# Antibiotic resistance of *Clostridium perfringens* isolates from broiler chickens in Egypt

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**Summary**

The use of antibiotic feed additives in broiler chickens results in a high prevalence of resistance among their enteric bacteria, with a consequent emergence of antibiotic resistance in zoonotic enteropathogens. Despite growing concerns about the emergence of antibiotic-resistant strains, which show varying prevalences in different geographic regions, little work has been done to investigate this issue in the Middle East. This study provides insight into one of the world's most common and financially crippling poultry diseases, necrotic enteritis caused by *Clostridium perfringens*. The study was designed to determine the prevalence of antibiotic resistance in *C. perfringens* isolates from clinical cases of necrotic enteritis in broiler chickens in Egypt. A total of 125 isolates were obtained from broiler flocks in 35 chicken coops on 17 farms and were tested using the disc diffusion method. All 125 isolates were resistant to gentamicin, streptomycin, oxolinic acid, lincomycin, erythromycin and spiramycin. The prevalence of resistance to other antibiotics was also high: rifampicin (34%), chloramphenicol (46%), spectinomycin (50%), tylosin-fosfomycin (52%), ciprofloxacin (58%), norfloxacin (67%), oxytetracycline (71%), flumequine (78%), enrofloxacin (82%), neomycin (93%), colistin (94%), pefloxacin (94%), doxycycline

(98%) and trimethoprim-sulfamethoxazole (98%). It is recommended that *C. perfringens* infections in Egypt should be treated with antibiotics for which resistant isolates are rare at present; namely, amoxicillin, ampicillin, cephradine, fosfomycin and florfenicol.

**Keywords**

Antibiotic resistance – Broiler chickens – *Clostridium perfringens* – Egypt – Middle East – Necrotic enteritis.

## Introduction

Necrotic enteritis is caused by the bacterium *Clostridium perfringens*, a soil-borne organism found on almost every poultry farm in dust, faeces, feed, poultry litter and intestinal contents, as well as in the soil (1). *Clostridium perfringens* is the aetiological agent of a wide range of diseases in humans and animals. Necrotic enteritis, one of the most economically important and financially crippling enteric poultry diseases in broiler chickens, causes the more commonly recognised fulminant infection which can result in outbreaks with mortality rates of up to 50% (2). In the global poultry industry, necrotic enteritis is considered an emerging billion-dollar disease (3, 4). The disease emerged in broiler chickens in many European Union (EU) countries as a consequence of the ban on growth-promoting antibiotics in animal feed (3, 5, 6) and usually affects chickens between two weeks and six months of age, with the majority of reports from broilers aged two to five weeks (7).

This ban, together with an increase in legislative restrictions and the voluntary removal of antibiotic growth promoters worldwide, has been accompanied by increasingly apparent adverse consequences to poultry production and animal health (6). These antibiotics had an important prophylactic effect and their withdrawal is now associated with a deterioration in animal health, seen as increased diarrhoea, weight loss and mortality (5) and a resurgence in the incidence of clostridial necrotic enteritis in broilers (8). At present, recommendations for the control and treatment of mild and subclinical necrotic enteritis are empirical and based on extensions of validated

approaches to control the classic form of the disease with approved antibiotics that have proven efficacy against *C. perfringens* (9, 10).

As the costs of disease to the broiler industry are great (3), the use of antibiotics to prevent large financial losses has become more widespread. Antibiotic use in animals differs considerably between geographic regions (11), and so this study aims to determine the *in-vitro* resistance of *C. perfringens* to some antibiotics which are relevant to poultry production (for treatment, prophylaxis and growth promotion), to obtain up-to-date information for Egypt.

## Methods

### Source of isolates

A total of 125 *C. perfringens* isolates from previous field studies were selected to represent the period between 2009 and 2010 (12). The samples were originally collected and archived as part of other research and surveillance projects conducted by investigators in the Animal Health Diagnostic Centre of the Department of Microbiology in the Faculty of Veterinary Medicine at Cairo University.

