Brazilian Journal of Microbiology (2012): 535-543
ISSN 1517-8382

TRANSMISSION OF *Campylobacter coli* IN CHICKEN EMBRYOS

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Submitted: December 08, 2010; Returned to authors for corrections: June 17, 2011; Approved: January 16, 2012.

ABSTRACT

Campylobacter coli is an important species involved in human cases of enteritis, and chickens are carriers of the pathogen mainly in developing country. The current study aimed to evaluate the transmission of *C. coli* and its pathogenic effects in chicken embryos. Breeder hens were inoculated intra-esophageally with *C. coli* isolated from chickens, and their eggs and embryos were analyzed for the presence of bacteria using real-time PCR and plate culture. The viability of embryos was verified. In parallel, SPF eggs were inoculated with *C. coli* in the air sac; after incubation, the embryos were submitted to the same analysis as the embryos from breeder hens. In embryos and fertile eggs from breeder hens, the bacterium was only identified by molecular methods; in the SPF eggs, however, the bacterium was detected by both techniques. The results showed no relationship between embryo mortality and positivity for *C. coli* in the embryos from breeder hens. However, the presence of bacteria is a cause of precocious mortality for SPF embryos. This study revealed that although the vertical transmission is a possible event, the bacteria can not grow in embryonic field samples.

Key words: *Campylobacter coli*. Viability. Transmission. Breeder hens.

INTRODUCTION

Campylobacter spp., mainly *C. jejuni* and *C. coli*, are among the most commonly reported bacterial causes of human enteritis in industrialized countries (26). *C. jejuni* is the principal bacterial species found in developed countries; however, more recent studies have shown the importance of *C. coli* (2, 7, 20). In developing countries, *C. coli* is also a common agent in human cases of enteritis, and chickens are important carriers of the pathogen.

Whether the transmission of *Campylobacter* spp. in chickens occurs mainly through of the horizontal via (19), the vertical route is a question that is still under discussion by the majority of authors and has not yet been fully answered. Moreover, this bacterium is considered nonpathogenic in birds, and the few reports available on the damage that it can cause to

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chicken embryos are limited to old work.

Due to the importance of *C. coli* and the scarce and old work on transmission and pathogenicity in chickens, this study aimed to evaluate the vertical transmission and pathogenic effects of *C. coli* in chicken embryos.

MATERIALS AND METHODS

Inoculation of *C. coli* in breeder hens and analysis of eggs and chicken embryos

Four breeder hens (Cobb Vantress) with 203 days old and confirmed to be negative for *Campylobacter* spp. by cloacal swab plate culture, were used in the experiments. These birds were kept until the age of 231 days in the experimental room at the Veterinary Hospital of the Universidade Federal de Uberlândia (UFU), MG, Brazil. They were inoculated intra-esophageally every three days with 10^3 CFU of *C. coli* isolated from chicken feces. During 28 days, the birds were monitored for the presence of *C. coli* by cloacal swab plate culture. Eggs were collected twice daily (early morning and late afternoon) and immediately disinfected with 1% formalin solution spray.

The eggs were sorted into each experimental group based on the order of collection. The first egg was analyzed immediately (no incubation) and the following five eggs were incubated and removed for analysis depending on the order in which they were collected, for 24 hours, six days, 13 days, 17 days or 21 days. The cycle was then reinitiated repeatedly, with next egg being collected immediately and the following five analyzed after 24 hours, six days, 13 days, 17 days, or 21 days of incubation. Incubation took place in the experimental hatchery located at the Centro de Bioterismo e Experimentação Animal of the UFU (CBEA - UFU).

Inoculation of *C. coli* in the eggs of specific pathogen-free (SPF) poultry

One hundred SPF eggs were used: 50 for the test group and 50 as controls. Test group eggs were artificially inoculated with 0.1 mL of 0.85% NaCl solution containing 10^3 CFU of *C. coli* isolated from birds. Bacteria were inoculated into the air sac without damaging the internal membrane, using sterile hypodermic needles (0.3 x 13 mm). Control group eggs were inoculated in same place but with 0.1 mL of 0.85% NaCl without bacteria.

