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Improvement of Modified Charcoal-Cefoperazone-Deoxycholate Agar by Supplementation with a High Concentration of Polymyxin B for Detection of *Campylobacter jejuni* and *C. coli* in Chicken Carcass Rinses

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Modified charcoal-cefoperazone-deoxycholate agar (mCCDA) was improved by supplementation with a high concentration of polymyxin B. The ability of the supplemented medium to isolate *Campylobacter jejuni* and *C. coli* from chicken carcass rinses was compared to that of Campy-Cefex agar and mCCDA. Modification of mCCDA with increased polymyxin B yielded a significantly ($P < 0.05$) higher isolation rate and greater selectivity than those achieved using Campy-Cefex agar and mCCDA.

Although various rapid detection methods have been used for detecting *Campylobacter*, culture with selective media is the standard method (7). Selective agars for the detection of *Campylobacter* contain various growth supplements and antibiotic agents to reduce the damage caused by oxidative toxins and eliminate competing flora (2, 12). However, it is difficult to isolate *Campylobacter* spp. from foods with numerous and diverse background microflora. Doyle and Roman (4) reported that it was much more difficult to obtain isolates of *Campylobacter* spp. from chickens because the indigenous flora of chicken skin is a better competitor for *Campylobacter* spp. than are the floras of other foods. In our previous study, we found that various selective media, such as Karmali agar and modified charcoal-cefoperazone-deoxycholate agar (mCCDA), showed poor sensitivity and selectivity for the detection of *Campylobacter*, especially in food samples with numerous background microflora, because they were not effective against Gram-negative bacteria (1).

Polymyxin B is an antibiotic derived from the bacterium *Bacillus polymyxa.* The antibiotic is bactericidal for Gram-negative bacteria, and its mode of action comprises two major steps, permeabilization of the outer membrane and induction of lethal leakage of cytoplasmic components (16). Although Karmali et al. (8, 9) reported that a high concentration of polymyxin B (50,000 IU/liter) improved the selectivity of media for the primary isolation of *Campylobacter jejuni*, the agent has not been used at high concentrations in recent times because *Campylobacter coli* was found to be highly susceptible when the agent was added to media such as Mueller-Hinton (MH) agar and Preston enrichment media (5, 7, 11). Polymyxin B has not been used at high concentrations in selective media containing a combination of cefoperazone and activated charcoal, such as mCCDA.

In this study, we modified mCCDA by adding polymyxin B. The aim of this study was to evaluate the effect of high concentrations of polymyxin B on the ability of mCCDA to isolate and select *C. jejuni* and *C. coli* from chicken carcass rinses.

In total, we used 44 strains of *C. jejuni* (ATCC 33560, NCTC 11168, 10 human isolates, and 32 food isolates) and 88 strains of *C. coli* (ATCC 33559, 12 human isolates, and 75 food isolates). Clinical strains isolated from sporadic cases of food poisoning were kindly provided by the Korea Centers for Disease Control and Prevention (Osong, South Korea). Food strains isolated from various meat products over the past 2 years, 2009 and 2010, include 32 strains of *C. jejuni* (chicken, 30; pork, 1; and beef, 1) and 75 strains of *C. coli* (chicken, 72; pork, 2; and beef, 1). All the cultures, which had been kept frozen at −70°C until used, were streaked onto blood agar (Oxoid, Basingstoke, United Kingdom) with 5% laked horse blood (Oxoid) and then microaerobically incubated at 42°C for 48 h for two passages.

Campy-Cefex agar (Acumedia, Baltimore, MD) and mCCDA (Oxoid) were prepared according to the manufacturers' recommendations. Modified mCCDA (polymyxin B-supplemented mCCDA; P-mCCDA) was made by adding two vials of polymyxin B (concentration, 50,000 IU/vial; Oxoid) to 1 liter of mCCDA. All plates were stored at 4°C and used within 3 days.

To evaluate the growth of *C. jejuni* and *C. coli* strains on selective media, 10^4 cells of each of 44 *C. jejuni* and 88 *C. coli* strains in 0.85% saline water were streaked onto each of the three different agars described above. All plates were microaerobically incubated at 42°C for 48 h. Plates with small, shiny, round, and gray colonies were considered positive for *Campylobacter*.

