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Antimicrobial activity of three wines against *Campylobacter jejuni* and the effect of low temperature on their survival ability

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Forty-three (43) *Campylobacter jejuni* isolated from sheep feces and carcass swab samples by conventional culture method were 100% confirmed by polymerase chain reaction (PCR) test. The nucleotide sequences of 362 bp showed 100% homology to the completed genome of *C. jejuni* subsp. M1 and 81116. Antimicrobial property of different types of wine against *C. jejuni* was investigated. White wine and two red wines with 11.5% ethanol significantly reduced the viability of *C. jejuni*. The minimum bactericidal concentration of white wine and for both red wines was 10 and 25%, respectively. *C. jejuni* lost their viability when frozen or refrigerated at -20 and 4°C. The reduction rates of *C. jejuni* were 0.54 - 1.02 logCFU/ml during storage at 4°C, whereas it exhibited 0.72 - 1.52 logCFU/ml during storage at -20°C.

Key words: Conventional culture, polymerase chain reaction (PCR), viability.

INTRODUCTION

Campylobacter is the most common cause of foodborne bacteria gastroenteritis in humans worldwide. Incidences of *Campylobacter* have increased steadily over the years. The European Food Safety Authority reported 212,064 cases for *Campylobacter* (EFSA, 2012). Previous studies showed that *Campylobacter jejuni* is the leading cause of bacterial food borne illness in the world, (Skirrow, 1994; Frost et al., 1998; Nayak et al., 2003; Wilson, 2003; EFSA, 2012). Hence, it may be feasible to implement multiple barriers along the farm to the processing stage of the product to reduce human campylobacteriosis and reliable identification method.

Wines have a potent antimicrobial property against *Campylobacter* which has been demonstrated under various experiments (Boban et al., 2010; Isohanni et al., 2010). Various studies described the antimicrobial properties of wine against different relevant food-borne pathogens. Some studies indicated that the strength of antimicrobial property of wine is attributed to different components of wines and a better antimicrobial proficiency associated with red wine than white wine because of high level of phenolic compound in red wine (Sugita-Konishi et al., 2001; Just and Daeschel, 2003; Moretro and Daeschel, 2004; Papadopoulou et al., 2005; Mónica et al., 2009). However, others studies reported that phenol-stripped wines have considerable antimicrobial activity that is not attributed to its phenolic and non-phenolic compound, demonstrating that the antimicrobial activity of wine cannot be expected only on the basis of the content of particular components of wine (Weisse et al., 1995; Boban et al., 2010). Meanwhile, other studies reported that the antimicrobial efficacy of phenolic com-

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pounds in wine may be also enhanced by other components of the inherent environmental factors such as low pH, the combination of ethanol and organic acids (Wen et al., 2003; Anabela et al., 2008; Mónica et al., 2009), on the other hand, Mónica et al. (2009) reported that wines having 11.5% ethanol significantly reduced the viability of *C. jejuni*. For these reasons, wines might be used as antimicrobial ingredients together with the addition of further antimicrobial agents to reduce the numbers of *Campylobacter*, thus lowering the risk of *Campylobacter* cross-contamination and transmission through food (Anabela et al., 2008; Isohanni et al., 2010).

The response of *C. jejuni* to low temperatures has been studied in some detail, and showed a remarkable and sudden growth rate decline near the lower temperature limit (Anonymous, 1996; Hazeleger et al., 1998). It is important to better understand the behavior of *C. jejuni* in the food production environment, and the methods to reduce/prevent their ability to survive in a certain food production processes. Therefore, we studied the effect of three types of wine and low temperature on the survival of *C. jejuni* that was isolated from sheep faeces and carcass swab samples by using the combination of conventional culture and polymerase chain reaction (PCR) methods for a rapid and sensitive finding.

