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Full Length Research Paper

Genetic Characterization of *Campylobacter jejuni* Isolated from Boiler Flocks

H. Abo Al-Yazeed¹, Amani M. Marwan¹, Attia, A. M.², A. Samir¹, Ammar, A. M³

¹Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

² Department of Bacteriology, Animal Health Research Institute, Dokki, Giza.

³ Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt.

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Campylobacter is a worldwide infection which has been estimated it as the most significant economic burden by EFSA and ECDC in 2016. It caused mainly by either *Campylobacter jejuni* or *Campylobacter coli*. Poultry are a natural reservoir for *Campylobacter* species, although it is insignificant for poultry health and is a leading cause of gastroenteritis for humans in developed and developing countries. The disease is endemic in Egypt and is a major cause for diarrhea in children. Good understanding of epidemiology and surveillance of *Campylobacter* will help in elimination and prevention of it among animals and humans. Many molecular typing techniques used to track the source of infection and reduce *Campylobacter* infection rate. To achieve that, 290 samples were collected from broiler flocks and slaughter market from Cairo governorate, Egypt. Bacteriological and molecular identification have been implemented based on genus and species level for *C. jejuni* then phylogenetic tree analysis of *flaA* gene to correlate the genetic relatedness and emphasize that chicken is the major source of infection.

Keywords: *Campylobacter jejuni*, genotyping, *flaA* gene, short variable region, chicken, Human.

INTRODUCTION

Avian host either domestic or wild birds are the main natural reservoir for *Campylobacters*, particularly thermophilic *Campylobacter jejuni* and *Campylobacter coli*. It is believed that the horizontal transmission plays an important role in broiler flock colonization which reaches 100% at slaughter age. However, its commensal nature, it

results in little or no distinguished signs in broiler flocks while in laying hens, results in high morbidity and mortality rates due to vibronic hepatitis (Burch 2005, Stephens et al. 1998).

Globally, public health services pay a significant interest to *Campylobacter* species because it found to be pathogenic for human and reported as one of the three common causes of foodborne outbreaks in Europe in 2010 (ECDC 2013). Exposure to poultry and poultry by-products through handling, preparing and consumption are the main

*Corresponding Author's Email: amanyanmarwan110@mail.com

risk factors of campylobacter infection in human. (Whiley et al. 2013; Wingstrand et al. 2006; Rosenquist et al. 2013).

Molecular typing methods aims to track the sources of campylobacterial infection, as well, monitor geographically specific strains and develop control strategies to *Campylobacter* infection within the food chain. Recently, several DNA based techniques are available for differentiation of *Campylobacter* species and offers higher type ability, greater discriminatory power in comparison with the phenotypic methods (Ioannidis et al. 2006; Pendleton et al. 2013).

The most common available genotypic techniques include pulsed-field gel electrophoresis (PFGE) which has been proven as a gold standard method (Gerner-Samidt et al. 2006; Ghorashi et al. 2013), randomly amplified polymorphic DNA (RAPD) fingerprinting, amplified fragment length polymorphism (AFLP) profiling, rpo typing, flagellin (*flaA*) typing (Meinersmann et al. 1997) and multilocus sequence typing (MLST).

The *flaA* gene is a common genotyping method used for *C. jejuni* and *C. coli* by PCR followed by restriction fragment length polymorphism (RFLP) and short variable region (SVR) sequencing method (Meinersmann et al. 1997; Nackamkin et al. 1993). The *flaA* typing thought to be widely used because of its speed and simplicity. The *flaA* gene is the main monomeric subunit of the flagellum which encodes the flagellin protein (Fitzgerald et al. 2001; Harrington et al. 1997; Nielsen et al. 2000; Shi et al. 2002). Short variable region sequencing of *flaA* gene believed to be the most convenient method for detection of *Campylobacter* by providing high level of discrimination of *C. jejuni* (Sails et al. 2003).

The epidemiology of *Campylobacter* species in the developed countries differs from that the developing world. Epidemiology and surveillance reports about *Campylobacter* species in developing countries are limited for both humans and animals, particularly genotypic information (Shobo et al. 2016; Ngulukun et al. 2016). Therefore, the aim of the current research is genotyping *Campylobacter jejuni* from broiler flocks in Cairo governorate, Egypt by sequencing *flaA* gene variable region, and genealogic analysis.