A total of 80 whole chickens were purchased from June to August 2011 from four different grocery stores in Seoul, South Korea. All experimental procedures for detecting *C. jejuni* and *C. coli* were performed in accordance with the method proposed by the U.S. Food Safety and Inspection Service (FSIS), with modifications (14). Chicken carcasses were rinsed with 400 ml of buffered peptone water (BPW; Difco, Sparks, MD). To ensure even distribution, we thoroughly mixed the carcass rinse by gently shaking the sample for 1 min. A 25-ml test portion from the 400 ml of rinse was enriched with 25 ml of 2× blood-free Bolton enrichment broth (Oxoid) in a 50-ml screw cap conical tube. Less

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TABLE 1 Growth of *C. jejuni* and *C. coli* isolated from 20 chicken carcass rinses on three different media

Organism	No. of plates with indicated media positive for *C. jejuni* or *C. coli*[a]														
	1st trial			2nd trial			3rd trial			4th trial			**Total**		
	P	M	C	P	M	C	P	M	C	P	M	C	**P**	**M**	**C**
C. jejuni	14	10	7	3	1	5	0	2	0	0	1	1	**17**	**14**	**13**
C. coli	0	0	0	4	6	0	6	7	3	5	2	2	**15**	**15**	**5**
C. jejuni and *C. coli* cocontamination	2	2	1	10	4	6	14	6	8	12	10	3	**38**	**22**	**18**
Total[b]	**16**	**12**	**8**	**17**	**11**	**11**	**20**	**15**	**11**	**17**	**13**	**6**	**70**^{A}	**51**^{B}	**36**^{C}

[a] P, P-mCCDA; M, mCCDA; C, Campy-Cefex agar.
[b] Different superior letters (A, B, C) within a row indicate significant differences ($P < 0.05$) in the numbers of positive samples.

than 0.5 cm of headspace was left in the tubes, and the tubes were tightly capped. Each sample (50 ml) was enriched at 42°C for 48 h. A loopful of the enrichment broth was streaked onto each of the three selective media and incubated microaerobically at 42°C for 48 h. Suspected colonies (maximum, 10) were removed and subcultured onto 5% horse blood agar. Presumptive *C. jejuni* and *C. coli* colonies were confirmed with colony PCR according to the method described by Denis et al. (3).

Using data from our previous study (1) as a reference, we compared the plates of *C. jejuni*/*C. coli* and competing organisms in terms of isolation rate and selectivity, respectively. The plates were compared in pairs by using Fisher's exact test with GraphPad InStat software (GraphPad Software, Inc., San Diego, CA, USA). Selectivity was also evaluated on the basis of the growth index of the competing flora (1, growth of a few colonies; 2, growth of colonies on about half of the plate; and 3, growth on most of the plate) according to the method described by Peterz (13). The growth index was determined and averaged only for contaminated media with competing flora.

All *C. jejuni* ($n = 44$) and *C. coli* ($n = 88$) strains grew on mCCDA, Campy-Cefex agar, and P-mCCDA. We used a two-times-higher concentration of polymyxin B (100,00 IU/liter) than that suggested by Karmali et al. (8, 9), but our results indicate that the addition of polymyxin B to mCCDA at this concentration does not inhibit the growth of either the *C. jejuni* or the *C. coli* strains tested in this study.

As stated above, *C. coli* was found to be more susceptible to antibiotics in the selective media, particularly to colistin (polymyxin E) and polymyxin B, than was *C. jejuni*. Goossens et al. (5) have reported that colistin (polymyxin E) and polymyxin B should not be included as a selective supplement because of the inhibitory effect of the agent on *C. coli*. Ng et al. (11) have also reported that *C. coli* strains are susceptible to polymyxin B present in selective media. However, they did not use polymyxin B with activated charcoal and cefoperazone-based selective media. In the preliminary study, we combined a high concentration of polymyxin B with various selective agars as well as mCCDA. Interestingly, upon the addition of polymyxin B, the growth of most *C. coli* strains tested in this study ($n = 88$) was greatly inhibited on blood-supplemented MH agar (85 strains), Preston agar (85 strains), and Campy-Cefex agar (78 strains), but not on mCCDA. The inhibitory effect of antibiotic agents might differ because of various factors, such as growth temperature, growth condition, antibiotic combination, and toxin-neutralizing supplements (9, 11). Activated charcoal and cefoperazone in mCCDA may harmonize with polymyxin B, reducing the inhibitory effect on *C. coli*; however, the underlying mechanism remains unclear.