The eggs were incubated similarly but in separate incubators for each experimental group (test and control), located at CBEA-UFU. Overall, six eggs were analyzed after 24 hours, ten eggs were analyzed after six days, 13 days and 17 days of incubation, and 14 eggs were analyzed after incubating to the day of hatching (21 days).

Laboratory Diagnostic

Samples were prepared in the Laboratório de Biotecnologia Animal Aplicada (LABIO). For SPF eggs incubated for 24 hours and eggs from intra esophageally inoculated breeder hens incubated for 24 hours and without incubation, 1 mL albumen and 1 mL yolk were collected and added to 9 mL of Bolton broth (Oxoid). For all others embryos (incubated for six days, 13 days, 17 days and 21 days), only the yolk was collected.

For intra-esophageally inoculated breeder hens, 1g of meconium of hatched broilers and of embryos that died after 18 days of incubation was collected. In SPF embryos inoculated in the air sac, 1 g of jejunum and 1 g of cecum from hatched broilers and from embryos that died after 18 days of incubation were collected and placed in 9 mL of Bolton broth.

All the samples in Bolton broth were analyzed to identify and quantify *C. coli* using the BAX System Real-Time PCR assay (Dupon Qualicon); afterwards, the samples were incubated at 37°C during 24 hours in microaerophilic atmosphere (Probac microaerobac generator) for further culture.

Real-time PCR was performed according to the manufacturer's protocol (24). *C. jejuni* ATCC 33291 was also used as a positive control.

***Campylobacter* spp. culture**

After enrichment in Bolton broth, the samples (yolk, meconium, jejunum and cecum) were plated in CCDA agar with CCDA antibiotic supplement (Oxoid) and 5% horse blood hemolysate and incubated at 37°C for 48 hours in a microaerophilic atmosphere (Probac microaerobic generator). *C. jejuni* ATCC 33291 was used as a positive control. Cloacal swabs from intra-esophagially inoculated breeder hens were plated directly without enrichment in Bolton broth. Characteristic colonies were confirmed as *Campylobacter* spp. by Gram staining and *Campylobacter* species were identified by multiplex PCR.

Multiplex PCR

Multiplex PCR was used to confirm the species of isolated colonies, as described by Harmon et al. (8). The heat-extracted DNA was amplified using the following primers (Invitrogen): Set I - *flaA* gene – *C. jejuni* and *C. coli* (460 bp) pg 3 and pg 50; and Set II - *C. jejuni* (160 bp) C1 and C4, described by Gillespie et al. (7). PCR amplification was carried out with 20 ng of DNA and the reagents (Invitrogen), as previously described (25). The PCR amplification was performed in a Thermal Controller (PTC 150 - MJ Research, Inc.) with cycles as previously described by Harmon et al. (8). PCR reaction products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide, with a 100 bp ladder as molecular weight marker (Invitrogen); the gels were visualized under UV light, in a transilluminator (Chemical Company T102 SIGMA).

Statistical analysis

Descriptive statistics were used to compare the results of real-time PCR and microbiological analysis and the number of viable embryos or chicks. The kappa coefficient ($p < 0.05$) was used to compare the results obtained in conventional culture plate analysis and those obtained by real-time PCR. The Biostat 5.0 program was used for these analyses (1).

Approval of an appropriate ethics committee

Chicken embryos and hatched broilers were sacrificed according Comitê de Ética na Utilização de Animais (CEUA) da Universidade Federal de Uberlândia, number 057/09 and number 323/09.

RESULTS AND DISCUSSION

Cloacal swabs of breeder hens artificially contaminated (intra-esophageal route) with *C. coli*

The positivity of *Campylobacter* spp. in cloacal swabs from breeder hens inoculated by the intra-esophageal route was of 54.5%, 36.3%, 54.5% and 45.4% in the breeder hens one, two, three and four, respectively, during the 11 days that were monitored. There was no pattern to the excretion of *Campylobacter* in breeder hens' cloacal swabs because in different days pos-inoculation there were *Campylobacter* spp negative animals. These results are in disagreement with those of Mbata (14), who found that the elimination of *Campylobacter* is persistent.

