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Development of a selective media for detecting *Campylobacter* spp. in chicken carcasses using avibactam supplemented mCCDA

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Abstract *Campylobacter* spp. are major causes of gastrointestinal infections worldwide, and are commonly identified using modified-charcoal-cefoperazone-deoxycholate agar (mCCDA). However, the efficacy of this screening technique is often hindered by overgrowth of competing flora, such as extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*. Thus, in the present study we supplemented mCCDA with a recently developed ESBL inhibitor, avibactam (A-mCCDA). We inoculated mCCDA and A-mCCDA plates with 25 strains each of *Campylobacter* spp. and ESBL-producing *E. coli*, and thereby determined that the optimum avibactam concentration required to inhibit ESBL-producing *E. coli* was 0.0625 mg/L. At this concentration, a significantly higher proportion of *Campylobacter* spp. was isolated using A-mCCDA compared to that using mCCDA ($P < 0.05$). Thus, the results of the present study support the use of A-mCCDA to improve current *Campylobacter* screening methods.

Keywords *Campylobacter* spp. · Chicken carcass rinse · Modified-charcoal-cefoperazone-deoxycholate agar · Avibactam

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Introduction

Campylobacter spp., particularly *C. jejuni* and *C. coli*, are leading causes of foodborne illnesses worldwide, such that the European Food Safety Authority and the European Centre for Disease Prevention and Control reported campylobacteriosis as the most prevalent zoonosis in 2016 (EFSA and ECDC, 2018). Similarly, 1.3 million cases of *Campylobacter*-associated illnesses have been reported to occur annually in the United States (Geissler et al., 2017). The consumption and/or handling of raw or inappropriately cooked poultry products are considered major sources of campylobacteriosis (Edwards et al., 2011).

Currently, the presence or absence of *Campylobacter* spp. in poultry meat samples has predominantly been determined using modified charcoal-cefoperazone-deoxycholate agar (mCCDA) (Chon et al., 2016a). This medium contains third-generation antibiotics, including cephalosporin and cefoperazone, which selectively isolate *Campylobacter* spp. However, the routine use of antibiotics in poultry farming has encouraged bacteria to develop resistance to broad-spectrum antibiotics (such as cephalosporins) by producing extended-spectrum β-lactamase (ESBL). This enzyme hydrolyzes essential molecular structures within antibiotics (Kim et al., 2016), thus inactivating them. As a result, ESBL-producing bacteria overgrow on mCCDA plates, and thereby mask the growth, and confound the detection, of *Campylobacter* colonies derived from poultry samples (Chon et al., 2016a; 2016b).

To date, various ESBL inhibitors have been added to mCCDA to improve its performance by suppressing the growth of ESBL-producing competing flora such as *Escherichia coli* (Chon et al., 2013; Chon et al., 2016a; 2016b). While some of these inhibitor-supplemented media enable *Campylobacter* spp. to be detected with increased

selectivity and sensitivity, they are not suitable for commercialization and/or application in either research or industrial contexts due to their high cost and/or complex production requirements. Moreover, bacteria resistant to previously developed ESBL-inhibitor/antibiotic combinations have now been identified; thus, novel ESBL inhibitors are urgently needed (Fernández-Martínez et al., 2015; Sharma et al., 2016; Stapleton et al., 1995).

Avibactam is an ESBL inhibitor that was developed in 2011, and has been shown in pure culture models and clinical trials to be capable of neutralizing a wider spectrum of β-lactamase-producing bacteria, even at low concentrations, compared to other ESBL inhibitors (e.g., clavulanic acid, tazobactam, and sulbactam) (Abboud et al., 2016; Ehmann et al., 2012). To the best of our knowledge, it has not yet been evaluated as a selective agent for the isolation of *Campylobacter* using culture media. Thus, the present study supplemented mCCDA with avibactam (A-mCCDA), and compared the performance of the supplemented media with that of standard (non-supplemented) mCCDA in detecting *Campylobacter* spp., and inhibiting the growth of competing flora derived from whole-chicken carcass-rinse samples.

