Research Note

Improvement of Bolton broth by supplementation with tazobactam for the isolation of *Campylobacter* from chicken rinses

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ABSTRACT Overgrowth of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) on *Campylobacter* media prevents the latter's selective isolation, thereby making the improvement of *Campylobacter*-selective media necessary. We evaluated tazobactam (an ESBL inhibitor) to supplement Bolton enrichment broth (Tz-Bolton broth) for the selective isolation of *Campylobacter* in chicken carcass rinses. First, using 20 strains of ESBL-producing *E. coli* and 13 *Campylobacter* strains, we found 4 μg/mL of tazobactam to be optimal for inhibiting the ESBL-producing *E. coli* while allowing the growth of all tested *Campylobacter* strains. Next, 80 whole chicken carcasses were rinsed with buffered peptone water (BPW), and 25 mL of BPW rinse was mixed with 2 × blood-free Bolton broth (25 mL) with or without tazobactam followed by incubation at 42°C for 48 h under microaerobic conditions. A loopful of the incubated broth was inoculated on modified charcoal-cefoperazone-deoxycholate agar (mCCDA) and microaerobically incubated at 42°C for 48 h. The tazobactam supplemented Bolton broth showed a higher *Campylobacter* isolation rate (38.8%, $p < 0.05$) than normal Bolton broth (15%). Moreover, the number of mCCDA plates with non-*Campylobacter* was much lower ($p < 0.05$) after enrichment in Tz-Bolton broth (0%) than in the normal Bolton broth (80%), suggesting that selectivity of the modified broth was superior to normal Bolton broth.

Key words: *Campylobacter*, Tazobactam, Bolton broth, Chicken

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INTRODUCTION

For several decades, various types of selective agars and broths have been used for the detection of *Campylobacter* from poultry products (Corry et al., 1995; Oyarzabal et al., 2005). Currently, *Campylobacter*-selective media that are used for the isolation of *Campylobacter* contain several antibacterial agents to exclude competing microbiota in poultry carcasses (Corry et al., 1995; Oyarzabal et al., 2005). For instance, cefoperazone, vancomycin, trimethoprim, polymyxin B, and rifampicin are known to be effective in inhibiting the growth of competing microbiota, while supporting *Campylobacter* growth (Corry et al., 1995). Of these, cefoperazone, a third-generation cephalosporin, is an antibacterial agent that suppresses both Gram-positive and -negative bacteria (Corry et al., 1995).

However, cefoperazone resistant microorganisms have increased in poultry, making selective *Campylobacter* isolation from raw poultry meat difficult. Extended-spectrum β-lactamase (ESBL)-producing *E. coli* is strongly resistant to cefoperazone and prevalent in poultry (Warren et al., 2008; Costa et al., 2010; Leverstein-van Hall et al., 2011). An overgrowth of ESBL-producing *Escherichia coli* (*E. coli*) in *Campylobacter* media containing cefoperazone has been shown to hamper the selective isolation of *Campylobacter* from poultry products (Jasson et al., 2009; Moran et al., 2011; Hayashi et al., 2013; Chon et al., 2014; Smith et al., 2015). In previous studies, potassium clavulanate, an ESBL inhibitor, was found to be very effective as a supplement in *Campylobacter* media (Moran et al., 2011; Chon et al., 2014).

In addition to potassium clavulanate, there are commercially available ESBL inhibitors such as tazobactam

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and sulbactam (Miller et al., 2001; Mohanty et al., 2005). These ESBL inhibitors bind to the active site of beta-lactamase enzyme and maintain the antibacterial effect of cephalosporins by inhibiting the hydrolysis of the beta-lactam ring (Smith et al., 2015). The addition of tazobactam (1, 2, 3, and 10 mg/L) in modified charcoal-cefoperazone-deoxycholate agar (mCCDA) has been shown to greatly inhibit the growth of ESBL-producing *E. coli*; without reducing *Campylobacter* cell number in pure culture and chicken cecal contents (Smith et al., 2015). In an earlier study, we also demonstrated that tazobactam is the most effective supplement added to mCCDA for suppressing ESBL-producing *E. coli* (Chon et al., 2016). However, the number of ESBL-producing *E. coli* increased during enrichment and to a lesser extent during culture on selective agar, posing a problem for qualitative detection (Jasson et al., 2009; Moran et al., 2011; Chon et al., 2013a; Chon et al., 2014). It is therefore necessary to use supplements with different modes of action than that of cefoperazone to suppress the growth of ESBL-producing *E. coli* during enrichment. There have been no studies in which tazobactam was used as an additional selective agent in broths used for enrichment of *Campylobacter*. In this study, we modified Bolton broth, one of the most commonly used *Campylobacter* enrichment broths containing cefoperzone, by supplementation with tazobactam (Tz-Bolton broth), and compared the isolation rate and selectivity of the modified medium with that of unmodified Bolton broth in chicken carcass rinses.

