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Work-related increases in titer of *Campylobacter jejuni* antibody among workers at a chicken processing plant in Miyazaki prefecture, Japan, independent of individual ingestion of edible raw chicken meat

Elisabet TANGKONDA[1,2], Meiko KUBO[3], Satoshi SEKIGUCHI[4,5],
Taisuke SHINKI[2], Satomi SASAKI[4], Kentaro YAMADA[2,4], Takako TANIGUCHI[4],
Torrung VETCHAPITAK[4]* and Naoaki MISAWA[1,2,4]*

[1] Graduate School of Medicine and Veterinary Medicine, University of Miyazaki, 5200 Kihara-Kiyotake-cho, Miyazaki 889-1692, Japan
[2] Laboratory of Veterinary Public Health, Department of Veterinary Science, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan
[3] Miyakonojo Meat Inspection Center, 38-1 Hirae-cho, Miyakonojo-shi, Miyazaki 885-0021, Japan
[4] Center for Animal Disease Control, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan
[5] Laboratory of Animal Infectious Disease and Prevention, Department of Veterinary Science, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

ABSTRACT. Workers in poultry abattoirs may be frequently exposed to *Campylobacter jejuni*, which is a leading cause of bacterial food poisoning in Japan. The present study was conducted to measure the titers of IgG and IgA antibodies against *C. jejuni* among 104 female workers in a chicken processing plant in Miyazaki prefecture, Japan. Information regarding habitual ingestion of raw chicken meat and potential occupational risk factors was collected using a questionnaire. Acid extracts of four *C. jejuni* strains representing the genotypes most dominant in Miyazaki were used as antigens. The levels of both immunoglobulins measured by ELISA were not correlated with ingestion of edible raw chicken meat, the amount consumed in one sitting, or its frequency. Although age was correlated with antibody levels, the length of employment was not. Furthermore, the IgG and IgA levels in workers at the evisceration step were significantly higher than those at other locations in the plant. To identify the bacterial proteins recognized by the workers' IgG and IgA antibodies, Western blotting followed by LC/MS was conducted. Flagellin was identified as the common protein recognized in the sera of workers for whom ELISA demonstrated both the highest and lowest antibody levels. We concluded that the titers of IgG and IgA against *C. jejuni* in workers at the processing plant had been increased by occupational exposure to *Campylobacter*, regardless of raw chicken meat ingestion.

KEY WORDS: antibody response, *Campylobacter jejuni*, chicken processing plant, ELISA, occupation

Campylobacter jejuni is one of the most important causes of bacterial foodborne illness in humans worldwide [9]. *C. jejuni* colonizes the intestinal tract of livestock animals, especially chickens, with a high prevalence in the range of 10^3–10^9 cfu/g [20]. Such high levels allow bacteria to spread easily in the environment of a chicken processing plant, resulting in contamination of chicken carcasses, equipment, and work surfaces [5, 15]. Therefore, consumption of raw and/or undercooked chicken meat contaminated with this organism has been considered to be a main route of transmission to humans [16, 23]. Furthermore, direct contact with infected animals and contaminated products has also been reported as a possible cause of infection in humans [22, 24].

In addition to the risk of *Campylobacter* infection in consumers by ingestion of contaminated foods, occupational exposure due

*Correspondence to: Misawa, N.: a0d901u@cc.miyazaki-u.ac.jp, Vetchapitak, T.: torrung.hong@cc.miyazaki-u.ac.jp

to frequent contact with infected animals and their products may occur in farmers, veterinarians, workers in poultry processing plants, and butchers [26]. However, since such individuals rarely develop campylobacteriosis [6], some specific immune responses due to frequent exposure to low levels of *Campylobacter* may effectively protect them against disease manifestations [8]. This protection may be associated with increased antibody levels [28]. In this context, however, another parameter may be potentially relevant, i.e. the relationship between the immune response and the location in a processing plant where work is performed. Since a previous microbiological survey of *Campylobacter* contamination in chicken carcasses has demonstrated a significant difference in contamination levels among various steps of processing [25], we hypothesized that the levels of immune response among workers may differ according to location, due to the differing degrees of exposure to this pathogen.

Another factor that needs to be considered is consumption of raw and/or undercooked chicken meat or liver. In the southern part of Japan including Miyazaki and Kagoshima prefectures, there is a tradition of eating sliced raw chicken meat (so-called *torisashi*) or undercooked chicken meat (so-called *tataki*) seared only at the surface, and epidemiological studies have shown that these are the main foods responsible for campylobacteriosis in Japan [29]. This has led to the hypothesis that Japanese individuals who consume raw and/or undercooked chicken meat may have a specific humoral immune response against *Campylobacter*. However, information on this phenomenon is still lacking.