MATERIAL AND METHODS

Sample collection:

In our study, a total of 290 samples were collected from broiler farm, slaughterhouse and chicken market in Cairo governorate. A one hundred cloacal swab samples were collected from live birds. Additionally, 50 samples from different edible organs such as; liver, gall bladder, gizzard, spleen and intestine were collected and transferred into sterile blender containing Preston enrichment broth. In

addition, 40 samples of drip wash and edible organs respectively from chicken market and 60 chicken meat samples (neck skin, thigh and breast muscle) were collected. All samples were kept on ice and processed within 4 hours after sampling.

Campylobacter isolation and identification

The samples were processed according to ISO 10272 (ISO 2006) to isolate *Campylobacter* species. Samples were examined by direct plating and selective enrichment methods. Meat and swab samples were inoculated into Bolton selective enrichment broth (Oxoid) and incubated for 24 hours at 37°C in microaerophilic conditions using a gas generating kit (Campygen, Oxoid, Lot: 13L08-C25-14). A loop of broth directly cultured onto mCCDA agar (Oxoid) supplemented with *Campylobacter* selective supplement. The plates were incubated at 42 °C for 48 to 72 hours in microaerophilic conditions (Hald et al. 2004).

Water samples were filtrated by using a 0.45 µm sterile membrane filter which was placed in Bolton broth and incubated at 42°C for 48 h in microaerophilic conditions. Later, the cultures were inoculated onto mCCDA agar and incubated under microaerophilic conditions at 42°C for 2 to 5 days.

After 72 hours, all cultured plates were checked for purity and the assumed *Campylobacter* colonies were confirmed for its characteristic corkscrew-like motility and spiral shaped cells by phase contrast microscopy. Additionally, identification of the *Campylobacter* strains was performed by performing catalase, oxidase (BBL, Becton, Dickinson and Co., Sparks, MD, USA) and hippurate hydrolysis tests (Remel, Lenexa, KS, USA). The hippurate-positive isolates were identified as *C. jejuni*, while, hippurate negative isolates were identified as *C. coli* (Hariharan et al. 2009).

Genetic identification

One single colony from the suspected positive sample was subcultured onto blood agar plate (CM0055; Oxoid, 7% sheep blood and 1000 mg of cyclohexamide per litre), and PCR method used for *Campylobacter* confirmation and species identification was described by Best et al., 2003 which is based on the detection of partial sequences of *map A* amplicon that allow the simultaneous identification of *C. jejuni* (Vidal et al., 2013).

A typical *Campylobacter* colony from each culture was propagated for DNA extraction. Genomic DNA was extracted by QIAamp DNA mini kit (QIAGEN, Lot No: 11872534, Kat No: 51306) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until used. The concentration of DNA was determined using a Nano Drop 1000 (Thermo Fisher Scientific), and its purity was estimated as described by Sambrook and Russell 2001.

Table 1: Oligonucleotide primers used for genus confirmation, species identification and virulence factors.

Primer	Target gene	Sequence (5'-3')	Amplicon size	Annealing Temperature	Reference
23SF	23S rRNA	TATACCGGTAAGGAGTGCTGGAG	650 pb	59°C 30 sec	Wang et al., 2002.
23SR		ATCAATTAACCTTCGAGCACCG			
MapA F	mapA	CTA TTT TAT TTT TGA GTG CTT GTG	589 pb	55 °C 45 sec	Eunju and Lee, 2009.
MapA R		GCT TTA TTT GCC ATT TGT TTT ATT A			
flaA 664	flaA	AATAAAAATGCTGATAAACAGGTG	855 pb	53°C 1 min	Datta et al., 2003.
flaA1494		TACCGAACCAATGTCTGCTCTGATT			
VirB-232	virB11	TCTTGTGAGTTGCCTTACCCCTTTT	494 pb	53°C 1 min	Datta et al., 2003.
VirB-701		CCTGCGTGTCCGTGTATTATTTACCC			
WlaN-DL39	wlaN	TTAAGAGCAAGATATGAAGGTG	672 pb	46 °C 45 sec	Linton et al., 2000.
WlaN-DL41		CCATTTGAATTGATATTTTTG			
VAT2	cdtB	GTAAAAATCCCCTGCTATCAACCA	495 pb	42°C 2 min	Bang et al., 2003.
WMI-R		GTTGGCACTTGGAATTTGCAAGGC			

