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Development and stability of bacteriocin resistance in *Campylobacter* spp

Ky Van Hoang[1], **Norman J. Stern**[2], and **Jun Lin**[1,*]

[1]Department of Animal Science, The University of Tennessee, Knoxville, TN 37996, USA

[2]USDA-ARS, Russell Research Center, Athens, GA 30604, USA

Abstract

Aims—Several bacteriocins (BCNs) that were identified from chicken commensal bacteria dramatically reduced *Campylobacter* colonization in poultry and are being directed toward on-farm control of this important foodborne human pathogen. A recent study has shown that BCN resistance in *C. jejuni* is very difficult to develop *in vitro*. In this study, *in vivo* development and stability of BCN resistance in *Campylobacter* was examined.

Methods and Results—Chickens infected with *C. jejuni* NCTC 11168 were treated with BCN E-760 at the dose of 5 mg/kg body weight/day via oral gavages for three consecutive days, which selected BCN-resistant (BCN^r) mutants in the treated birds. However, all the *in vivo*-selected mutants only displayed low-levels of resistance to BCN (MIC = 2–8 mg/L) when compared to parent strain (MIC = 0.5 mg/L). Inactivation of CmeABC efflux pump of the BCN^r mutants led to increased susceptibility to BCN (8–32 fold MIC reduction). Three different BCN^r *Campylobacter* strains (*in vitro*- or *in vivo*-derived) were examined for the stability of BCN resistance using both *in vitro* and *in vivo* systems. The low-level of BCN resistance in these strains was not stable *in vitro* or *in vivo* in the absence of BCN selection pressure.

Conclusions—Usage of BCN E-760 only selected low-level BCN^r *C. jejuni* mutants *in vivo* and the low-level BCN resistance was not stable *in vitro* and *in vivo*.

Significance and Impact of the Study—The study provides helpful information for risk assessment of the future practical application of the anti-*Campylobacter* BCNs in animals.

Keywords

Campylobacter; bacteriocin; resistance

INTRODUCTION

Campylobacter species, the epsilon class of proteobacteria, are the leading bacterial causes of human gastroenteritis in developed countries (Allos, 2001). In addition to watery diarrhea and/or hemorrhagic colitis, infection with *Campylobacter* spp can result in post-infectious manifestations such as Guillain Barre syndrome, an acute immune mediated disorder that may lead to respiratory muscle compromise and death (Nachamkin et al., 1998). *Campylobacter* spp are considered to be commensal organisms in the intestinal tracts of wild and domestic animals including chickens and other avian species (Diker et al., 2000). Epidemiological studies demostrated that consumption of contaminated poultry meat is the major cause of human campylobacteriosis (Stern et al., 2001, Stern et al., 2004). Thus, on-

*Corresponding author: Dr. Jun Lin, Department of Animal Science, The University of Tennessee, 2640 Morgan Circle Drive, Knoxville, TN 37996-4574, Phone: (865) 974-5598. Fax: (865) 974-7297, jlin6@utk.edu.

farm control of *Campylobacter* spp could reduce the risk of human *Campylobacter* infections. Of the several proposed strategies to reduce this risk, anti-*Campylobacter* bacteriocins (BCNs) are considered a promising strategy to protect food safety and public health (Lin, 2009).

BCNs are short cationic antimicrobial peptides (AMPs) naturally produced by diverse microbes in different environments (Willey and van der Donk, 2007). Despite significant structural and characteristic differences, BCNs display potent antimicrobial activities against a wide range of viruses, bacteria, and fungi and have been recognized as a novel class of antimicrobials to control food borne pathogens (Settanni and Corsetti, 2008, Zasloff, 2002, Hugas et al., 1998, Galvez et al., 2007). As a group of naturally nontoxic antimicrobials, some BCNs, such as nisin, have long been applied for food preservation (Willey and van der Donk, 2007). Many bacteria including intestinal commensals could make at least one bacteriocin (Klaenhammer, 1988, Riley and Gordon, 1992). Therefore, the intestinal BCN-producing bacteria may achieve a competitive advantage and function as innate barriers against pathogens in the hosts. In addition, the natural and low-toxic BCNs have been proposed as promising candidates for novel antimicrobials against microbial infections (Joerger, 2003, Asaduzzaman and Sonomoto, 2009). Several anti-*Campylobacter* BCNs have been isolated and characterized from chicken commensal bacteria, which includes OR-7 from *Lactobacillus salivarius* (Stern et al., 2006), E-760 and E50–52 from *Enterococcus faecium* (Line et al., 2008b, Svetoch et al., 2008), and bacillocins from *Paenibacillus polymyxa* (Stern et al., 2005). Animal studies have demonstrated that these BCNs greatly reduced *C. jejuni* colonization in chicken intestine. Therefore, these natural anti-*Campylobacter* BCNs are being developed for on-farm control of *Campylobacter* to protect public health.