In the present study, we aimed to evaluate the levels of immunoglobulin G (IgG) and IgA against *C. jejuni* in serum samples from workers at a chicken processing plant who were occupationally exposed to *Campylobacter* spp. contamination on chicken carcasses throughout the year. A questionnaire, including items on age, consumption of raw chicken meat, work locations within the plant, length of employment, etc., was completed by each worker to evaluate the factors that influenced the detectable antibody responses. Furthermore, specific antigens of *C. jejuni* recognized in the workers' sera were also identified. To our knowledge, this is the first study to have measured the levels of antibodies against *C. jejuni* among workers at a chicken processing plant in Japan, and evaluated risk factors affecting the humoral immune response.

MATERIALS AND METHODS

Chicken carcasses

A total of 65 broiler carcasses were obtained from a commercial chicken processing plant in Miyazaki prefecture, Japan, where the present seroepidemiological investigation was conducted. The carcasses examined were collected randomly after evisceration between July 2010 and November 2011. Each individual carcass was placed in a sterilized plastic bag and delivered at 4°C to our laboratory within 24 hr.

Skin sampling and microbiological tests for Campylobacter spp.

To examine the level of *Campylobacter* spp. contamination on the skin of chicken carcasses, approximately 10-g samples of breast and back skin were removed from three different areas (total 6 pieces) with sterilized scalpel and forceps, and transferred to a sterilized stomaching bag. The skin samples were stomached for 1 min in 90 ml of Preston enrichment medium containing nutrient broth (Oxoid, Basingstoke, UK) with Preston *Campylobacter* selective supplement SR0117 (Oxoid) and 5% defibrinated horse whole blood (Nippon Bio-Sipp Center, Tokyo, Japan). Serial ten-fold dilutions of these suspensions were made in Preston enrichment medium. After microaerophilic culture at 42°C for 48 hr (under mixed gas: 80% N_2, 10% CO_2, 5% O_2, and 5% H_2), 10 μl of the culture solution in each tube for the most probable number (MPN) was inoculated onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA; Oxoid) supplemented with CCDA selective supplement SR0155 (Oxoid), then incubated again under the same microaerophilic conditions as those described above. The number of campylobacters was obtained by applying the common 3-tubes MPN procedure at each dilution [30].

Study design and participants

A total of 123 workers in the same chicken processing plant described above were invited to participate in this study. All of them were healthy and agreed to answer questionnaires and to take blood samples for measurement of IgG and IgA antibodies to *C. jejuni*. The questionnaire included the following items: gender, age in years (<30, 30–50 or >50), length of employment (<1 year or ≥1 year), work locations in the plant (slaughtering, evisceration, cutting, deboning, trimming, packaging or shipping), habitual ingestion of raw and/or undercooked chicken meat or its product(s), amount in one meal (>100 g, 50–100 g or <50 g) and frequency (weekly to monthly or yearly), and food poisoning symptoms after eating raw and/or undercooked chicken meat. Blood samples were collected from each participant by sterile venipuncture, and serum samples were stored at −20°C until testing. This study was approved by the Faculty of Agriculture ethics committee, University of Miyazaki (acceptation number A-1).

Bacterial strains and growth conditions

C. jejuni strain 81–176, originally isolated from a milk-borne outbreak in Minnesota [12], was used for preliminary tests to determine the most appropriate conditions for ELISA and Western blotting as described below. Four *C. jejuni* strains (Penner serotypes and origins), AK1 (HS:3, chicken meat), B19 (untypable, cecum content of a broiler chicken), C65 (HS:4, rectal content of cattle) and H96 (HS:3, feces of a patient with enteritis), were used as a cocktail antigen for the ELISA and Western blotting assay. These strains were selected as representing the most dominant clonal complex (CC65) of multilocus sequence typing (MLST) observed in Miyazaki prefecture [11]. They were grown in Brucella agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at 37°C for 18–24 hr under microaerobic conditions.

Preparation of acid extracts of C. jejuni cells

An acid extract (AE) of *C. jejuni* strains was prepared as described elsewhere with minor modification [14]. In brief, bacterial cells grown on Brucella agar plates were harvested in 10 mM phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 8,000 × *g* at 4°C for 10 min. The pellet was washed three times with PBS. Then, 3 ml of 100 mM glycine-hydrogen chloride (Nacalai Tesque, Kyoto, Japan) buffer (pH 2.2) was added. The suspensions were stirred with a magnetic stirrer for 30 min, and centrifuged at 18,000 × *g* at 4°C for 10 min. Then, the supernatant was neutralized using Econo-Pac® 10DG Columns (Bio-Rad Laboratories, Tokyo, Japan). The antigen concentration was determined using a Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories) in accordance with the manufacturer's instructions. Each AE from 4 strains was adjusted to the same concentration and 10 µg/ml AE was prepared as the ELISA antigen mixture. The pooled AEs were stored at −80°C until further work.