Fresh eggs, embryos and chicks of breeder hens artificially contaminated (intra-esophageal route) with *C. coli*

C. coli was absent from embryos incubated for 24 hours, 13 days and 17 days, but it was found in fresh eggs, embryos incubated for six days and newly hatched chicks (Table 1).

The real-time PCR showed that 25.0% (2/8) of fresh eggs contained *C. coli*. The bacteria was found only in the albumen of eggs with count of 1.9×10^4 CFU/g and 8.2×10^5 CFU/g. *C. coli* was not isolated by plate culture method.

Among chicken embryos analyzed with six days of incubation, 12.5% (2/16) were positive for *C. coli*; of these, only one died, after five days of incubation. In chicken embryos or newly hatched eggs analyzed with 21 days of incubation, a 20.0% (2/10) positivity of *C. coli* was detected by *real-time* PCR. The bacteria were found in the yolk in one case, and in the meconium in the other case (Table 1).

However, the bacterium was not isolated by the method of plate culture in the periods analyzed.

There was not relationship between embryonic mortality and *C. coli* positivity in chicken embryos from breeder hens inoculated intra-esophageally.

In this study, bacteria in the yolk of intra-esophageally inoculated hens were detected only by *real-time* PCR. *C. coli* was absent from embryos incubated for 24 hours, 13 days or 17 days, but was present in fresh eggs, embryos incubated for six days and newly hatched chicks (Table 1). The concentrations of the PCR samples ranged from $1.9x10^4$ CFU/g to $1.7x10^6$ CFU/g, values greater than inoculated concentration of bacterium ($1x10^3$ CFU). Thus, these results indicate that replication of the bacteria occurred after inoculation (Table 1). Despite the samples had been negative by culture, it is important emphasize the existence of forms of bacterium called viable but non-cultivable form (VNC), which is not recoverable in plate culture.

Table 1. Viability and presence of Campylobacter in six-day-old chicken embryos and chicken embryos or newly hatched in 21 day-old incubation from hens inoculated by intra-esophageal with *C. coli.*

Age of incubation	Identification	Sample	Protocol analysis			Viability
			real time PCR			
			Result	Specie	CFU/g	
six-day-old chicken embryos	8',9',10',11',12'13',14',16', 17',18',19',20'*	yolk	N	-	-	Viable
	15'	yolk	P	*C. coli*	$1.7x10^6$	Viable
	21'	yolk	N	-	-	EM 4 days
	22'	yolk	N	-	-	EM 2 days
	23'	yolk	P	*C. coli*	$4.4x10^4$	EM 5 days
chicken embryos or newly hatched in 21 day-old incubation	24',25',26',29'*	yolk	N	--	--	Viable
	24',25',26',29'*	meconium	N	--	--	
	27'	yolk	P	*C. coli*	$2.8x10^4$	Viable
	27'	meconium	N	--	--	
	28'	yolk	N	--	--	EM 21 days
	28'	meconium	N	--	--	
	30'	yolk	N	--	--	EM 4 days
	31'	meconium	N	--	--	EM 19 days
	31'	yolk	N	--	--	
	32'	yolk	N	--	--	EM 3 days
	33'	yolk	N	--	--	Viable
	33'	meconium	P	*C. coli*	$4.7x10^4$	

N: Negative; P: Positive; *Analysis individual with similar results; EM: embryo mortality

Depending on the severity and type of stress, the microorganisms present survival strategies and thus, can enter the state VNC, where the cells retain metabolic activity, but are not cultivated by conventional methods available. According to Murphy et al. (16), state VNC is a major concern for public health agencies, since these cells can return to normal vegetative metabolism. This process is called resuscitation, thus increasing the potential risk of infection caused by food for consumption.