To develop the BCN-based intervention strategy against *Campylobacter*, several important issues (e.g. BCN resistance development, mechanism, stability) need to be addressed for future regulatory approval and public acceptability. Recently, we examined prevalence, development, and molecular mechanisms of BCN resistance in *Campylobacter* using molecular and genomic approaches (Hoang et al., 2011). In this study, susceptibilities of 137 *C. jejuni* and 20 *C. coli* isolates to the anti-*Campylobacter* BCNs OR-7 and E-760 were examined. Only one *C. coli* strain displayed resistance to the BCNs (MIC = 64mg/L) while others were susceptible with MIC ranging from 0.25 to 4 mg/L. The *C. coli* mutants resistant to BCN OR-7 also were obtained by *in vitro* selection but all displayed only low-level resistance to OR-7 (MIC = 8 to 16 mg/L). We also observed that *in vitro* BCN resistance in *C. jejuni* is very difficult to develop. However, it is still unknown if usage of bacteriocins will promote the emergence of BCN-resistant (BCN^r) *Campylobacter* mutants *in vivo*. In addition, it is unclear if the BCN^r *Campylobacter* can persist in the absence of selective pressure. To address these questions, in this study, *in vivo* emergence of BCN^r *Campylobacter* was examined using a chicken model system and the stability of BCN resistance was determined using both *in vitro* and *in vivo* systems. This study provides helpful information for risk assessment of the future practical application of the anti-*Campylobacter* BCNs in poultry.

MATERIALS AND METHODS

Bacteriocin, bacterial strains and growth conditions

The E-760 was purified from *E. faecium* NRRL B-30745 as described in a recent publication (Line et al. 2008), which includes two general purification steps: 1) crude E-760 (~9% purity) preparation from the supernatant using ammonium sulfate precipitation, and 2) E-760 purification from the crude preparation using two different chromatography columns which finally results in E-760 purity up to 98.8%. The E-760 was dissolved in sterile

distilled H_2O and stored at −20°C prior to use. The amino acid sequences of E-760 (62 aa residues) was consistent with class IIa bacteriocins based on its N-terminal region of the peptide and the E-760 were also resistant to high temperature (e.g. 100 °C, 5 min) and a wide pH range (e.g. 5.0–8.7) (Line, Svetoch et al. 2008).

C. jejuni NCTC 11168, a BCN sensitive (BCN^s) strain (E-760 MIC = 0.5 mg/L), was purchased from NCTC and was used as a parent strain in this study for selecting BCN^r mutants *in vitro*. JL106, a natural BCN^r *C. coli* strain isolated from human (E-760 MIC = 64 mg/L), was examined in a recent study (Hoang et al, 2011) and was also used for stability testing in this study. JL 341, a BCN^r mutant (E-760 MIC = 8 mg/L) derived from NCTC 11168 by natural transformation using genomic DNA of *C. coli* JL106 (Hoang et al, 2011), was also chosen for stability testing in this study. The *Campylobacter* strains were grown routinely on MH agar plates or in MH broth at 42 °C under microaerobic conditions generated by using CampyGen Plus gas pack (Oxoid, Lenxa, KS) in an enclosed jar. *Campylobacter*-specific growth supplements and selective agents (SR084E and SR117E; Oxoid) were added to the media when needed. When required, the MH media were also supplemented with various amounts of E-760.

