuperCARBA™ plates these were prepared for the participating laboratories by the MAST group (MAST DIAGNOSTICS, Amiens, France). Table 3 lists commercial agar plates included and whether the plate detects OXA-48 CPE, non-OXA-48 CPE or both.

2.4. Protocol

Fig. 1 outlines the protocol used in this multicenter evaluation study. In brief, all samples were diluted 1:10 in buffered peptone water (BPW) and incubated overnight at 37 °C. Per lab, three control strains, one strain without a carbapenemase gene, one with an OXA-48-like carbapenemase gene and one with a non-OXA-48-like carbapenemase gene (Table 4) were included to validate growth on the selective agar plates according to the validation protocol described by the EURL-AR (Hasman et al., 2015). The next day, 10 μL of each enrichment was plated on each of the six agar plates. After overnight incubation at 35 °C or 37 °C (depending on the recommendation for the selective agar plate) a maximum of three suspected CPE colonies were picked from each plate according to the colony morphology described by the manufacturer. Suspected colonies were sub-streaked on the respective selective agar plate and blood agar. On single colonies species identification was performed according to the methodology available at the participating laboratory (see Supplementary file 3), with Matrix-assisted laser desorption ionization – time-of-flight mass spectrometry (MALDI-TOF MS) being the most common method used (nine out of eleven laboratories). On isolates with the correct species, i.e. *Klebsiella pneumoniae*, *Salmonella* spp. or *E. coli,* susceptibility testing was performed by either broth microdilution or disk diffusion, with the minimum criteria of including meropenem. On all presumptive pure cultured CPE isolates, genotypic identification was performed by either PCR or real-time PCR, using established protocols at the participating laboratories, which at a minimum included the following genotypes: bla_{IMP}, bla_{NDM}, bla_{KPC}, bla_{VIM} and bla_{OXA-48} (for details on PCRs see Supplementary file 3). For reporting the results of each step, all participants were provided with a pre-prepared report scheme.

Table 3
Selective agar plates included in the multicenter evaluation study.

Name	Producer	Ready-to-use or In-house media	CPE detected
Brilliance™ CRE Agar	Oxoid	Ready-to-use	Non-OXA-48 and OXA-48
CHROMID® CARBA Agar	bioMérieux	Ready-to-use	Only non-OXA-48
CHROMID® OXA-48 Agar	bioMérieux	Ready-to-use	Only OXA-48
Chromatic™ CRE	Liofilchem	Ready-to-use	Non-OXA-48 and OXA-48
Chromatic™ OXA-48	Liofilchem	Ready-to-use	Only OXA-48
CHROMagar™ mSuperCARBA™	CHROMagar	In-house*	Non-OXA-48 and OXA-48

* Manufactured by MAST DIAGNOSTICS, Amiens, France, for the multicenter evaluation study.

3. Results

3.1. Questionnaire

In total eleven institutes returned the questionnaire (see Supplementary file 1). Eight laboratories normally used pre-enrichment of the samples overnight in BPW at 37 °C and one laboratory used Tryptic Soy Broth (TSB), while one laboratory normally did not use pre-enrichment and one laboratory never cultured samples for CPE. Two of the eight laboratories performing the BPW pre-enrichment, also reported the use of a second selective enrichment step. One of these two laboratories followed the BPW pre-enrichment using a lysogenic broth with 1 mg/L cefotaxime incubated at 44 °C prior to plating on selective agars, while the other laboratory followed the BPW pre-enrichment using BPW with 0.25 mg/L ertapenem and 50 mg/L vancomycin incubated at 37 °C prior to their PCR detection procedure. Regarding selective agar plates used at the different laboratories, all laboratories reported that they used agar plates from bioMérieux, either both the CHROMID® CARBA and the CHROMID® OXA-48 as separate plates, or the combined bi/dual plate CHROMID® CARBA SMART®. In addition, one laboratory also used the CHROMagar™ KPC.

3.2. Validation of CPE selective agar plates using reference strains

To validate the selective agar plates, all laboratories used the *E. coli* ATCC 25922 as a carbapenem-susceptible control strain, and none of the eleven participants reported growth of this strain on any of the six selective agar plates (Table 4). All data from the multicenter study is presented in Supplementary file 4.

