Occurrence and antimicrobial resistance of *Campylobacter jejuni* isolates from poultry in Casablanca-Settat, Morocco

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Abstract

Campylobacteriosis and *Campylobacter* spp. resistance to antibiotics represents a serious worldwide public health problem thermophilic Campylobacters, in particular, are major causes of gastroenteritis in humans. The aim of this study was to determine the prevalence and antimicrobial resistance of *Campylobacter jejuni* isolated from chicken droppings, of commercial poultry in the city of Casablanca, Morocco. Between February and September 2017, 140 samples of chicken droppings were collected and analyzed by classical bacteriology methods for isolation and identification according to Moroccan Standard NM ISO/TS 10272-3 (2013), followed by molecular identification (PCR: polymerase chain reaction). Among the 140 samples, 102 (73%) were positive by *Campylobacter* spp. tests and 38 (27.14 %) were negative to *Campylobacter* spp. Among the positive colonies, 41 (40, 2%) were *C. jejuni*. Of the 41 *C. jejuni* isolates, resistance was detected to tetracycline (100%), erythromycin (97%), ampicillin (85%), ciprofloxacin (77%), amoxicillin/clavulanic acid (61.4%), and gentamicin (12.0%). In conclusion, the data obtained in the current study demonstrate that the majority of *C. jejuni* isolates evaluated were resistant to antimicrobials of the cycline, macrolide, and fluoroquinolone families, and all of the isolates were susceptible to gentamicin. Fluoroquinolone is the drug of choice for treating *Campylobacter* infections. These results underline the need for prudent use of antibiotics in poultry production to minimize the spread of antibiotic-resistant *Campylobacter* spp.

Introduction

Campylobacter infections are among the leading zoonotic agents causing acute gastroenteritis in developed countries (Parsons *et al*., 2010; Verma *et al*., 2014; Campagnolo *et al.*, 2017). Among Campylobacter spp, *Campylobacter jejuni* is mostly associated with human and other domesticated animal enteritis, followed by *Campylobacter coli*, *Campylobacter upsaliensis*, and other species (Moore *et al*., 2005; Parsons *et al*., 2010; Campagnolo *et al.*, 2017; Leahy *et al*., 2017). Campylobacteriosis is the most reported foodborne illness annually in the European Union since 2005. According to the European Food Safety Authority (EFSA), 246,571 cases of verified human campylobacterioses were reported in 2018. In the United States, campylobacteriosis affects a million people a year, and in Canada, there are over 200 thousand cases registered each year (Ravel *et al.,* 2016; Rosenberg *et al.,2016*). Cases of campylobacteriosis have become common also in Africa, Asia, and the Middle East, particularly in children (Johnson *et al.,* 2017). Poultry is the main reservoir of these pathogens and harbors them without clinical manifestations. Transmission of *Campylobacter spp* to humans occurs mainly through cross contamination in the kitchen as well as the consumption of contaminated raw/undercooked poultry products or close contact with infected animals (Migura-Garcia *et al*, 2017; Suzuki *et al.,* 2009). A small proportion of campylobacteriosis cases may be attributed to other animals or environmental sources (Chen *et al*., 2014). The gastrointestinal manifestations of Campylobacteriosis include cramps, fever, myalgia, weight loss, and acute watery or bloody diarrhea; however, the infection may result in severe extra-intestinal sequelae, especially acute neurological symptoms such as Guillain-Barre syndrome and Miller Fisher syndrome, due to a cross-reactivity between the bacterial lipooligosaccharides and nervous system gangliosides (Ma. SM, 2011). Antibiotic treatment is suggested for patients with severe campylobacteriosis (Kaakoush *et al.*, 2015). There are several common antimicrobial agents for campylobacteriosis therapy: erythromycin, ciprofloxacin, tetracycline, and doxycycline (Wang *et al.,* 2013). But overconsumption, misuse and use of antibiotics by the veterinary industry, to fatten animals and promote their growth, contributes to increased resistance to antibiotics. In Morocco, we are in a therapeutic dead end. In fact, in more than a decade, the consumption of antibiotics has gone from 134.5 million doses to almost 224 million (2003-2016). The consumption of antibiotics in Morocco is dominated by volume by amoxicillin alone or combined with clavulanic acid (more than 50%). But Quinolones, followed by combinations of antibiotics (Spiramycin/Metronidazole) and other betalactamines are the most progressive. The consumption of beta lactams (including penicillin derivatives) is largely dominant (61% in 2016). For example, penicillin resistance increased from 60.3% in 1999 to over 70% in 2004, according to