MATERIALS AND METHODS

Culture of *C. jejuni*

Forty three *C. jejuni* were isolated from sheep fecal and carcass swab samples by microbiological conventional method. The culture was grown onto modified Charcoal, Cefoperazone, Desoxycholate Agar (mCCDA CM739B Oxoid Ltd. Basingstoke, Hampshire UK). The mCCDA is blood free selective medium with CCDA selective supplement SR155E recommended for the isolation of thermophilic *Campylobacter* spp. from clinical and environmental samples. The plates were incubated under a microaerophilic atmosphere (85% N_2, 10% CO_2, 5% O_2) using CampyGen^{TM} gas generating kits (Oxoid) at 42°C for 48 h. *Campylobacter* spp. was identified based on the characteristics of colony appearance, Gram staining reactions and positive test for oxidase and catalase reactions, hippurate hydrolysis, susceptibility to nalidixic acid (30 µg) and cephalothin (30 µg (Oxoid). These parameters formed the basis for the identification of *C. jejuni* as proposed by On (1996). The type strains *C. jejuni* (NCTC 11351) were included as positive control. The isolated *C. jejuni* were used to be confirmed by PCR technique for the subsequent tests.

DNA isolation

The pellet of *C. jejuni* was used to extract DNA using the method of Chen and Kuo (1993) with modification. The pellets were placed in 300 µl of solution I [100 ml of solution I contained 0.2 M sucrose, 0.1 M NaCl, 0.03 M Tris-HCl, 0.01 M EDTA, pH 8.0] and 600 µl of solution II was added [100 ml of solution II contained 50 mM Tris-HCl, 50 mM EDTA, 5 g of 10% SDS]. After incubated at 65°C for 30 min, 300 µl of solution III [100 ml of solution III contained 5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml and 28.5 ml of distilled water] and 200 µl CH_2Cl, IAA were added, and were incubated at -20°C for 30 min and centrifuged at 16,000 x*g* at 4°C for 10 min, the supernatant was carefully transferred into a new PCR tube followed by the addition of 400 µl isopropanol and centrifuged at 16,000 xg at 4°C for 10 min. The DNA pellet was washed with 70% ethanol then centrifuged at 16,000 xg for 10 min at 4°C. Finally, the DNA obtained was dried at room temperature, and dissolved with 15 to 20 µl of distilled water.

Polymerase chain reaction (PCR)

Oligonucleotide primers specific for *Campylobacter* species 5'TGACGCTAGTGTTGTAGGAG 3' and 5'GTTGCACTTAGCGATGATGG 3' (CL2-CR3) described by Ng et al. (1997) for *C. jejuni* (*C. jejuni* ORF1 gene partial cds, 1418 bp [GenBank accession number U27272]) were used to amplify DNA fragment. The total volume 25 µl of PCR reaction mixture contain 2 µl of DNA template, 2.5 µl of 10x PCR buffer, 2.5 µl of 50 mM of MgCl, 1.0 µl tag DNA polymerase, 1.0 µl of 10mM dNTPs, 2.5 µl of each primer and 11 µl of deionized water. The PCR tubes were placed in a thermocycler and the reaction started by denaturation for 10 min at 95°C followed by 25 cycles of denaturation at 95°C for 15 s, annealing at 48°C for 15 s and extension at 72°C for 30 s, a final 10 min incubation at 72°C was allowed for the completion of primer extension after the last cycle. The amplified product was electrophoresed in 1.2% agarose gel at 100 V for 30 min stained with ethidium bromide and photographed by UV light.

DNA sequencing

The PCR products were purified using the Wizard^{R} SV Gel and PCR Clean-UP System. The purified PCR product was used to long read sequencing by sequence analyzer at DNA technology laboratory in 1st BASE DNA sequencing company Malaysia.

Antimicrobial activity of different types of wine against *C. jejuni*

Three types of wine: white wine (Kemila) and two red wines (Aksumit and Gouder, 2010) with the same alcohol content (11.5%) were used in this study. *Campylobacter* inoculums were suspended in phosphate buffered solution (PBS) and wine (red and white) in a concentration of 1, 10, 25, 50, 75 and 100% then were serially diluted and were incubated for 10 min at 150 rpm in a shaker. Then, the inoculums was plated on to Mueller-Hinton agar with 5% sheep blood and incubated under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) for 48 h at 42°C for the subsequent analysis.

Effect of low temperature on the viability of *C. jejuni*

Approximately, the same size of *C. jejuni* colonies were chosen from mCCDA blood free agar plate and were inoculated into 10 ml of 0.1% peptone broth + 0.9% NaCl (obtaining 2.5 logCFU/ml concentration). One milliliter of the cultures were added to 9 ml of Brucella Broth (BB) and placed at 4 and -20°C for 2, 7 and 14 days. Then, subsequent cultivation was spreaded on Muller Hinton agar with 5% sheep blood plate and incubated in a microaerophilic atmosphere at 42°C for 48 h.