To validate growth of OXA-48 strains on the selective agar plates, ten of eleven laboratories used the strain *E. coli* 16874 bla_{OXA-48} distributed by the EURL-AR, which is used for validation of the selective agars in the
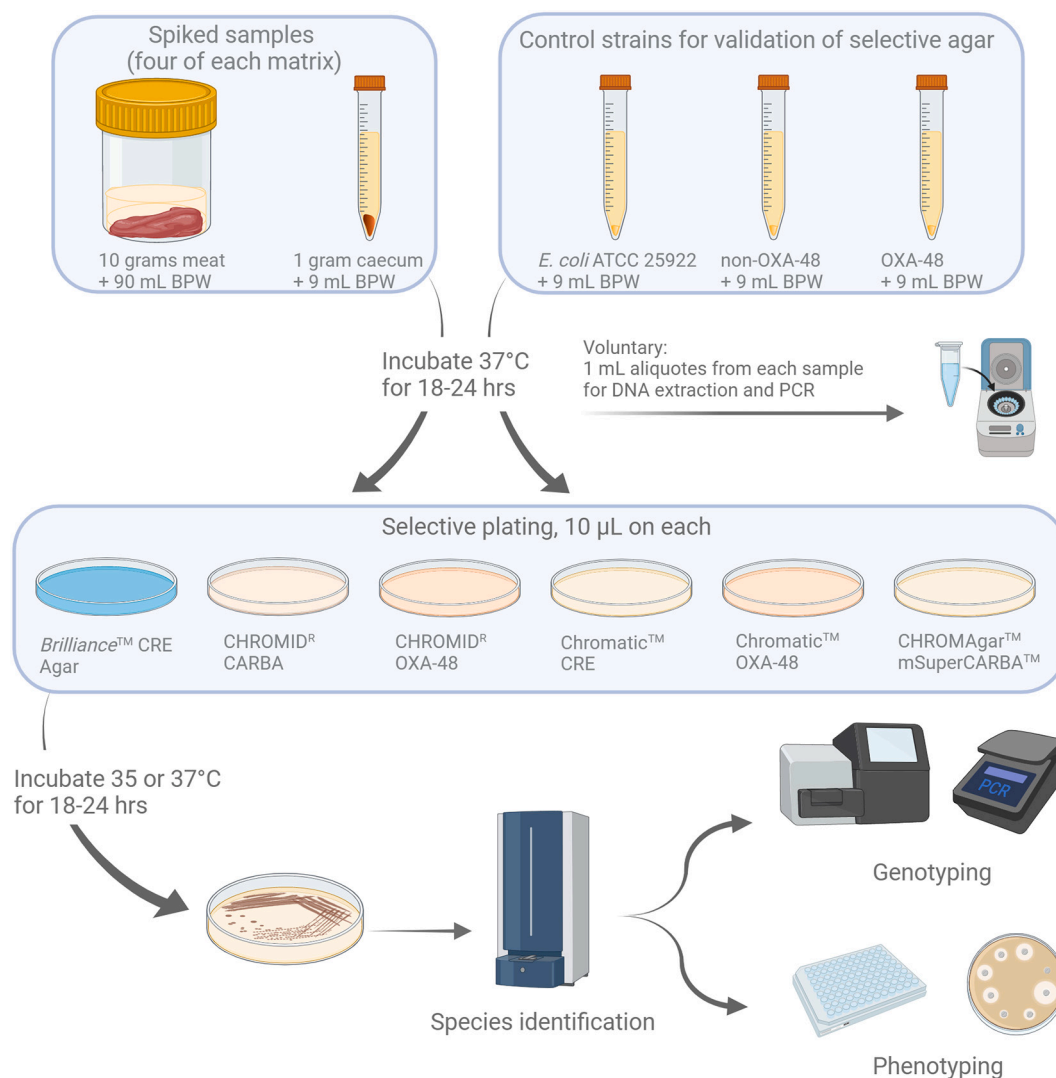
Table 2
Overview of samples, matrices and bacteria/gene combinations.