Enzyme-linked immunosorbent assay (ELISA)

Optimal conditions for ELISA assay were predetermined, and the protocol was established before testing based on a previous study with minor modification [8]. Briefly, 75 µl of the pooled AEs was added to each well of a 96-well ELISA microplate assay plate (Corning incorporated, Kennebunk, ME, USA). The assay was performed in triplicate and the microplate was incubated for 60 min at 37°C, and then allowed to stand at 4°C overnight. The unbound antigens were washed five times with PBS containing 0.05% Tween 20 (Nacalai Tesque) (PBS-T) using a 96-well washer machine (Bio-Rad Laboratories), and blocked at 37°C for 2 hr with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in PBS. The plates were washed five times with PBS-T, and incubated at 37°C for 90 min with 75 µl/well sera diluted 1:200 for IgG or 1:400 for IgA detection in PBS-T with 1% BSA. After washing, 75 µl/well goat anti-human IgG (γ-chain) or IgA (α-chain) conjugated to horseradish peroxidase (HRP; Southern Biotech Associate Inc., Birmingham, AL, USA) diluted 1:4,000 in PBS-T with 1% BSA was added to detect immunoglobulin, and incubated at 37°C for 90 min. The plates were washed again, and then 75 µl of substrate solution containing 20 mM citric acid monohydrate (Nacalai Tesque), 20 mM sodium hydrogen phosphate (Nacalai Tesque), and 3 mM *o*-phenylenediamine (Nacalai Tesque) was added to each well and incubated in a dark environment at 37°C for 15 min. The reaction was stopped by adding 75 µl of 2.5 M sulfuric acid (Nacalai Tesque), and the optical density (OD) at 492 nm wavelength was determined using the ELISA microplate reader machine (Benchmark Plus microplate spectrophotometer; Bio-Rad Laboratories).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Protein profiles of the pooled AEs were examined by SDS-PAGE as described previously [27]. Briefly, approximately 5 µg of AEs was boiled in a sample buffer containing 140 mM SDS (Kanto Kagaku), 50 mM 2-mercaptoethanol (Nacalai Tesque), 3 mM bromophenol blue (Nacalai Tesque), 325 mM glycerol (Nacalai Tesque), and 31.4 mM Tris at pH 6.8, at 100°C for 5 min. Then, the AE samples were loaded onto 4% stacking gel and 12.5% separating gel, and electrophoresed at 200V for 30 min. SDS-PAGE patterns were visualized by staining with Coomassie brilliant blue (CBB) G-250 (Bio-Rad Laboratories). For Western blot analysis, the proteins separated by SDS-PAGE were transferred onto nitrocellulose sheets (NCSs) (Bio-Rad Laboratories). NCSs were immersed in 4% block ace in PBS-T at 37°C for 60 min to block protein-unbound sites, and then cut into individual strips. Five serum samples showing the highest (individuals Nos. 93, 195, 92, 163, and 116 for IgG, and individuals Nos.195, 93, 92, 105, and 103 for IgA) and lowest (individuals Nos.147, 208, 217, 129, and 159 for IgG, and individuals Nos. 154, 165, 159, 168, and 161 for IgA) ODs in ELISA among the sera examined were incubated at 37°C for 60 min in serum diluted 1:500 in PBS-T. The membrane was then washed three times for 5 min each in PBS-T. Immune reactions were identified using goat-anti-human IgG (γ-chain) conjugated to HRP (1:3,000) (Southern Biotech Associate Inc.) for IgG or goat-anti-human IgA (α-chain) conjugated to HRP for IgA (1:3,000) (Southern Biotech Associate Inc.). Color development was observed after addition of 3,3′-diaminobenzidine (DAB) (Dako Inc., Carpinteria, CA, USA).

Two-dimensional gel electrophoresis and Western blotting

A 100-µg portion of the AEs was prepared with a ReadyPrep 2-D Cleanup kit (Bio-Rad Laboratories) and used for two-dimensional gel electrophoresis (2-DE). First-dimensional isoelectric focusing (IEF) and second dimensional SDS-PAGE were performed in accordance with the manufacturer's instructions (Bio-Rad Laboratories). Samples were applied to rehydrated immobilized pH gradient (IPG) strips (7-cm long, pH range 3–10, Japan Bio-Rad Laboratories) in a focusing tray. Then, the focusing IPG strips were run (Step 1: 250 V, 30 min; Step 2: 4,000 V, 60 min; Step 3: 4,000 V, 10,000 V-h) at 20°C under mineral oil. After focusing, the strips were equilibrated, first in equilibration buffer I (Bio-Rad Laboratories) for 20 min, and then in equilibration buffer II (Bio-Rad Laboratories) for 10 min. The strips were placed on top of the second-dimension gel (Bio-Rad Laboratories), and electrophoresis was performed at 200 V for 30 min. The gel was visualized by staining with CBB, and the other gel was transferred to NCS for Western blotting as described above. Sera of individuals Nos. 93 and 195 working in the plant showing the highest OD in IgG and IgA ELISA were used for detecting the recognized antigens, respectively.