Materials and methods

Bacterial strains

In total, 25 *Campylobacter* strains, comprising 11 *C. jejuni* (NCTC 11168, ATCC 33560; two human, and seven food isolates), and 14 *C. coli* (ATCC 33559; three human, and 10 food isolates), were used in this study. All human isolates were kindly provided by the Korea Centers for Disease Control and Prevention (KCDC; Cheongju, South Korea). Food isolates were collected in our laboratory (between 2014 and 2016) from meat products. Bacteria were grown at 42 °C for 48 h under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) on Columbia blood agar (Oxoid, Hampshire, UK) that was supplemented with 5% (v/v) laked horse blood (Oxoid). Microaerobic conditions were achieved using an AnaeroPack jar (Mitsubishi Gas Chemical, Tokyo, Japan), with a GENbox microaer gas generator (bioMérieux, Marcy-l'Étoile, France). The 25 utilized ESBL-producing *E. coli* strains were isolated from chicken carcass samples, and incubated at 37 °C on tryptic soy agar (Oxoid) for 24 h prior to use.

mCCDA and A-mCCDA preparation and inoculation

Control mCCDA (Oxoid) plates were prepared according to the manufacturer's instructions as described in our previous study (Chon et al., 2016a). To determine the optimal concentration of avibactam (Avention, Incheon, South Korea) required for mCCDA supplementation to both inhibit the growth of ESBL-producing *E. coli,* and support the recovery of *Campylobacter* spp., avibactam solutions prepared with various concentrations were added into a 20 ml of mCCDA agar plate to final concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 ug/mL as described in our previous study (Chon et al., 2016b). Once prepared, mCCDA and A-mCCDA plates were inoculated with approximately 1×10^4 cells each of the *Campylobacter* and ESBL-producing *E. coli* strains (suspended in phosphate-buffered saline), and incubated at 42 °C for 48 h, as described above.

Isolation of *Campylobacter* spp. from chicken-carcass rinses inoculated on mCCDA and A-mCCDA plates

Eighty whole chicken carcasses were purchased between May and September 2017 from four different retail stores (20 samples/store) located in Seoul, South Korea. *Campylobacter* spp. were detected in chicken samples as previously described (Chon et al., 2016b). Briefly, chicken carcasses were rinsed with 400 mL of buffered peptone water (Oxoid), and shaken gently for 1 min. A 25-mL aliquot of each rinse was supplemented with 2× blood-free Bolton enrichment broth (Oxoid) in a 50-mL cell-culture flask with a filter cap (SPL Life Sciences, Gyeonggi-do, South Korea), and incubated at 42 °C for 48 h under microaerobic conditions. A loopful of the rinse-inoculated enrichment broth was then inoculated onto mCCDA or A-mCCDA (0.0625 mg/L) plates, which were then incubated (42 °C for 48 h) under microaerobic conditions. Suspected *Campylobacter* colonies were selected and sub-cultured on Columbia blood agar, before their identities were confirmed via a previously described polymerase chain reaction (PCR) method (Denis et al., 1999).

Identification of competing flora

To identify the predominant competing flora on each medium, 40 suspected non-*Campylobacter* colonies (i.e., 20 colonies each isolated from mCCDA and A-mCCDA plates) were randomly selected, and identified via biochemical-identification and ESBL-production analyses that were conducted using a VITEK® 2 GN ID Card and VITEK® antimicrobial susceptibility testing (AST)-N224 Card (bioMérieux), respectively, according to the manufacturers' instructions.

Statistical data analysis

The number of plates found to be positive for *Campylobacter* and non-*Campylobacter* colonies was compared using Fisher's exact test, which was performed using GraphPad InStat 3 software (GraphPad Software, San Diego, CA, USA). As previously described (Chon et al., 2016b), a growth index was determined and analyzed only for media that were contaminated with non-*Campylobacter* colonies. A score of 1 was used to indicate the growth of a small number of colonies, while scores of 2 and 3 were used to indicate the growth of colonies on approximately half, and most of the plate, respectively. A P value < 0.05 was considered to indicate statistical significance.

Results and discussion

Optimal avibactam concentration required to produce effective A-mCCDA

Growth data for the 25 *Campylobacter* (11 *C. jejuni*, and 14 *C. coli*) and 25 ESBL-producing *E. coli* isolates that were grown on the mCCDA and A-mCCDA plates (at various concentrations of avibactam) are provided in Table 1.