### Bacterial identification

Caecal contents were inoculated into phosphate-buffered saline (PBS: 0.01M, pH7.2) and plated on Shahidi–Ferguson *perfringens* (SFP) selective media plates (SFP agar base, Becton Dickinson Microbiology Systems), containing 400 µg D-cycloserine (Sigma–Aldrich, Saint Louis, Missouri, United States [USA]) as a further selective agent. Plates were incubated anaerobically in an atmosphere of $H_2$ and $CO_2$ (GasPak; BBL) for 24 h at 37°C. Colonies with dark centres and/or diameters larger than 1 mm were selected in a systematic manner (12) to eliminate sampling bias and were sub-cultured on SFP plates. Colonies were further sub-cultured on sheep blood agar and checked visually for a typical *C. perfringens* double-haemolysis zone surrounding the colonies. A reverse Christie, Atkins, Munch-Petersen (CAMP) test (13) was used to confirm *C.*

*perfringens*' identity. Bacterial suspensions were then frozen in brain heart infusion (BHI) broth with 20% glycerol at –70°C.

### Identification of *Clostridium perfringens* isolates using polymerase chain reaction assay

The 125 *C. perfringens* isolates, including *C. perfringens* ATCC 13124 as a positive control, were examined using polymerase chain reaction (PCR) assay. *Staphylococcus aureus* ATCC 29737 was used as a negative control. The boiling technique (14) was used to extract DNA from the isolates and multiplex PCR assay was used for toxinotyping (15).

### Antibiotic susceptibility testing

At any one sampling time, a maximum of two isolates per chicken coop were selected for antibiotic-resistance testing. The disc diffusion method was used, according to the recommendations of the British Society for Antimicrobial Chemotherapy (BSAC) (16), and was chosen for its simplicity and reproducibility. The 25 most cost-effective antibiotics routinely used to treat *C. perfringens* infections (Table I) were tested (17). Antibiotic discs (BBL: Becton, Dickinson and Company, New Jersey, USA 07417) were purchased from Fisher Scientific (Pittsburgh, Philadelphia, USA) and Mast Diagnostics (Mast Group, Merseyside, UK). Susceptibility testing and interpretation took place in the Department of Microbiology in the Faculty of Veterinary Medicine at Cairo University.

Isolates ($1 \times 10^5$ colony-forming units) were plated on Columbia blood agar, using a Steers inoculum replicator, and incubated anaerobically overnight at 37°C. Colonies were then suspended in 0.9% NaCl to a 0.5 McFarland standard and diluted 40-fold. Cultures were incubated anaerobically for 24 h, with *C. perfringens* ATCC 13124 as a control. To examine antibiotic resistance, logarithmically growing organisms (0.5 McFarland standard) were streaked on 150 × 15 mm Mueller-Hinton agar plates (Remel, Lenexa, Kansas, USA) and the antibiotic discs were applied at the doses shown in Table I. The plates were incubated at 37°C for 24 h. The inhibition zone was

measured for each antibiotic and resistance breakpoints were determined according to BSAC methods for antimicrobial susceptibility testing (Version 10.2, May 2011). To facilitate analysis of the data, isolates classified as intermediate were considered susceptible. Multidrug resistance was defined as resistance to more than two antibiotics (18, 19).

## Results

All 125 *C. perfringens* isolates were classified as toxinotype A in the multiplex PCR assay.

### Antibiotic resistance of *Clostridium perfringens* isolates

The resistance of 125 *C. perfringens* isolates to 13 types of antibiotic used to treat infections caused by this microorganism is shown in Table I. High prevalences of resistance were observed in: spectinomycin (50%), neomycin (93%), colistin (94%), pefloxacin (94%), trimethoprim-sulfamethoxazole (98%), gentamicin (100%), streptomycin (100%), lincomycin (100%), oxalinic acid (100%), erythromycin (100%) and spiramycin (100%). However, resistance to fosfomycin, florfenicol and cephradine was negligible at 2% to 3% and resistance to amoxicillin and ampicillin did not exceed 7%. In all, 20 resistance profiles were identified (Table II).

