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Short communication

Presence of *Clostridium difficile* in poultry and poultry meat in Egypt

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ABSTRACT

C. difficile has been recognized as a potential zoonotic agent encouraging investigations of *C. difficile* prevalence and ribotypes in animals. Here we report the prevalence and diversity of Egyptian *C. difficile* in I) samples from healthy poultry (n = 50), II) samples from diseased poultry (n = 54), and III) poultry meat (n = 150). Thirteen isolates were obtained from seven healthy and five diseased animals, but no *C. difficile* was cultured from poultry meat. The isolated *C. difficile* strains belonged to 3 different PCR-ribotypes (039/2, 205 and 001/FLI01). The detection of strains related to RT 001 known for its ability to cause disease in humans makes poultry a potential reservoir for pathogenic *C. difficile*.

Clostridium difficile, a Gram-positive, anaerobic, spore-forming bacterium, is an important cause of antimicrobial-associated nosocomial diarrhea in humans [1]. It was recently reclassified as *Clostridioides* (*C.*) *difficile* by Lawson et al. [2]. Infection with *C. difficile* (CDI) is affecting mostly elderly individuals receiving antibiotics under hospital settings [1]. However, the emergence of novel, so-called hypervirulent strains, such as e.g. ribotype 027 and 078 in North America and Europe, resulted in an increase in the incidence, severity and number of relapses of the disease in humans with a change in its epidemiology [3]. CDI now affects also non-hospitalized individuals and patients, who were earlier considered to have a low risk. Further, remarkable rates of probable community-acquired CDI were reported for the USA (30–120 cases per 100,000 persons per year) and the Netherlands (390–730 per 100,000 person years) [3]. Besides humans some companion and farm animal species including birds are also susceptible for CDI and can develop lesions comparable to that seen in humans [4–8]. Animals may act also as carriers for *C. difficile* without showing clinical symptoms [9]. It is of interest that both, humans and animals, share a subset of similar *C. difficile* PCR ribotypes (RT), a finding that suggests possible zoonotic transmission of the organism [9].

In poultry feces, a high proportion of toxigenic *C. difficile* was described in two studies from Zimbabwe (17.4%, 29%) [10,11]. However, the highest prevalence recorded was found in a layer farm in Slovenia (62.3%) with a high genotypic diversity of the isolates, most of them nontoxigenic [12]. High genetic diversity but low prevalence in poultry was observed in India (prevalence = 14%, RTs = 13) [13], Austria (prevalence = 5%, RTs = 3) [14] and the Netherlands (prevalence = 5.8%, RTs = 5) [15]. Retail poultry meat may act as a source of *C. difficile* and therefore several studies were carried out to estimate the occurrence in these products. A higher frequency was observed in studies from USA and Canada with prevalences of 11%, 12.5%, 12.8% and 44% [16–19] when compared to prevalences reported from European countries reviewed in Ref. [20], i.e. 0% from Sweden [21], and Austria [14] and 2.7% from

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Table 1
Summary of reports on *Clostridium difficile* in poultry and retail poultry products.

