Clostridium perfringens occurrence and ribotypes in healthy broilers reared in different European countries

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ABSTRACT The main aim of this study was to investigate the occurrence and ribotypes of *Clostridium perfringens* in broiler flocks reared in 2 European countries that apply European Union Regulation 1831/2003. A total of 1,532 cecum contents were collected between June 2005 and November 2006 from birds belonging to 51 intensively reared flocks produced in the Czech Republic and 41 intensive production, organic, and free-range flocks reared in Italy. *Clostridium perfringens* was detected in 64.7 and 82.9% of the Czech Republic and Italian flocks, respectively, at mean loads ranging between 3.65 and 4.77 $\log_{10}$ cfu per gram of cecum content. More than 1 ribotype was identified among isolates belonging to the same flock in 57.1 and 76.5% of the Czech Republic and Italian flocks, respectively. Moreover, common ribotypes were identified between strains belonging to 2 up to 8 different flocks. In particular, 4 ribotypes were shared between strains isolated in the 2 European countries. The results of this study report on *C. perfringens* occurrence and mean populations in broilers reared on diets devoid of antibiotic growth promoters. Moreover, these findings show for the first time the presence of common ribotyping profiles among isolates collected from birds reared more than 1,000 km apart.

Key words: *Clostridium perfringens*, broiler, occurrence, ribotype

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INTRODUCTION

Clostridium perfringens is a gram-positive, anaerobic, rod-shaped, spore-forming bacterium that is capable of causing gastrointestinal and enterotoxemic diseases in both humans and animals (Hatheway, 1990; Rood and Cole, 1991). *Clostridium perfringens* does not invade healthy cells but produces various toxins and enzymes that are responsible for the associated lesions and symptoms (Petit et al., 1999).

Clostridium perfringens is frequently found in the intestinal tract of healthy poultry, usually at levels lower than 10^4 organisms/g (Craven et al., 2001b). However, its presence is a concern for the poultry industry because it can cause necrotic enteritis (**NE**) following an excessive growth of the organism in the intestinal tract leading to toxin production and ultimately to gut lesions. The predisposing factors for NE in poultry include intestinal damage caused by coccidial pathogens; diets with high levels of undigestible, water-soluble nonstarch polysaccharides (e.g., rye, wheat, barley); diets with a high protein content (e.g., fish meal, meat, or bone meal); and animal fat (Van Immerseel et al., 2004). Physical damage to the intestinal mucosa caused by poultry bedding litters high in fiber content is also a recognized predisposing factor for NE (Truscott and Al-Sheikhly, 1977) as well as litter types having high moisture retention capacity (Hermans and Morgan, 2007).

The disease has been controlled by the prophylactic supplementation of feed or water with a variety of antibacterial drugs, but it is expected to become more widespread in the European Union as a consequence of the mandated withdrawal of antibiotic growth promoters and prophylactic antibiotics in chicken feed (Bedford, 2000).

Some scientists have suggested that healthy chickens can carry several different *C. perfringens* strains within a single flock and even within individual birds, whereas flocks suffering from NE only carry 1 or 2 strains (Engström et al., 2003; Nauerby et al., 2003). The reasons for the selective proliferation of those strains are not known and it is not yet possible to relate any specific subtype of *C. perfringens* to the development of NE. However, recently Keyburn et al. (2008) reported the

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discovery of a previously unidentified pore-forming toxin of *C. perfringens* named NetB and the encoding gene, *net*B, only from strains recovered from chickens with NE.

Several subtyping methods have been used to differentiate *C. perfringens* strains. They include serotyping (Gross et al., 1989); use of bacteriocins, bacteriophages, plasmid analysis (Schalch et al., 1998); SDS-PAGE (Klein et al., 1996); pyrolysis mass spectroscopy (Sisson et al., 1992); ribotyping (Forsblom et al., 1995; Schalch et al., 1997); macrorestriction enzyme analysis by pulsed field gel electrophoresis (Klein et al., 1996; Ridell et al., 1998); amplified fragment length polymorphism analysis (McLauchlin et al., 2000); multilocus enzyme electrophoresis (Urwin and Maiden, 2003); and repetitive-element PCR (Siragusa et al., 2006).

A subtyping method to recommend to poultry companies for monitoring *C. perfringens* strains circulating in production facilities should combine high typing performance and discriminatory power with ease of use. Automated *Eco*RI ribotyping meets these requirements because it is a completely automated technique and produces results within 8 h.