Protein identification by Nano-LC-ESI-IT-TOFMS

Protein identification was performed as described previously [28]. In brief, the CBB protein bands of interest based on the Western blot results were excised from the 2-DE SDS-PAGE gels, and washed with a solution of 50 mM ammonium bicarbonate (Nacalai Tesque) and 30% acetonitrile (ACN) (Nacalai Tesque) at room temperature for 10 min. Dithiothreitol (10 mM) (Nacalai Tesque) in 50 mM ammonium bicarbonate was added, and each sample was incubated at 56°C for 60 min. The gels were then

alkylated with 55 mM iodoacetamide (Wako Pure Chemical Industries, Tokyo, Japan) and 50 mM ammonium bicarbonate at room temperature for 45 min. The protein in the gel was digested with trypsin (10 ng/µl, Sigma-Aldrich Japan) at 37°C for 16 hr. The resulting peptides were extracted from the gel pieces by adding 5% trifluoroacetic acid (TFA) (Nacalai Tesque) and 50% ACN. The supernatants were pooled and concentrated in a vacuum centrifuge to a final volume of 10 µl, and then finally acidified by addition of 10 µl of 0.1% TFA. The peptides were identified using electrospray ionization ion trap time-of-flight multistage mass spectrometry (ESI-IT-TOFMS; Shimadzu, Tokyo, Japan) with a NanoLC system DiNa (KYA Technologies, Tokyo, Japan) via a nanoelectrospray ion source. A 5–10-µl portion of peptide digests was loaded on a HiQ sil C18W column (75 µmφ × 100 mm × 200 mm) (KYA Technologies). The mobile phase consisted of two components: component A was 0.1% formic acid (Nacalai Tesque) in 2% ACN, and component B was 0.1% formic acid in 95% ACN, and running at 300 nl/min. The gradient started at 0% B, and B was increased to 50% in 40 min. Data were generated in MGF file format and subjected to database searching on the MASCOT server.

Statistical analysis

ELISA data were analyzed by RStudio software version 1.2.1335 (RStudio, Boston, MA, USA). Differences in the mean OD values among the workers were analyzed using Pairwise Wilcoxon test. Statistical significance was defined as $P<0.05$.

RESULTS

Enumeration of campylobacters contaminating the skins of chicken carcasses

The numbers of *Campylobacter* spp. naturally contaminating the back and breast skins of chicken carcasses collected after the evisceration process during the 15-month period (July 2010−November 2011) are shown in Table 1. *Campylobacter* was recovered from the back skin (65/65; 100%) and breast skin (64/65; 98.5%), with an average yield of 1.33 log MPN/10 g and 1.05 log MPN/10 g, respectively. The highest numbers of *Campylobacter* contaminating the carcasses were observed in August, being 2.62 log MPN/10 g and 2.55 log MPN/10g for back and breast skin, respectively.

Relationship of each questionnaire parameter to ELISA antibody responses among workers

Of the 123 workers who consented to participate, 104 were female and 19 were male. Female workers considerably outnumbered male workers, and all the males worked only at locations where slaughtering and shipping were performed (Table 2). Therefore, to avoid any statistical bias, only serum samples from female workers were used for analysis of each immunoglobulin response. Based on their answers in the questionnaire, none of the participants showed any clinical signs or symptoms suggestive of food poisoning (data not shown).

Table 3 shows the results of ELISA for each parameter as a risk factor, including age, working period, raw meat consumption, the amount of raw meat eaten at one sitting, and the frequency of raw meat ingestion. Overall, no significant differences were

Table 1. Counts of *Campylobacter* spp. on chicken carcasses after the evisceration in a chicken processing plant in Miyazaki Prefecture, Japan during July 2010–November 2011

Year	Month	n [a]	Mean no. of *Campylobacter* spp. [b] [log MPN/10g (SD)]	
			Back skin	Breast skin
2010	July	8	1.22 (1.25)	0.95 (1.07)
	August	6	2.62 (0.52)	2.55 (0.65)
	September	1	1.39 (0.23)	0.97 (0.38)
	October	8	1.58 (0.53)	1.40 (0.64)
	November	3	1.73 (1.52)	1.49 (1.41)
	December	4	1.39 (1.17)	0.82 (0.67)
2011	January	6	1.83 (1.23)	1.49 (0.92)
	February	4	1.02 (0.71)	0.56 (0.65)
	March	1	0.65 (0.68)	0.65 (0.68)
	April	3	0.17 (0.30)	0
	May	ND	ND	ND
	June	ND	ND	ND
	July	4	1.25 (0.86)	0.91 (0.70)
	August	8	1.25 (0.41)	0.80 (0.58)
	September	6	1.60 (0.74)	1.25 (0.65)
	October	2	1.13 (0.48)	1.06 (0.07)
	November	1	1.05 (0.42)	0.84 (0.47)

[a] Number of carcasses; [b] three skin samples each from the back and breast of the carcasses were examined; ND: not done.

