VIABILITY OF *Campylobacter jejuni* IN COMMERCIAL EGGS

VIABILIDADE DE Campylobacter jejuni *EM OVOS COMERCIAIS*

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ABSTRACT: The aim of this study was to verify the penetration and permanence of *C. jejuni* inside commercial eggs. Thirty eggs were submerged in peptone water artificially contaminated with 10^5 UFC.mL^{-1} of *C. jejuni.* An equal number of eggs were submerged in an identical solution, however, without addition of the microorganism. Parallel to this, 60 eggs were inoculated by means of a small incision in the larger pole, without lesion to the internal membrane, inserted into the air chamber with a sterile insulin needle containing 10^5 CFU.mL^{-1} of *C. jejuni*. Thirty eggs were inoculated with a sterile solution of 0.85% sodium chloride and kept as negative control. After storage at 25ºC, and after 1, 6 and 24 hours, 10 eggs from each of the two treatments were separated into the albumen and yolk contents, and each portion was individually analyzed by the traditional culture method for the presence/absence of *Campylobacter*. In another nine eggs, obtained from the same lot of animals, the albumens were separated to verify their inhibitory activity against the microorganism. The albumen in different concentrations was incorporated into Bolton broth and inoculated with 10^6 CFU.mL^{-1} of *C. jejuni*, and after incubation at 37ºC for 24 hours, striated in selective agar. In none of the analyzed samples (treatments and controls) was the growth of *Campylobacter* observed. The results demonstrated that under the experimental conditions used, *Campylobacter jejuni* is incapable of surviving, colonizing and multiplying inside commercial eggs. The white of the eggs, as an isolated factor, even at the concentration of 84.9% was incapable of completely inhibiting the growth of the microorganism in Bolton broth at 37ºC, in a microaerophilic atmosphere. The consumption of commercial eggs is not a risk factor for the acquisition of Campylobacteriosis by humans.

KEYWORDS: *Campylobacter jejuni*. White commercial eggs. Viability. Consumption. Albumen.

INTRODUCTION

Campylobacter jejuni is a common cause of gastroenteritis and enterocolitis in humans, and has been outstanding as an emergent microorganism of food origin in various parts of the world (FRIEDMAN et al., 2000; AQUINO et al., 2002; CDC, 2003; EFSA, 2009). According to Germano and Germano (2001), *Campylobacter* sp can infect man by means of direct contact with contaminated animals or carcasses, or indirectly by the ingestion of contaminated food. Infection mainly occurs by the consumption of contaminated water and foods of animal origin, such as drinking raw milk and its derivatives, raw or poorly processed poultry, pork and beef meat.

There are numerous studies on the isolation of *Campylobacter* sp in chickens, and these point out that the intestinal tract of domestic birds is the main reservoir of this microorganism (MACHADO, TOSIN; LEITÃO, 1994). Approximately 75% of the broiler birds present *C. jejuni* and/or *C. coli* (HUMPHREY, 1999). However, the epidemiology of the disease has not yet been completely explained, and it is not known, for example, whether the consumption of commercial eggs represents a risk of infection to humans.

Campylobacter sp is found as a commensal organism in the gastrointestinal and reproductive tracts of birds, and thus can contaminate the eggs (SHANE, 2002); consumption of this food can be a risk to consumers and must be evaluated.

The aim of this study was to verify the passage and permanence of *Campylobacter jejuni* inside commercial eggs; analyze *in vitro* whether the albumen of the egg inhibits the viability of *Campylobacter jejuni* and thus determine whether the consumption of commercial eggs represents a risk of human campylobacteriosis.

METHODOLOGY

The study was conducted in the period from December 2007 to January 2008, and used 159 white commercial eggs from a commercial poultry farm. The eggs were obtained from the second collection of the day, from 74 week-old birds, removed aseptically from cages to avoid environmental contamination. The strain used for artificial contamination was BH13, of *Campylobacter jejuni*, isolated from chicken, and donated by the Oswaldo Cruz Foundation.