In vivo development of BCN resistance in *Campylobacter*

The *in vivo* development of BCN resistance in *Campylobacter* was examined using a chicken model system. In this experiment, day-old broiler chicks (a kind gift from commercial company Hubbard Hatchery, Pikeville, TN) were randomly assigned to either treatment (10 chicks) or control groups (10 chicks). All birds were placed in sanitized wire cages with unlimited access to feed and water. Nutritionally complete feed was prepared in the feed mill at the Johnson Animal Research and Teaching Unit. Prior to inoculation with *C. jejuni* NCTC 11168, all birds were confirmed to be free of *Campylobacter* by culture of cloacal swabs. However, these birds have not been tested to examine which BCNs are present naturally in the intestine. At 2 days of age, all birds in treatment and control groups were inoculated with fresh *C. jejuni* NCTC 11168 cultures (10^7 CFU/bird) via oral gavages. For the treatment group, at 9 days of age when each bird was fully colonized by the *C. jejuni* 11168, all birds were treated with BCN E-760 at the dose of 5 mg/kg body weight/day via oral gavages for three consecutive days. Birds in the control group were gavaged with water. Cloacal swabs were collected from all birds in both groups at day 1, 2, 3, 5, and 7 after the initial BCN treatment. Samples from each bird were spread onto MH agar selective plates containing 8 mg/L of E-760 to select for *in vivo* emerged BCN^r mutants. Individual colonies from BCN E-760 containing plates were randomly selected to identify level of BCN E-760 resistance using MIC testing as described below. Multiple isolates with different E-760 MICs were analyzed by PCR to confirm their genetic identities. The PCR was done using primers specific for the *cmp* gene encoding the major outer membrane protein as previously described by (Huang et al., 2005) which revealed no difference between input strain and output isolates.

In vitro stability of BCN resistance

Three BCN^r mutants were examined for the *in vitro* stability of acquired BCN resistance, which include the human clinical isolate JL106, the *in vitro*-derived mutant JL341, and the *in vivo*-selected mutant K58 (E-760 MIC = 8 mg/L) obtained from the above chicken experiment using *C. jejuni* NCTC 11168 as a parent strain. Briefly, the three strains were inoculated in BCN-free MH broth and grown under microaerobic conditions at 42°C. The *Campylobacter* cultures were sub-cultured every 2 days in fresh MH broth (1:400 dilutions) for 70 days in the absence of any antimicrobials. Following passages 10, 15, 20, 25, 30, and 35, the cultures were serially diluted (10-fold dilutions) in MH broth and plated onto both MH agar plates and MH agar plates supplemented with E-760 at a concentration of 8 mg/L.

The plates were then incubated under microaerobic conditions at 42°C for two days. The total numbers of colonies on each type of plates were counted and compared at each time point. In addition, for passage 35, 20 colonies for each mutant were randomly selected from BCN-free MH agar plates and were subjected to MIC testing.

In vivo stability of BCN resistance using a chicken model system

The same BCN resistant mutants (JL106, JL341, and K58) were used for *in vivo* stability test using chicken model system. Bird source and maintenance were the same as those used in the *in vivo* development of BCN resistance experiment described above. Forty one-day old chicken were randomly assigned into four groups (10 chickens each group). Prior to inoculation with *Campylobacter*, all birds were confirmed free of *Campylobacter* by cultured cloacal swabs. Birds in each group received corresponding *Campylobacter* strain at a dose of 10^7 CFU/bird via oral gavages at 3 days old. Birds in control group were inoculated with BCN^s *C. jejuni* NCTC11168. Birds in the other three treatment groups were inoculated with JL106, JL341, and K58, respectively. Birds in all groups received BCN-free feed and water throughout the trial. Cloacal swabs were collected from birds in all groups at day 6, 22, and 42 after *Campylobacter* inoculation. Samples from each bird were serially diluted and spread onto two different types of MH agar plates to recover total *C. jejuni* 11168 populations (normal selective plates), and BCN^r populations (selective plates containing 8 mg/L of E-760). *Campylobacter* colonies were enumerated following 48 hours of incubation at 42°C under microaerobic conditions. At each time point, representative colonies from chicken in each group were chosen for E-760 MIC test.