Table 2. Gender distribution of workers in the chicken processing plant at each work location

Working location	Gender	
	Male	Female
Slaughtering	7	0
Evisceration	0	16
Cutting	0	13
Deboning	1	31
Trimming	0	27
Packaging	0	17
Shipping	11	0
Total	19	104

observed among the parameters, except for age: the level of IgA, but not that of IgG, was significantly higher ($P<0.05$) in the older group (>50 years old) than in the younger group (<30 years old).

Next, the antibody levels measured by ELISA among the various work locations in the plant were compared (Fig. 1). The mean OD values were 0.623 and 0.372 for IgG and IgA, respectively. The highest OD value for IgG was observed in workers located at the evisceration processing step (0.886), followed in order by packaging (0.609), deboning (0.589), trimming (0.569), and cutting (0.539) (Fig. 1A). For IgA levels, the highest OD was observed in workers located at the evisceration step (0.633), followed in order by cutting (0.410), trimming (0.353), packaging (0.317), and deboning (0.268) (Fig. 1B). The IgG level in workers at the evisceration step was significantly higher than those in workers involved in cutting ($P<0.05$), deboning ($P<0.01$) and trimming ($P<0.01$) (Fig. 1A). Similarly, the IgA level in workers at the evisceration step was significantly higher than those in workers involved in deboning ($P<0.001$), trimming ($P<0.001$), and packaging ($P<0.01$) (Fig. 1B).

Table 3. Titers of IgG and IGA antibody against pooled *Campylobacter jejuni* strains measured by ELISA in workers at the chicken processing plant in relation to questionnaire items

Group [a]		n [b]	IgG (SD) [c]	IgA (SD) [c]
Age				
	Young (<30)	3	0.452 (0.211) [A]	0.152 (0.068) [A]
	Adult (30–50)	73	0.639 (0.320) [A]	0.378 (0.379) [AB]
	Old (>50)	28	0.614 (0.204) [A]	0.380 (0.201) [B]
Length of employment				
	<1 year	10	0.598 (0.036) [A]	0.300 (0.179) [A]
	≥1 year	94	0.630 (0.301) [A]	0.380 (0.348) [A]
Raw meat consumption				
	Yes	46	0.639 (0.308) [A]	0.380 (0.445) [A]
	No	58	0.617 (0.278) [A]	0.366 (0.217) [A]
Amount of raw meat eaten per time				
	Lot (>100 g)	3	0.711 (0.576) [A]	0.551 (0.554) [A]
	Medium (50–100 g)	21	0.664 (0.329) [A]	0.447 (0.614) [A]
	Little (<50 g)	22	0.606 (0.257) [A]	0.293 (0.138) [A]
Frequency of eating raw chicken meat				
	Weekly to monthly	18	0.543 (0.192) [A]	0.283 (0.164) [A]
	Yearly	28	0.702 (0.353) [A]	0.443 (0.550) [A]

[a] Parameter of risk obtained from the questionnaire; [b] numbers of workers; [c] the same letter within a group (A–B) indicates no significant difference at the 5% level ($P>0.05$).
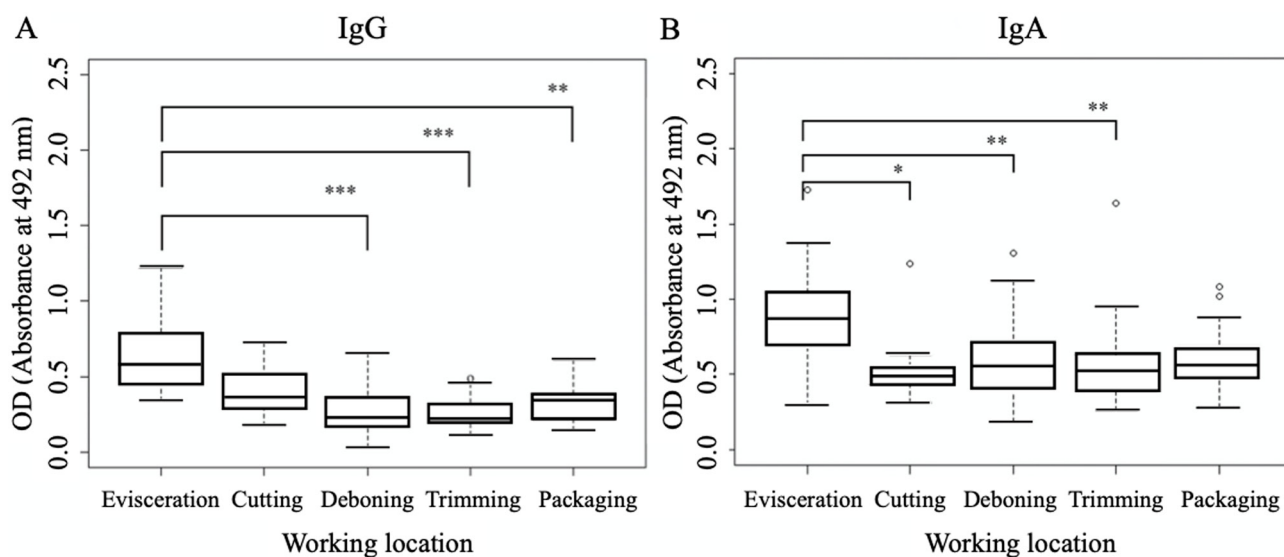