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Like many other countries, Morocco has insufficient capacity to cope with antimicrobial resistance. If there is not yet a national multisectoral coordination plan allowing an effective response, Morocco follows many positive approaches revealing its means of combating the problem and notably has several vertical programs endowed with the laboratory capacities necessary to detect antimicrobial resistance and a national protocol to ban the use of antibiotics in animal husbandry from July 2017. despite the fact that broiler producers are required to sign management contracts with veterinary clinics involved in activities related to poultry diseases, in accordance with Moroccan law 49.99 (Law 49-99; 2002) (Rahmatallah *et al*., 2018). And ONSSA has also taken various measures, including restricting the use of antibiotics in animals. The present study's aim was a comprehensive identification and molecular characterization of *C. jejuni* isolated from chickens, using the classical bacteriology method. Moreover, the strains were identified by molecular identification using the PCR technique and their resistance to several antimicrobials was investigated.

Materials and Methods

Validation of the method for isolation and identification of Campylobacter in chickens they purchased on a large market of living birds from different farms was undertaken between February and September 2017 during which, 140 samples of chicken droppings were collected from chickens purchased on a large market.

The classical bacteriology method was used for isolation and identification according to the Moroccan Standard NM ISO/TS 10272-3 (2013) is similar to the ISO method for Campylobacter used as reference worldwide just the normalization code. We then carried out the validation of *C. jejuni* by PCR with a positive control (*C. jejuni* ATCC 33560) and negative control (*C.coli* 33559).

Study area and sample preparation

This study was conducted in Casablanca, MOROCCO. From February to September 2017, 140 samples of chicken droppings were collected from chickens purchased on a large market from different farms and different flocks in the same site. The sample was taken in a legal and regular manner from different site, twice a month, 10 samples were taken in each sampling precisely on Thursday, which represents the peak of the sale on the market: 5 samples from red chicken (laying chicken) and 5 others from white chicken (broiler chicken) with bird age do not exceed 3 weeks. The droppings are collected by sterile swabs containing 5 mL of alkaline peptone water (this is the transport medium), then rapidly transported to the laboratory in a cooler.

Isolation and biochemical identification

Isolation of *Campylobacter spp* was performed after enrichment as proposed by NM ISO / TS 10272-3 (2013) Food microbiology Horizontal method for the search and enumeration of Campylobacter spp, Part 3: Semi quantitative method. Briefly, 1 g of dropping contents was transferred to 9 mL of Preston enrichment broth base (Oxoid CM 0067, Oxoid Ltd., Basingstoke, Hampshire, UK) containing Campylobacter growth factor (Oxoid SR 0232E, Oxoid Ltd.) and 7% (v/v) defibrinated sheep blood. Incubation was performed in a jar containing a microaerobic atmosphere packet generator (5% oxygen, 10% carbon dioxide, 85% nitrogen) type CAMPYGen (Oxoid CN0025A, Oxoid Ltd.) during 24 h at 37◦C. After enrichment, each sample, was directly streaked onto a Campylobacter Selective Agar (Oxoid Ltd.) containing 5% fresh sterile defibrinated sheep blood and Campylobacter supplement III (Sigma, St. Louis, MO, USA) for primary isolation. Incubation was carried out for 72h at 37◦C. One presumptive *C. jejuni* colony from each agar plate was streaked on Colombia blood agar plates and incubated under microaerobic conditions for 24 h at 37°C. The isolated colonies were identified by their characteristic morphology on testing of the absence of growth at 25°C under aerobic conditions, motility and Gram staining by 100 × 10 magnification microscope, open diaphragm and capacitor at maximum with a drop of oil immersion, as well as by oxidase testing. The plates were incubated in an environment of 5% oxygen, 10% carbon dioxide, and 85% nitrogen for 36–48 h at 37° C. These tests were followed by confirmation tests using API Campy (bioMérieux, Marcy-l'Etoile, France). All isolates were stored in 25% (v/v) glycerol-peptone broth at -70°C.