None of the tested *Campylobacter* strains were inhibited on A-mCCDA containing 0.0625–4 mg/L of avibactam; however, they were inhibited on A-mCCDA containing $\geq$ 8 mg/L of avibactam, indicating that high concentrations of avibactam prevent *Campylobacter* growth (Table 1). Notably, the 14 *C. coli* strains analyzed survived on A-mCCDA supplemented with avibactam at all concentrations, while two and one *C. jejuni* strains survived on A-mCCDA supplemented with 8 mg/L and 16 mg/L, respectively (data not shown). The observed higher level of avibactam resistance exhibited by the *C. coli* compared to that exhibited by the *C. jejuni* strains is consistent with the results of previous studies, in which *C. coli* strains generally exhibited low levels of antibiotic-binding activity (Pezzotti et al., 2003; Tajada et al., 1996).

In contrast, all tested ESBL-producing *E. coli* strains were inhibited on A-mCCDA containing even the lowest concentration of avibactam (0.0625 mg/L). Thus, to minimize the potential inhibitory effect of avibactam against *Campylobacter*, this concentration of avibactam was determined to be optimal for *Campylobacter* screening.

In terms of cost-effectiveness, the supplementation of mCCDA with avibactam was estimated to be superior or comparable to supplementation with other ESBL inhibitors (Table 2). In fact, the calculated cost of supplementing mCCDA with a sufficient concentration of an alternative ESBL inhibitor to suppress the growth of ESBL-producing *E. coli* (Chon et al., 2013; 2016a; 2016b) was shown to be much higher than that of supplementing mCCDA with avibactam, with the exception of clavulanic acid. In addition, A-mCCDA displayed the lowest growth index of 1.6 (Table 2) of the selective agars analyzed, indicating that it exhibited the lowest level of contamination by competing flora. Together, these findings suggest that A-mCCDA is more appropriate for use in commercial settings than previously reported ESBL inhibitors.

Capacity of mCCDA and A-mCCDA to enable the selective isolation of *Campylobacter* from chicken-carcass rinses

The number of A-mCCDA plates that were positive for *Campylobacter* growth (10/80; 12.5%) was significantly

Table 1 Comparison of growth of 25 *Campylobacter* and 25 ESBL-producing *Escherichia coli* strains on A-mCCDA plates containing various concentrations of avibactam

Selective agar	Avibactam conc. (mg/L)	No. positive strains/total no. strains (%)	
		Campylobacter	ESBL-producing *E. coli*
mCCDA	0	25/25 (100)[a]	25/25 (100)[a]
A-mCCDA	0.0625	25/25 (100)[a]	0/25 (0)[b]
	0.125	25/25 (100)[a]	0/25 (0)[b]
	0.25	25/25 (100)[a]	0/25 (0)[b]
	0.5	25/25 (100)[a]	0/25 (0)[b]
	1	25/25 (100)[a]	0/25 (0)[b]
	2	25/25 (100)[a]	0/25 (0)[b]
	4	25/25 (100)[a]	0/25 (0)[b]
	8	16/25 (64)[a]	0/25 (0)[b]
	16	15/25 (60)[a]	0/25 (0)[b]

ESBL, extended-spectrum β-lactamase; mCCDA, modified-Charcoal-Cefoperazone-Deoxycholate-Agar; A-mCCDA, avibactam-supplemented mCCDA

[ab]Different letters within a row indicate a significant difference ($p < 0.05$)

Table 2 Comparison between cost and performance of supplementing mCCDA with ESBL inhibitors to suppress the growth of ESBL-producing *Escherichia coli*

Agent	Retail price ($/mg)	Concentration required to suppress ESBL-producing *E. coli* growth (mg/L)	Price of supplementation ($/L)	Un-supplemented versus supplemented media			References
				Campylobacter isolation (%)	Contamination by competing flora (%)	Growth index of competing flora	
Avibactam	47.6	0.0625	3.0	0 versus 13%	100 versus 40%	3.0 versus 1.6	Current study
Clavulanic	2.5	1	2.5	32 versus 56%	92 versus 37%	2.8 versus 1.8	Chon et al. (2013)
Tazobactam	12.9	4	51.6	31 versus 57%	83 versus 1%	NA	Chon et al. (2016b)
Cefotetan	1.1	32	35.2	26 versus 60%	100 versus 29%	3.0 versus 2.1	Chon et al. (2016a)
Cefoxitin	0.7	32	22.4	26 versus 57%	100 versus 30%	3.0 versus 2.3	Chon et al. (2016a)

mCCDA, modified-charcoal-cefoperazone-deoxycholate-agar; ESBL, extended-spectrum β-lactamase; NA, not analyzed

