High Prevalence of Hyper-Aerotolerant Campylobacter jejuni in Retail Poultry with Potential Implication in Human Infection

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Campylobacter jejuni is a leading cause of foodborne illnesses around the world. Since C. jejuni is microaerophilic and sensitive to oxygen, aerotolerance is important in the transmission of C. jejuni to humans via foods under aerobic conditions. In this study, 70 C. jejuni strains were isolated from retail raw chicken meats and were subject to multilocus sequence typing (MLST) analysis. In the aerotolerance testing by aerobic shaking at 200 rpm, 50 (71.4%) isolates survived after 12 h (i.e., aerotolerant), whereas 20 (28.6%) isolates did not (i.e., aerosensitive). Interestingly, further aerobic cultivation showed that 25 (35.7%) isolates still survived even after 24 h of vigorous aerobic shaking (i.e., hyper-aerotolerant). Compared to aerosensitive strains, the hyper-aerotolerant strains exhibited increased resistance to oxidative stress, both peroxide and superoxide. A mutation of ahpC in hyper-aerotolerant strains significantly impaired aerotolerance, indicating oxidative stress defense plays an important role in hyper-aerotolerance. The aerotolerant and hyper-aerotolerant strains were primarily classified into MLST clonal complexes (CCs)-21 and -45, which are known to be the major CCs implicated in human gastroenteritis. Compared to the aerosensitive strains, CC-21 was more dominant than CC-45 in aerotolerant and hyper-aerotolerant strains. The findings in this study revealed that hyper-aerotolerant C. jejuni is highly prevalent in raw chicken meats. The enhanced aerotolerance in C. jejuni would impact human infection by increasing possibilities of the foodborne transmission of C. jejuni under aerobic conditions.

Keywords: Campylobacter jejuni, aerotolerance, chicken isolates, MLST-genotyping, oxidative stress

INTRODUCTION

Campylobacter jejuni is one of the leading bacterial causes of gastroenteritis (Altekruse et al., 1999), annually causing approximately 400–500 million infection cases worldwide (Ruiz-Palacios, 2007). C. jejuni is a commensal bacterium in a wide range of animals and is zoonotically transmitted to humans mainly by the consumption of contaminated animal products (Nielsen et al., 2006; Wilson et al., 2008). Particularly, high colonization levels of C. jejuni in the poultry intestines often result in the contamination of poultry products during processing, and contaminated poultry is the major source of transferring C. jejuni to humans (Silva et al., 2011). In Canada, 62% of retail raw chicken legs are contaminated with Campylobacter (Bohaychuk et al., 2006). In UK and US, similarly, Campylobacter is found in 76 and 57.3% of retail
poultry products, respectively (Cui et al., 2005; Little et al., 2008). The transmission of _Campylobacter_ is also caused by contamination through food handlers and cross-contamination involving contaminated kitchen equipment (Medeiros et al., 2008; Kennedy et al., 2011). _Campylobacter_ is also isolated from various environmental sources, such as wildlife, sewage, and manure, which may work as vehicles to disseminate _Campylobacter_ in food production systems and to humans (Whiley et al., 2013). In both foodborne and environmental routes of _C. jejuni_ transmission to humans, _C. jejuni_ should overcome harsh environmental conditions, particularly high oxygen tensions in atmosphere, which may directly impact the viability of _C. jejuni_.

As a thermotolerant and microaerophilic bacterium, _C. jejuni_ grows optimally at 42°C under low oxygen environments (Young et al., 2007). Although _C. jejuni_ is sensitive to high oxygen tensions in atmosphere, it exhibits a certain level of aerotolerance to survive under oxygen-rich conditions (Kaakoush et al., 2007; Luber and Bartelt, 2007). Aerotolerance is closely related to oxidative stress defense, since aerobic exposure results in the accumulation of toxic reactive oxygen species (ROS) that may give damage to proteins and lipids in _C. jejuni_ (Oh et al., 2015). A few genes of oxidative stress defense have been shown to affect aerotolerance in _C. jejuni_. For instance, a mutation of _fdxA_ encoding the ferredoxin FdxA significantly reduces aerotolerance in _C. jejuni_ (van Vliet et al., 2001). A double mutation of bacterioferritin comigratory protein (i.e., Bcp) and thiol peroxidase (i.e., Tpx) impairs the aerotolerance of _C. jejuni_ (Atack et al., 2008). Recently, we reported that alkyl hydroperoxide reductase (AhpC) plays a more important role in the aerotolerance of _C. jejuni_ than other key ROS-detoxification enzymes, such as catalase (KatA) and superoxide dismutase (SodB; Oh et al., 2015).