Country	Study period (Month/Year)	Sample material	No of samples	Positive culture (%)	Molecular typing and characteristics		Reference
					Method	Reported types (No. of isolates)	
Slovenia	08/2009 - 2010	wild passerine birds, cloacal samples	465	0	–	–	[40]
Slovenia	10/2007 -04/2008	Chicken feces	61	38 (62.3%)	Toxinotyping Ribotyping	Toxinotype IV (3), 0 (2), nontoxigenic (most) RT023(3); other 11 RTs	[12]
The Netherlands	10/2008 - 03/2009	Chicken meat	257	7 (2.7%)	Toxin genes-PCR PCR-ribotyping	A + B + CD-(4); A-B-CD-(3) RT 001(1); 003(2); 071(1); 087(1) and US[#] (2)	[22]
The Netherlands	2009–2010	Poultry feces	121	7 (5.8%)	Toxin genes-PCR PCR-ribotyping	A + B + CD-(4); A-B-CD-(3) RT003(1); 014(2); 056(1); 010(2); US(1)	[15]
Sweden	04-09/2008	Poultry meat and sausage	4	0	–	–	[21]
Turkey	10/2012 -04/2013	Chicken meat parts and livers	310	25 (8%)	Toxin genes-PCR	A+(8), B+(5), CDT -	[41]
Zimbabwe	–	Chicken feces	100	29 (29%)	Enzyme immunoassay for Toxin A & B	A+ or B + (26)	[11]
		Soils (market places)	100	22 (22%)		A+ or B +(21)	
Zimbabwe	–	Chicken feces	115	20 (17%)	Enzyme immunoassay for Toxin A & B	A+ or B + (11)	[10]
		Pigeon feces	8	0		–	
		Duck feces	4	0		–	
		Turkey feces	3	0		–	
Canada	11/2008 -06/2009	Chicken meat parts	203	26 (13%)	PCR ribotyping Toxin genes-PCR	RT 078 (26) A + B + CD+ (26)	[30]
USA, Texas	2009	Chicken feces	300	7 (2.3%)	Toxinotyping PFGE	Toxinotype V PFGE-NAP7-variant (91% similarity)	[33]
	07/2010	Poultry meat	32	4 (12.5%)[*]	Toxinotyping PFGE	Toxinotype V PFGE-NAP7(3) or NAP7-variant(4)	
Austria	03 to 07/2008	Broiler gut/feces	59	3 (5%)	Toxin genes-PCR PCR-ribotyping	A + B+ (2), A-B-(1) RT001(1), 446(1), AI-79(1)	([14]
	02 to 04/2008	Chicken meat	6	0	–	–	
USA, Pennsylvania	10/2011 - 09/2012	Ground turkey meat	76	11 (14.5%)	Toxin genes-PCR[**]	A + B + CD+ (3); A + B + CD-(1); A + B-CD+(1); A-B-CD+(4); A-B-CD-(2)	[18]
					PCR-ribotyping	RT027(1); 078 (2); PA01(3); PA05(1); PA07(1); PA14(2); PA18(1)	
		Chicken thighs	77	6 (8%)	Toxin genes-PCR PCR-ribotyping	A + B + CD+ (4); A-B-CD+(1); A-B-CD-(1) PA05(1); PA07(1); PA11(1); PA16(1); PA17(2)	
USA, Arizona	01 - 04/2007	Ground turkey	9	4 (44%)	Toxin genes-PCR[***] PCR-ribotyping Toxinotyping PFGE	A + B + CD+ RT078 Toxinotype V NAP7	[16]
USA (many states)	2009–2011	Ground turkey	614	0	–	–	[42]
		Chicken breast	259	0	–	–	
USA, Ohio	2008	Poultry feces	340	1	Toxin genes-PCR & toxin detection by ELISA	Non-toxigenic	[43]
India	03/2012 -07/2014	Poultry feces	165	23 (14%)	Toxin genes-PCR PCR-ribotyping Toxinotyping PFGE	A + B+ (6), A-B-(17) RT014; 087; SLO 134; SLO 160; ACD 012; ACD 014; 084(CE); SLO 002; SLO 131; ACD 013; ACD 015; ACD 016 (each 1 isolate); 032(CE) (10) Toxinotype 0 –	[44]
Costa Rica	11/2009 -04/2010	Poultry meat	67	1 (1.4%)	Toxin genes-PCR PCR-ribotyping	A + B + C + CD- RT029	[34]

#US = unspecified.
*Three different methods were compared for *C. difficile* isolation; the best recovery rate was 12.5% representing 4 out of 32 samples positive.
**Deletion within *tcdC* was assessed, 39bp Δ*tcdC* observed in 2 and 8 chicken and turkey isolates, 18 bp Δ*tcdC* in 3 and 1 chicken and turkey isolates and no deletion in one and two chicken and turkey isolates, respectively.
***All isolates have a 39 bp deletion within *tcdC*.

the Netherlands [22]. The frequent isolation of ribotypes which are also found in humans constitutes a substantial overlap and makes poultry meat a potential source for *C. difficile* infection in humans.

Despite recent reports, CDI remains neglected in countries of the Middle East and only few studies describe prevalent RTs [23–26]. In particular, no data are available on the prevalence of *C. difficile* in any Egyptian animal species, nor on their molecular characteristics. Therefore, the current preliminary study was conducted to investigate the presence and PCR-ribotypes of *C. difficile* in healthy and diseased poultry, meat and edible offal.