Statistical analysis

The SAS software version 9.00 (TS-MO) was used to determine the significance differences of the effect of different type of wine and low temperature on the survival of *C. jejuni*. The data was determined using GLM procedure. A value of $p \leq 0.05$ was an

Table 1. *C. jejuni* isolated by conventional culture method and confirmed by polymerase chain reaction.

Diagnostic method	Tests used to detect *Campylobacter jejuni*						
	Morphology	**Catalase**	**Oxidase**	**NA**	**Cephalothin**	**Hippurate**	***C. jejuni* detected**
Conventional	Spiral, curved	+	+	S	R	+	43
PCR	-	-	-	-	-	-	43

S, Susceptible; R, resistance; PCR, polymerase chain reaction.

indicative of statistically significant difference.

RESULTS

Isolation of *C. jejuni* by conventional culture method and PCR

A total of 43 *C. jejuni* were isolated from sheep faeces and carcass swab samples by conventional culture method, and was confirmed 100% by PCR test as shown in Table 1. The confirmation of *C. jejuni* by PCR with the set of primer CL2-CR3 forward and reverse, respectively, yielded 362 bp.

Nucleotide analysis

The PCR products of *C. jejuni* isolates (KC433407) were sequenced and analyzed. For the nucleotide sequences of 362 bp obtained in this study. The sequence of *C. jejuni* exhibits 100% homology with the sequence from *C. jejuni* subsp. *jejuni* M1 and *C. jejuni* subsp. jejuni 81116 completed genome (362 bp when compared with GenBank accession numbers CP001900 and CP000814). In addition, the sequence of *C. jejuni* (KC433407) showed 99% homology to *C. jejuni* subsp. *jejuni* S3 and RM1221 (362 bp when compared with GenBank accession numbers CP001960 and CP00025).

The nucleotide sequences were aligned together with the sequences of *C. jejuni* completed genome and the reference sequence for primer design (Figure 1). The results showed that the sequence of *C. jejuni* (KC433407, 362 bp) was exactly similar to CP001900 and CP000814 whereas three position differences were found when compared with CP001960 and CP000025 and 12 position different from U27272 (Primer reference) (Ng et al., 1997).

Antimicrobial property of wine against *C. jejuni*

The results show that all the three tested wine with 11.5% ethanol exhibited significant ($p \leq 0.05$) antimicrobial activity against *C. jejuni*. Table 2 shows the viability of *C. jejuni* for the different concentrations of wine. The results showed that white wine and red wines had high microbicidal effect against *C. jejuni*, the minimum microbicidal concentration of white wine and for both red wines were 10 and 25%, respectively. The results suggest that white wine has a higher microbicidal effect against *C. jejuni* than red wine. There was no growth of *C. jejuni* at 25% and above for white wine whereas the two red wines completely inhibited the growth of *C. jejuni* at 50%.

Effect of low temperature on the viability of *C. jejuni*

C. jejuni cells lost their viability when frozen or refrigerated at -20or 4°C as shown in Table 3. The rate of reduction of *C. jejuni* was 0.54, 0.74 and 1.02 logCFU/ml during storage at 4°C for 2, 7 and 14 days, respectively. On the other hand, when stored at -20°C for 2, 7 and 14 days, the reduction Log CFU/ml were 0.72, 1.17 and 1.52, respectively. Storage by freezing at -20°C for more than 2 days showed statistically significantly different ($p \leq 0.05$) on the reduction of *C. jejuni* count when compared with refrigerating at 4°C for 7 days.