PCR primers were used to confirm the genus and species level of *Campylobacter*. The specific regions of the 23S rRNA (Wang et al., 2002), and mapA genes were amplified by PCR as described by Denis et al., 1999; Wieczorek and Osek, 2005. Moreover, *Campylobacter jejuni* isolates were tested for the presence of the most common described virulence genes such as; flaA, virB11, wlaN and cdtB. The PCR conditions for the assessed genes was performed as described by (Wieczorek 2010). The primer sequences of all the genes, size of PCR amplicon and PCR condition used in our study are presented in table 1.

FlaA typing

Flagellin gene typing was performed as described by Nachamkin et al. 1996. The QIAquick PCR product purification kit (Qiagen Inc. Valencia CA) used to purify the PCR product. DNA strands were sequenced using Big Dye Terminator 3.1 kit (Applied Bio-system) and flaA primers. according to Nishimura et al. 1996, the forward fla A primer 5'-TA CTA CAG GAG TTC AAG CTT-3' or reverse fla A primer 5'-GT TGA TGT AAC TTG ATT TTG-3' that represent the variable (V1) region used as PCR primers. Sequencing reactions were carried out by using 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The automatic sequencer ABI 377 (Applied Bio-system) used for analysis and the obtained sequences were determined by BLAST analysis with sequences available at Genbank (NCBI).

RESULTS

In our study, the prevalence rate of *Campylobacter species* in cloacal swab samples from live birds was 30% and 50%

from different organs of dead birds with diarrhea. From the chicken outlet, the highest incidence of *Campylobacter* from chicken market samples were 45% from skin samples, 40% and 30% out of meat samples (thigh and breast muscles) respectively. Moreover, isolation rate of *Campylobacter* was 17.5% from different edible organs, 22.5% from drip wash samples as shown in Table 2.

Here in the study, molecular identification of thermophilic *campylobacter* species using *Campylobacter* 23S rRNA gene evidenced only 94 (32.4%) *campylobacter* spp. isolates in all the examined samples (Table 2). In addition, it was found that 70 *C. jejuni* isolates with an incidence of 24.13% were confirmed to species level by polymerase chain reaction through detection of mapA gene (Table 2).

Conventional PCR targeting the species specific virulence gene flaA, virB11, wlaN and cdtB was performed using 10 *C. jejuni* isolates (Table 3). Two isolates harbored both flaA and virB11 genes which were selected for molecular sub typing by using flaA gene sequencing.

Detection of four different presumptive virulence and toxin genes by PCR. among *C. jejuni* strains isolated from different chicken samples are summarized in Table 3.

The sequence of flaA gene was performed. The sequences were aligned by using Clustal W meg 5. The phylogenetic tree was constructed and bio Edit, software was used for Clustal W Multiple alignment and sequences identity matrix as well as MEGA6 software version (6.06) was used for construction of phylogenetic by neighbor-joining method (Fig. 3). It was detected that isolate number 5 had high percentage similarity 98.8% with *C. jejuni* strain 12567 (Accession number CP028909.1) and isolate number 7 showed the high similarity 98.1% with *C. jejuni* D42a (Accession number CP007751.1).

Table 2: Distribution of *Campylobacter* species in different types of samples.

Source	Sample type	No. of Samples	<i>Campylobacter species</i>		<i>C. jejuni</i>	
			No.	%	No.	%
Chicken Farm	Cloacal swab from live birds	100	30	30%	25	25%
Dead Birds	Liver	10	5	50%	4	40%
	Gizzard	10	3	30%	1	10%
	Gall bladder	10	6	60%	4	40%
	Spleen	10	3	30%	2	20%
	Intestine	10	8	80%	5	50%
Chicken Market	Drip wash	40	9	22.5%	6	15%
	Edible organs	40	7	17.5%	6	15%
	Neck skin	20	9	45%	5	25%
	Thigh muscle	20	8	40%	7	35%
	Breast muscle	20	6	30%	5	25%
Total		290	94	32.4%	70	24.13%

Table 3: Results of virulence genes characterization of some *C. jejuni* isolates.