The detection limit of the plating method was 100 $CFUg^{-1}$ of feces. The significant difference in *Campylobacter* colonization levels (log_{10} transformed $CFUg^{-1}$ of feces) at each sampling point between groups was calculated using Student's *t* test. A *P*-value of <0.05 was considered significant.

E-760 susceptibility test

The susceptibilities of *Campylobacter* strains to BCN E-760 was determined by standard microtitre broth dilution method in MH broth with an inocula of 10^6 bacterial cells per mL as described by Jorgensen and Turnidge (2003). Minimum inhibitory concentration (MIC) was determined by the lowest concentration of E-760 showing complete inhibition of *Campylobacter* growth after 24 hours of incubation at 42°C. Duplicate experiments were performed to confirm the consistency of MIC results.

Inactivation of the *cmeB* gene in E-760 resistant mutants

Chromosomal DNA was isolated from a *cmeB* mutant (Lin et al., 2002) using the Wizard Genomic Purification Kit (Promega) according to the manufacturer's instructions. The insertional mutation of *cmeB* in the extracted genomic DNA was transferred to the *in vivo*-selected E-760 resistant mutants by natural transformation. Natural transformation (biphasic method) was performed following standard procedure (Davis et al., 2008).

RESULTS

Effect of BCN E-760 treatment on the emergence of E-760 resistant *Campylobacter* in chickens

All chickens in both groups were successfully colonized by *C. jejuni* NCTC 11168 prior to E-760 treatment at 9 days of age. BCN^r mutants were emerged in only one chicken one day after the first E-760 treatment (Fig. 1). Total 80% of chickens were observed to shed $E\text{-}760^r$ mutants at 5 days after the first treatment (Fig 1). However, BCN^r mutants were soon cleared in majority of chickens (80%) after cessation of E-760 treatment (7 days after the

first treatment), strongly suggesting the instability of BCN resistance *in vivo.* MIC test of 17 randomly selected E-760^r resistant mutants indicated that all the selected mutants only displayed low-level resistance to E-760 with MIC ranging from 2 mg/L to 8 mg/L. No E-760^r mutants were selected on the selective plates containing 8 mg/L E-760 for the fecal samples from all chickens in control group.

CmeABC multidrug efflux system contributes to *in vivo* acquired BCN resistance

Our recent study (Hoang et al., 2011) has shown that the multidrug efflux pump CmeABC contributed to both intrinsic and *in vitro* acquired BCN resistance in *Campylobacter*. In this study, we examined the role of CmeABC in the *in vivo* acquired BCN resistance using the mutants obtained from above chicken experiment. As shown in Table 1, regardless of resistance level, inactivation of *cmeB* significantly reduced E-760 MIC of all mutants to the level of 0.125 mg/L, which is also lower than the MIC level of wild-type parent strain 11168 for E-760 (Table 1).

E-760 resistance is not stable *in vitro*

As shown in Fig 2, less than 10% of JL341 populations could be still selected on MH agar plates containing 8 mg/L of E-760 after 10 passages in the absence of E-760 selective pressure. Following 35 passages, only a very small population of JL341 (0.0005%) were recovered on the E-760-containing plates. Although JL106 and K58 showed higher stability than JL341 *in vitro*, less than 0.1% and 1% of JL106 and K58, respectively, were selected on the E-760-containing plates after 35 passages. Consistent with the result from differential plating, MIC tests of randomly selected colonies (20 for each mutant) after 35 passages showed all strains displayed significantly lower MIC for E-760 MIC (≤ 1 mg/L) than parent strain JL341.

in vivo instability of E-760 resistance in *Campylobacter*

All chickens were successfully colonized by *Campylobacter* in either control group inoculated with *C. jejuni* NCTC 11168 or in treatment groups inoculated with *C. jejuni* JL341, K58, or *C. coli* JL106; the shedding level is approximately 7 $\log_{10}$ units per gram feces at 6 days post inoculation (Fig. 3A). The shedding levels of *Campylobacter* in colonized chickens were also slightly reduced in both control and treatment groups at 22 and 42 days post inoculation when compared to those at 6 days post inoculation (Fig. 3A).