Sample	Matrix	Matrix origin	Bacterial species	Carbapenemase gene	MEM[#] MIC (mg/L)	ERT[#] MIC (mg/L)	IMI[#] MIC (mg/L)	Reference
C-1	Caecal	Pig	BLANK	–	–	–	–	–
C-2	Caecal	Pig	*E. coli* NCTC 13476	bla_{IMP}	2	>2	2	NCTC
C-3	Caecal	Pig	*K. pneumoniae* NCTC 13442	bla_{OXA-48}	4	>2	16	NCTC
C-4	Caecal	Pig	*E. coli* R1180	bla_{VIM-1}	0.25	0.06	4	(Irrgang et al., 2017)
M-1	Meat	SPF* Turkey	BLANK	–	–	–	–	–
M-2	Meat	SPF* Turkey	*K. pneumoniae* ATCC BAA-1705	bla_{KPC-2}	>16	>2	16	ATCC
M-3	Meat	SPF* Turkey	*E. coli* 16874	bla_{OXA-48}	1	2	2	(Hasman et al., 2015)
M-4	Meat	SPF* Turkey	*S.* Kentucky 2014LSAL00827	bla_{NDM-1}	2	1	4	(Hawkey et al., 2019)

* SPF - specific pathogen free.
[#] All MICs were performed at Anses, Fougères Laboratory, France. (MEM) indicates meropenem, (ERT) indicates ertapenem, and (IMI) indicates imipenem.

Fig. 1. Schematic outline of the multicenter evaluation study.
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EU monitoring program (European Commission, 2020; Hasman et al., 2015). One laboratory used a *K. pneumoniae* 32516 *bla*$_{OXA-48}$ strain. *E. coli* 16874 strain was detected on the plates expected to detect *bla*$_{OXA-48}$ isolates (i.e. CHROMID® OXA-48, Chromatic™ OXA-48, Chromatic™ CRE, and CHROMagar™ mSuperCARBA™), but not on *Brilliance*™ CRE Agar in any of the ten laboratories using this strain. However, this strain should not be able to grow on the included non-OXA-48 selective agar CHROMID® CARBA Agar growth was recorded in four laboratories, although three of them reported growth of only a few colonies. *K. pneumoniae* 32516 has a higher meropenem MIC value compared to *E. coli* 16874, 4 mg/L and 1 mg/L, respectively, and grew on all plates tested, including CHROMID® CARBA Agar where it was not expected to grow (Table 4).

To validate growth of non-OXA-48 CPE strains, nine of eleven laboratories used the strain *E. coli* TZ 3638 *bla*$_{GES-5}$ distributed by the EURL-AR used for validation of the selective agars in the EU monitoring program. Further, one laboratory used *E. coli* 40336 *bla*$_{KPC-3}$ strain (Mani et al., 2017) and one laboratory *E. coli* TZ 116 *bla*$_{VIM-1}$ strain which was also distributed by the EURL-AR as a second choice for validation of selective agar plates. On all plates the detection of the non-OXA-48 CPE were seen, except for *Brilliance*™ CRE Agar which could not detect the *E. coli* TZ 3638 *bla*$_{GES-5}$, (Table 4). One laboratory reported growth of *E. coli* TZ 3638 *bla*$_{GES-5}$ on CHROMID® OXA-48 Agar, and two

laboratories reported growth of this strain on Chromatic™ OXA-48. *E. coli* TZ 116 was also reported to grow on Chromatic™ OXA-48 (Table 4). None of these strains were expected to grow on the two OXA-48 selective agars.

3.3. Performance of selective agar plates using spiked and blank samples

In this part of the study, four laboratories were excluded and the results from only seven out of the eleven laboratories were included. The reason for this exclusion was that only the results of laboratories that performed species identification, susceptibility testing and genotyping on isolates from all selective agar plates were included.

None of the seven laboratories was able to culture the *bla*$_{VIM-1}$ positive *E. coli* strain spiked in caecum sample C-4. From meat sample M-4 spiked with *Salmonella* Kentucky carrying *bla*$_{NDM-1}$ growth of *E. coli* and/or *Enterobacter* spp. carrying a *bla*$_{NDM}$ gene (gene variant not determined) was detected by five of the participating laboratories, and one laboratory reported detecting an *E. coli* carrying both *bla*$_{NDM}$ and *bla*$_{VIM}$. The homogeneity and stability studies at Agency for Food, Environmental and Occupational Health & Safety (Anses) Fougères laboratory before and after shipping the samples verified the findings of meropenem-resistant *E. coli* and *Enterobacter* spp. together with *S.* Kentucky. Further, no *E. coli bla*$_{VIM-1}$ was detected from sample C-4 after

Table 4
Number of laboratories showing growth of control strains on different selective agars.