Fig. 1. IgG **(A)** and IgA **(B)** levels in workers determined from pooled acid extracts of *Campylobacter jejuni* strains according to work locations in the processing plant, including evisceration ($n=16$), cutting ($n=13$), deboning ($n=31$), trimming ($n=27$), and packaging ($n=17$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Recognized antigens in acid extracts of serum from workers

AEs prepared from 4 different strains of *C. jejuni* were separated by SDS-PAGE (Fig. 2A) and transferred to NCS, then immunoblotting was performed using each of the five serum samples showing the highest and lowest ODs for both IgG and IgA by ELISA. The groups with the highest and lowest ODs for IgG and IgA ELISA bound to AEs showed a range of 15–95 kDa (Fig. 2B) and 40–95 kDa (Fig. 2C), respectively. Several common bands were recognized in sera for both the highest- and lowest-OD groups for IgG and IgA, while others appeared to be unique to a specific individual. A strong positive band with an apparent molecular mass of 43 and 60 kDa was detected for both IgG and IgA in the highest-OD serum samples (Fig. 2B and 2C). Similarly, two bands of 43 and 60 kDa were also recognized for both IgG and IgA in the lowest-OD serum samples (Fig. 2B and 2C).

Identification of C. jejuni antigens recognized by antibodies in plant workers

The pooled AEs of *C. jejuni* were separated by 2-DE and their degrees of binding to both IgG (individual No. 93) and IgA (individual No. 195) in serum samples which showed the highest titers by ELISA were examined by Western blotting. The positive spots were then subjected to protein identification using NanoLC-ESI-IT-TOFMS. The strongest spots shown by Western blotting by IgG and IgA antibodies showing the highest ODs in the ELISA were identified as flagellins: flagellin A (FlaA, 59.6 kDa) and flagellin B (FlaB, 59.7 kDa). However, other spots that had been demonstrated by one-dimensional Western blotting (Fig. 2) were not detected clearly by 2-DE followed by Western blotting (data not shown).

DISCUSSION

Workers in chicken processing plants are considered to be at high risk of *Campylobacter* infection due to frequent exposure to the organism present on contaminated carcasses as well as in the working environment [5, 6]. Previous studies of immunity against
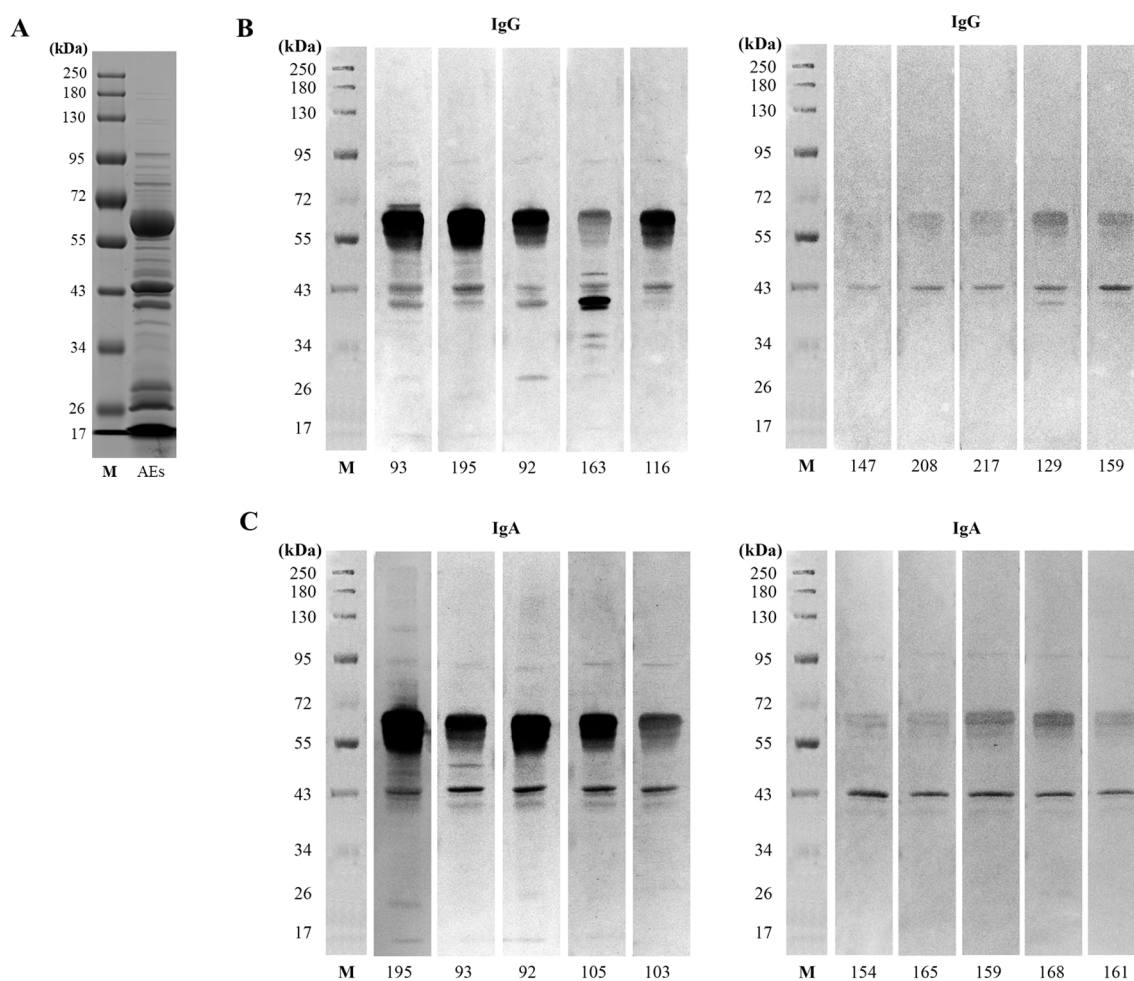

