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The influence of age on *Campylobacter jejuni* infection in chicken

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ABSTRACT

Campylobacter jejuni (*C. jejuni*)-host-interaction may be affected by the maturation stage of the chicken's immune system and the developing gut microbiota composition. We compared these parameters between birds *C. jejuni*-inoculated at day one, 10, 22 and 31 post hatch. The highest *C. jejuni*-colonization rate and numbers of colony forming units (CFU) were detected in caecal content of day-one-inoculated birds while the lowest was detected in 22-days-old birds. The low bacterial colonization of 22-days-old chickens correlated with the most prominent immune reactions in this age group in comparison to other age groups. Age and *C. jejuni*-inoculation had a significant effect on lymphocyte numbers and cytokine expression levels in caecum as well as on gut flora composition. Overall, the immune response to *C. jejuni* is significantly influenced by the age of the infected chickens leading to differences in *C. jejuni*-colonization pattern between age goups.

1. Introduction

Campylobacter species, in particular *Campylobacter jejuni* (*C. jejuni*), are the most common causes of human food-borne bacterial gastroenteritis in the industrialized world (Bronzwaer et al., 2009; Frost, 2001). It is generally accepted that chickens are natural hosts for *C. jejuni*, and colonized commercial broiler chickens are the main vector for transmitting this pathogen to humans (Hermans et al., 2011).

C. jejuni infections in mammals are associated with an adaptive local T cell-immune response at the gastro-intestinal mucosa (Spiller et al., 2000) and a highly polarized proinflammatory and Th1 cytokine response. This is characterized by up-regulation of the proinflammatory cytokines such as IL-8, IL-6 and Th1 cytokines including IFN-γ, as well as the production of immunoglobulin (Ig) A and G (Baqar et al., 2001; Edwards et al., 2010). It has also been demonstrated that T lymphocytes, especially $CD4^+$ T cells release IFN-γ in response to *C. jejuni* infection in humans (Fimlaid et al., 2014). The activation of innate pattern recognition receptors and the release of proinflammatory cytokines after *C. jejuni* infection have been shown to induce acute intestinal inflammation (de Zoete et al., 2010; Zilbauer et al., 2007). Latest findings also indicate that *C. jejuni* may induce inflammasome activation in both murine and human cells without apparent cytotoxicity (Bouwman et al., 2014).

Previous studies suggested a Th1 polarization of the immune response in avian species following *C. jejuni* infection (Shaughnessy et al., 2009). Innate immune responses were induced by *C. jejuni* in different avian cell lines, including HD 11 macrophages, primary chicken kidney cells and primary chicken embryo intestinal cells (Li et al., 2008; Smith et al., 2005). These studies demonstrated an increase of proinflammatory cytokines after *C. jejuni* infection. *In vivo*, *C. jejuni*-inoculated birds showed an increase in mRNA expression of IL-6 and the chicken IL-8-homolog in ileal and caecal tissue (Smith et al., 2008) and developed both a local and systemic humoral immune response (Cawthraw et al., 1994). However, this immune response remains largely undefined (Wigley, 2013) and may vary depending on the *C. jejuni* strain, age and genotype of birds (Li et al., 2010; Smith et al., 2008) Most studies are difficult to compare because different *C. jejuni* strains and dosages, different genotypes of birds and age groups were used (Humphrey et al., 2014; Jones et al., 2004; Li et al., 2010; Smith et al., 2008).

Age plays a vital role in the outcome of an infection (Kretzschmar-McCluskey et al., 2008; Zhang et al., 2014). The age effect is mainly associated with increasing maturation of the immune system. Interaction between physical host barriers and the pathogen's ability of replication in a changing microenvironment may also contribute to this effect (Jilbert et al., 1998). It was demonstrated that adult birds may mount a more protective immune response than newly hatched chicks after *Salmonella*

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infection (Crhanova et al., 2011). Under field conditions, *C. jejuni* may colonize birds at all ages but most of the broiler flocks become *C. jejuni* positive around three-weeks post hatch (ph) (Bull et al., 2006). The minimum number of colony forming units (CFU) required for successful *C. jejuni*-colonization varied between two and 14 days old birds (Ringoir et al., 2007; Smith et al., 2008). However, at present, only limited information is available on the underlying mechanisms of these age effects in chicken. It is speculated that maternal antibodies may interfere with colonization although data on this protective effect are controversial (Cawthraw and Newell, 2010). On the other hand, changes in the developing gut microflora composition may also contribute to the *C. jejuni* colonization pattern (O'Loughlin et al., 2015).

The goal of this study was to determine the impact of the developing immune system and gut microflora composition on the colonization pattern of *C. jejuni*. This possible role of age for the outcome of *C. jejuni*-colonization and infection in chickens had not been investigated to our knowledge under comparable experimental conditions before. Commercial broilers from the same parent flock, which were raised under the same management conditions and feed, were inoculated at the age of one, 10, 22 and 31 days ph with either of two different *C. jejuni* strains from which one was previously shown to invade the liver of chickens (strain 0097) while the other (Lior6) did not (Pielsticker et al., 2016). Local and systemic immune reactions were compared as well as the difference in the gut flora composition for two selected age groups.