	Strain	CP-gene	No. of labs (n)*	MIC (mg/L)			Detection of OXA-48 CPE isolates		Detection of OXA-48 and non-OXA-48 CPE isolates			Detection of non-OXA-48 CPE isolates
				MEM#	IMI#	ERT#	CHROMID® OXA-48	Chromatic™ OXA-48	Brilliance™ CRE Agar	CHROMagar™ mSuperCARBA™	Chromatic™ CRE	CHROMID® CARBA
Non-CPE	E. coli ATCC 25922	–	11	≤0.03	≤0.12	≤0.015	0	0	0	0	0	0
OXA-48 CPE	E. coli 16874	bla_{OXA-48}	10	1	2	2	10	10	0	10	10	4
	K. pneumoniae 32516	bla_{OXA-48}	1	4	>2	>32	1	1	1	1	1	1
Non-OXA-48 CPE	E. coli TZ 3638	bla_{GES-5}	9	0.5	1	0.5	1	2	0	9	9	9
	E. coli 40336	bla_{KPC-3}	1	3	2	1.5	0	0	1	1	1	1
	E. coli TZ 116	bla_{VIM-1}	1	2	4		0	1	1	1	1	1

* Number of laboratories that used the strain. (MEM) indicates meropenem, (IMI) indicates imipenem, and (ERT) indicates ertapenem.
# MICs were derived from the EURL-AR, except for E. coli 40336 (Mani et al., 2017) and K. pneumoniae 32516 (Anses, Fougères Laboratory, France).

the homogeneity and stability studies.

When including samples C-4 and M-4 in the analyses, sensitivity ranged from 43% to 100% (Table 5). The only agar-plate that performed with 100% sensitivity was the CHROMID® OXA-48 when examining the bla_{OXA-48} positive strains. However, if C-4 and M-4 were excluded from the analyses, CHROMID® OXA-48, CHROMID® CARBA Agar and Chromatic™ CRE performed with 100% sensitivity, and CHROMagar™ mSuperCARBA™ performed with 96% sensitivity. Chromatic™ OXA-48 and *Brilliance*™ CRE Agar performed with a sensitivity of 43% and 75%, respectively.

In at least one sample, all seven laboratories reported growth of other bacteria than the expected one, i.e. background flora. Overall, unspecific growth was reported more frequently from meat samples than from caecal samples (Fig. 2A). *Pseudomonas* spp. were repeatedly identified, but other Gram-negative bacteria like *Acinetobacter* spp., *Stenotrophomonas* spp., *Enterobacter* spp., and *Ochrobactrum* spp., were also reported. In sample M-4, spiked with bla_{NDM-1} positive *S.* Kentucky, other bla_{NDM-1} positive species (*E. coli* and/or *Enterobacter* spp.) were also detected. The bla_{NDM} gene was found in one *Enterobacter* and in nine *E. coli* isolated from sample M-4.

All selective agar plates showed growth of background flora. However, difference between plates exists, e.g. CHROMagar™ mSuperCARBA™ displayed the most background flora, while *Brilliance*™ CRE Agar displayed the least background flora of all media (Fig. 2B).

In sample C-3, four laboratories observed either different colony sizes on the selective agar plates or mucoid and non-mucoid colonies of *K. pneumoniae* colonies after sub culturing on blood agar plates from all selective agar plates with growth. MICs of these different colonies ranged from 1 to 16 mg/L, but OXA-48 was confirmed in all isolates. Supplementary file 4.

Fig. 2A and B show a qualitative overview of the background flora sorted by sample (A) and by agar plate (B).