The presence of bacterium although *real-time* PCR over 10^3 CFU/g shows that transovarian transmission is possible and that the microorganism can penetrate the egg shell pores and survive during the incubation period. This can be explained by the size of the egg shell pores (11 μm to 12 μm) (21), although the albumen is not an appropriate environment for growth (3, 11). Fonseca et al. (6) found that *Campylobacter* is not viable in infertile eggs inoculated by air sac but albumen doesn't avoid the bacterial growth when inoculated the bacterium in

the albumen.

There are no reports in the literature regarding the use of PCR and direct plate culture for *C. coli.* For *C. jejuni*, however, Hiett et al. (9) found results similar to ours, with 70.0% of eggs positive by direct PCR but negative by plate culture. Fonseca et al. (5) found 80.0% positivity in the meconium of broilers from breeder hens naturally contaminated with *Campylobacter* using PCR, but not by plate culture.

Other authors (12, 27) have found embryonic mortality in birds artificially inoculated with *C. jejuni* but there are no such reports for birds naturally contaminated with *C. coli* or *C. jejuni.*

C. coli in the yolk of embryos or in the meconium of broilers from breeder hens inoculated intra-esophageally with *C. coli* did not kill the embryos ($p > 0.05$). There were 17 viable embryos and negative in *real-time* PCR and 1 embryos precocity died, which was positive in *real-time* PCR. Most researchers claim that vertical transmission is not possible, but older works mention that, under experimental conditions, the bacteria caused embryonic mortality when inoculated in eggs (12, 13, 27).

Fresh eggs, embryos and chicks from SPF eggs inoculated with *C. coli* in the air sac

Among eggs incubated for 24 hours, 83.3% (5/6) in the test group were positive for *C. coli* in the albumen or yolk (Table 2). The control group samples were negative.

In embryos incubated for six, 13 and 17 days, *C. coli* was found with at least one of the techniques used in 50.0% (5/10), 100.0% (10/10) and 80.0% (8/10) of samples, respectively (Table 3).

All chicken embryos in the control group were negative with both detection methods. Of the ten eggs assigned to six days of incubation, six were alive, one was infertile, two died within three days and one died within two days. Of the ten embryos assigned to 13 days of incubation, six were alive, one was infertile, two died within two days and one died within three days. Of the eggs incubated for 17 days, six were alive, two died within four days, one died within three days and another died within two days.

Among eggs incubated until the day of hatching (21 days of incubation), *Campylobacter* sp. was not found in the jejunum, cecum or yolk in the test group. *C. coli* was present only in embryos with early mortality (less than six days of incubation), with a prevalence of 35.7% (5/14) in this subgroup (Table 4).

Table 2. *Campylobacter* sp. in albumen and yolk from SPF eggs air chamber contaminated with *C.coli* after 24 hours.

Egg	Sample	Protocol analysis			
		Real time PCR			Microbiology[1] (mpcr[2])
		Result	Specie	CFU/g	
1	Albumen	N	--	--	N
1	yolk	N	--	--	N
2	Albumen	N	--	--	N
2	yolk	P	*C. coli*	9.6×10^6	P (*C.coli*)
3	Albumen	P	*C. coli*	1.0×10^4	P (*C.coli*)
3	yolk	P	*C. coli*	3.9×10^7	P (*C.coli*)
4	Albumen	P	*C. coli*	5.2×10^4	N
4	yolk	P	*C. coli*	7.9×10^6	P (*C.coli*)
5	Albumen	P	*C. coli*	1.9×10^4	P (*C.coli*)
5	yolk	N	--	--	N
6	Albumen	P	*C. coli*	2.4×10^7	P (*C.coli*)
6	yolk	P	*C. coli*	7.9×10^6	N

[1] plate culture; [2] multiplex PCR; N: Negative; P: Positive

Table 3. Viability and presence of Campylobacter in six-day-old, 13-day-old and 17-day-old chicken embryos from SPF eggs inoculated with *C. coli*.