Presumably due to our traditional notion that _C. jejuni_ is sensitive to oxygen, aerotolerance has not been investigated extensively in _C. jejuni_ isolates from poultry. To examine the level of aerotolerance in _C. jejuni_, in the present study, we isolated 70 _C. jejuni_ strains from retail chicken meats in Edmonton, Alberta, and analyzed their aerotolerance, revealing that aerotolerant and hyper-aerotolerant _C. jejuni_ strains are highly prevalent in chicken. Moreover, most hyper-aerotolerant _C. jejuni_ isolates belong to the clonal complexes (CCs) of multilocus sequence typing (MLST) that are frequently implicated in human infection and outbreaks.

**MATERIALS AND METHODS**

**Isolation of _C. jejuni_ from Retail Chicken Meats**

Chicken meats of different brands and products were purchased from seven different retail stores in Edmonton, Canada. _C. jejuni_ was isolated as described by Chon et al. (2012) with some modifications. Briefly, the chicken meats were submerged in buffered peptone water (Oxoid, UK) at 37°C overnight and then inoculated in Bolton _Campylobacter_ selective broth (Oxoid) at 42°C for 24 h under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂). Aliquots (100 µl) were serially diluted and spread on Mueller-Hinton (MH) agar plates supplemented with Preston _Campylobacter_ selective supplements (Oxoid). Cultures were incubated at 42°C for 48 h under microaerobic conditions. _C. jejuni_ colonies were confirmed by multiplex PCR as described previously (Wang et al., 2002). The primer sequences were described in Table 1. All _C. jejuni_ strains were grown on MH media at 42°C under microaerobic conditions. Occasionally, culture media were supplemented with kanamycin (50 µg/ml).

**MLST Analysis**

MLST analysis of _C. jejuni_ isolates was performed based on the method outlined in pubMLST (pubmlst.org) and a previous report (Dingle et al., 2001) by using seven housekeeping genes, including _aspA_, _glnA_, _glyA_, _pgm_, _tkt_, and _uncA_ (Table 1). Overnight culture of _C. jejuni_ on MH agar plates was harvested in 1 ml of PBS, and then 10 µl of _C. jejuni_ suspension was mixed with 90 µl of PBS and boiled for 10 min. After centrifugation, pellets were removed, and supernatant was used as template. PCR was carried out with ExTaq polymerase (Takara, Japan). PCR amplicons were commercially sequenced by Macrogen (Seoul, Korea), and the sequences were analyzed in the _Campylobacter_ PubMLST database (http://pubmlst.org/campylobacter/).

**Aerotolerance Test**

Aerotolerance test was carried out according to our previous report (Oh et al., 2015). Briefly, _C. jejuni_ strains were grown...
TABLE 2 | Clonal complexes of C. jejuni isolates from raw chicken meats.

| Clonal complex | ST no. | Isolate number | Sources      |
|----------------|--------|----------------|--------------|
| 21             | 13     | 66             | Whole chicken|
| 21             | 23     | Whole chicken  |
| 24             | Whole chicken |
| 32             | Whole chicken |
| 33             | Whole chicken |
| 34             | Whole chicken |
| 36             | Whole chicken |
| 37             | Whole chicken |
| 43             | 29     | Thigh          |
| 44             | Whole chicken |
| 50             | 25     | Whole chicken  |
| 30             | Whole chicken |
| 31             | Whole chicken |
| 35             | Whole chicken |
| 38             | Whole chicken |
| 506            | 62     | Whole chicken  |
| 63             | Whole chicken |
| 64             | Whole chicken |
| 65             | Whole chicken |
| 1086           | 8      | Whole chicken  |
| 43             | Whole chicken |
| 2375           | 13     | Whole chicken  |
| 2377           | 68     | Drumstick      |
| 3794           | 10     | Whole chicken  |
| 4663           | 15     | Drumstick      |
| 4881           | 11     | Whole chicken  |
| 4911           | 67     | Whole chicken  |
| 6261           | 7      | Whole chicken  |
| LA             | 48     | Drumstick      |
| 49             | Drumstick |
| 50             | Drumstick |
| 69             | Drumstick |
| 70             | Drumstick |
| 934            | 54     | Whole chicken  |
| 158            | 28     | Whole chicken  |
| 1352           | 55     | Thigh          |
| 45             | 52     | Whole chicken  |
| 53             | Whole chicken |
| 56             | Whole chicken |
| 57             | Whole chicken |
| 58             | Whole chicken |
| 137            | 2      | Whole chicken  |
| 4              | Whole chicken |
| 659            | 1      | Whole chicken  |
| 998            | 27     | Drumstick      |
| 1818           | 21     | Whole chicken  |
| 22             | Whole chicken |
| 6193           | 26     | Whole chicken  |
| 59             | Whole chicken |
| 60             | Whole chicken |
| 61             | Whole chicken |