Sample No	Results			
	<i>VirB11</i>	<i>WlaN</i>	<i>flaA</i>	<i>cdtB</i>
5	+	-	+	-
1	-	-	-	-
7	+	-	+	-
3	-	-	-	-
4	-	-	-	-
2	-	-	-	-
6	-	-	-	-
8	-	-	-	-
9	-	+	-	-
10	-	-	-	-

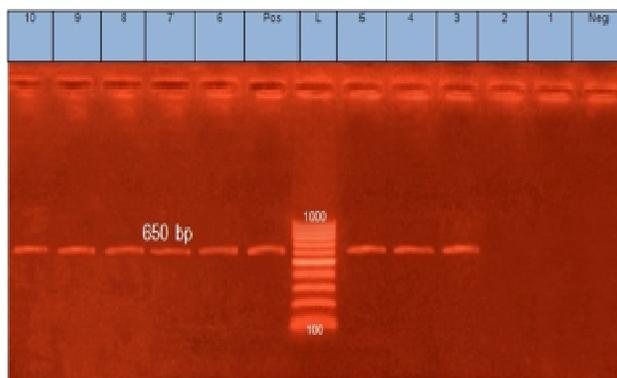


Figure 1: Agarose gel electrophoresis of conventional PCR for detection of 23S rRNA gene for *Campylobacter* spp. (650-bp fragment) in examined samples.

Lanes 3-10: positive results for *Campylobacter* spp.

Pos: positive control.

Neg: negative control

L: Lane marker (Gel Pilot 100 bp. ladder)

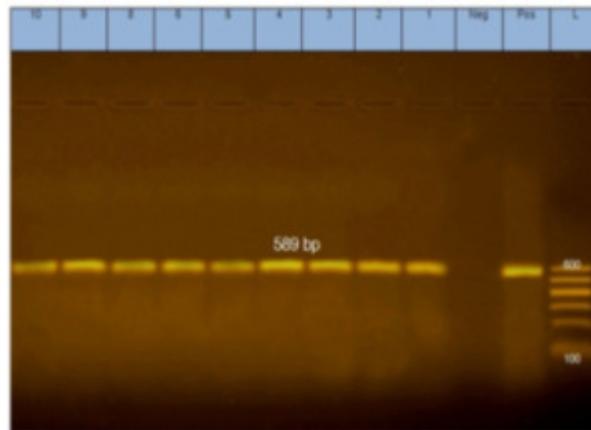


Figure 2: Agarose gel electrophoresis of conventional PCR for detection of *mapA* gene for *C. jejuni*. (589-bp fragment) in examined samples.

Lanes 1-10: positive results for *C. jejuni*.

Pos: positive control.

Neg: negative control

L: Lane marker (Gel Pilot 100 bp. ladder)

DISCUSSION

Campylobacter has been known as one of the most common foodborne pathogen worldwide and resulting in diarrheal illness for human for over three decades (Bolton 2015). According to the Foodborne Diseases Active Surveillance Network (Food Net), it has been reported about 14 and 71 cases per 100,000 populations annually are diagnosed with campylobacteriosis in U. Sand and European Union respectively (CDC 2017; EFSA 2016). It has been reported that the infection caused by *Campylobacter* is more frequent in comparison with other infections such as; *Salmonella* species, *Shigella* species and *E. coli* O157:H7 (CDC 2013). *Campylobacter* assumed to be the most common food borne disease among human (Man 2011 and Dasti et al., 2010). Furthermore, *Campylobacter enteritis* is estimated as a multibillion-dollar disease by the centers for Disease Control and Prevention and consumption of poultry meat is the main source of infection (Hiatt 2002).