Age of incubation	Identification	Protocol analysis				
		Real time PCR			Microbiology[1] (mpcr[2])	Viability
		Result	Specie	CFU/g		
Six day old chicken embrios	7	P	*C. coli*	$1.8x10^7$	P (*C.coli*)	EM 5 days
	8	P	*C. coli*	$3.4x10^4$	P (*C.coli*)	Viable
	9,10,11,12*	N	-	-	N	Viable
	13	P	*C. coli*	$1.8x10^7$	P (*C.coli*)	Infertile
	14	P	*C. coli*	$1.4x10^8$	P (*C.coli*)	Viable
	15	P	*C. coli*	$9.6x10^5$	P (*C.coli*)	EM 2 days
	16	N	--	--	N	EM 3 days
13 day old chicken embryos	17	P	*C. coli*	$3.0x10^7$	P (*C.coli*)	EM 2 days
	18	P	*C. coli*	$8.9x10^6$	P (*C.coli*)	EM 2 days
	19	P	*C. coli*	$4.6x10^6$	P (*C.coli*)	EM 2 days
	20	P	*C. coli*	$3.2x10^6$	P (*C.coli*)	Infertile
	21	P	*C. coli*	$1.1x10^6$	P (*C.coli*)	EM 3 days
	22	P	*C. coli*	$2.4x10^6$	P (*C.coli*)	EM 3 days
	23	P	*C. coli*	$2.0x10^7$	P (*C.coli*)	EM 2 days
	24	P	*C. coli*	$2.0x10^7$	P (*C.coli*)	EM 2 days
	25	P	*C. coli*	$3.9x10^5$	N	Viable
	26	P	*C. coli*	$1.1x10^6$	P (*C.coli*)	Viable
17 day old chicken embryos	27	P	*C. coli*	$<1.0x10^4$	P (*C.coli*)	EM 2 days
	28	P	*C. coli*	$5.5x10^4$	P (*C.coli*)	EM 1 day
	29	P	*C. coli*	$6.8x10^6$	P (*C.coli*)	EM 4 days
	30	N	--	--	N	Viable
	31	N	--	--	N	EM 2 days
	32	P	*C. coli*	$1.5x10^7$	P (*C.coli*)	EM 3 days
	33	P	*C. coli*	$2.0x10^6$	P (*C.coli*)	EM 3 days
	34	P	*C. coli*	$3.8x10^6$	P (*C.coli*)	EM 3 days
	35	P	*C. coli*	$9.7x10^5$	P (*C.coli*)	EM 2 days
	36	P	*C. coli*	$8.6x10^6$	P (*C.coli*)	EM 2 days

[1] plate culture; [2] multiplex PCR; N: Negative; P: Positive; * Analysis individual with similar results; EM: embryo mortality

Table 4. Viability and presence of Campylobacter in embryos or chicks hatched on the day of hatching from SPF eggs inoculated with *C. coli*.

Identification	Sample	Protocol analysis				
		PCR Real time			Microbiology[1] (mpcr[2])	Viability
		Result	Specie	CFU/g		
37	Yolk	N	--	--	N	EM 20 days
	Jejunum	N	--	--	N	
	Cecum	N	--	--	N	
38, 39,46,40, 49, 50*	Yolk	N	--	--	N	Viable
	Jejunum	N	--	--	N	
	Cecum	N	--	--	N	
41	Yolk	N	-	-	N	EM 3 days
42	Yolk	N	--	--	N	EM 20 days
	Jejunum	N	--	--	N	
	Cecum	N	--	--	N	
43	Yolk	P	*C. coli*	$3.3x10^6$	P (*C.coli*)	EM 2 days
44	Yolk	P	*C. coli*	$1.2x10^6$	P (*C.coli*)	EM 2 days
45	Yolk	P	*C. coli*	$5.3x10^6$	P (*C.coli*)	EM 3 days
47	Yolk	P	*C. coli*	$2.5x10^7$	P (*C.coli*)	EM 6 days
48	Yolk	P	*C. coli*	$1.1x10^7$	N	EM 2 days

[1] plate culture; [2] multiplex PCR; N: Negative; P: Positive; * Analysis individual with similar results; EM: embryo mortality

Of the 14 eggs incubated for 21 days in the control group, six chicks hatched, three were alive but remained inside the partially broken egg shell, one was infertile, one died within one day, one died within four days, one died within six days and another died within seven days.