In total, we tested 54 enteritis-affected birds raised in 27 different farms, 50 healthy birds from 8 different slaughterhouses and 150 meat/offal samples collected from 7 slaughterhouses, 2 local retail outlets and 5 private households (Table 2). The slaughterhouses were small slaughterhouses with a capacity of less than 100 birds per day. Samples from chickens with enteritis (necrotic enteric lesions or abnormal intestinal fluid contents) were collected at private veterinary clinics in the governorates of Sharkia and Dakahliyah in 2014 and 2015. According to the available information, flock sizes differed from 3000 to 25,000, the age of the animals ranged between 10 days and 6 weeks in case of broilers and was up to 64 weeks in case of layer hens. All chickens were raised conventionally without restrictions on the use of in-feed antibiotics. Samples submitted comprised 54 birds from 27 flocks (8 layer, 18 broiler and 1 breeder). Intestinal parts of each bird, and in some cases liver tissue, were separated (2–4 samples per bird) and cultured individually (n = 123 samples). Eight different local slaughterhouses were visited and the caeca of 50 asymptomatic healthy birds (broiler = 40, ducks = 10) were collected. 150 food samples were collected between October and December 2015 from 14 different sites in Dakahliyah. Samples included retail chicken meat parts (n = 76) and chicken edible internal organs (n = 52) which were purchased from local slaughterhouses (n = 7) and retail outlets (n = 2). Voluntary participants (n = 5) provided duck meat parts (n = 8) and ducks' edible internal organs (n = 14) from house reared birds. All samples were stored frozen at −20 °C until processing.

Poultry samples were processed for the isolation of *C. difficile* as described previously [27]. For the food samples, 1 g was thoroughly blended into 9 ml phosphate buffer saline in a bag mixer for 2 min. 100 μl from this mixture were directly plated on CDMN agar (2–3 days) and 1 ml of this mixture was inoculated in 9 ml of TCDMN broth for enrichment (7–10 days). The culturing method after enrichment, identification and strain isolation was done as described elsewhere [27].

Bacterial DNA was prepared using DNeasy Blood and Tissue kit (Qiagen-Germany). Isolates were confirmed as *C. difficile* and screened for toxins-encoding genes, by PCR as described [12,28]. Capillary gel electrophoresis-based PCR ribotyping was done according to the protocol of Indra et al., 2015 [29] applying conditions previously described [27]. PCR ribotypes were assigned using the Webribo database (https://webribo.ages.at/). Cases of an incomplete match were designated by adding the suffix (/FLI01) to the best matching Webribo PCR-ribotype.

Thirteen isolates were obtained from chickens after enrichment and confirmed as *C. difficile* by PCR [28]. Six *C. difficile* strains were detected in five enteritis-affected chickens (5 positive of 54 tested; 9.3%), from four different flocks; five of these isolates were recovered from cecum and one from duodenum (Table 2). Additional, seven *C. difficile* were obtained from the cecum of healthy birds (7 positive of 50 tested; 14%), six of these isolates originated from the same slaughterhouse (Table 3).

The prevalence of *C. difficile* in birds was 11.53% (12 out of 104), and positive samples were detected in broiler chicken only. This finding could be caused by small sample size of layer hens (n = 14) and ducks (n = 10) compared to broilers (n = 78) or by the age of the sampled animals as low prevalence is seen in older birds [12]. Generally, the sampled animals were not young and layer hens and ducks in this study were older than broilers. The unrestricted use of antimicrobials in animal feedstuff in Egypt may also contribute to low *C. difficile* positivity rates. However, the prevalence found in this study is comparable to those of previous investigations done on poultry feces (Table 1).

C. difficile was not cultured from poultry meat samples. This could either reflect the actual prevalence of *C. difficile* in the samples or a limitation in our detection protocol. However, the CDMN based cultivation used in the present study has proven successful in many previous reports [30–32]. This study and others (Table 1) report zero or a very low prevalence of *C. difficile* in poultry meat indicating that poultry food products may not be an important source for *C. difficile* transmission to humans. On the other hand, North American studies frequently reported the isolation of NAP7/078 *C. difficile* strains, a hypervirulent type that can cause human CDI [16,18,30,33]. Other human-associated toxigenic strains were also recorded in poultry meat such as RT 027 in US [18], RT 001 in the Netherlands [22] and RT 029 in Costa Rica [34].

Non-toxigenic *C. difficile* (A–B–CDT–; n = 10) and toxigenic *C. difficile* (A + B + CDT–; n = 3) isolates were confirmed by PCR as PCR-ribotypes 039/2, 205 and 001/FLI01 (Table 3). The non-toxigenic RT 039/2 was predominant i.e. six strains from healthy

Table 2
Isolation of *Clostridium difficile* from different poultry samples.