In the present, the overall prevalence of *Campylobacter* in broiler flocks were around 32.4% from different sources as shown in table 2. Similarly, Haruna et al, 2012 who reported the prevalence of *Campylobacter* was 47.2% in broiler flocks in Japan. Additionally, Carron 2017 and his collaborators found that the prevalence of *Campylobacter* in broiler farms varied between 33 % to 44% whereas the prevalence were 60% to 64% in chicken retailers of Kenya. Also, in Egypt it has been recorded that overall occurrence of *Campylobacter* was 24.9% (56 out of 225) in 2017 by Asmaa et al, 2017 and Mostafa Abushahbain and his colleagues in 2018 estimated the overall prevalence of *Campylobacter* species detected by biochemical reactions and multiplex PCR assay was found to be 23.51% and

22.46%, respectively. Further study reported an overall prevalence of *Campylobacter* 69.5% in Nairobi chickens (Mageto et al. 2018).

C. jejuni was detected in 70 out of 290 (24.13%) of the examined samples. In our study the results are comparable with Zhang in 2018 where *C. jejuni* incidence rate is 33.6% was obtained from 348 samples collected in the slaughterhouse. Higher results were estimated by the European Union, where 60.8% of broiler samples were positive for *C. jejuni* (EFSA 2010).

Campylobacter isolates were recovered from live birds with 30% from cloacal samples. These results are similar with Van Asselt and his colleagues in 2008 where the prevalence rate was ranged from 20 to 31%. Another Tanzanian study recorded 42.5% of cloacal swab samples of chickens (Chuma et al. 2016). On the other hand, Brazil and Costa Rica estimated 100.0% and 80.0% of their flocks were positive for *Campylobacter* when cecal samples were studied (Giombelli and Gloria, 2014; Zumbaco-Gutiérrez et al., 2014). Although different prevalence rates are recorded in developing countries, these fluctuations due to different methodologies of culturing and identification which make the direct comparison of results very difficult.

According to USDA researchers, the retail chicken is a highly contaminated with *C. jejuni* with an isolation rate 98% for trade chicken meat and the *Campylobacter* count exceeds 10^3 in skin and offal (USDA 2008). In our study, we found that incidence of *C. jejuni* isolated were 15%, 25% 35% and 25% from edible organs, chicken skin, thigh and breast muscle respectively. These findings are closely similar to a study conducted in Zagazig where they reported the rate of *C. jejuni* were 47.5%, 25.9% and

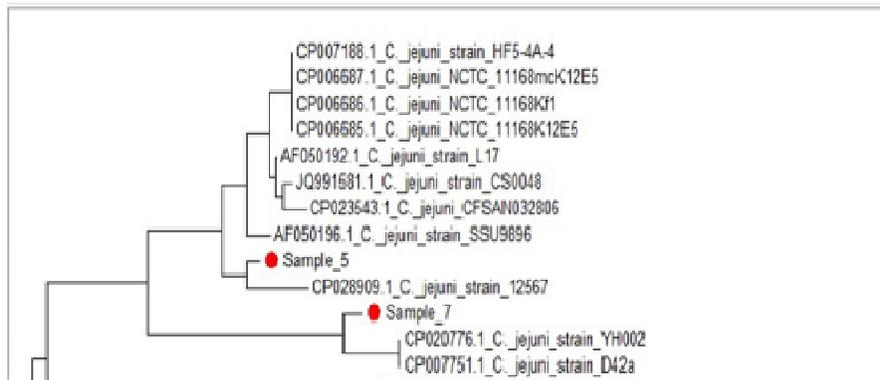


Figure 3: Phylogenetic tree of *flaA* gene sequences of *Campylobacter jejuni* recovered from broiler chicken. The tree was generated based on the neighbor-joining method.

47.5% from skin, breast and thigh muscle respectively (Awadallah et al., 2014).

High incidence of *C. jejuni* isolated from different organs in dead birds like; intestinal content and liver with 50% and 40%. A closely findings were detected in liver samples (53.3%) which obtained by Stoyanchev 2004 while lower percentages (24% and 21%) of *Campylobacter* species in liver were obtained by Bartkowiak-Higgo et al. 2006 and Vashin et al. 2009 respectively. Higher rates of *C. jejuni* isolation were obtained by El-fadaly et al., 2016 where they collected intestinal and liver samples from five governorates in Egypt with 72.1% and 52.8% respectively.

This is maybe attributed to transportation of live birds to the slaughterhouse results in possibility of contamination, especially if cleaning and disinfection is insufficient and the same cages are used for several different flocks. On top of that, the fundamental infection and contamination carried out during the feathering and evisceration steps in poultry processing (Sasaki et al., 2013; Gruntar et al., 2015).