The rate of *C. coli* positivity was high with *real-time* PCR and traditional plate culture for SPF eggs inoculated in the air sac. The concentrations of bacteria in the albumen and yolk ranged from $1.0x10^4$ CFU/g to $3.9x10^7$ CFU/g indicating that replication occurred inside the eggs.

Unlike the embryos from breeder hens inoculated intra-esophageally with *C. coli*, SPF embryos inoculated in the air sac had higher embryonic mortality in the test group than in the control group. Therefore, embryonic mortality was associated with the presence of bacteria in the test group.

In embryos from breeder hens contaminated intra-esophagially with *C. coli,* only *real-time* PCR yielded positive results. However, for SPF embryos, there was an excellent replicability between *real-time* PCR and culture plate methods, based on the kappa coefficient ($K=0.8921$, $p<0.0001$).

The kappa test comparing the results of plate culture and *real-time* PCR as well as the presence of the positive control *C. jejuni* ATCC 33291 shows that the techniques used in this study did not present problem on isolation of the bacterium viable cultivable. However, it is important consider the VNC forms, which are not recoverable in plate culture.

Embryonic mortality occurred early in SPF embryos inoculated in the air sac (less than seven days of hatching). Early mortality is probably due to the embryo's active immunity, because embryos incubated for 14 days acquire the functional capacity to detect the entry of an antigen (28).

Research on embryonic mortality and vertical transmission of *C. coli* is unusual. However, there have been some studies with *C. jejuni*. The embryonic mortality in *C. coli* contaminated eggs in this study is comparable to previously published results (12, 13), in which the inoculation of some strains of *C. jejuni* in the corioalantoid membrane was found to be lethal to embryos. Zaki e Reda (27) found 0.7% early mortality in chicken embryos positive for *C. jejuni* after inoculation*;* late embryonic mortality was 1.8% and 2.1% of chicks broke the shell but did not fully hatch.

Vertical transmission is commonly discussed as an epidemiologically important route of infection in birds. Most authors mention that this is not the main route of transmission (2, 10) but Pearson et al. (18), using epidemiological analysis, and Cox et al. (4), by comparing the genetic patterns of bacteria isolated in breeding and their progeny, agreed that vertical transmission is an important route of *Campylobacter* transmission in birds. Based on this study, we can affirm that vertical transmission is a possible route of infection.

In other findings from Melo et al. (15), *C. jejuni* quickly penetrated egg shell pores and reached the yolk of eggs from fertile SPF when the egg matrix was in contact with wood-shavings contaminated with *C. jejuni*.

The high rates of embryonic mortality in *Campylobacter*-positive SPF eggs along with the lower mortality in *Campylobacter*-positive eggs from intra-esophagially inoculated breeder hens leads to speculation that some factor prevents the survival of *C. coli* in breeder hen eggs.

The non-viability of *C. coli* in the eggs of inoculated breeder hens may be associated with the birds' immunology. Sahin et al. (19) found high levels of antibodies against *Campylobacter* in broilers in the first and second week of life, but the antibodies declined or even disappeared in the third and fourth week. The absence of antibodies in the third week coincides with peaks of *Campylobacter* infection in many chicken farms (17, 22, 28), showing that maternal immunity is an important barrier to infection. In this work however, we did not use immunological tools that could support this hypothesis.

Another possible explanation is that the bacteria adopt some sort of protection mechanism until the maternal antibodies disappear, after which they become viable and replicate, similarly to what occurs in the laboratory (23). Under extreme temperature and pH conditions, the bacteria adopt

forms that are VNC.

CONCLUSION

Campylobcater coli are pathogenic to SPF chicken embryos and causes high early embryonic mortality. However, in embryos from breeder hens, vertical transmission is a possible route of infection but the bacterium would be either VNC form or nonviable in plate culture and the bacterium isn't a pathogenic in chicken embryos.

ACKNOWLEDGEMENTS

This study was financed by the "Fundação de Amparo a Pesquisa de Minas Gerais" (FAPEMIG) (Minas Gerais Research Support Foundation) and "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) (Federal Brazilian Research Support Foundation).

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