4. Discussion

Compared to earlier studies performed on evaluating selective agar plates for the detection of CPE, the current study had the large advantage that it was a multicenter cross-country study focusing on spiked samples instead of already purified bacterial strains. Hence, the study more closely mimicked the experimental conditions in a routine surveillance. However, this setup also resulted in that only a relatively small number of samples were included, due to the number of participants and the combination of matrices, genes, and bacterial strains needed to be tested. In our study, *Brilliance*™ CRE Agar was shown to support growth of both bla_{IMP} and bla_{KPC}, but could not detect the bla_{OXA-48} strain with low carbapenem MIC (meropenem 1 mg/L). In addition, all nine laboratories using the *E. coli* TZ 3638 bla_{GES-5} strain for validation of the selective agars, provided as a positive control by the EURL-AR, reported that this strain did not grow on the *Brilliance*™ CRE Agar. Cohen Stuart et al. (2013) arrived at a similar conclusion that *Brilliance*™ CRE Agar possessed a high sensitivity but a low specificity, with the difference that they tested a high number of isolates: 95 CPE and 160 non-CPE strains, including AmpC beta-lactamase and ESBL-producing bacteria. The low specificity for this agar was mainly due to growth of AmpC beta-lactamase or ESBL-producing bacterial strains and that it was less sensitive for bla_{OXA-48} positive strains. The *Brilliance*™ CRE Agar was also tested in a study by Girlich et al. (2013b) using 131 CPE strains, where growth was described for both OXA-48 producers as well as other carbapenemase producers (KPC, VIM and IMP). However, in that study, the SuperCARBA medium, from which the CHROMagar™ mSuperCARBA™ had been derived (Amar et al., 2017), performed better than the *Brilliance*™ CRE Agar in detecting strains tested. This was also comparable to our study in which the CHROMagar™ mSuperCARBA™ performed better than the *Brilliance*™ CRE Agar. The better performance of the CHROMagar™ mSuperCARBA™ plate was mainly due to the detection of the *E. coli* 16874 bla_{OXA-48} strain by all

Table 5
Number of laboratories detecting growth of the expected strain.

		OXA-48 detection		OXA-48 and non-OXA-48 CPE detection			non-OXA-48 detection
Material	Strain	CHROMID® OXA-48	Chromatic™ OXA-48	*Brilliance*™ CRE Agar	CHROMagar™ mSuperCARBA™	Chromatic™ CRE	CHROMID® CARBA
Caecal	C-1 unspiked	0*	0*	0*	0*	0*	0*
	C-2 EC IMP	0*	0*	7	7	7	7
	C-3 KP OXA-48	7	1	7	7	7	0*
	C-4 EC VIM-1	0*	0*	0	0	0	0
Meat	M-1 unspiked	0*	0*	0*	0*	0*	0*
	M-2 KP KPC-2	0*	0*	7	7	7	7
	M-3 EC OXA-48	7	5	0	6	7	0*
	M-4 SK NDM-1	0*	0*	6	2	4	1
Total score	n	14	6	27	29	32	15
Specificity	%	100	100	100	100	100	100
Sensitivity	%	100	43	64	69	76	54
Sensitivity (excluding sample C-4 and M-4)	%	100	43	75	96	100	100

(EC) indicates *E. coli*, (KP) indicates *K. pneumoniae*, (SK) indicates *Salmonella* Kentucky. (*) indicates that the strain should not grow on this plate. Sample C-4 contained a strain with very low meropenem MIC, which made it difficult to detect. In sample M-4, plasmid conjugation or contamination had occurred, as different bacterial species were detected positive for bla_{NDM}-gene, which resulted that the correct gene group, bla_{NDM}, but an incorrect species determination were reported from eight of eleven participants.