Countries using pluck-shop based markets have higher contamination rates of *Campylobacter* spp. from poultry than countries using modern processing plants (Parkar et al., 2013). Manual slaughtering and evisceration lead to fecal contamination of carcasses, which in turn may be responsible for increased numbers of *Campylobacter* spp. in poultry meat (Parkar et al., 2013).

Moreover, rate of *Campylobacter* species in drip wash water was 22.5% and 15% is estimated for *C. jejuni*. These findings proved that rinsing steps during processing may further worsen the problem of contaminated carcasses by introducing *Campylobacter* organisms even to uncontaminated carcasses (Bashor, 2004; Wagenaar, 2013).

The phenotypic markers failed to differentiate the *Campylobacter* isolates on family and genus level, thus allowed the molecular techniques for species identification among the *Campylobacter* members (Achtman and

Wagner 2008). Molecular techniques are a gold standard method with high sensitivity and specificity for some pathogens (Amar et al. 2007 and Quinn, 1995). Variable PCR techniques targeting variety of genes such as; *hipO*, *ceuE* and *mapA* genes to confirm *C. jejuni* (Khalifa et al. 2013; Persson et al. 2005; Nayak et al. 2005 and Gomes et al., 2016).

In the current study, 10 representative isolates were chosen from different sources to be molecularly identified and confirmed on genus and species level by targeting 23S rRNA and *mapA* genes and only 8 isolates were confirmed as *C. jejuni*. While two isolates are not confirmed as *Campylobacter* and it is thought to be due to difficulty in identifying the correct colony or over growth of neighboring colonies (Jensen et al., 2005). Additionally, gene mutation has been previously reported as a role of PCR failure in targeting the specific genes (On and Jordan, 2003).

Moreover, Banowary et al., 2015 reported that PCR technique could be affected by various factors like; quality and quantity of DNA, annealing temperature between and DNA and primers, self-annealing between PCR products and different copy numbers of the targeted genes.

The isolates showed wide variation for the presence of pathogenic genes; however, presence of virulence genes revealed the pathogenic potential of the isolates. The findings are in agreement with earlier observations regarding the presence of *flaA* genes in *C. jejuni* species isolated from human as well as chicken (Bang et al., 2003; Datta et al., 2003; Rozynek et al., 2005). The products of these genes are responsible for the expression of adherence and colonization (Nuijten et al., 2000; Ziprin et al., 2001).

In fact, the overall lack of surveillance report about *Campylobacter* species in chicken and human result in the development of many molecular typing and sub typing techniques which offers free availability of an electronic database to facilitate the results of phylogenetic analysis

(EFSA, ECDC 2016; Magana et al. 2017). Furthermore, genotyping is the most common and have higher discriminatory power and indicate genetic relatedness between different *Campylobacter* species including; pulse field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), *flaA* short variable region (SVR), as well as multi-locus sequence typing (MLST). (Colles and Maiden 2012).

In our study, *flaA* typing was used to identify the genetic relatedness and phylogenetic tree is constructed using *flaA* gene sequence (Fig. 3). Sequence analysis of sample number 5 in our study showed that high similarity (98.8%) with chicken sample isolated from UK (CP028909). While, sample number 7 showed high identity (98.8%) with two isolates (CP020776) and (CP007751) which isolated from liver calf and chicken cecum from USA respectively. These finding proved that chicken is the main source of infection to human as well as genetic identity between human, chicken and farm animals. It is extensively described that the discrimination level of *flaA* typing technique is greater than stereotyping while it is lower than PFGE that's why it should be supported by other genotyping methods like; MLST (Ribot et al. 2001; Dingle et al. 2005).

In conclusion, poultry contamination by *Campylobacter* species plays a significant role in human infection. So, further intensive studies needed to implement the effective intervention strategy for day old chick to reduce the colonization rate and human risk.

Conflict of interest

Authors declare that they have no competing interest.

Ethics approval

All procedures performed in our study involving animals were in accordance with the ethical standards of the "Research Ethics Committee" Faculty of Veterinary Medicine, Cairo University, Egypt.

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