2. Materials and methods

2.1. Animals

One day old commercial broiler chickens were obtained from the BWE Hatchery Weser-Ems GmbH & Co. KG, Visbek, Rechterfeld, Germany. Birds were progenies of the same parent flock and were all delivered at day old to the clinic at the same day. Chickens were raised at the Clinic of Poultry, University of Veterinary Medicine Hannover, under the same management conditions and feed, securing a *Campylobacter*-free environment. Broilers were kept in the same isolation room till the age of infection. Afterwards, infected and non-infected experimental groups were moved to different isolation rooms (one for each infected and one for non-infected control birds). Both units had comparable management conditions (raised on wood shavings until the day of inoculation, and subsequently moved to units on wire floor) and same feed source. The animal experiments were conducted in accordance to the Animal Welfare Regulation of Lower Saxony and were approved by the Lower Saxony State Office for Customer Protection and Food Safety (LAVES). Commercial broiler feed (Table 1) and water were provided *ad libitum*. Broilers were fed a standard starter diet up to 14 days of age and then received a grower diet till the end of the experiment. Broilers were observed daily for the presence of clinical signs. Broiler chicken are generally free of *C. jejuni*, when they are delivered to the farm (Ingresa-Capaccioni et al., 2015; Cox et al., 2012). All birds in our experiments tested negative for *C. jejuni* by clocal swabs directly platied on CCDA plates at the day of *C. jejuni* inoculation. The animals did not receive any vaccination.

Table 1
Ingredients and nutrient contents of the experimental diets.

Components, per kg	Broiler-feed	
	Starter	Grower
Crude protein (g)	215	210
Crude lipids (g)	52	67
Crude fiber (g)	31	35
Crude ash (g)	56	51
MJ ME	12.4	12.4
Ca (g)	9	8
P (g)	6	5.5
Na (g)	1.4	1.4
Methionine (g)	5.5	5.0
Lysine (g)	12.5	11.5
Monensin-Na (mg)	100	100

MJ ME = megajoule metabolizable energy.

2.2. Bacterial strains, C. jejuni inoculum preparation and detection of C. jejuni in experimental samples

Two different *C. jejuni* strains were chosen for this study: one invasive one, which had been also detected in the liver and a non-invasive strain. Both were shown to colonize chicken caecum in high numbers between three and 21 days pi (Pielsticker et al., 2016). The *C. jejuni* strain of serogroup Lior6 had been isolated from a chicken at the Clinic of Poultry, University of Veterinary Medicine Hannover, Germany and was stored in skim milk at −70 °C. The *C. jejuni* strain 0097 which had been isolated from a laying hen, was kindly provided by Dr. Ingrid Hänel, Friedrich-Löffler-Institute, Jena, Germany.

The cryopreserved strains were thawed and plated on Charcoal Cefoperazone dexoxycholate agar (CCDA, Oxoid, Basingstoke, England) for 48 h under microaerophilic conditions (10% CO_2, 5% O_2, 85% hydrogen) at 38 °C. After two days, one *C. jejuni* colony was transferred to three ml Standard-I-Bouillon (Merck, Damstadt, Germany) and incubated for another 48 h under microaerophilic conditions at 38 °C to increase bacterial growth.

One ml of the bacterial suspension was diluted with sterile phosphate buffered saline (PBS) to achieve approximately 10^4 CFU/(Colony Forming Units) for oral inoculation. To confirm the CFU of *C. jejuni* in the inocula, the bacterial suspension was serially diluted in a 10-fold dilution series, spread on CCDA plates and incubated for 48 h at 38 °C. After incubation the colonies were counted to calculate the CFU (Smith et al., 2008) (Supplemental Table 1), the number of CFU was comparable between experimental days.

For the detection of *C. jejuni* in caecal and cloacal swabs collected from inoculated and non-inoculated chickens during the experiments, samples were plated on CCDA plate either with pre-enrichtment for non-inoculated groups or without pre-enrichment for inoculated birds. After 48 h plates were evaluated for *C. jejuni*-specific colonies. Detection of *C. jejuni*-positive colonies was confirmed by gram-staining.

2.3. Isolation of intraepithelial lymphocytes and flow cytometry analysis

Single cell suspensions of intraepithelial lymphocytes (IEL) were prepared as previously described (Schwarz et al., 2011).

10^6 IEL of the ileum and caecum were triple stained with a combination of the following antibodies (final concentrations per ml): mouse-anti-chicken-CD3 (2 μg) (Chen et al., 1986) conjugated to phycoerythrin (R-PE), biotinylated mouse-anti-chicken-CD8β (5 μg) (Chan et al., 1988) used in conjunction with streptavidin conjugated to SpectralRed™ (SPRD) (5 μg) and fluorescein (FITC)-conjugated mouse-anti-chicken CD4 (5 μg). All antibodies were obtained from Southern Biotech, provided by Biozol, Eching, Germany. The stained cells were analyzed using the Beckman Coulter Epics XL© flow cytometer and EXPO 32 ADC software program (Beckman Coulter Company, Miami, FL). The lymphocyte population was gated for $CD3^+$ IELs according to size and granularity and 200,000 events in ileum and caecum samples were measured. $CD3^+$ IELs were then analyzed for positive staining with anti-CD4-FITC and anti-CD8-SPRD. Presented is the percentage of $CD4^+$ and