The *in vivo* stability of BCN E-760 resistance was monitored by differential plating method as well as MIC test of randomly selected colonies for E-760 (40 colonies per time point per group). Differential plating method indicated that percentage of E-760 resistant mutants in total *Campylobacter* population in fecal sample from individual chicken dramatically decreased over the long-term growth of three E-760 resistant *Campylobacter* strains in chickens without E-760 selection pressure (Fig. 3B). By 42 days postinoculation, approximately 0.02%, 0.17%, and 0.49% of *Campylobacter* populations from chickens inoculated with JL341, JL106, and K58, respectively, grew on E-760-containing selective plates (Fig. 3B). MIC test of selected isolates confirmed the same trend of instability of E-760 resistance *in vivo*. The resistance levels of all randomly selected colonies were reduced to ≤ 1 mg/L at 42 days postinoculation.

DISCUSSION

Several anti-*Campylobacter* BCNs have successfully been identified and characterized from chicken commensal bacteria (Stern et al., 2006, Line et al., 2008a, Stern et al., 2005, Svetoch et al., 2008, Svetoch et al., 2005). Feeding these anti-*Campylobacter* BCNs to poultry at pre-slaughter stage eliminated *Campylobacter* colonization and these BCNs have

been proposed to control *Campylobacter* spp in poultry (Stern et al., 2006, Line et al., 2008a, Stern et al., 2005, Svetoch et al., 2008, Svetoch et al., 2005). Although these BCNs are effective in reducing *Campylobacter* spp colonization in poultry, the use of these anti-*Campylobacter* BCNs in poultry may lead to emergence of BCN^r isolates which may affect sustainable application of BCNs in poultry for *Campylobacter* control. Therefore, studying BCN resistance including development and stability of resistance are crucially important for future regulatory approval and public acceptability of this intervention measure.

In our previous study, we demonstrated that *Campylobacter* spp could develop low level BCN resistance *in vitro* but high-level of BCN resistance failed to develop *in vitro* despite extensive efforts (Hoang *et al.* 2011). In this study, we examined the *in vivo* development E-760 resistance in *Campylobacter* using a chicken model. We have used the similar chicken model system to study *in vivo* development of *Campylobacter* resistance to various clinical antibiotics (Lin et al., 2007, Luo et al., 2003, Han et al., 2008, Caldwell et al., 2008). Our data showed that in response to E-760 treatment, limited BCN^r *C. jejuni* emerged as early as one day after the E-760 treatment. However, E-760 resistance level in the *in vivo*-selected *Campylobacter* was low throughout the whole study. This emergence pattern is different from *in vivo* development of fluoroquinolone resistance in *Campylobacter* (Luo et al, 2003) in which the mutants with high-level resistance emerged rapidly in all treated chickens as early as 1 day after the initiation of enrofloxacin treatment and also was different from macrolide resistance development (Lin, Yan et al. 2007) in which *in vivo* development of macrolide resistance was only observed after long-term exposure to tylosin (> 31 days). Our findings suggest that among *Campylobacter* there is limited development of resistance to the anti-*Campylobacter* BCNs, such as E-760. These findings support a recent theory that bacteria have not developed a highly effective mechanism to resist BCNs and other endogenous AMPs during evolution, which is likely due to multiple targets of natural AMPs (Preschel and Sahl, 2006). However, the results from this study should be interpreted cautiously because the experiment was conducted in a laboratory environment using a small number of chickens which may not represent the production conditions in poultry farms. In addition, only one representative *C. jejuni* strain alongside one BCN (E-760) was chosen to study in vivo emergence of BCN resistance in this study. The development of antimicrobial resistance development on farms is complex and influenced by multiple factors such as animal species, production environment, genetic backgrounds of bacterial species, and management practices. Furthermore, due to the lack of sufficient E-760 for a dosing experiment, it is still unknown if higher selection pressure would promote the development of mutants with high-level resistance to BCN E-760.