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using pooled acid extracts (AEs) of *Campylobacter jejuni* strains; **(A)** SDS-PAGE gel stained with CBB G-250. AEs were prepared from 4 different strains of *C. jejuni* and separated by SDS-PAGE; **(B)** Western blotting probed with serum samples containing the highest (left) and lowest (right) IgG titers; **(C)** Western blotting probed with serum samples containing the highest (left) and lowest (right) IgA titers. The numbers below the strips indicate the numbers of individual workers. M; marker.

C. jejuni have focused on the detection and/or characterization of antibodies persisting in the sera of workers who developed foodborne illnesses [2, 28]. In the present study, we examined whether workers in a chicken processing plant in Miyazaki prefecture have developed an acquired immune response to *C. jejuni*. To clarify the factors that might lead to an increase in antibody levels in plant workers, we distributed a questionnaire to obtain data on their age, duration of work, work locations in the plant, and any food poisoning symptoms they might have suffered after eating raw chicken meat, as the latter is a common dietary habit in Miyazaki prefecture and we needed to examine whether it might have affected the immune response elicited during work in the chicken processing plant. For this purpose, we asked workers who have eaten raw and/or undercooked chicken meat about the amount they consumed in one sitting, and how often they did so. Furthermore, before starting the study, we confirmed that the chicken carcasses in the processing plant examined were indeed contaminated with *Campylobacter* throughout the year (Table 1).

ELISA was conducted to assess the IgG and IgA responses of female workers against *C. jejuni*. Due to the diversity of *C. jejuni* antigens, we used pooled samples of local strains that were the dominant MLST in Miyazaki prefecture among various sources [11] to provide soluble antigens for this assay. The use of such pooled samples has been standard practice for the development of broadly specific serological assays with considerable serotype heterogeneity [10]. Glycine acid extraction was used for production of the antigen, since this method releases major surface antigens such as flagella and their component proteins, which appear to be common to many *C. jejuni* strains [4].

Surprisingly, no significant differences in IgG and IgA levels were observed between the groups of workers who stated that they ate and did not eat raw and/or undercooked chicken meat (Table 3). Furthermore, none of the participants stated that they had suffered symptoms suggestive of enteritis after ingestion of such meat. One possible explanation is that all of the participants may have acquired protective immunity conferring resistance to campylobacteriosis as a result of exposure to *C. jejuni* in the chicken processing plant. A previous report of campylobacteriosis in a developing country has indicated that symptomatic infection rarely occurred in individuals over 2 years of age but asymptomatic cases were frequent [1]. In developed or industrialized countries, clinical severity may vary among individuals due to factors such as occupational exposure in chicken processing plants or ingestion of contaminated foods or water by individuals lacking immunity [17]. In the present study, we demonstrated that consumption of raw chicken meat did not affect the humoral levels of both IgG and IgA. However, since eating raw and/or undercooked chicken meat is considered the most significant risk factor for *C. jejuni* infection [31], it is strongly recommended that chicken meat should only be eaten after thorough cooking. Furthermore, we confirmed that there is no significant difference in the IgG and IgA levels between the groups of workers who stated that they ate or did not eat raw and/or undercooked chicken meat among the workers at evisceration step (data not shown).

Unfortunately, we did not set negative control in the present ELISA assay. Normally, healthy subject has been often used as negative control to detect confounding, recall bias, and selection bias in the epidemiological research [13]. Ideally control group including healthy individuals should be determined in the same area. Nevertheless, it is very difficult to include healthy individuals because of ethical issue. Furthermore, it is nearly impossible to collect sera from people who have never expose to *Campylobacter* such as neonatal. Therefore, in this study, we solely compared the antibodies levels among the workers.