One hundred and fifty eggs were divided into the following experimental groups:

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T1 (treatment 1) - A receptacle containing 3000mL of sterile peptone water was artificially contaminated with 10^5 CFU.mL^{-1}*Campylobacter jejuni* BH13. In it, 30 eggs were deposited, and left to rest at a temperature of 25ºC, protected in plastic film. After 1 hour, 6 hours and 24 hours, for each selected hour, 10 eggs were collected for analysis.

C1 (Control 1) – The identical procedure as that for T1 was followed, but without the inoculation of *C. jejuni* BH13.

T2 (Treatment 2) - In 60 eggs a small perforation was made through the membrane of the shell in the larger pole, and using a sterile syringe 0.1mL of a *Campylobacter jejuni* BH13 culture was inoculated. The concentration inoculated was 10^5 CFU.mL^{-1}. After 1 hour, 6 hours and 24 hours, for each selected hour, 20 eggs were collected for analysis.

C2 (Control 2) – The same procedure as followed in T2 was performed in 30 eggs, but the inoculation was 0.1mL of 0.85% sterile sodium chloride solution.

The number of colony forming units (10^5 CFU.mL^{-1}) used was sufficient to ensure a high pressure of contamination since the dose of infection is low for this bacterium.

In each of the periods in which the eggs were sampled, an aliquot of peptone water from groups T1 and C1 was removed for analysis.

To verify whether albumen exerts an inhibitory power on the BH13 strain of *Campylobacter jejuni*, the whites of nine eggs from the same place were used under the same conditions described above. In the laboratory, the eggs were externally cleaned with ethyl alcohol and the albumen and yoke were aseptically separated. The yolks were discarded and the albumen homogenized and used for the test.

Different concentrations of albumen (9.4% to 84.9%) were incorporated into Bolton broth (Oxoid®), added from the selective supplement (Oxoid®) (20mg/L sodium cefoperazone, 20mg/L vancomycin, 20mg/L trimethropim and 50mg/L cyclohexamine), and 5% of hemolyzed horse blood (Table 1). After this, each of the different concentrations were inoculated with 0.1mL of the BH13 strain (10^6 CFU.mL^{-1}) and homogenized.

Immediately after inoculation, the samples were incubated at 37ºC for 24 hours in a microaerophilic atmosphere.

Table 1. Preparation of the different concentrations of albumen in Bolton broth for the antimicrobial test

Egg white mL/ (%)[1]	Bolton (mL)	Blood (mL)	*C. jejuni* BH13 (mL)
1 (9.4)	9	0.5	0.1
2 (18.9)	8	0.5	0.1
3 (28.4)	7	0.5	0.1
4 (37.7)	6	0.5	0.1
5 (47.2)	5	0.5	0.1
6 (56.6)	4	0.5	0.1
7 (66.0)	3	0.5	0.1
8 (75.5)	2	0.5	0.1
9 (84.9)	1	0.5	0.1

– percentage of albumen tested, calculated in relation to the final volume of 10.6mL

The eggs sampled in the different periods, taken from treatments T1, C1, T2 and C2 were externally cleaned with 70% ethyl alcohol and broken in a laminar flow chapel. The albumen and yoke were aseptically separated and individually analyzed.

The presence of *Campylobacter* was verified using the traditional analysis protocol, according to Karmali et al. (1986) and Bolton and Robertson (1982). The Bolton broth was used for pre-enriching the samples and the solid medium, Cefoperazone deoxycholate Agar-(CCDA), was used for isolation.

Individually 1mL of the albumen and 1mL of the yoke samples were pipetted into identified tubes containing 9mL of Bolton broth (Oxoid®), and these were incubated at 37ºC for 24 hours in a microaerophilic atmosphere (Probac generator®). After enrichment, aliquots of each culture were seeded on CCDA agar plates (Oxoid®), in addition to the selective supplement CCDA (Oxoid®) and 5% of hemolyzed horse blood. The plates were incubated in anaerobiosis jars in a microaerophilic atmosphere (Probac generator®) at 37ºC for 48 hours.

The colonies were identified under light microscopy, using the 100X magnification

objective. The morphology was evaluated after performing smears and modified differential Gram staining, with the substitution of safranin by 0.8% carbolfuchin to confirm the comma or "seagull wing" morphology. Pendant drop microscopy was performed to verify the "corkscrew" movement.