To obtain important information for the risk assessment of on-farm control of *Campylobacter* using the anti-*Campylobacter* BCNs, it is important to examine whether BCN^r *Campylobacter* can persist in the absence of selection pressure. Antibiotic resistance in *Campylobacter* has displayed unique features with respect to fitness cost and stability of resistance when compared with other microorganisms. Our previous studies demonstrated that low levels of macrolide resistance in *Campylobacter* which was associated with mutations in ribosomal proteins L4 and L22 were not stable *in vitro* and *in vivo*. In contrast, high levels of macrolide resistance due to mutations at A2074G and A2075G in 23S rRNA were very stable in the absence of macrolide selection pressure in both *in vitro* and *in vivo* systems (Lin et al., 2007, Caldwell et al., 2008). Fluoroquilonone (FQ) resistance in *Campylobacter* associated with *gyrA* mutation is more intriguing (Luo et al., 2005). Specifically, the FQ resistant strains did not show fitness cost and the FQ resistance is highly persistent *in vitro* and *in vivo* (Luo et al., 2005). Notably, FQ resistance even enhances ecological fitness of *Campylobacter* and make FQ-resistant *C. jejuni* outcompete parent sensitive strain in the absence of selective pressure (Luo et al., 2005). Using both *in vitro* and *in vivo* systems, in this study we showed that E-760 resistance was not stable in

Campylobacter regardless of specific species (*C. jejuni* or *C. coli*) and resistance levels (E-760 MIC = 8 or 64 mg/L). This information provides additional information supporting feasibility of practical application of BCN for *Campylobacter* control in poultry. Based on the findings from this study, the BCN resistance trait of resistant mutants could be dramatically lost after long-term colonization of the mutants in new flocks that receive BCN-free feed. Consequently, emergence of low-level BCN resistance in *Campylobacter* may have little effect on the efficacy of later BCN treatment prior to slaughter.

Revealing molecular mechanisms of BCN resistance in *Campylobacter* may facilitate us to develop more effective BCN-based intervention measure to reduce *Campylobacter* load in poultry. Multidrug resistance efflux pump CmeABC plays a critical role in *Campylobacter* resistance to structurally diverse antimicrobials (Lin et al., 2002, 2003). Our recent study (Hoang et al., 2011) has revealed that CmeABC also contributed to both intrinsic and acquired resistance to BCN for the mutants selected *in vitro*. In this study, we further demonstrated that active transport of CmeABC efflux pump confers resistance of *in vivo*-selected mutants to BCN (Table 1). Given a limited number of strains examined in this study, it is suggested that a greater number of strains should be used for *cmeABC* sequencing and function analyses in the future. Based on these findings, inhibition of the CmeABC pump by efflux pump inhibitors should significantly increase susceptibility of *Campylobacter* to BCN and reduce the frequency of emergence of BCN resistance in *Campylobacter*, as what we have observed for the effect of efflux pump inhibitors on *Campylobacter* resistance to clinical antibiotics (Martinez and Lin, 2006). Consequently, specific efflux pump inhibitors may be used in a combination treatment to enhance the efficacy of oral administration of the anti-*Campylobacter* BCNs for reducing *Campylobacter* load in poultry. This speculation remains to be examined in the future.

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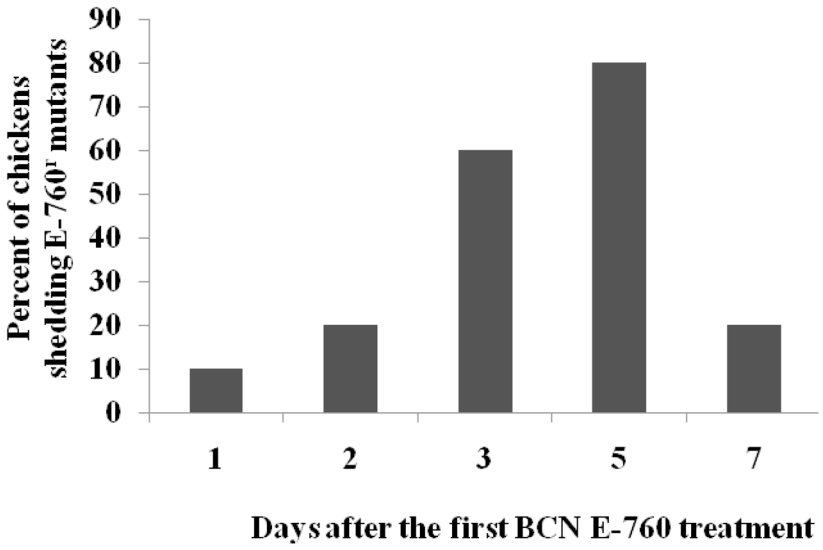