DNA extraction and PCR conditions

Presumptive *Campylobacter spp* isolates after purification, one presumptive *C. jejuni* colony from each agar plate was streaked on Colombia blood agar and saved in glycerol-peptone broth at -70°C, are revitalized and grown on nutrient agar overnight at 37°C. After incubation, 3 to 5 colonies of pure strain were harvested and suspended in 100 µL in hypotonic water. Cell suspensions were heated at 100°C for 10 min, and then cooled to room temperature. Thereafter, cell suspensions were pelleted by centrifugation at 12,500 rpm/min for 10 min. The supernatant was collected and transferred to a new tube, then stored in 25% (v/v) glycerol-peptone broth at -70°C.

The PCR was carried out within a thermal cycler under a final volume of 25 µL containing 0.5 µL $MgCl_2$(25 mM), 4 µL of extracted DNA, 12.5 µL RedMixte of Taq DNA polymerase, 0.2 µL of each primer (25mM) for gene amplification (CJ-6027(F) 5'gaatgaaattttagaatgggg3' and CJ-6028(R) 5'gatatgtatgattttatcctgc3'). Amplification conditions for the genes specific to *C. jejuni* were as follows: 95°C for 5 min, followed by 30 amplification cycles; denaturation at 95°C for 45 sec, annealing at 48°C for 30 sec, and extension at 72°C for 30 sec. An additional extension step (5 min, 72°C) was performed. Finally, amplicons were electrophoresed on 2% agarose gel, colored with ethidium bromide (0.5 µg/mL) and visualized under UV light. The *C. jejuni* PCR product was 358 bp in length (Stonnet *et al*., 1993). Because we developed a PCR test specific for *C. jejuni* based on the use of oligonucleotide primers that were specifically directed to the synthesis of a 358 bp DNA fragment from the *C. jejuni* genome (Manel *et al*., 2018). *C. jejuni subsp. jejuni* ATCC 33560 was used as the reference strain (positif control) and C.coli 33559 (negative control).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method using Mueller-Hinton agar (Oxoid Ltd.) supplemented with 5% defibrinated sheep blood, according to the French Institute of Susceptibility (Manel *et al*., 2018). Disks impregnated with antibiotics (bioMérieux, Marcy-l'Etoile, France) and their corresponding concentrations were as follows: ciprofloxacin (CIP: 5 µg); erythromycin (E: 30 µg); gentamicin (GM: 10 µg); ampicillin (AMP: 30 µg); amoxicillin/clavulanic acid (AMC: 30 µg); tetracycline (TE: 30 µg). Briefly, well-isolated colonies of the same morphological type were selected from an agar culture plate and transferred into 10 mL of sterile saline buffer (NaCl 0.9%). After homogenization and a 1/10 dilution, 1 mL of the mixture were flooded onto the surface of a Mueller-Hinton agar (Oxoid Ltd.) containing 5% defibrinated sheep blood. The inoculum was allowed to dry for 5 min and antibiotic disks were placed on the plate. After 48 h of microaerobic incubation at 37°C according to the European Committee for Antibiotic Susceptibility Testing (EUCAST), the inhibition zone diameters were measured with calipers. *C.*

Jejuni ATCC 33560 was used as the reference stress. The isolates were classified as susceptible, intermediate, and resistant according to the EUCAST.

Results

Prevalence

Overall, among these 140 analyzed samples, 102 (73%) *Campylobacter spp* were identified by a conventional biochemical method according to the Moroccan standard and confirmed by the "API Campy". The colonies appeared smooth and transparent, with a regular border; microscopic observation of colonies in the fresh state revealed bacteria with *Campylobacter* characteristic motility in the flight of midges and Gram staining (microscopic immersion observation, X 1000) revealed the presence of Gram-negative, S-curved or spiral bacilli and some coccoïdal degenerative forms. Furthermore, these bacteria showed catalase and oxidase-positive activities compatible with the *Campylobacter* genus. After the biochemical confirmation, we performed a molecular genotypic confirmation by PCR. We developed a PCR test specific for *C. jejuni* based on the use of oligonucleotide primers that were specifically directed to the synthesis of a 358 bp DNA fragment from the *C. jejuni* genome (Manel *et al*., 2018). In the present study, 102 isolates were analyzed by this PCR, which was carried out blindly. The results are shown in Figure 1. Positive controls were performed by testing DNA from *C. jejuni* reference strains. The negative control consisted of all the necessary PCR reagents but without the target DNA which was replaced by 10 µl sterile water.