MATERIALS AND METHODS

Bacterial Strains

A total of seven strains of *Campylobacter jejuni* [*C. jejuni*; 81–176, A74C, three human isolates (*CJclin_12*, *CJclin_4*, and *CCA_8*), one chicken isolate (*GD_1*) and one beef isolate (*SS_4*)] and six strains of *Campylobacter coli* [*C. coli*; American Type Culture Collection (ATCC; Manassas, VA, USA) 33559, ATCC 49941, one chicken isolate (*C_213*), one human isolate (*CCA_9*), one canine isolate (*C_CAM*), and one beef isolate (*CCH_B4*)] were used in the present study. Clinical outbreak strains were kindly provided by the Korea Centers for Disease Control and Prevention (Cheongju, South Korea). Food strains were isolated from meat products from retail stores in Seoul (during 2009 and 2010).

A total of 25 non-*Campylobacter* strains [20 ESBL-producing *E. coli* (collected from chicken meat from 2011 to 2013 in Korea), *Lactobacillus salivarius* ATCC 11741, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 7002, *Acinobacter* spp. *ACB911* (kindly provided by Dr. Mohamed Nawaz), and *Ochrobactrum anthropi HAC7* (kindly provided by Dr. Mark Hart) were used in this study. *Campylobacter* strains were streaked onto blood agar and incubated microaerobically (5% O_2, 10% CO_2, and 85% N_2) at 42 °C for 24–48 h. Non-*Campylobacter* strains were inoculated onto blood agar and incubated at 37 °C for 24–48 h before use.

Preparation of Bolton Broth and Tz-Bolton Broth

Bolton broth was prepared according to the manufacturer's recommendations. Briefly, 13.8 g Bolton broth base (Oxoid, Hampshire, UK) was suspended in 500 mL distilled water and the suspension was autoclaved at 121 °C for 15 min. The broth was cooled, and 1 vial of Bolton broth antibiotic supplement (10 mg cefoperazone, 10 mg trimethoprim, 10 mg vancomycin, and 5 mg amphotericin B per vial; Oxoid) were added to the cooled broth. Tazobactam sodium salt (Sigma-Aldrich, St. Louis, MO) was filtered using a 0.45-mm syringe filter and added to Bolton broth in amounts as described below.

Growth of ESBL-producing E. coli in Various Concentration of Tazobactam in Tz-Bolton Broth

We inoculated ESBL-producing *E. coli* strains into Bolton broth with various concentrations of tazobactam to determine the minimal inhibitory concentration (MIC) of tazobactam required for suppression of ESBL-producing *E. coli* strains. Tazobactam dissolved in distilled water was diluted with a two-fold dilution series and inoculated into 200 μL Bolton broth in a 96-well plate to concentrations of 0.25 to 64 μg/mL, as well as Bolton broth without tazobactam. Approximately 10^4 cells of each tested strain were inoculated into normal and modified Tz-Bolton broth and incubated at 42°C for 48 h microaerobically. Using a plate reader, bacterial growth was measured as the absorbance level at 600 nm.

Growth of Campylobacter Cultures in Normal and Tz-Bolton Broth

The minimal concentration of tazobactam in Bolton broth that could sufficiently inhibit all tested ESBL-producing *E. coli* isolates was 4 μg/mL. A total of seven *C. jejuni* and six *C. coli* strains were enriched in 4 μg/mL Tz-Bolton broth to investigate the effect of this concentration of tazobactam on the growth of *Campylobacter*. Approximately 10^2 cells from each strain were inoculated into 20 mL normal or Tz-Bolton broth containing 4 μg/mL tazobactam, and then incubated at 42°C for 48 h under microaerobic conditions. The number of *Campylobacter* in the enriched broths was enumerated on the blood agar plates by using the plate count method.