In this study, the occurrence of *C. perfringens* among flocks and cecal contents of broilers reared on farms in the Czech Republic and Italy that adhered to European Regulation 1831/2003, banning antibiotic growth promoters and prophylactic antibiotics in chicken feed, were quantified. Moreover, *Eco*RI automated ribotyping was performed on selected *C. perfringens* isolates obtained from broilers and resulting ribotypes were compared.

MATERIALS AND METHODS

Samples Tested

Between June 2005 and November 2006, a total of 1,338 cecum content samples were collected from multiple slaughterhouses from healthy broiler chickens belonging to 51 intensively reared flocks (i.e., average of 27 ceca per flock) in the Czech Republic. In the same period of time, 194 ceca were collected at the slaughterhouse from healthy broiler chickens belonging to 41 flocks produced in Italy (i.e., average of 5 ceca per flock). Specifically, 104 ceca were collected from 23 intensively reared flocks, 45 from 9 organic flocks, and 45 from 9 free-range flocks. All birds belonging to the tested flocks in the Czech Republic and Italy were fed diets without antibiotic growth promoters.

According to the Council Directive 2007/43/EC, intensively reared flocks were raised within environmentally controlled poultry houses, with daily free access to water and feed. The maximum stocking density did not exceed 42 kg of live weight/m^2.

According to Regulation EEC 1538/91, free-range birds were reared in poultry houses with free access to open air runs. Each house was provided with pop-holes of a combined length equal to or greater than 4 m per 100 m^2 of surface of the house. The maximum stocking density did not exceed 27.5 kg of live weight/m^2. The open-air runs comprised an area mainly covered by vegetation of not less than 1 m^2 per chicken. Moreover, the feed formulation used in the growing phase contained at least 70% cereals. The birds were slaughtered at a minimum age of 56 d or later.

According to Council Regulation (EC) No. 1804/99, birds comprising the organic flocks were reared under organic growing conditions. Poultry houses had at least one-third of their solid floors covered with bedding litter and contained exit-entry pop-holes of a combined length of at least 4 m per 100 m^2 area of the house. The open-air runs were mainly covered with vegetation and permitted birds to have easy access to adequate numbers of drinking and feeding troughs. Birds were fed ad libitum organic diets. The stocking density did not exceed 21 kg of live weight/m^2. The birds were slaughtered at a minimum age of 81 d.

Among the Italian flocks, the effect of different feeding and litter types (Table 1) on the occurrence of *C. perfringens* was analyzed. After collection at the slaughterhouse, all samples were placed in single sterile plastic bags and transferred within 6 h to the laboratory under refrigeration conditions.

Sampling Procedures

The contents of each cecum were diluted 1:10 (wt/vol) in sterile physiological saline solution (0.85% wt/vol NaCl) and homogenized for 1 min with a stomacher. Subsequently, 1:100 and 1:1,000 dilutions were made and triplicate pour plates prepared using Per-

Table 1. Rearing parameters of the Italian flocks tested

	Flock type		
Item	Intensive (n = 23)	Free range (n = 9)	Organic (n = 9)
Feeding type	Vegetable diet[1] (7) Animal diet[2] (16)	Vegetable origin (9)	Vegetable origin (9)
Litter type	Straw (10) Wood shavings (10) Rice hulls (3)	Straw (9)	Straw (6) Wood shavings (3)

[1]Vegetable diet = proteins and fats of vegetable origin.

[2]Animal diet = proteins of animal or vegetable origin and fats of animal origin.

Table 2. *Clostridium perfringens* occurrence among broiler flocks tested

Country	Rearing type	Positive flocks/flocks tested (%)	Positive ceca/ceca tested (%)	Mean *C. perfringens* population per gram of cecum content (log_{10} cfu)
Czech Republic	Intensive	33/51 (64.7)	167/1,338 (12.4)	4.91[c]
Italy	Intensive	18/23 (78.2)	64/104 (61.5)	4.56[b]
Italy	Organic	8/9 (88.9)	27/45 (60)	4.47[b]
Italy	Free range	8/9 (88.9)	23/45 (51.1)	3.36[a]

[a–c]Numbers without common superscripts differ significantly ($P \leq 0.05$).

fringens Agar Base (Oxoid, Basingstoke, UK) supplemented with D-cycloserine (400 mg/L; **TSC**; i.e., TSC agar; Oxoid) and 5% of egg yolk emulsion (Oxoid). After the inoculum was absorbed (about 5 min), plates were overlaid with 10 mL of TSC agar without egg yolk emulsion and incubated at 37°C for 22 ± 2 h in an AnaeroGen Atmosphere Generation System (Oxoid).