laboratories when validating the agar plates (Table 4), while none of these laboratories detected this strain with the *Brilliance*™ CRE Agar. It should, however, be mentioned that the *Brilliance*™ CRE Agar did perform better in a recent study by Gottig et al. (2020) in which it was compared to Chromatic™ CRE, CHROMID® CARBA, CHROMID® OXA-48 and two in-house made media called McCARB and McCARB-T. In that study, the better sensitivity of *Brilliance*™ CRE Agar (98.6%) compared to Chromatic™ CRE (94.2%) was based primarily on the failure to detect two *K. pneumoniae* strains with bla_{KPC-2} by Chromatic™ CRE, and that the CHROMID® CARBA agar failed to detect one of the *K. pneumoniae* strains together with a *Providencia stuartii* strain with bla_{NDM-1}. The authors also pointed out that the CHROMID® CARBA agar again missed to detect eight out of twenty investigated bla_{OXA-48} strains resulting in a sensitivity of 85.5%. However, sensitivity raises to 96% when bla_{OXA-48} strains are excluded, as the CHROMID® CARBA agar is not designed to detect bla_{OXA-48} strains. In summary, performance of the studied plates have been described to vary depending on the choice of strains tested, but all included plates in the current study have previously been described to perform overall well in detecting CPE (Girlich et al., 2013a; Girlich et al., 2013b). Although in our current study the Chromatic™ CRE, CHROMID® CARBA agar and CHROMID® OXA-48 agar performed slightly better than the other included agars (Table 5). However, this better performance could be biased by the strains that were included. In addition, the CHROMID® CARBA agar and CHROMID® OXA-48 were also the agars that most participating laboratories reported to use routinely. Thus, improved performance may be because these laboratories have more experience in handling and interpreting suspected growth on these plates. Despite the better performance by CHROMID® CARBA agar in our study a recent German study did highlight that this agar have difficulties detecting CPE with low MICs (Pauly et al., 2020). Based on the German experience on detecting CPE in food producing animals where mostly CPE with low MICs has been detected it was also concluded that if CHROMID® CARBA agar are used in screening it might underestimate the occurrence of CPE.

Unfortunately, the strains chosen for the current study might have influenced some inconsistencies in the outcome of the evaluation of the agar plates. One large issue was that caecal sample C-4, which was reported as negative by all laboratories. This sample was spiked with an *E. coli* bla_{VIM-1} isolate originating from German fattening pigs that already had proved challenging to detect using the non-selective enrichment in BPW (Irrgang et al., 2019). Another issue that arose during the evaluation was that in meat sample M-4 spiked with a bla_{NDM-1} carrying *S.* Kentucky, a potential plasmid transfer might have occurred as eight out of the eleven laboratories reported findings of *Enterobacter* spp. and/or *E. coli* isolates carrying bla_{NDM}. An alternative, but a more unlikely, explanation could be that a contamination of the sample could have occurred. The detection of the *S.* Kentucky strain was also likely further hampered due to the difficulty of separating it from the background flora as it, like most of the environmental bacteria, is non-chromogenic. There was a higher presence of background flora on the selective agar plates cultured with enriched turkey meat samples compared to the pig caeca samples, which was not unexpected as bacterial contamination of poultry meat at slaughter has been well described (Rouger et al., 2017). Because of the difficulty to detect bla_{VIM-1} carrying *E. coli* and bla_{NDM-1} carrying *S.* Kentucky in samples C-4 and M-4, respectively, these samples were excluded from the comparison in the outcome of the evaluation.

Another difficulty arose in sample C-3 with the *K. pneumoniae* bla_{OXA-48} strain. This sample resulted in growth of mucoid and non-mucoid colonies of *Klebsiella* with different meropenem MICs. This phenomenon is well described by Chiarelli et al. (2020). As this strain was detected in all seven laboratories on all except one agar plate (Table 5), it is therefore very unlikely that this has influenced the interpretation of the growth on these plates.