*Growth of non-*Campylobacter *Cultures in Normal and Tz-Bolton Broth*

Five non-*Campylobacter* strains (*Lactobacillus salivarius* ATCC 11741, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 7002, *Acinobacter* spp. *ACB911*, and *Ochrobactrum anthropi HAC7*) were grown on original and modified broth to determine the exclusivity of the tested media. Approximately 10^4 cells from each stain were inoculated into 2 mL normal or Tz-Bolton broth containing 4 μg/mL tazobactam in multi-well plates, and then incubated at 42 °C for 48 h under microaerobic conditions. To determine the growth, the OD value was read as described above.

Detection of Campylobacter *in Chicken Carcass Rinse*

A total of 80 chicken carcasses were purchased from two retail stores in Seoul, South Korea. *Campylobacter* was detected according to the protocols described in our previous study (Chon et al., 2014). Briefly, chicken carcasses were rinsed in 400 mL buffered peptone water (Difco), which was then gently shaken for 1 min. For the test, 25 mL from the 400 mL rinsate and 25 mL 2 × blood-free Bolton broth with or without tazobactam (4 μg/mL) were mixed in a 50-mL conical tube and then enriched at 42°C for 48 h microaerobically. A loopful of enrichments was inoculated onto mCCDA plates followed by incubation at 42°C for 48 h microaerobically. Suspicious colonies were selected and subcultured on blood agar. The presence of *Campylobacter* was confirmed by using a colony PCR, according to the method described in Denis et al. (1999).

RESULTS AND DISCUSSION

Growth of Pure Cultures of Campylobacter *in Normal and Tz-Bolton Broth*

Because a high concentration of antibacterial agent would affect the normal growth of *Campylobacter*, we determined the minimal concentration of tazobactam needed to suppress ESBL-producing-*E. coli*, while supporting *Campylobacter* spp. growth. Considering all tested strains were inhibited in Bolton broth with a tazobactam concentration of 4.0 μg/mL. The concentration was enough to inhibit the growth of tested *E. coli* strains (Table 1). The comparisons of cell counts for the seven *Campylobacter jejuni* and six *C. coli* strains enriched in the two types of Bolton broths indicated no statistical difference in cell numbers between normal (8.54 ± 0.50 Log CFU/mL) and modified Bolton broth (8.51 ± 0.53 Log CFU/mL) containing 4.0 μg/mL tazobactam (Table 2). The data clearly indicate that this concentration of tazobactam allows the growth of *Campylobacter* without adverse effect. Therefore, in all subsequent experiments on the detection of *Campylobacter* from chicken carcass rinses, we used 4.0 μg/mL tazobactam in Bolton broth.

Table 1. Minimum inhibitory concentration (MIC) of tazobactam in Bolton broth of 20 extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* strains.

Concentration of tazobactam in Bolton broth (μg/mL)	Number of strains grown on plates/total number of strains of ESBL-producing *E. coli* (%)
0	20 (100)
0.25	18 (90)
0.5	12 (60)
1	5 (25)
2	1 (5)
4	0 (0)
8	0 (0)
16	0 (0)
32	0 (0)
64	0 (0)

Table 2. Number of cells of seven *Campylobacter jejuni* and six *C. coli* strains enriched in normal Bolton broth or Tz-Bolton broth containing 4 μg/mL tazobactam.

		Number of cells (Log CFU/mL)	
Species	Strain No.	Bolton broth	Tz-Bolton broth
C. jejuni	81–176	7.82	7.69
	A74/C	7.91	7.74
	CCA_8	8.98	8.81
	GD_1	8.76	8.80
	SS_4	8.89	8.59
	CJclin_12	8.61	8.79
	CJclin_4	7.85	8.28
C. coli	ATCC 33559	8.28	7.90
	ATCC 49941	8.03	7.96
	CCA_9	8.76	8.80
	C_CAM	9.20	9.18
	CCH_B4	9.18	9.18
	C_213	8.69	8.92
	Mean value ± SD[a]	8.54 ± 0.50 A	8.51 ± 0.53 A

[a]The numbers of cells enriched in the two broths were statistically compared by Student's *t*-test. Different letters (A, B) within a row indicate a significant difference ($p < 0.05$) in the number of positive samples. CFU, colony forming units

*Growth of Non-*Campylobacter *Cultures in Normal and Tz-Bolton Broth*

The growth of microorganisms such as *Lactobacillus salivarius*, *Acinetobacter* spp., *Proteus* spp., *Ochrobactrum* spp., and *Pseudomonas* spp., as well as ESBL-producing *E. coli* on agars and broths used for detection and isolation of *Campylobacter* has been reported by many researchers (Baylis et al., 2000; Hu and Kuo, 2011; Ahmed et al., 2012; Chon et al., 2012; Vaz et al., 2014; Yoo et al., 2014). To determine if the addition of tazobactam influences the growth of other competing microbiota as well as ESBL-producing *E. coli*, the selectivity of normal and modified media against those competing microbiota was evaluated in this study. All other tested bacteria were inhibited in both media.