TABLE 2 | Continued

| Clonal complex | ST no. | Isolate number | Sources      |
|----------------|--------|----------------|--------------|
| 362           | 587    | 3              | Whole chicken|
| 14            | Whole chicken |
| 39            | Whole chicken |
| 40            | Whole chicken |
| 41            | Whole chicken |
| 42            | Whole chicken |
| 353           | 452    | 6              | Whole chicken|
| 3             | Whole chicken |
| 9             | Whole chicken |
| 3484          | 45     | Whole chicken  |
| 354           | 2359   | 5              | Whole chicken|
| 48            | 142    | 51             | Thigh        |
| NTb           | 17     | Whole chicken  |
| 18            | Whole chicken |
| 19            | Whole chicken |
| 20            | Whole chicken |
| 46            | Drumstick |
| 47            | Drumstick |

a LA: isolates that were unassigned to any CC defined.
b NT: not typable.

on MH agar at 42°C for 18 h under microaerobic conditions. C. jejuni strains were resuspended in fresh MH broth and diluted to an OD600 of 0.07, and then bacterial suspensions were incubated at 42°C with shaking at 200 rpm under aerobic conditions. Samples were taken after 0, 12, and 24 h for serial dilution and CFU counting.

Susceptibility to Oxidative Stress

Campylobacter jejuni strains were inoculated in MH broth at 42°C for 8 h with agitation under microaerobic conditions, and then bacterial cultures were exposed for 1 h to oxidants, including 100 µM of cumene hydroperoxide (CHP), 1 mM of hydrogen peroxide (H2O2) and 100 µM menadione (MND; a superoxide generator). The viability was determined by serial dilution and CFU counting.

Construction of ahpC Mutants

A suicide plasmid for an ahpC mutation in C. jejuni was described in our previous study (Oh and Jeon, 2014). The ahpC suicide vector was introduced to C. jejuni strains by electroporation, and ahpC mutants were selected by growing on MH agar supplemented with kanamycin (50 µg/ml). The ahpC mutation was also confirmed by PCR with mahpC-F and mahpC-R primers (Table 1).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, inc., USA). Statistical significance of differences between the groups was compared by using two-way analysis of variance (ANOVA). The frequency distribution was determined as described elsewhere (Callicott et al., 2008) by performing the Mann–Whitney test followed by one-way
RESULTS

MLST Analysis of C. jejuni Isolates from Retail Chicken Meats

We isolated 70 C. jejuni strains from 19 raw chicken meats from seven different retail stores in Edmonton, Alberta. The majority (77.1%) of C. jejuni strains was isolated from whole chicken samples, and 18.6% from drumsticks, and 4.3% from thighs (Table 2). The MLST results showed that the 70 strains were distributed in six different CCs. The major CCs were 21 and 45 that constituted 42.86 and 21.43% of C. jejuni isolates from chickens, respectively (Table 2 and Supplementary Figure S1).

Differential Aerotolerance Levels in C. jejuni Chicken Isolates

The aerotolerance of the 70 C. jejuni isolates from chickens was investigated by growing them aerobically with vigorous shaking...
at 200 rpm. Depending on the levels of aerotolerance, the 70 isolates were clustered into three groups: (i) the aerosensitive group that lost viability before 12 h (Figure 1A), (ii) the aerotolerant group in which C. jejuni strains maintained viability for 12~24 h (Figure 1B), and (iii) the hyper-aerotolerant group where C. jejuni strains remained viable even after 24 h of aerobic shaking (Figure 1C). Whereas a total of 20 strains were aerosensitive, most isolates were aerotolerant or hyper-aerotolerant (Figure 1). Interestingly, 25 C. jejuni strains were hyper-aerotolerant and maintained viability even after 24 h of vigorous shaking (at 200 rpm) under aerobic conditions (Figure 2C). The major CCs were CC-45 (35%) and CC-21 (30%) in the aerosensitive group (Figure 2A). However, CC-21 was predominant in both aerotolerant and hyper-aerotolerant groups; 48% in each (Figures 2B,C). No C. jejuni isolates in CC-353 and CC-362 were aerosensitive, but they constituted about 16% of hyper-aerotolerant C. jejuni isolates (Figures 2A,C). These data show high prevalence of hyper-aerotolerant C. jejuni in retail chicken meats, which clusters mainly in a few major CCs.