The aliquots of sterile peptone and contaminated water used for submerging the eggs were striated directly onto the CCDA agar (Oxoid®) and the colonies were identified as described above.

The different cultures added from the different concentrations of albumen after incubation in a microaerophilic atmosphere at 37ºC for 24 hours, were cultivated in CCDA agar (Oxoid®) and the colonies were identified as previously described.

Statistic analysis used was only description.

RESULTS AND DISCUSSION

No positivity was observed for *Campylobacter jejuni* in the eggs researched in this study. The type of artificial inoculation used, by submersion or direct inoculation into the internal content, did not influence the results. Monitoring of the peptone water used to contaminate the eggs demonstrated that the inoculated microorganisms remained viable during the entire experimental period, and that the peptone water used to submerge the control group presented no contamination at any time whatever.

Negativity for *Campylobacter jejuni* in the submerged eggs or those inoculated with the BH13 strain, obtained in this study is in agreement with the experiments of different authors. Doyle (1984) found no *C. jejuni* in the internal content of eggs, even after they had remained in contact with the *Campylobacter* sp culture at three different temperatures. A study conducted in the "Triângulo Mineiro" region in the State of Minas Gerais, in fresh infertile eggs from lots of breeder hens positive for *Campylobacter* sp, also presented no positivity (FONSECA, 2006). Zaki and Redá (1995) and Rabie (1992) analyzed infertile commercial eggs, and all the samples presented negative results. Analysis performed in eggs after immersion in contaminated feces determined final concentrations of 107 to 108 cells ml"1. *C. jejuni* on the SPF egg shell, but the microorganism was restricted to the membrane of the shell, and was not found in the internal content (NEILL, CAMPBELL and O'BRIEN, 1985).

Studies conducted by Sahin et al. (2003) in SPF (Specific Pathogen Free) chicken eggs and by Maruyama et al. (1995) in quail eggs immersed in suspension containing log 10 CFU/ml of *C. jejuni* showed that the viability of *C. jejuni* is dramatically diminished when the bacteria is inoculated into the albumen or aerial sac.

The results of this study are in disagreement with those obtained by Shane et al. (1986). The authors contaminated commercial eggs with feces containing *Campylobacter jejuni*, and verified the presence of microorganisms in 4.28% (3/70) and 1.43% (1/70), in the shells and internal content, respectively. Nevertheless, the viability of *C. jejuni* in the shells of eggs was maintained for only 16 hours. Hanninen et al. (1984) separated the yolks from the albumen and inoculated *C. jejuni* into the yolks of fresh eggs, and submitted them to storage for different times and at different temperatures. The authors observed that at the temperature of 37ºC in the period of 48 hours, the microorganism survived and increased in number of cells, but at 20ºC and 4ºC, although the microorganism survived, there was a reduction in the number of cells.

Analyses of different studies, with and without isolation of *Campylobacter* sp in infertile eggs, demonstrated that the recovery of the microorganism only occurred in artificially contaminated eggs, and even so in a small percentage of the samples. Furthermore, they showed that the storage temperature is an important factor for the viability of this bacterias.

The bacteria of the genus *Campylobacter* measure 0.2µm to 0.8µm wide by 0.5 µm to 5µm long, a significantly smaller size than the pores of the egg shell, which are from 11µm to 12µm. Moreover, *Campylobacter jejuni* is motile, by means of corkscrew movements (VANDAME et al., 1992). Although it is physiologically possible for *Campylobacter* sp to enter through egg shells and remain there, in this study no bacteria were isolated in the researched eggs. From the negativity observed, it is possible to suggest some intrinsic factor of the eggs or storage environment inhibited the viability of the microorganism.

Another possibility to explain the results obtained is *Campylobacter's* capacity of generating VNC forms in adverse environments, which are viable but not culturable (TRACHOO et al. 2002). According to Rowe et al. (1998), the VNC form in *Campylobacter* sp is induced by factors such as stress due to scarcity of nutrients, and represents the organism's survival strategy in the natural environment. It is possible that the storage temperature, allied to intrinsic factors of the egg induced the VNC forms, which are not detected by traditional culture method used as diagnosis in this study.