Table 3. Comparison of normal Bolton broth or Tz-Bolton broth containing 4 μg/mL tazobactam for the detection of *Campylobacter* on modified charcoal-cefoperazone-deoxycholate agar plates following the selective culture of 80 chicken carcass rinses.

	Number of positive plates/Total number of plates tested (%)[a]	
	Bolton broth	Tz-Bolton broth
Campylobacter	12/80 (15.0) A	31/80 (38.8%) B
non-*Campylobacter*	64/80 (80.0%) A	0/80 (0) B

[a]Different letters (A, B) within a row indicate a significant difference ($p < 0.05$) in the number of positive samples. The number of positive plates was compared by using Fisher's exact test in pairs.

Comparison of Normal and Tz-Bolton Broth in Chicken Carcass Rinse

Qualitative detection of *Campylobacter* from chicken samples using normal and modified broth is compared in Table 3. Selectivity of the two broths was also compared by counting the number of agar plates with non-*Campylobacter* species in Table 3. A significantly greater ($p < 0.05$) number of plates from Tz-Bolton broth were *Campylobacter*-positive (31 out of 80, 38.8%) compared to normal Bolton broth (12 out of 80, 15%). Furthermore, no mCCDA plates from Tz-Bolton broth (0 out of 80, 0%) were positive for non-*Campylobacter* microbiota. However, 80% (64 out of 80) of mCCDA plates streaked with normal Bolton broth were positive for non-*Campylobacter* ($p < 0.05$). These results indicated that the qualitative detection and selectivity of modified Bolton broth were superior to normal Bolton broth.

To inhibit the growth of ESBL-producing *E. coli*, several modified versions of *Campylobacter* enrichment broth supplemented with various antibiotics, such as potassium clavulanate, polymyxin B, and triclosan have been developed (Moran et al., 2011; Chon et al., 2013a,b; Chon et al., 2014). However, no previously developed media showed zero percent of contamination with non-*Campylobacter* colonies, suggesting that tazobactam would be a more suitable supplement than other selective agents for *Campylobacter* broth. In concordance with the results of Smith *et al.* (2015), tazobactam is very effective as an additional supplementation for its ESBL-inhibition ability, and it is chemically stable.

Because *Campylobacter* growth on mCCDA plates from normal Bolton broth was highly inhibited by the dense growth of competing microbiota, the identification of competing microbiota on mCCDA plates from normal Bolton broth is necessary. We therefore randomly selected the predominant non-*Campylobacter* colonies that grew on 16 mCCDA plates from normal Bolton broth. We picked those colonies and inoculated them onto ESBL chromogenic medium (*Brilliance*^{TM} *ESBL*; Oxoid) followed by incubation at 37°C for 48 h to determine whether ESBL-producing *E. coli* indeed hampered the isolation of *Campylobacter* from normal Bolton broth. After incubation, we found that tested contaminants showed the typical morphology of an ESBL-producing *E. coli* colony on the chromogenic medium. Consistent with previous studies, ESBL-producing *E. coli* are the most common competing microbiota that hampers the selective isolation of *Campylobacter* (Jasson et al., 2009; Moran et al., 2011; Chon et al., 2014). The highly dense growth of ESBL-producing *E. coli* masks the growth of *Campylobacter*, making isolation of target bacteria especially difficult in poultry samples.

Many researchers in Asian and European countries have reported that a high prevalence of ESBL-producing *E. coli* in chicken makes the selective isolation of *Campylobacter* in poultry difficult (Jasson et al., 2009; Moran et al., 2011; Hayashi et al., 2013; Chon et al., 2014; Smith et al., 2015). In the present study, we demonstrated that Bolton broth modified with tazobactam resulted in a better detection ability than unmodified Bolton broth that is conventionally used for qualitative isolation of *Campylobacter* from chicken samples.

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