Figure 1.
Effect of BCN E-760 usage on the emergence of E-760 resistant *C. jejuni*. Each bar represents the percentage of chickens that shed E-760 resistant mutants in the treatment group.

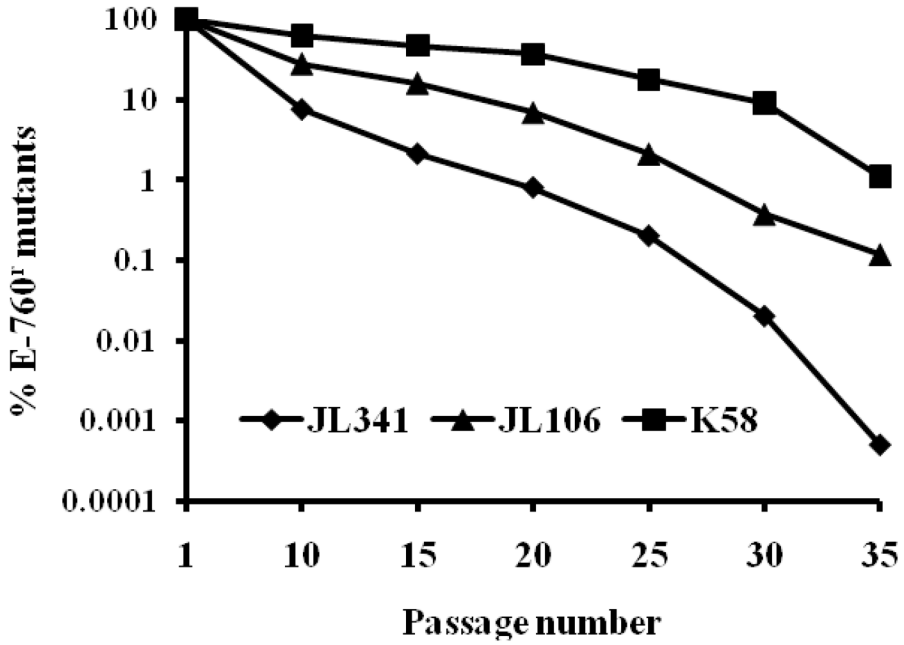

Figure 2.

Stability of BCN E-760 resistance *in vitro*. Three strains were passed in MH broth without E-760 selection pressure as described in Materials and Methods. The percentage of BCN^r population was calculated based on differential plating using plates containing 8 mg/L of BCN E-760.