A comparison of the performances of the three selective media in detecting *C. jejuni* and *C. coli* in whole-chicken rinses is shown in Tables 1 and 2. The number of P-mCCDA plates positive for both *C. jejuni* and *C. coli* was significantly higher than those of positive mCCDA and Campy-Cefex plates ($P < 0.05$) (Table 1). Furthermore, there were fewer contaminated P-mCCDA plates than for the other media, and P-mCCDA plates had the lowest growth index of competing flora, indicating the superior selectivity of P-mCCDA (Table 2).

Chon et al. (1) have reported that inhibition of Gram-negative competing flora may enhance the detection ability of the media. Polymyxin B, which inhibits only Gram-negative bacteria, may effectively eliminate background microflora in the chicken carcass rinses. In each trial, we biochemically confirmed two or three contaminants that appeared on each selective agar by using Vitek 2 (bioMérieux, Marcy l'Etoile, France), and most of the contaminants were identified as *Escherichia coli*, followed by *Acinetobacter*

TABLE 2 Growth of competing flora isolated from 20 chicken carcass rinses on three different media

Parameter	Growth on indicated media[a]														
	1st trial			2nd trial			3rd trial			4th trial			**Total**		
	P	M	C	P	M	C	P	M	C	P	M	C	**P**	**M**	**C**
No. of plates with competing flora[b]	8	9	20	10	9	20	8	8	20	5	8	19	**31**^{A}	**34**^{A}	**79**^{B}
Average growth index of the competing flora[c]	2.1	3.0	2.9	1.3	3.0	2.7	1.1	3.0	3.0	1.4	2.9	2.9	**1.5**	**3.0**	**2.9**

[a] P, P-mCCDA; M, mCCDA; C, Campy-Cefex agar.
[b] Different superior letters (A, B) within a row indicate a significant difference ($P < 0.05$) in the number of contaminated plates.
[c] Scoring of growth index: 1, growth of a few colonies; 2, growth of colonies on about half of the plate; 3, growth on most of the plate.

spp. and *Proteus* spp. (data not shown). *Campylobacter* colonies may be covered by colonies of these bacteria, which grew densely in the mCCDA and Campy-Cefex media. P-mCCDA yielded the lowest number of plates with contaminating flora, and the growth index of the competing flora was lower on the P-mCCDA plates, thereby making isolation and differentiation of suspicious colonies easier than on other selective media. In contrast, the Campy-Cefex agar recommended by the U.S. FSIS for its high level of quantitative detection (12, 14) was extensively contaminated with the competing flora and showed the lowest isolation rate and least selectivity in this study. Our findings suggest that the Campy-Cefex agar would not be effective in qualitative detection using enriched samples with numerous competing floras.

The presence of background microflora in foods is one of the most common factors that interfere with the isolation of pathogens on selective media (1, 6, 10). In a study by Wesley et al., overgrowth of indigenous poultry flora necessitated the addition of agents to inhibit the growth of contaminants and enhance the growth of *Campylobacter* (15). Enhanced selectivity may increase the isolation rate of selective agar (4). To enhance selective agars for the detection of *Campylobacter* spp. in chicken samples, we used a high concentration of polymyxin B as an additional supplement for mCCDA in this study. Our data indicate that using polymyxin B as a supplement in mCCDA effectively eliminates competing microfloras that are resistant to cefoperazone, resulting in higher isolation rates for *C. coli* as well as for *C. jejuni*. In summary, P-mCCDA is superior to other selective media used by many food authorities for the selective isolation of *C. jejuni* and *C. coli* from chicken carcass rinses.

However, this study has some limitations, because only one detection protocol and enrichment time were used. Per the U.S. FSIS guideline, the enrichment time for qualitative detection of *Campylobacter* in chicken carcass rinses is 24 to 48 h. When a shorter enrichment time (24 h) was applied in this study, the results were found to be different; a longer incubation time (48 h) may result in the overgrowth of competing bacteria (1). Therefore, the effectiveness of P-mCCDA should be validated in other growth conditions and by using more *Campylobacter* strains.

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