### Multiple resistance patterns and distribution

The resistance patterns and distribution of the *C. perfringens* isolates indicated that all 125 isolates demonstrated multiple resistance (i.e. resistance to more than 2 antibiotics) (Table III). All the isolates were resistant to between 8 and 11 types of antibiotic: 59% were resistant to 9 of the 11 types of antibiotic, 30% to 10 types and 11% to 8 types. None of the isolates was resistant to all 25 antibiotics tested.

The predominant multi-resistance profile comprised gentamicin, streptomycin, oxalinic acid, lincomycin, erythromycin and spiramycin, which accounted for all 125 isolates. The isolates also exhibited an aminoglycoside, glycopeptide, lincosamide and/or macrolide resistance profile.

## Discussion

The findings of this study are consistent with the conclusion that *C. perfringens* type A is the most common cause of poultry necrotic enteritis (14). In humans, food poisoning, antibiotic-associated diarrhoea, sporadic diarrhoea and even some cases of sudden infant death syndrome are caused by this subtype (20).

Multiple resistance to most of the antibiotics tested was revealed. However, some extreme variations were noticed and a higher prevalence of resistance was observed to commonly used antibiotics (gentamicin, streptomycin, oxolinic acid, lincomycin, erythromycin, spiramycin, neomycin, trimethoprim-sulfamethoxazole, pefloxacin, colistin and doxycycline), compared with antibiotics used in specific cases (e.g. tylosin).

*In-vitro* studies on the antibiotic resistance of *C. perfringens* are diverse and numerous: surveys have been carried out in Japan (21, 22), the USA (23), Belgium (11, 24), Bulgaria (25), Sweden (26, 27), Brazil (28), Jordan (29), Iran (30) and Canada (31). Resistance of *C. perfringens* animal isolates to tetracycline, lincomycin and erythromycin has been reported in several countries (11, 26, 32). The resistance phenotypes recorded in the present study (Table IV) differ from earlier studies examining *C. perfringens*. A study in Belgium found that *C. perfringens* resistance to penicillins was very rare and beta- (β-) lactamase was not demonstrated; therapy with antibiotics such as penicillin, amoxicillin, ampicillin, erythromycin, dihydrostreptomycin and tetracycline provided an adequate clinical response (33). A recent examination of *C. perfringens* isolates from commercial turkey flocks in Germany found no resistance to β-lactam antibiotics (amoxicillin, oxacillin, penicillin), lincospectin, tylosin, doxycycline, tetracycline, enrofloxacin, trimethoprim-sulfamethoxazole or lincomycin, whereas a low prevalence of resistance was detected against erythromycin and the highest against spectinomycin, neomycin and colistin (34).

A low prevalence (%) of resistance to ampicillin and chloramphenicol has been reported in several studies (23, 35, 36). Resistance to

macrolide, lincosamide and streptogramin B (MLS) antibiotics is also a common trait (37). *Clostridium perfringens* strains isolated from Belgian broilers were sensitive to enrofloxacin, erythromycin, tylosin, florfenicol and bacitracin (38).

In another study in Belgium, strains appeared uniformly susceptible to amoxicillin and tylosin (24). Low and moderate prevalences (%) of resistance have been reported for lincomycin (11, 24), whereas other reports describe a greater number of lincomycin-resistant strains in broiler chicken and turkey isolates (23, 28) and in cattle, dog and human isolates (11, 26, 36, 39, 40, 41). In contrast, 100% of the *C. perfringens* isolates in the present study were resistant to lincomycin. Swine isolates predominantly showed an increased prevalence of resistance to clindamycin (72%) and erythromycin (69%), whereas bovine isolates had an increased resistance to clindamycin (90%) and florfenicol (90%) (31). An increased prevalence of resistance to tetracycline was spread across cattle, swine, turkey and chicken *C. perfringens* isolates (31).