‡A score of 1 indicates the growth of several colonies, while scores of 2 and 3 indicate colony growth on approximately half, and most of the plate, respectively

higher than that of mCCDA plates (0/80; 0%)($P < 0.05$), and furthermore, significantly fewer A-mCCDA (32/80; 40%) than mCCDA (80/80; 100%) plates were contaminated with non-*Campylobacter* colonies ($P < 0.05$), indicating that the selectivity of A-mCCDA was superior to that of mCCDA. In addition, the growth index (1.62) of the non-*Campylobacter* spp. that were observed on A-mCCDA was approximately two times lower than that observed on mCCDA (2.98). As a result, suspected *Campylobacter* colonies were covered by overgrown ESBL-producing *E. coli* colonies on the mCCDA agar plates, whereas only *Campylobacter* colonies were detected on the A-mCCDA plates, allowing single *Campylobacter* colonies to be easily identified and isolated (Table 3, Fig. 1).

A limitation of this study is that the performance of A-mCCDA was not compared to those of other previously developed ESBL inhibitor (e.g., clavulanic acid or tazobactam)-supplemented mCCDAs. Moreover, given that the experiments were not performed simultaneously, direct comparisons between A-mCCDA and other previously developed and investigated ESBL-supplemented mCCDAs may be inaccurate. Nevertheless, this is the first study to evaluate avibactam as a selective agent for the isolation of *Campylobacter*. Further studies are required to simultaneously compare the performance of additionally modified *Campylobacter*-selection media in assessing various clinical, environmental, and food samples.

In conclusion, A-mCCDA enabled the highly selective identification and isolation of *Campylobacter* spp. compared to mCCDA, as demonstrated by the fact that the A-mCCDA plates analyzed exhibited an approximately 2.0- and 2.5-fold lower non-*Campylobacter* contamination rate and growth index, respectively, than the mCCDA plates. Thus, by eliminating masking colonies, the application of A-mCCDA may enable the prevalence of *Campylobacter* in raw poultry to be more accurately

Table 3 Comparison of *Campylobacter* and non-*Campylobacter* colony growth on mCCDA and A-mCCDA plates, as indicated by the number of positive plates and observed growth indices

	No. positive plates/total no. plates tested (%)	
	mCCDA	A-mCCDA
Campylobacter colonies	0/80 (0)	10/80 (12.5)*
Non-*Campylobacter* colonies	80/80 (100)	32/80 (40.0)*
Non-*Campylobacter* colony growth index†	2.98	1.62

mCCDA, modified-charcoal-cefoperazone-deoxycholate-agar; A-mCCDA, avibactam-supplemented mCCDA

†A score of 1 indicates the growth of several colonies, while scores of 2 and 3 indicate colony growth on approximately half, and most of the plate, respectively

*$P < 0.05$ versus mCCDA

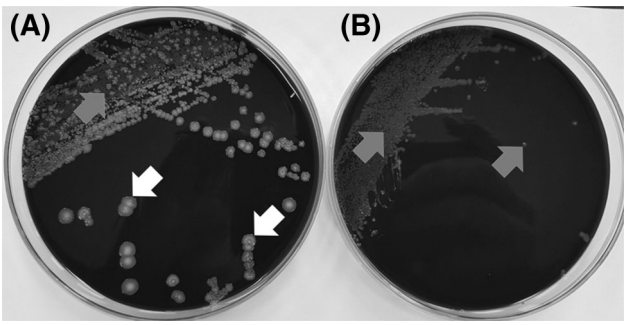

Fig. 1 Representative images showing bacterial growth on modified-Charcoal-Cefoperazone-Deoxycholate-Agar (mCCDA) and avibactam-supplemented mCCDA (A-mCCDA) plates inoculated with the same whole-chicken carcass rinse sample. (**A**) Both non-*Campylobacter* colonies (white arrows) and suspected *Campylobacter* colonies (gray arrows) were observed on the mCCDA plates. (**B**) No non-*Campylobacter* colonies were detected on the A-mCCDA plates; thus, suspected *Campylobacter* colonies were easily identified

determined, especially in samples contaminated with ESBL-producing *E. coli*. Furthermore, supplementing mCCDA with avibactam rather than clavulanic acid or tazobactam has the advantage of achieving a wide antibiotic spectrum at an optimal concentration, while incurring lower or comparable costs.

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