**Oxidative Stress Resistance in C. jejuni Isolates**

Since oxidative stress defense plays an important role in the aerotolerance of C. jejuni (Oh et al., 2015), oxidative stress resistance was compared between the different aerotolerance groups. Two strains from each aerotolerance group were randomly chosen and exposed to different kinds of oxidants for 1 h for viability testing. The strains from the aerosensitive group easily lost viability by exposure to H₂O₂, CHP, and menadione (MND). However, two strains from the hyper-aerotolerant group demonstrated significantly enhanced resistance to oxidative stress, both peroxide and superoxide (Figure 3). The strains from the aerotolerant group were more resistant to oxidants than the strains from the aerosensitive group but more susceptible than those from the hyper-aerotolerant group (Figure 3). The results clearly indicate that hyper-aerotolerant strains are more resistant to oxidative stress than aerosensitive strains.
Role of \( \text{ahpC} \) in the Aerotolerance of Hyper-aerotolerant \( C. \text{jejuni} \) Isolates

Our previous study showed that \( \text{ahpC} \) plays a key role in \( C. \text{jejuni} \) survival under aerobic conditions, compared to other ROS-detoxification genes, such as \( \text{katA} \) and \( \text{sodB} \) (Oh et al., 2015); thus, we decided to investigate how \( \text{ahpC} \) contributes to hyper-aerotolerance in \( C. \text{jejuni} \). We constructed \( \text{ahpC} \) knockout mutants of the six \( C. \text{jejuni} \) strains from the three different aerotolerance groups. The \( \text{ahpC} \) mutation in the strains from the aerosensitive group resulted in approximately 3 log CFU reduction compared to wild type after 6 h of aerobic culture (Figure 4). Interestingly, \( \text{ahpC} \) mutants of the hyper-aerotolerant strains did not survive after 24 h, indicating the \( \text{ahpC} \) mutation reduced the aerotolerance level from hyper-aerotolerance to aerotolerance. These findings clearly showed that oxidative stress defense, particularly \( \text{ahpC} \), is critical for the hyper-aerotolerance in \( C. \text{jejuni} \).

DISCUSSION

The poultry intestines provide optimal growth conditions for \( C. \text{jejuni} \), such as high body temperature (42°C), high nutrients, and low oxygen levels. However, \( C. \text{jejuni} \) is exposed to oxygen-rich conditions during food processing and preservation. Thus, oxidative stress is an unavoidable stress that \( C. \text{jejuni} \) should overcome to survive during its foodborne transmission to humans (Kim et al., 2015). Despite our common perception that \( C. \text{jejuni} \) is sensitive to oxygen, in this study, we revealed that hyper-aerotolerant \( C. \text{jejuni} \) is highly prevalent in raw chicken meats. \( \text{Arcobacter} \) sp. are similar to \( \text{Campylobacter} \) and previously described as an aerotolerant \( \text{Campylobacter} \)-like organism. \( \text{Arcobacter} \) is associated with animals and humans, and is frequently isolated in poultry (Vandamme et al., 1992; Snelling et al., 2006). Therefore, specific identification methods are needed to differentiate between \( \text{Campylobacter} \) and \( \text{Arcobacter} \) (Call et al., 2003), and multiplex PCR is often used for this purpose (Mandrell and Wachtelt, 1999). The \( \text{hipO} \) gene encoding hipuricase is present only in \( C. \text{jejuni} \) but not in any other \( \text{Campylobacter} \) sp. and \( \text{Arcobacter} \) sp. (Wang et al., 2002; Jensen et al., 2005). Whereas \( C. \text{jejuni} \) preferably grows at 42°C, \( \text{Arcobacter} \) grows optimally 24~30°C (Phillips, 2001). In this study, \( C. \text{jejuni} \) was isolated at 42°C, at which \( \text{Arcobacter} \) cannot grow (Bhunia, 2008). In addition, the isolates were confirmed by PCR with primers for the \( \text{hipO} \) gene and classified by the MLST scheme for \( C. \text{jejuni} \).