Among the 102 *Campylobacter* positive isolates analyzed, 41 (40, 2%) were *C. jejuni* and the rest were *C. coli* and others. Moreover, in this study, the prevalence of *Campylobacter spp* infection was found to be the highest in the summer season (40%) and the lowest during the cold season (9%) (Figure 2).

Statistical analysis

The statistics concerning the prevalence of Campylobacters between the different seasons, using Chi-square tests, shows that the Chi-square value is 20.365 higher than the critical value (Chi-square critical= 7.8417) with a significant threshold of 0.05 and degree of freedom of 3. We therefore conclude that the difference between seasonal prevalence was statistically significant ($P<0.05$).

Antimicrobial susceptibility testing of isolated strains

The results of antimicrobial susceptibility of *C. jejuni* isolates from chicken droppings against the seven tested antimicrobial agents are shown in Table 1.

All isolates 100% were resistant to tetracycline, and a very high resistance was observed against erythromycin 97%, ampicillin 85%, and fluoroquinolone (ciprofloxacin) 77%. To a lesser extent, resistance rate to gentamicin 12.0%. Similar resistance rates were also observed for amoxicillin/clavulanic acid (61.4%).

Discussion and Conclusions

Poultry is the main reservoir of *Campylobacter spp* and poultry consumption represents a major cause of human campylobacteriosis. The presence of such pathogens in poultry is of great concern for

Table 1. Percentages of antibiotic resistance of *Campylobacter jejuni* (41 isolates) and *Campylobacter spp.* (102 isolates) isolated from poultry sources in Morocco.

Antibiotics	*Campylobacter jejuni* (n) %	*Campylobacter spp.* (n) %
Tetracycline (30 µg)	(41) 100	(93) 91
Ciprofloxacin (5 µg)	(32) 77	(71) 70
Ampicillin (30 µg)	(35) 85	(84) 81.9
Erythromycin (30 µg)	(40) 97	(91) 89
Amoxicillin + Clavulanic acid (30µg)	(25) 61.4	(57) 55.5
Nalidixic acid (30µg)	(19) 46.2	(41) 40
Gentamicin (10 µg)	(5) 12	(11) 10.3

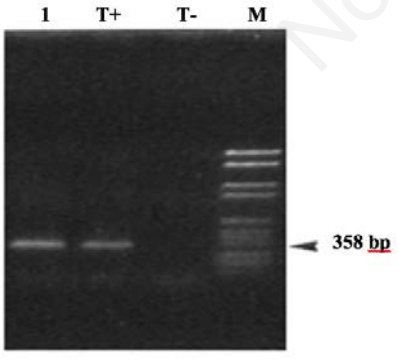

Figure 1. Detection of *Campylobacter jejuni* DNA by PCR and gel electrophoresis. The arrow indicates the *Campylobacter jejuni*-specific 358bp DNA fragment. M: molecular weight amrker VI (Boehringer, Germany); T +: positive control (*C. jejuni* ATCC33560); T-: negative control; strains (1): *C. jejuni*.

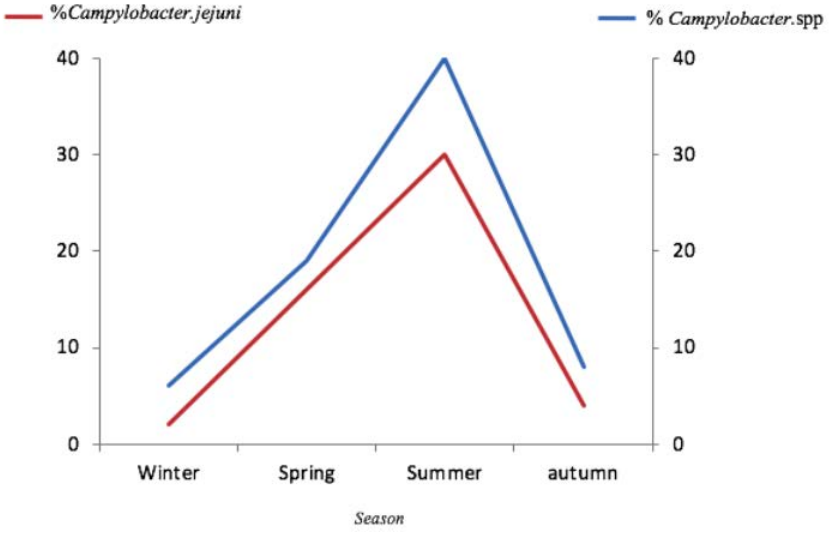

Figure 2. Prevalence of *Campylobacter spp* and *Campylobacter jejuni* isolates in poultry according to the different seasons.