Presumptive *C. perfringens* colonies, characterized by a black color and surrounded by an opaque zone, were confirmed microscopically by gram staining and then the colonies were enumerated.

One to 5 colonies per positive sample were purified through 3 subsequent culture steps in blood agar (Oxoid) plates supplemented with 7% of defribrinated sheep blood (Oxoid) and incubated as described previously. All *C. perfringens* purified colonies were biochemically identified using the commercial available kit ANAEROtest23 test kit according to the instructions of the manufacturer (Pliva-Lachema, Brno, Czech Republic) and then stored at −70°C in cooked meat medium broth (Oxoid) for further characterizations.

Positive ceca were considered those colonized by 10 or more colony-forming units of *C. perfringens* per gram of cecal content. *Clostridium perfringens*-positive flocks were considered those in which at least 1 cecum was positive.

Ribotyping

Ribotyping was performed on purified 24-h cultures grown on brain heart infusion agar (Oxoid) using a RiboPrinter (DuPont Qualicon, Wilmington, DE) according to the instructions of the manufacturer (Bruce, 1996) and *Eco*RI was selected as the restriction enzyme (DuPont Qualicon). The ribotype pattern for each isolate was automatically compared with patterns stored in the RiboPrinter database. The identification of each isolate was predicted when the corresponding pattern matched with a similarity ≥0.86 one of the patterns of the DuPont RiboPrinter Identification library. The characterization consisted of combining profiles within a similarity range, as calculated using the proprietary algorithm of the RiboPrinter, greater than 0.93 to form a dynamic ribotype or ribogroup that reflected the genetic relatedness of the isolates. Ribotyping profiles were imported into Bionumerics 4.61 software (Applied Maths, Saint-Martens-Latem, Belgium). Normalized profiles were compared using the Dice correlation coefficient and clustered by the unweighted pair group method with arithmetic mean according to a similarity ≥93% and a 1% band position tolerance.

Discriminatory Index

Simpson's discriminatory index (**D**) as described by Hunter and Gaston (1988) was calculated for ribotyped strains. This index represents the probability that 2 randomly chosen isolates would be distinguished by *Eco*RI ribotyping. As the numerical index approaches the maximum value of 1 (representing 100% discriminatory ability), the greater the probability that *Eco*RI ribotyping would be able to discriminate between 2 unrelated isolates.

Statistical Analysis

The data collected were analyzed with the Statgraphics package (version 5.1; StatSoft Inc., Tulsa, OK). The occurrence of *C. perfringens* within single cecum contents among broiler flocks and between and within countries was compared using the Pearson's χ^2 test. A *P*-value ≤0.05 was considered statistically significant. The mean cecal populations of *C. perfringens* from birds reared using different housing parameters were compared using the ANOVA. A *P*-value ≤0.05 was considered statistically significant.

RESULTS

Occurrence of C. perfringens

Sixty-four percent of the intensively reared broiler flocks produced in the Czech Republic were *C. perfringens*-positive (Table 2). One to 18 positive ceca were detected in each of 33 positive flocks. The positive ceca were 12.4%. *Clostridium perfringens* was enumerated in 93 of 167 positive ceca (56.7%) and the populations ranged between 2.77 and 5.51 log_{10} cfu/g with a mean value of 4.91 log_{10} cfu/g (Table 2). Overall, 36.4% of the ceca had *C. perfringens* populations lower than 4 log_{10} cfu/g.

A total of 78.2% of the Italian intensively reared flocks had at least 1 *C. perfringens*-positive cecal sample, whereas 88.9% of the organic and free-range flocks were positive (Table 2). A total of 61.5% of the cecal samples from intensively reared flocks were *C. perfrin-*

Table 3. *Clostridium perfringens* occurrence and mean populations from broiler ceca (Italian flocks) as influenced by rearing parameters

Item	Intensive	Free range	Organic
	% positive ceca		
Vegetable diet	58.6	51.1	60
Animal diet	62.6	—	—
Litter			
Straw	56	51	57
Wood shavings	60	—	67
Rice hulls	80	—	—
	Mean populations (log cfu/g)		
Vegetable diet	3.34[a]	3.36[a]	4.47[b]
Animal diet	4.69[b]	—	—
Litter			
Straw	4.50	3.36	4.61
Wood shavings	4.71	—	3.95
Rice hulls	3.20	—	—

[a,b]Numbers without common superscripts differ significantly ($P \leq 0.05$).