Most \( C. \text{jejuni} \) isolates from raw chicken meats belonged to CC-21 (42%) and CC-45 (21%; Table 2 and Supplementary Figure S1). This is consistent with previous reports from Canada and other countries. The ST-21 and ST-45 complexes are most dominant in a variety of sources around the world, accounting for 39 and 14% \( C. \text{jejuni} \) isolates deposited in pubMLST, where 76% of isolates in the CC ST-21 and 60% in the CC ST-45 are of human origin, such as stool and blood (Colles and Maiden, 2012). In the UK, CCs ST-21 and ST-45 are commonly detected in veterinary (i.e., cow, pet, sheep, and poultry) and human sources (Manning et al., 2003). CCs 21, 45, 353, and 354 are also highly prevalent in sources from humans and foods in Eastern China (Zhang et al., 2015). According to the results of an MLST analysis of 122 \( C. \text{jejuni} \) isolates from Danish patients with symptoms of gastroenteritis, reactive arthritis, and Guillain–Barré syndrome, CCs ST-21, -45, and -22 are highly prevalent, accounting for 64% of all \( C. \text{jejuni} \) isolates from humans. Whereas ST-22 is significantly linked to Guillain–Barré syndrome, ST-21 and -45 are primarily associated with gastroenteritis (Nielsen et al., 2010). An extensive MLST analysis of 289 \( C. \text{jejuni} \) isolates in Canada showed that CCs 21, 45, and 353 constitute the major \( C. \text{jejuni} \) population (CC-21: 26%, CC-45: 18%, and CC-353: 11%) in a variety of sources, including humans, chickens, raw milk, and environmental water. Interestingly, more than 50% of isolates that were classified in CC-21 were from humans (Lévesque et al., 2008), indicating high frequencies of human infection by \( C. \text{jejuni} \) strains in CC-21. In the US, an MLST analysis of 47 \( C. \text{jejuni} \)
isolates from 12 outbreaks exhibited that CC ST-21 is commonly involved in human epidemics (Sails et al., 2003). Whereas CC-21 and CC-45 were distributed at similar levels in the aerosensitive C. jejuni isolates (CC-21: 30%, CC-45: 35%; Figure 2A), CC-21 is predominant in both aerotolerant (48%) and hyper-aerotolerant (48%) isolates (Figures 2B,C), and the frequencies of CC-21 distribution in aerotolerant and hyper-aerotolerant groups were statistically significant (Supplementary Figure S2).

Hyper-aerotolerant C. jejuni strains were more resistant to peroxide (i.e., H2O2 and CHP) and superoxide (i.e., menadione) stresses than aerosensitive strains (Figure 3), suggesting that increased resistance to oxidative stress would contribute to hyper-aerotolerance in C. jejuni. Our previous study showed that ahpC is the most important ROS-detoxification gene in the aerotolerance of C. jejuni (Oh et al., 2015). In this study, we demonstrated that ahpC is also a key player in C. jejuni’s hyper-aerotolerance (Figure 4). Enhanced resistance to oxidative stress may result in hyper-aerotolerance; this enables hyper-aerotolerant C. jejuni to survive easily under aerobic conditions during food processing, increasing risks of foodborne transmission to humans. Interestingly, it has been reported that C. jejuni strains in CC-21 from poultry and human clinical samples frequently exhibit hyper-invasiveness than strains in other CCs (Fearnley et al., 2008). C. jejuni strains in CC-21 usually (85.7%) have the sialylated lipooligosaccharide (LOS) class C and are highly invasive than isolates from other CCs (Habib et al., 2009). Taken our findings and these previous reports together, C. jejuni strains in CC-21 are often hyper-aerotolerant and likely to be invasive. Presumably, this would be why CC-21 is frequently involved in human infection and outbreaks (Sails et al., 2003; Nielsen et al., 2010).

To be the best of our knowledge, this is the first report about the prevalence of hyper-aerotolerant C. jejuni in chicken meats. Importantly, hyper-aerotolerant C. jejuni isolates are distributed mostly in the CCs that are often implicated in human infection, suggesting potential impact of hyper-aerotolerance on food safety and public health. Although we showed oxidative stress defense contributes to hyper-aerotolerance in C. jejuni in this study, there seems to be other unknown factors associated with hyper-aerotolerance since ahpC reduced aerotolerance, but not completely eliminated it (Figure 4). Based on the differential clustering of the LOS class in CC-21 (Habib et al., 2009), possibly some other genetic variations might also be involved in hyper-aerotolerance. Therefore, an extensive future study, such as whole genome sequencing, will be needed to further characterize hyper-aerotolerant C. jejuni and to reveal its implication in human infection.

AUTHOR CONTRIBUTIONS

Design of the project: EO, LM, and BJ; Performance of the experiments: EO; Data analysis: EO, LM, and BJ; Writing of the manuscript: EO, LM, and BJ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01263

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