The inability of the current EURL-AR protocol used in the EFSA AMR surveillance program, and the current study, in combination with all investigated agar plates to detect the bla_{VIM-1} positive strain in sample C-4 is worrisome, since this gene has first been identified in an *E. coli* strain isolated from food producing animals in Germany in 2013 (Fischer et al., 2013), and afterwards in a *Salmonella* Infantis and in an *E. coli* isolated from a minced pork meat sample in Germany and Belgium, respectively (Borowiak et al., 2017; Garcia-Graells et al., 2020). All three isolates did have a meropenem MIC equal to or below 1 mg/L. In addition, two *E. coli* isolates with bla_{VIM-1} were also detected from fattening pigs in the 2017–2018 German monitoring program (European Food Safety Authority and European Centre for Disease Prevention and Control, 2019.).
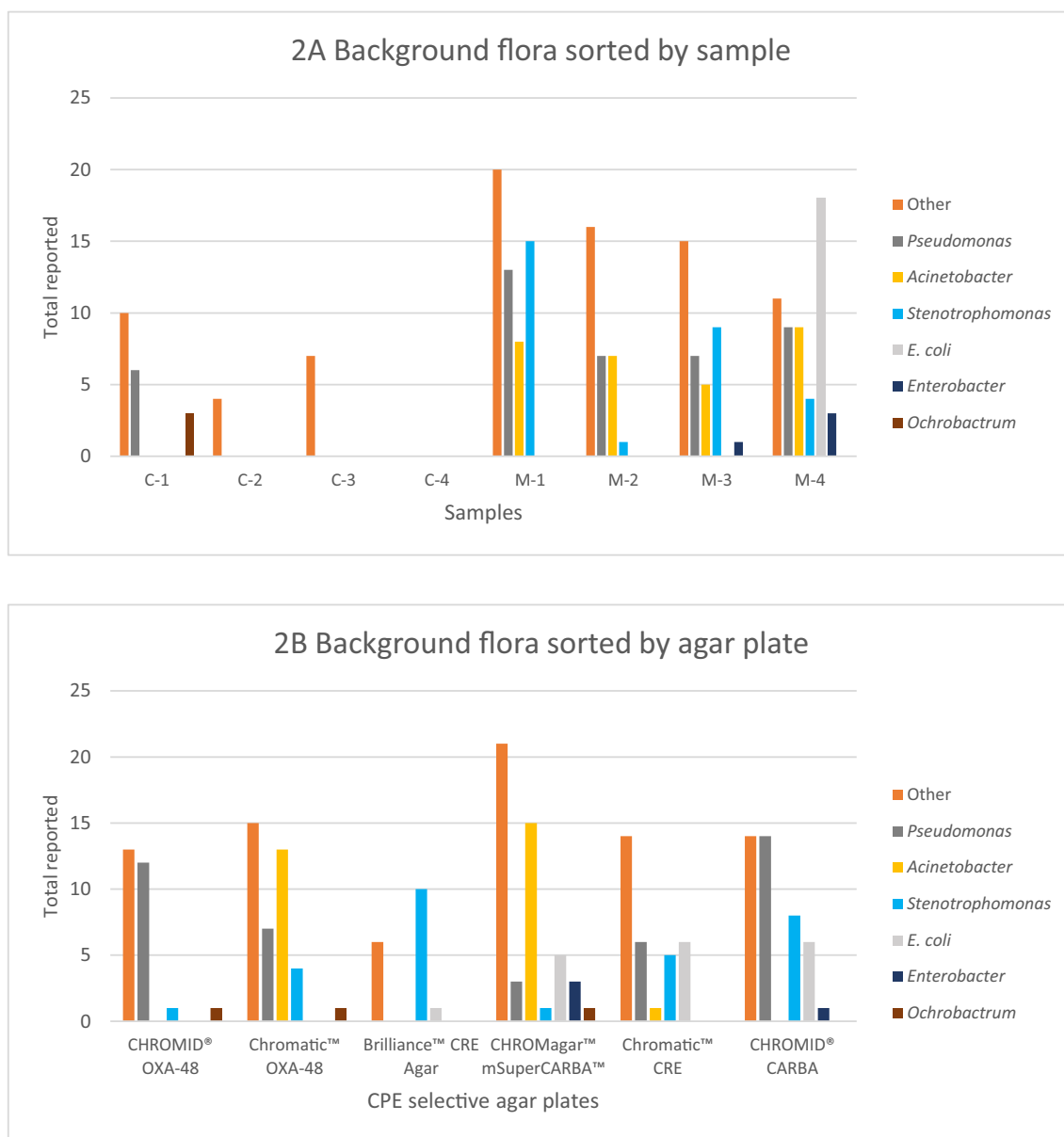
Fig. 2. Overview of background flora (organism/genus) total reported by sample (2A) and total reported by agar plate (2B). Presence and absence of accompanying flora were scored as "1" or "0" and sorted according to samples (2A) or selective media (2B). The scoring is qualitative only and presence of a genus in a sample was scored as "1" for the specific genus and sample. The y-axis presents total reported of the identified species per participant. "Other" was registered when: a) mixed culture with no name, b) pure culture with no name, or c) correct identification but mixed culture. From the eighteen *E. coli* isolates reported in sample M-4, ten were confirmed to carry a bla_{NDM} gene, and from the three *Enterobacter* isolates reported in sample M-4, one was confirmed for a bla_{NDM} gene. *E. coli* with bla_{NDM} was reported from *Brilliance*™ CRE Agar ($n = 1$), CHROMID® CARBA agar ($n = 3$), Chromatic™ CRE (n = 3) and the CHROMagar™ mSuperCARBA™ ($n = 3$). The *Enterobacter* carrying bla_{NDM} was reported from the CHROMagar™ mSuperCARBA™ plate.