gens-positive, whereas among organic and free-range flocks, the occurrence was 60 and 51.1%, respectively. *Clostridium perfringens* populations from intensively reared flocks ranged between 2 and 5.72 $\log_{10}$ cfu/g, with a mean value of 4.56 $\log_{10}$ cfu/g; between 2.78 and 5.46 $\log_{10}$ cfu/g, with a mean load of 4.47 $\log_{10}$ cfu/g in organic flocks; and between 2.13 and 4.36 $\log_{10}$ cfu/g, with a mean load of 3.36 $\log_{10}$ cfu/g in free range-flocks (Table 2). A total of 63.1, 56, and 77.7% of ceca collected within intensive, organic, and free-range flocks, respectively, were colonized by less than 4 $\log_{10}$ cfu of *C. perfringens* per gram of cecal content.

The occurrence of *C. perfringens* among cecum contents of Italian birds was not influenced by feeding or litter type (Table 3). In contrast, the *C. perfringens* mean population among ceca from intensively reared birds was influenced by the feeding type. Birds fed a vegetable diet had significantly lower *C. perfringens* mean populations per gram of cecal content in comparison to birds fed diets containing animal by-products (i.e., 3.34 vs. 4.69 $\log_{10}$ cfu/g). This same positive diet effect was also observed for free-range flock birds (i.e., 3.36 $\log_{10}$ cfu/g) but not for the organic flocks (4.47 $\log_{10}$ cfu/g). Housing litter types did not influence the mean cecal *C. perfringens* populations (Table 3).

Ribotypes of C. perfringens Isolates

A total of 93 sample isolates from the Czech Republic (n = 38) and Italy (n = 55) were submitted to automated ribotyping. The Czech Republic strains were collected from 19 positive flocks. In particular, a single isolate was obtained from 5 different flocks, whereas 2 or 3 isolates were obtained from the remaining 14 different flocks. In 57.1% of these 14 flocks, the isolates had more than 1 ribotyping profile. For the Italian isolates, 34 were collected from 15 intensively reared flocks, 11 from 6 organic flocks, and 10 from 5 free-range flocks. A single isolate was obtained within 9 flocks, whereas 2 to 5 isolates were obtained from the remaining 17 flocks. In 76.5% of these flocks, the isolates showed more than 1 ribotyping profile.

Table 4. Origin of the isolates belonging to common ribotypes

Ribotype (no. of isolates)	No. of isolates from Czech Republic	No. of isolates from Italy	No. and type of flock from which the isolates were collected	Cluster label
323-S-8 (2)	—	2	1 intensive	1
322-S-7 (2)	—	2	1 free range	2
321-S-1 (2)	—	2	2 intensives	3
397-S-4 (6)	6	—	4 intensives	4
309-S-5 a (11)	11	—	8 intensives	5
309-S-5 b (3)	2	1	3 intensives	6
404-S-2 (4)	1	3	1 intensive, 2 organics	7
397-S-3-a (2)	2	—	1 intensive	8
322-S-4 (15)	3	12	8 intensives	9
326-S-7 (2)	—	2	1 organic	10
397-S-3 (3)	3	—	2 intensives	11
399-S-7 (2)	2	—	2 intensives	12
397-S-3-b (2)	2	—	1 intensive	13
404-S-5 (3)	—	3	2 organics	14
322-S-5 (2)	1	1	1 intensive, 1 organic	15
322-S-6 a (2)	—	2	1 intensive, 1 free range	16
322-S-6 b (2)	—	2	1 intensive	17

A total of 45 different ribotypes were obtained from the ribotyped strains. Overall, 28 profiles were associated with single isolates, whereas 17 were shared between 2 to 15 isolates (Table 4). Both unique as well as common ribotyping profiles were characterized by 9 to 15 bands, with a molecular weight ranging between 1 to 45 kbp. The overall similarity among different ribotyping profiles ranged between 20 and 90%.

The 38 isolates from the Czech Republic farms were classified in 15 different ribotypes. Overall, 8 were ribotypes shared between 2 and 11 isolates, whereas 7 were unique ribotypes even though 2 of them (i.e., 404-S-2 and 322-S-5) were shared with Italian isolates (Table 4). The 55 Italian isolates were classified in 34 different ribotypes. Overall, 9 were ribotypes shared between 2 and 12 isolates, whereas 25 were unique ribotypes, even though 2 of them (i.e., 309-S-5 b and 322-S-5) were shared with isolates collected in the Czech Republic. The majority of shared ribotypes were associated with isolates from broilers reared using the same rearing practice (Table 4). The 3 exceptions were ribotypes 404-S-2 and 322-S-5, which were shared between isolates from birds reared within intensive and organic flocks, and ribotype 322-S-6 a isolates from birds reared using intensive and free-range practices.