Here we examined the relationship between work location in the plant and individual levels of antibodies to *C. jejuni*. This revealed that individuals who worked at the evisceration step had levels of both IgG and IgA that were significantly higher than those of workers at the other processing steps (Fig. 1). The evisceration process is known to have a high potential for *Campylobacter* contamination due to rupture and/or leakage of intestinal contents containing the organism, which can account for as much as 10^{10} cfu/g of fecal content [21]. Here we demonstrated that after the evisceration process, 100% of carcasses were contaminated with *Campylobacter*, up to a maximum of 2.62 log MPN/10 g and 2.55 log MPN/10 g for back and breast skin, respectively (Table 1), indicating that workers could be easily exposed with the bacterium during work, especially at the evisceration step. Although it is desirable to examine the levels of *Campylobacter* contamination on chicken carcasses at each processing step for more precise enumeration of the organism and levels of worker immunity, it is difficult to collect carcasses at each step due to the high speed of the chicken processing line.

For other parameters including age, duration of work, and consumption of raw chicken meat, there were no significant difference in IgG and IgA levels between any of the groups except for age (Table 3). The level of IgA, but not that of IgG, in the oldest group (>50 years old) was significantly higher than that in the younger group (<30 years old) (Table 3). However, only serum IgA, and not IgG, tends to increase with age. Therefore, the differences in antibody levels observed between the old and young groups in this study are in line with previous reports and may not have been a consequence of *Campylobacter* infection.

In general, kinetics of humoral immunity of IgA after *Campylobacter* infection in humans increase in serum in the acute phase of infection (1-week post-infection) and rapidly decline to normal level after 14–20 days from the onset of symptoms [7, 10]. In contrast, serum IgG antibodies are produced during convalescent phase of infection or antigen exposure (1 week to 2 months), rising a few weeks after it begins, then decreasing and stabilizing for long period (1 year), and provide protection against subsequent *Campylobacter* infection [7]. Therefore, the estimation of IgA-specific antibodies is suitable for the diagnosis of recent infection with *C. jejuni*, while IgG-specific antibodies are useful for a study of prevalence of campylobacteriosis in the general population [10]. High levels of IgA were detected in the sera of asymptomatic workers in this study, indicating that the frequent exposure to *C. jejuni* possibly provide the protective immunity.

A previous study have demonstrated that campylobacters can be isolated from not only chicken carcasses but also other locations in processing plants, such as work surfaces, equipment, and cotton gloves [32]. To prevent cross-contamination, processing plants need to employ the Hazard Analysis and Critical Control Point (HACCP) system to identify critical control points where hazards such as microbial contamination can occur, and establish appropriate control measures. The Ministry of Health, Labor and Welfare

of Japan revised the Poultry Slaughtering Business Control and Poultry Meat Inspection Act in 2014 to introduce the HACCP system to poultry processing plants in a stepwise manner. In line with this amendment, all poultry processing facilities in Japan are required to have introduced the HACCP system by May 2021 [29]. Therefore, based on the HACCP system, it is necessary to provide information to food business operators and workers about food safety practices and hygiene management to reduce the risk of exposure to pathogens.

In this study, we also performed Western blotting to detect the specific protein(s) recognized by antibodies in the sera of workers using the five samples showing the highest and lowest OD values measured by ELISA for IgG and IgA antibodies against pooled AEs of *C. jejuni*. It has been reported that only a few protein antigens may be important for induction of protective immunity to *Campylobacter* infection [19]. We found that two major antigens of 43 and 60 kDa were recognized by antibodies in sera of workers in these groups (Fig. 2). One protein among the positive spots was identified as flagellin using 2-DE followed by Nano-LC-ESI-IT-TOFMS. Flagella of *C. jejuni* are considered to be one of the most important virulence factors that help the organism to invade into the intestinal cells of the host [18], and have potential to elicit an immune response [2]. Cawthraw and collaborators demonstrated that flagellin was recognized commonly and strongly by antibodies among patients infected with *Campylobacter jejuni* [3]. Our finding in this study agreed with the previous study. To further confirm the recognized antigen by serum antibodies among workers, recombinant flagellin but not crude antigen (AEs) from *C. jejuni* should be used for Western blotting and ELISA assays. On the other hand, spots within the range 40–43 kDa were not clearly detected by Western blotting after 2-DE, possibly due to the low concentration of each protein. Therefore, we were unable to identify these proteins, and further work will be necessary to identify and characterize antigens other than flagellin.

Our present findings suggest that titers of IgG and IgA against *C. jejuni* in processing plant workers are increased by exposure to *Campylobacter*, regardless of individual consumption of raw chicken meat, even though the antibody level varies according to work location in the plant. We have also demonstrated that a long-term exposure of workers to *C. jejuni* may elicit protective immunity against campylobacteriosis. Further studies will be required to gain further insight into how *Campylobacter* food poisoning can be further minimized.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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