One of the isolates had a meropenem MIC of 2 mg/L, ertapenem MIC of 0.5 mg/L and imipenem MIC of 4 mg/L. In Pauly et al. (2021), it was described that this isolate was not detected using the EURL-AR protocol for CP *E. coli* and the CHROMID® CARBA agar plate, but instead it was detected using the EURL-AR protocol for the detection of ESBL/AmpC producing *E. coli* on a MacConkey agar plate with 1 mg/L cefotaxime. The reason it was identified in the ESBL/AmpC screening was likely due to the fact that the bla_{VIM-1} was co-located on a plasmid with a bla_{SHV-12} gene. Thus, it might be a risk that the current method is underestimating the incidence of CPE in European livestock, and meats thereof, based on the current methodology used in the harmonized monitoring protocol suggested by Decision 2013/652/EU (European Commission, 2013). The study by Irrgang et al. (2019) was also unable to detect the *E. coli* TZ 116 bla_{VIM-1} strain using the EURL-AR protocol, as we were in the

current study, and came to a similar conclusion: "*For monitoring programs according to the guideline 2013/652/EU it might be suitable to use two serial enrichment steps: first an unselective pre-enrichment step in buffered peptone water, using this culture for plating on selective OXA-48 media. In a second step, cultivating an inoculum of the unselective pre-enrichment culture in LB supplemented with 1 mg/L Cefotaxime at 44°C (for isolation of E. coli) or 37°C (for Enterobacteriaceae in general).*". The combined results of the current study and the Irrgang et al. (2019) study, highlights the need for further evaluation of the suitability of using BPW without antibiotics regarding the sensitivity and specificity of identifying CPEs. The urgency of performing this evaluation is high as the selective screening for CP *E. coli* in the harmonized monitoring in European livestock became mandatory as of 2021 according to the Decision 2020/1729/EU (European Commission, 2020). However, one should be aware that the

reasoning behind choosing the current EURL-AR protocol included other criteria than sensitivity and specificity. For example, the protocol needed to be cheap and easy to implement in all member states, so it was preferable that the protocol could be combined with already utilized protocols, such as the isolation of *Salmonella,* and not requiring additional sample collection for the national reference laboratories.

5. Conclusions

The evaluation showed that the investigated agars were not suitable for detection of non-chromogenic CPE strains nor for the detection of CPE with low meropenem MICs. All plates showed 100% specificity, and when including all eight samples the Chromatic™ CRE and the CHRO-Magar™ mSuperCARBA™ showed the greatest sensitivity, 76% and 69% respectively, while the CHROMID® CARBA performed less well with 54% sensitivity. However, when excluding the meat sample with the *Salmonella* Kentucky carrying bla_{NDM-1} and the caecal sample with the *E. coli* carrying bla_{VIM-1}, the CHROMID® CARBA, Chromatic™ CRE and CHROMagar™ mSuperCARBA™ showed 96–100% sensitivity. For the OXA-48 agars, the CHROMID® OXA-48 agar performed with 100% sensitivity compared to the Chromatic™ OXA-48 at 47%. The results emphasize that when choosing a selective agar one should always consider which carbapenemase and which bacterial species to detect.

The results of the current study also show the need for further development of the pre-enrichment step in the current EURL-AR protocol for detection of CPE`s. However, the consequence of such a change would likely be that the protocol would only be applicable for the specific monitoring of CP *E. coli,* instead of as now when used for several screenings.

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Declaration of Competing Interest

None.

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