DISCUSSION

Forty three *C. jejuni* isolated from sheep faeces and carcass swab samples by conventional culture method found 100% positive by PCR technique. This result was comparable with the finding of 100% detection of *C. jejuni* by both conventional culture and PCR method by Ertas et al. (2003) and Rahimi et al. (2010). The effectiveness of the combination of conventional culture and PCR methods for the rapid and sensitive finding of such bacteria was reported previously (Magistrado et al., 2001). Therefore, PCR method is a suitable, sensitive test and more rapid genetic assay for the identification and differentiation of *Campylobacter* spp. The sequence of *C. jejuni* (362 bp) in current study exhibited 100% homology with the sequence from *C. jejuni* subsp. *jejuni* M1 (1616648 bp) and *C. jejuni* subsp. jejuni 81116 (1628115 bp) completed genome. However, the nucleotide analysis of this result cannot be suggested as sup-species M1 or 81116 because it only represented small region of 362 bp when we compared it with completed genome. This finding is interesting for further study of *C. jejuni* sub-species in Ethiopia.

The current study show that wines (white and red) had

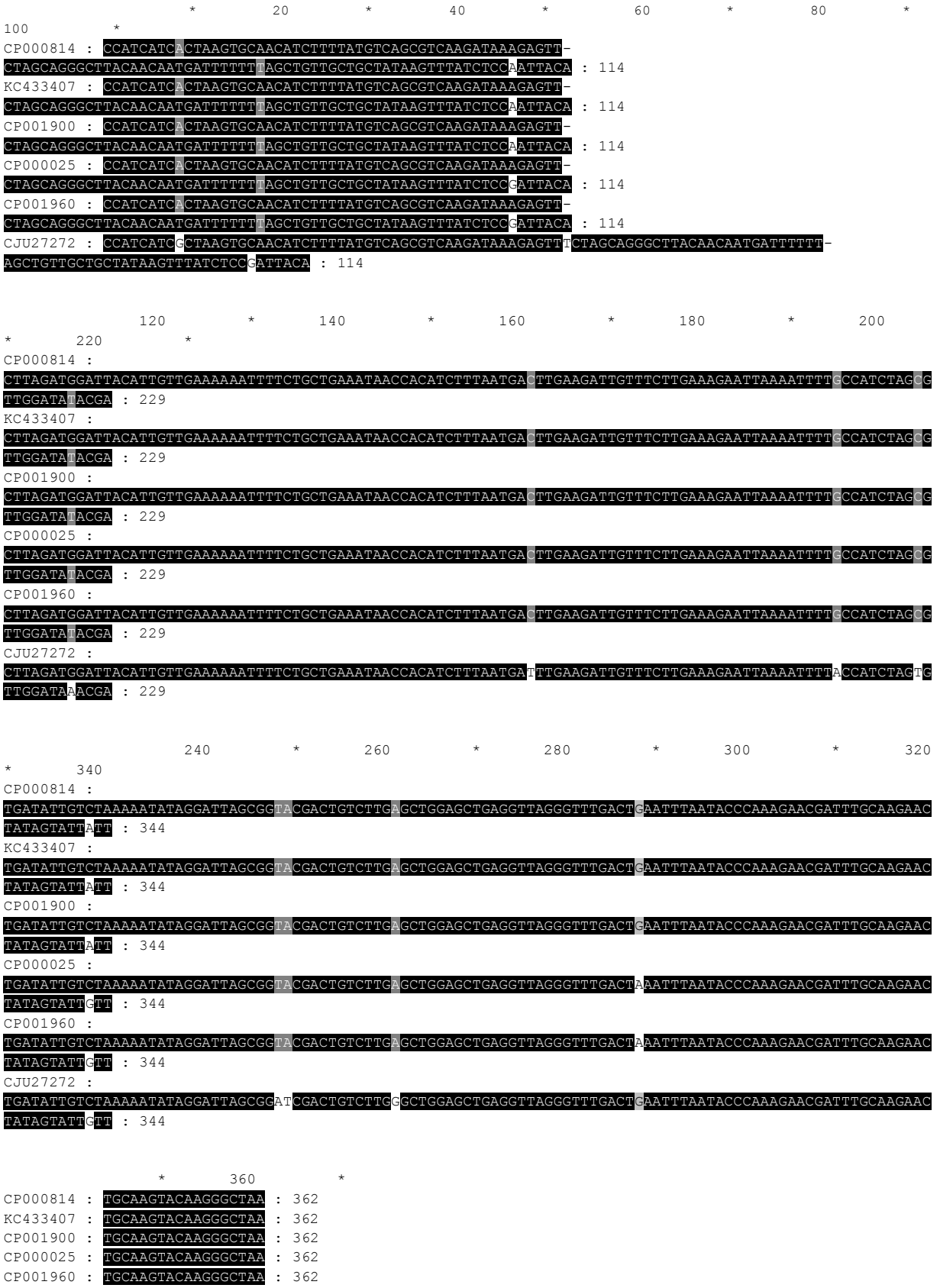

Figure 1. The nucleotide sequence alignment of *C. jejuni* (CP000814, CP001900, CP000025, CP001960 and CJU27272) and *C. jejuni* isolated (KC433407).