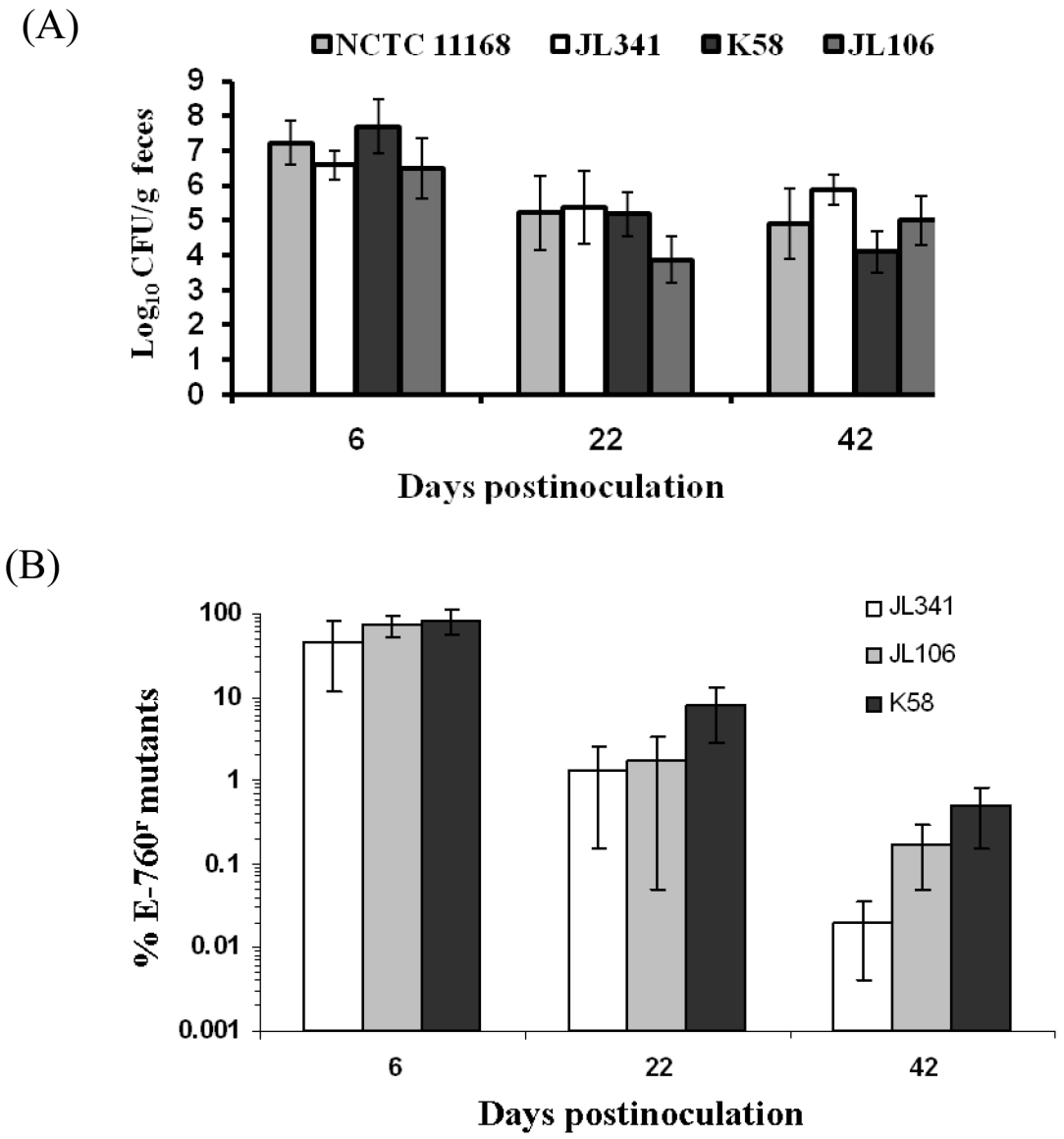

Figure 3.

Stability of BCN E-760 resistance *in vivo*. (A) Shedding levels of various *Campylobacter* strains in chickens. Chickens in each group (10 birds/group) were inoculated with E-760 sensitive strain NCTC 11168, or one of E-760^r mutants (JL341, K58, or JL106). All chickens received non medicated feed throughout the study. Each bar represents the mean of $\log_{10}$ CFUgram^{-1} feces ± standard deviation of *Campylobacter* colonized chickens in each group; (B) Instability of E-760 resistance *in vivo*. The percentage of E-760^r population was calculated based on differential plating as detailed in Materials and Methods. Each bar represents the mean of percentage of the E-760^r population of individual chickens ± standard deviation in each group.

Table 1

E-760 MICs of the *in vivo*-selected BCN^r *C. jejuni* isolates and their isogenic *cmeB* mutants

Strain	E-760 MIC (mg/L)[a]	E-760 MIC of isogenic *cmeB* mutant(mg/L)
C. jejuni NCTC 11168	0.5	0.125
E-760^r mutants selected *in vivo*	2 (3)	0.125
	4 (4)	0.125
	8 (10)	0.125

[a]Numbers in parentheses indicate the total number of isolates corresponding to each MIC.