The above speculation is reinforced by the conclusions of Cogan (2001) and Jay (2000). who related that enzymes present in the egg white, such as lysozime, avidin, egg inhibitory protein, egg flavoprotein and ovotransferrin have direct or indirect antimicrobial action against different microorganisms. In addition to this, albumen presents a low iron content (approximately 0.4mg/100g), which is necessary for bacterial metabolism. Moreover, the pH of albumen, which is approximately 9.0, does not represent a favorable environment for *Campylobacter jejuni*.

Although no *in vivo* growth of *Campylobacter* was observed, the tests conducted *in vitro* were also not sufficient to explain the effectiveness of the inhibitory action of albumen (Table 1). The addition of different concentrations of egg white (9.4% to 84.9%) to cultures of *Campylobacter* in Bolton broth was not capable of preventing the growth. An albumen concentration as high as 84.9%, still allowed the specimen to grow. Microscopic observation of the typical morphology and motility allowed one to affirm that *Campylobacter jejuni* BH13 remained in its typical motile spirochete form even after contact with high concentrations of albumen for 24 hours.

The non recovery of *Campylobacter* inoculated *in vivo* directly into albumen, and the survival of the same specimen *in vitro,* even at high concentrations of albumen, indicates that this factor in isolation is not capable of impeding the survival of *Campylobacter* in eggs. Thus, there is probably some factor on the immunology of the commercial chicken egg which prevents the viability of the bacteria.

CONCLUSIONS

The results of this study allowed one to conclude that under the experimental conditions used, *Campylobacter jejuni* BH13 is incapable of crossing the egg shell, surviving, colonizing and multiplying inside commercial eggs in its culturable form.

The consumption of commercial eggs stored at a temperature of 25ºC is not a risk factor for the acquisition of human Campylobacteriosis.

The albumen of eggs, when added to Bolton broth with hemolyzed blood, at adjusted pH and maintained at a temperature of 37ºC, in a microaerophilic atmosphere, is not capable of completely inhibiting the growth of the microorganism.

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RESUMO: O objetivo desse estudo foi verificar a passagem e a permanência de *C. jejuni* no interior de ovos comerciais. Trinta ovos foram submersos em água peptona artificialmente contaminada com 10^5 CFU.mL^{-1} de *C. jejuni* e igual número de ovos foi submerso em solução idêntica, porém, sem adição do microrganismo. Em paralelo, 60 ovos foram inoculados por meio de uma pequena incisão no pólo maior, sem lesionar a membrana interna, inseridos na câmara de ar com uma agulha de insulina estéril contendo 10^5 CFU.mL^{-1} de *C. jejuni*. Trinta ovos foram inoculados com solução estéril de cloreto de sódio 0,85% e mantidos como controle negativo. Após armazenamento a 25ºC, e após 1, 6 e 24 horas, 10 ovos de cada um dos dois tratamentos foram separados nos conteúdos de albume e gema, e cada porção individualmente analisada pelo método tradicional de cultivo para presença/ausência de *Campylobacter*. Em outros nove ovos, obtidos do mesmo lote de animais, foram separados albume, para verificar sua atividade inibitória frente ao microrganismo. O albume em diferentes concentrações foi incorporada em caldo Bolton e inoculada com 10^6 CFU.mL^{-1} de *C. jejuni*, e após incubação a 37ºC por 24 horas, estriadas em ágar seletivo. Em nenhuma das amostras analisadas (tratamentos e controle) foi observado o crescimento de *Campylobacter*. Os resultados demonstraram que nas condições experimentais utilizadas, *Campylobacter jejuni* não é capaz de sobreviver, colonizar e se multiplicar no interior de ovos comerciais. O albume, como fator isolado, mesmo na concentração de 84,9% não foi capaz de inibir completamente o crescimento do microrganismo em caldo Bolton a 37ºC em microaerofilia. O consumo de ovos não é um fator de risco para a aquisição de campilobacteriose por humanos.

PALAVRAS-CHAVE: *Campylobacter jejuni*. Ovos brancos comerciais. Viabilidade. Consumo. Albume.

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