Sample category	Source of sample (No.)	Bird or food type (No.)	Samples investigated (No.)	No. of positive samples (source)
Birds with enteritis	Layer flocks (8)	Layer (14)	Dou/Jej/Cecum/Liver (41)	0
	Broiler flocks (18)	Broiler (38)	Dou/Jej/Cecum/Liver (79)	5 (cecum = 4 and duodenum = 1)
	Breeder flocks (1)	Breeder (2)	Dou/Jej/Cecum (3)	0
Total	27	54	123	6
Asymptomatic birds	Slaughterhouses (8)	Broiler (40)	Caecum (40)	7 (caecum)
		Duck (10)	Caecum (10)	0
Total	8	50	50	7
Poultry meat parts	Slaughterhouses (7)	Retail meat parts* (84)	Retail meat parts (84)	0
	Households (5)	Liver (25)	Liver (25)	0
	Local retail outlets (2)	Gizzard (26)	Gizzard (26)	0
		Heart (15)	Heart (15)	0
Total	14	150	150	0

*Retail meat parts include chicken thighs, breast, leg muscle or wings, Dou: Duodenum, Jej: Jejunum.

Table 3
PCR-ribotyping of *Clostridium difficile* from healthy birds and birds with enteritis.

	Farm/slaughterhouse	Bird No.	Isolate	Place[#]	Toxin genes			PCR-ribotype
					A	B	CDT	
Healthy birds	A	1	16S0076	Dk	−	−	−	039/2
		2	16S0082	Dk	−	−	−	039/2
		3	16S0090	Dk	−	−	−	039/2
		4	16S0091	Dk	−	−	−	039/2
		5	16S0093	Dk	−	−	−	039/2
		6	16S0095	Dk	−	−	−	039/2
	B	7	16S0109	Dk	−	−	−	205
Birds with enteritis	C	8	15S0067	Dk	−	−	−	205
		8	15S0068	Dk	−	−	−	039/2
	D	9	16S0049	Sh	+	+	−	001/FLI01
		10[*]	16S0051	Sh	+	+	−	001/FLI01
	E	11	16S0060	Dk	−	−	−	039/2
	F	12	16S0063	Sh	+	+	−	001/FLI01
Total	6	12	13					

[*]Isolate from Doudenum.
#Dk: Dakahliyah, Sh: Sharkia.

animals in one slaughterhouse and two from birds with enteritis symptoms. To our knowledge, this ribotype was not detected before in poultry (Table 1), but was often observed in pet [15,27] and wild animals [35]. Interestingly, non-toxigenic RT 039/2, along with RT 097 and RT 078, was prevalent among hospitalized diarrheic patients in Kuwait [36]. Additionally, two strains belonging to non-toxigenic (A−B−CDT−) RT 205 were recovered from a healthy broiler and a broiler with enteritis. RT 205 and RT 039/2 together, were detected in the cecal sample of a broiler. Three potentially toxigenic isolates (A + B + CDT−) closely matching RT 001 (RT 001/FLI01) were found in chicken with enteritis. RT 001 was among the strains most frequently associated with human CDI in European countries in 2005 [37] and 2008 [38]. Prior studies reported the presence of RT 001 in gut samples of healthy poultry in Austria [14] and also in poultry meat samples in the Netherlands [22], objecting a connection of this RT to poultry enteritis cases. However, the detection of RT 001 in the present and previous studies point to the fact that poultry can be a reservoir of human pathogenic *C. difficile* strains. The limited diversity among the cultured *C. difficile* observed in this study is remarkable and in contrast to previous reports from Europe [12,15]. Unlike in Europe, Egyptian farms can use antimicrobials in feed, which may contribute to a limited heterogeneity of isolates by altering the intestinal microbiota [39].

In summary, this is the first report to describe the prevalence of different RTs of *C. difficile* in poultry in Egypt. Non-toxigenic RT 039/2 was the most abundant RT detected; but detection of RT 001/FLI01 strains in broilers corroborates poultry as a potential reservoir for human pathogenic strains. On the other hand, an involvement of poultry retail meat and edible internal organs in the epidemiology of *C. difficile* in Egypt could not be confirmed by this study.

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