The aim of the study was to obtain an overview of antibiotic resistance in *C. perfringens* on broiler farms in Egypt. Isolates from broiler chickens suffering from necrotic enteritis were found to be sensitive to amoxicillin, ampicillin, ciprofloxacin, rifampicin, cephradine, fosfomycin and florfenicol but all were resistant to colistin, trimethoprim-sulfamethoxazole, oxolinic acid, enrofloxacin, streptomycin, norfloxacin, gentamicin, lincomycin, doxycycline, flumequine, erythromycin, spiramycin and pefloxacin.

In a study in three Scandinavian countries (27), all *C. perfringens* isolates were classified as susceptible to ampicillin, vancomycin, avilamycin and erythromycin, but a high prevalence of resistance to tetracycline was found: in Sweden (76%), Norway (29%) and Denmark (10%). Only small amounts of tetracycline are used in poultry in Sweden, so the higher prevalence observed in the Swedish isolates cannot be attributed to a greater use of tetracycline in Sweden than in Denmark and Norway (42, 43, 44). A high prevalence (%) of

resistance to tetracycline is the most commonly observed phenotype of antibiotic resistance in *C. perfringens* (11, 28, 36, 40, 45).

Isolates of *C. perfringens* have been found to be highly susceptible to penicillin (28). Despite the increased resistance of *C. perfringens* cattle isolates to β-lactam antibiotics (36), in the present study penicillin was found to inhibit the growth of all broiler strains at the lowest concentration tested (0.25 mg/L), in agreement with earlier studies on *C. perfringens* poultry isolates (11, 23, 26, 39).

This study provides a baseline for the prevalence of antibiotic resistance in *C. perfringens* isolated from broiler chickens in Egypt. As is commonly the case in other bacterial species, increased antibiotic resistance was widespread, suggesting the potential for therapeutic challenges in the future unless care is taken to avoid the selection of multi-resistant organisms. It is advisable to periodically monitor the trends in resistance patterns of *C. perfringens* isolates, because of the possibility that this organism is a source of resistance genes transferring to other species of bacteria (45), including animal and human pathogens.

In Egypt, it is recommended that *C. perfringens* infections should be treated with amoxicillin, ampicillin, cephradine, fosfomycin or florfenicol. This is not the case in Jordan, where treatment is recommended with only three antibiotics (amoxicillin, lincomycin and tylosin) (24). A separate study (29), on the other hand, has recommended that treatment be carried out with penicillins or tetracyclines, especially amoxicillin and oxytetracycline.

## Conclusion

A widespread resistance to multiple antibiotics was observed in *C. perfringens* isolates from broiler chickens in this study. Periodic monitoring of trends in resistance patterns is advisable, because of the possibility that *C. perfringens* is a source of resistance genes transferring to other species of bacteria. There is significant potential for therapeutic challenges in the future unless care is taken to avoid the selection of multi-resistant organisms.

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**Table I**

**Prevalence (%) of antibiotic resistance in 125 strains of *Clostridium perfringens*, isolated from cases of necrotic enteritis in broiler chickens in Egypt**