human health, and their control in poultry has a great impact on public health. In Morocco, as in many developing countries, there is limited information regarding Campylobacter status in the conventional poultry sector. Our findings in Casablanca demonstrated that the prevalence of *Campylobacter spp* was 73% in chicken dropping samples, including 40.1% *C. jejuni* according to biochemical and molecular identification. This prevalence can be considered as moderate compared to those reported in other studies in different countries. Indeed, studies carried out in Brazil, Costa Rica, and Sri Lanka showed that 100%, 80%, and 63.8% of flocks respectively, were Campylobacter positive (Giacomelli *et al.,* 2014; Kalupahana *et al*., 2018). The European baseline study on Campylobacter in broilers indicated that the mean EU prevalence was 71.2%, ranging from 2% in Estonia to a maximum of 100% in Luxembourg (EFSA, 2010). The prevalence assessed in the present study is higher than the prevalence shown in the European Nordic countries, such as Sweden (13%), Finland (3.9%), and Denmark (10.3%), but lower than the prevalence reported in Spain (88%), Portugal (82%), and France (76.1%). Regarding *Campylobacter* species distribution, *C. jejuni* was the most common species found (40.1%) followed by *C. coli* and other species. These results are similar to other investigations reporting *C. jejuni* and *C. coli* as the most frequently isolated species in poultry (Giombelli *et al*., 2014; Sahin *et al*., 2015) and their distribution is comparable to our results in Abidjan, 63.75% in 2010 (Gouali *et al*., 2010), and in the North of Tunisia a predominance of *C. jejuni* (68.9%), followed by *C. coli* (31.1%) in 2018 (Manel *et al*., 2018).

The prevalence of Campylobacter in poultry in the different areas of Casablanca in Morocco ranged from 70% to 73%. No significant difference was found related to region distribution, since the samples belong to the same geographic region and have similar climatic conditions. Regarding the seasonal distribution, the highest positive rate was found in poultry sampled in the summer (40%), while a lower prevalence was found in autumn (8%) and spring (19%) (Table 2). Thus, we can conclude that the high prevalence of *Campylobacter* is related to the season rather than to other factors. In fact, in the spring and summer, the weather in Morocco is warm and humid and the highest heat index (HI) values are noted in the summer. These weather conditions combined with the type of poultry breeding (in the house) seem to be favorable for the survival of *Campylobacter* in the environment and enhance their spread in flocks (Kalupahana *et al*., 2018). This positive association is in agreement with data reported by the literature and is supported by the seasonality feature of this disease in humans, with a peak incidence always observed during warm and humid seasons (Nylen *et al*.,2002).

Table 2. Prevalence of *Campylobacter spp.* and *Campylobacter jejuni* isolates in poultry according to the different seasons.

Season	% *Campylobacter spp.*
Winter	6
Spring	19
Summer	40
Autumn	8
Total	73

This seasonality is well illustrated in the study conducted in Switzerland in 2008 (Nylen *et al*., 2002) where a prevalence of 59.2% was recorded in the summer season and 46.8% in the cold season. Another study in the North of Tunisia in 2018 (Manel *et al*., 2018) shown the difference in the prevalence of Campylobacter according to the season, we find that the highest prevalence of Campylobacter spp infection, was found in the autumn season (52%) and the lowest during the winter season (3.5%). In spring and summer, they obtained the same rate of infection (11%). The difference between seasonal prevalence was statistically significant (P<0.001).