Table 2. Effect of different proportions (0, 1, 10, 25, 50, 75 and 100%) of white and two types of red wine on the viability of *C. jejuni*.

Wine (%)	0	1	10	25	50	75	100
White wine /Kemila	2.37±1.035	1.95±1.088	0.75±0.91	0.00	0.00	0.00	0.00
Red wine /Axumit	2.38±1.035	1.98±1.067	1.04±1.004	0.06±0.978	0.00	0.00	0.00
Red wine /Gouder	2.38±0.96	2.01±1.155	1.19±1.067	0.59±0.992	0.00	0.00	0.00

Results are expressed as mean ± standard deviation LogCFU/ml (n = 3).

Table 3. The effect of low temperature on the viability of *C. jejuni*.

Temperature	Day 0	Day 2	Day 7	Day 14
4°C	2.5±1.15	1.96±1.175	1.76±1.097	1.48±1.097
-20°C	2.5±1.255	1.78±1.072	1.33±1.097	0.98±1.076

Results are expressed as mean ± standard deviation Log cfu/ml (n = 3).

high antimicrobial activity against *C. jejuni*. which has been demonstrated under various experiment (Wen et al., 2003; Puupponen-Pimia et al., 2005; Rodríguez-Vaquero et al., 2007a, b; Monica et al., 2009; Boban et al., 2010; Isohanni et al., 2010). Murray et al. (2002) showed that the consumption of wine reduces health risk by 6% demonstrating that wines have potent antimicrobial activity against foodborne pathogens. In this study, we found that white and red wine with 11.5% alcohol had high antimicrobial activity against *C. jejuni* with the minimum microbicidal concentrations of 10% dilution for white and 25% for two red wines while Monica et al. (2009) reported higher antimicrobial activity of red wine (10%) than white wine (25%) by giving credit to the high level of phenolic compound that red wine contain. However, Boban et al. (2010) reported that phenol-stripped wine have significant antimicrobial activity after intact wine and the potency of antimicrobial power of intact wine is not attributed to its phenolic and non-phenolic compound constituents. Weisse et al. (1995) also demonstrated that white wine reduced the number of organisms more rapidly than the red wine dilutions. Some studies give emphasis to the role of wine phenolic compound, some others accentuating the role of non-phenolic constituents of wine and some others to the combination of ethanol with organic acids (Anabela et al., 2008; Mónica et al., 2009). Moreover, the results of this study had significant importance in Ethiopia, in the country where raw meat consumption is widely accustomed, increasing the possibility of pathogen transmission to human.

The results of effect of low temperature on the viability of *C. jejuni* showed that the number of *C. jejuni* decrease greatly during freezing. Our results showed higher reduction rate during freezing at -20°C than refrigerating at 4°C, similar to observation by Franklın et al. (2006). Different studies showed *Campylobacter* cell reduction in beef, lamb and poultry meat storage at -19 and -20°C from 1 to 2 log units during short and long term storage (Barrel, 1984; Blankenship and Craven, 1982; Franklın et al., 2006). The reduction rate between 2 and 14 days storage in this study at 4°C showed important change in the number (0.49 logCFU/ml) of *C. jejuni*.

Conclusion

The results suggest that wines could be used as antimicrobial ingredients in food to reduce *C. jejuni*. Wine consumption especially with food may decrease the number of *C. jejuni* persisting further in the alimentary tract. The exposure of *C. jejuni* to white and red wines significantly reduced the number of viable cells. To reduce the disease burden of human campylobacteriosis accredited to meat production stage, it may be feasible to implement multiple barriers along the farm to the processing stage of the product. This finding may warrant consideration of the public health benefit relating to freezing food of animal origin especially meat to reduce *Campylobacter* exposure level.

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