| Antibiotics | Dose (µg) per disc | Distribution of resistance to antibiotics | |
|---|---|---|---|
| | | Number of resistant isolates | Percentage of resistant isolates |
| **Aminoglycosides** | | | |
| Spectinomycin | 10 | 62/125 | 50 |
| Gentamicin | 10 | 125/125 | 100 |
| Neomycin | 30 | 116/125 | 93 |
| Streptomycin | 10 | 125/125 | 100 |
| **Penicillins** | | | |
| Amoxicillin | 20 | 9/125 | 7 |
| Ampicillin | 10 | 9/125 | 7 |
| **Polymyxin** | | | |
| Colistin | 10 | 117/125 | 94 |
| **Glycopeptides** | | | |
| Oxolinic acid | 10 | 125/125 | 100 |
| **Fluoroquinolones** | | | |
| Ciprofloxacin | 5 | 72/125 | 58 |
| Norfloxacin | 10 | 84/125 | 67 |
| Enrofloxacin | 5 | 102/125 | 82 |
| Flumequine | 30 | 98/125 | 78 |
| Pefloxacin | 5 | 118/125 | 94 |
| **Lincosamides** | | | |
| Lincomycin | 30 | 125/125 | 100 |
| **Phenicols** | | | |
| Chloramphenicol | 30 | 57/125 | 46 |
| Florfenicol | 30 | 2/125 | 2 |
| **Tetracyclines** | | | |
| Doxycycline | 30 | 123/125 | 98 |
| Oxytetracycline | 30 | 89/125 | 71 |
| **Macrolides** | | | |
| Erythromycin | 15 | 125/125 | 100 |
| Spiramycin | 100 | 125/125 | 100 |
| **Phosphonic acids and derivatives** | | | |
| Fosfomycin | | 2/125 | 2 |
| Tylosin-fosfomycin | 30 + 50 | 65/125 | 52 |
| **Cephalosporins** | | | |
| Cephradine | 30 | 4/125 | 3 |
| **Rifampin** | | | |
| Rifampicin | 5 | 43/125 | 34 |
| **Sulfonamides** | | | |
| Trimethoprim-sulfamethoxazole | 1.25 + 23.75 | 123/125 | 98 |

**Table II**

**Profiles of antibiotic resistance in *Clostridium perfringens* isolates from broiler chickens in Egypt**

| Antibiotics | Antibiotic resistance profiles | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spectinomycin | S | R | S | R | R | S | R | S | R | S | R | S | R | S | S | R | R | R | R | S | R |
| Gentamicin | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Neomycin | S | R | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Streptomycin | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Amoxicillin | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | R | S |
| Ampicillin | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | R | S |
| Colistin | S | S | R | R | S | R | S | R | S | R | R | R | R | R | R | R | R | R | R | R | R |
| Oxolinic acid | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Ciprofloxacin | S | R | S | S | S | R | S | R | R | R | S | S | S | R | S | S | S | S | S | R | S |
| Norfloxacin | S | S | R | R | R | S | S | S | R | S | R | R | R | S | R | R | S | R | R | S | R |
| Enrofloxacin | S | S | R | S | S | S | R | S | R | S | R | R | R | S | R | R | R | R | R | R | R |
| Flumequine | S | R | R | S | S | S | S | S | R | R | R | R | R | S | R | S | R | R | R | R | R |
| Pefloxacin | S | R | R | R | R | R | R | R | S | R | R | R | R | R | R | R | S | R | R | R | R |
| Lincomycin | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Chloramphenicol | S | S | R | R | R | R | R | R | R | R | S | R | S | R | S | S | S | R | S | R | S |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Florfenicol | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **R** |
| Doxycycline | **S** | **R** | **R** | **S** | **R** | **R** | **R** | **R** | **S** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** |
| Oxytetracycline | **S** | **R** | **S** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **S** | **S** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** |
| Erythromycin | **S** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** |
| Spiramycin | **S** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** |
| Fosfomycin | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **R** |
| Tylosin-fosfomycin | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **S** | **R** | **R** | **R** | **R** | **R** | **S** | **S** | **R** | **S** | **R** |
| Rifampicin | **S** | **R** | **S** | **R** | **R** | **S** | **R** | **R** | **R** | **R** | **S** | **S** | **S** | **R** | **S** | **S** | **S** | **S** | **S** | **R** | **S** |
| Trimethoprim-sulfamethoxazole | **S** | **R** | **R** | **S** | **S** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** |
| Number of isolates | **0** | 1 | 9 | 1 | 1 | 5 | 2 | 12 | 1 | 6 | 12 | 18 | 9 | 6 | 4 | 5 | 6 | 1 | 21 | 3 | 2 |
| Percentage (%) of isolates | **0** | 0.8 | 7.2 | 0.8 | 0.8 | 4.0 | 1.6 | 9.6 | 0.8 | 4.8 | 9.6 | 14.4 | 7.2 | 4.8 | 3.2 | 4.0 | 4.8 | 0.8 | 16.8 | 2.4 | 1.6 |
| Number of antibiotics | **0** | 14 | 14 | 14 | 14 | 14 | 15 | 15 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 17 | 17 | 18 | 19 |