The high prevalence of Campylobacter in poultry may be considered a public health problem since consumers might be exposed to this biological risk. Moreover, Campylobacter strains have developed resistance to several antimicrobial agents over the years, including fluoro quinolones and macrolides, which are the drugs of choice in treatments (di Giannatale *et al*. ,2014) In this study, our findings revealed the high prevalence (>77%). of fluoroquinolone and macrolide resistant Campylobacters in poultry in Morocco. The prevalence of fluoroquinolone-resistant Campylobacters varies greatly between different countries. In Algeria, over 90% are resistant to quinolones, in particular to ciprofloxacin (95%). According to a WHO report (WHO, 2001), no fluoroquinolone-resistant isolate was detected in Norway (Hariharan *et al.,* 2009) and a 9.4% ciprofloxacin resistance rate was reported in Grenada (Jain *et al.,* 2005). In contrast to the low resistance reported in the above two studies, the rate of ciprofloxacin-resistant Campylobacters was high in India (77.1%) (Sonnevend *et al.,* 2006), the United Arab Emirates (85.4%) (Manel *et al*., 2018), and South Africa (91%) (Bsteer *et al.,* 2008). The high fluoroquinolone and macrolide resistance rates of Campylobacter in our study may be attributed to the widespread use of fluoroquinolones and macrolides in poultry production in Morocco. These classes of antibiotics are used for both the prevention and control of poultry diseases. However, the frequency of resistance to ampicillin (85%), and amoxicillin + clavulanic acid (61.4%), tetracycline (100%), erythromycin (97%), and gentamicin (12%) was comparable or lower than in the reports from most of the European countries (Kim *et al*., 2010; Stonnet *et al.,* 1993), in Italy 27.9% of the isolates were resistant to gentamicin and 20.9% were susceptible to both ciprofloxacin and erythromycin. In Tunisia, the following frequencies of resistance were found: erythromycin (100%), tetracycline (100%), and ciprofloxacin (99.2%) (Giacomelli *et al.,* 2014; Oporto *et al*., 2009; Skarp *et al*., 2016).To the contrary, in countries not permitting the use of fluoroquinolones in poultry production, such as Australia and the European Nordic countries, few resistant Campylobacter isolates were found in chickens and humans: in Australia 15.4% were resistant to ampicillin, 5.1% to tetracycline and 13.7% to ciprofloxacin (Mattheus *et al.,* 2012).

The relatively high percentage of resistance to most antimicrobial agents tested in our study due to the wide use of these agents as growth promoters or in animal treatment. In fact, in Morocco, as in most developing countries (Bsteer *et al.,* 2008), the use of antibiotics for humans and animals is relatively unrestricted. Because Moroccan producers and veterinarians involved in the production of broilers are regular users of antibiotics and it is well known that the use antimicrobials has become a common measure to ensure the economic profitability of broiler producers, In addition, Antibacterial growth activators are still allowed in Morocco and are regularly used by veterinary prescription by animal feed manufacturers. However, studies previous have shown treatment failures associated with reports of increased antibiotic resistance (Rahmatallah *et al*., 2018). In addition, although the Moroccan authorities apply a surveillance program for certain antimicrobials used in poultry, such as chloramphenicol and gentamicin, since 2001 (ONSSA, 2015). they do not effectively control the use and consumption of antimicrobials in food animals compared to Danish Programme for surveillance of antimicrobial consumption and resistance in bacteria from animals (DANMAP) is a

German program for monitoring the consumption of antimicrobials and the extent of resistances against antimicrobials in human and veterinary Medicine (GERMAP) in Europe or in the National Animal Health Monitoring System (NAHMS) in États-Unis. These programs are responsible for collecting, analyzing and of the publication of data on the sale and consumption of antibiotics among farm animals (Merle *et al*., 2012; FDA, 2014). Today, in the European Union, antibiotics are only used in animals as veterinary medicinal products, subject to veterinary prescription. No more antibiotic molecule is not used in animal production as growth promoters. (Sanders *et al.*, 2011). However, in the États-Unis a large number of antibiotics remains licensed at low dose as growth factors (Thai *et al.*, 2012).

In conclusion, we report the presence of a high contamination of thermophilic Campylobacters in poultry in Morocco and a high resistance of these bacteria to antimicrobials of the fluoroquinolone and macrolide family. These results show the need to strengthen the implementation of specific control procedures to decrease the contamination of poultry meat by Campylobacter and the necessity to reduce the use of antibiotics in the poultry sector. This study also shows the need to establish an efficient system for the control of Campylobacter infection in chickens.

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