R: resistant

S: susceptible

**Table III**

**Numbers and percentages of *Clostridium perfringens* isolates (*n* = 125) exhibiting resistance to various classes of antibiotic (*n* = 11)**

| Number (%) of resistant isolates | Number of antibiotic classes to which isolates are resistant |
|---|---|
| 8 (6.4%) | 8 |
| 74 (59.2%) | 9 |
| 37 (29.6%) | 10 |
| 6 (4.8%) | 11 |

**Table IV**

**Summary of antibiotic resistance rates (%) of *Clostridium perfringens* isolates in broiler chickens examined in the present study, and of resistance rates (%) reported in other studies, using the disc diffusion technique**

| Antibiotics | Samples* | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| **Spectinomycin** | **50** | ND | ND | ND | ND | ND |
| Amoxicillin | **7** | ND | ND | ND | ND | ND |
| Ampicillin | **7** | **14–49** | ND | **28** | **0** | **0** |
| Colistin | **94** | ND | ND | **40** | ND | ND |
| Oxolinic acid | **100** | ND | ND | ND | ND | ND |
| Gentamicin | **100** | **16–36** | ND | **53** | ND | ND |
| Neomycin | **93** | ND | ND | **88** | ND | ND |
| Streptomycin | **100** | ND | ND | ND | ND | ND |
| Ciprofloxacin | **58** | **14–35** | ND | ND | ND | ND |
| Norfloxacin | **67** | ND | ND | **23** | ND | ND |
| Enrofloxacin | **82** | ND | ND | **33** | ND | **0** |
| Flumequine | **78** | ND | ND | **40** | ND | ND |
| Pefloxacin | **94** | ND | ND | ND | ND | ND |
| Lincomycin | **100** | ND | ND | **80** | ND | **62** |
| Chloramphenicol | **46** | ND | ND | **0** | **0** | ND |
| Florfenicol | **2** | ND | **0–10** | ND | ND | **0** |
| Doxycycline | **98** | **12–27** | ND | ND | ND | ND |
| Oxytetracycline | **71** | **12–30** | ND | ND | ND | ND |
| Erythromycin | **100** | **9–25** | **0–31** | **30** | **1** | **0** |
| Spiramycin | **100** | ND | ND | ND | ND | ND |
| Fosfomycin | **2** | ND | ND | ND | ND | ND |
| Tylosin | ND | **18-42** | ND | ND | ND | **0** |
| Fosfomycin | ND | ND | ND | ND | ND | ND |
| Fosbac plus (tylosin-fosfomycin) | **52** | ND | ND | ND | ND | ND |
| Cephradine | **3** | ND | ND | ND | ND | ND |
| Rifampicin | **34** | ND | ND | ND | ND | ND |
| Trimethoprim-sulfamethoxazole | **98** | **13–30** | ND | **18** | ND | ND |

*Samples:

A: broiler chickens in the present study

B: boiled turkey, boiled pork/ham, smoked turkey and smoked pork/ham, study by Voidarou *et al.* (17)

C: cattle, swine, chickens and turkeys, study by Slavić *et al.* (31)

D: broiler chickens, study by Shojadoost *et al.* (30)

E: human specimens in Bulgaria, 1983–2007, study by Marina *et al.* (25)

F: broiler chickens in Belgium, study by Gholamiandehkordi *et al.* (38)

ND: not determined