Four different ribotyping profiles, corresponding to ribotypes 309-S-5 b, 404-S-2, 322-S-4, and 322-S-5, were identified from among Italian as well as Czech Republic isolates (Table 4). In particular, ribotypes 309-S-5 b and 322-S-4 were associated with isolates from intensively reared flocks in both countries.

The ribotyping profiles were grouped within 17 different clusters and assigned a number (Figure 1 and Table 4). Clusters 4, 5, 8, 11, 12, and 13 grouped only strains isolated in the Czech Republic, labeled as PFG CP CZ, whereas clusters 1, 2, 3, 10, 14, 16, and 17 were from Italian strains labeled as PFG CP. In particular, clusters 1, 3, and 17 grouped isolates from birds intensively reared; clusters 10 and 14 isolates from birds reared within organic flocks; cluster 2 isolates from free-range flocks; and cluster 16 isolates from intensively reared and free-range flocks. Isolates collected in both the Czech Republic and Italy were grouped within clusters 6, 7, 9, and 15 (Figure 1).

The D achieved using *Eco*RI ribotyping for the 93 strains tested was 0.953. It was found that *Eco*RI ribotyping confirmed the biochemical identification of all ribotyped isolates within the species *C. perfringens.*

DISCUSSION

This paper describes the occurrence of *C. perfringens* in broilers reared in 2 different European countries and fed without antibiotic growth promoters. The data collected did not show any statistically significant differences between *C. perfringens* occurrence among the Czech Republic and Italian intensively reared broiler flocks ($P = 0.249$). However, considering the total number of cecal samples collected within each intensively reared flock tested, the occurrence of *C. perfringens* detected in Italy was significantly higher than that found in the Czech Republic ($P < 0.001$). These findings might be explained by the longer bird rearing period applied in Italy in comparison to the Czech Republic (60 vs. 38 d). However, in comparison to the Czech Republic, the mean *C. perfringens* population per gram of cecum content was significantly lower not only among Italian birds reared within intensively reared flocks but also among broilers reared within the free-range and organic flocks (Table 2). It is interesting to note that 63.6% of the ceca collected from birds in the Czech Republic had populations greater than the threshold of 4 $\log_{10}$ cfu/g of cecum content usually found in healthy poultry (Craven et al., 2001b). Conversely, 63.1, 56, and 77.7% of ceca collected within intensively reared, organic, and free-range Italian flocks, respectively, were colonized by less than 4 $\log_{10}$ cfu of *C. perfringens* per gram of cecum content.

The cecal *C. perfringens* occurrence values obtained in this study were lower than those reported in the literature before adoption of Regulation 1831/2003, corresponding to 75 to 95% of intestinal contents of broiler chickens *C. perfringens*-positive (Craven et al., 2001a,b). Tschirdewahn et al. (1991) reported *C. perfringens* in 80% of 59 broiler chickens tested, with mean populations ranging between 3.65 and 4.77 $\log_{10}$ cfu/g. After the issue of European Regulation 1831/2003, Mitsch et al. (2004) detected *C. perfringens* in 87.5% of ceca collected from intensively reared broiler chickens, with a mean population of 3.73 ± 1.68 $\log_{10}$ cfu/g. In contrast, the occurrence of *C. perfringens* among broiler ceca detected in the present study was higher than those reported by Kalender and Ertas (2005) and Bjerrum et al. (2006), who found that 5 and 30% of intestinal contents from intensively reared broiler chickens were *C. perfringens*-positive. Furthermore, the occurrence detected in this study from among Italian organic broiler flocks was 60%, with a mean population of 4.47 $\log_{10}$ cfu/g, whereas Bjerrum et al. (2006) reported 100% positive ceca from organic broilers, with a mean population of 6.7 $\log_{10}$ cfu/g.

The higher *C. perfringens* mean loads detected among ceca of birds fed with animal diets confirmed the data reported by Kocher (2003) that animal protein ingredients, such as fish meal, increase the risk of occurrence of NE. Moreover, according to the results reported on broiler ileum samples by Knarreborg et al. (2002), including animal fat in the diet could lead to higher populations of *C. perfringens* compared with vegetable oil.

The 93 *C. perfringens* submitted in this study to automated *Eco*RI ribotyping were successfully discriminated with an overall D value of 0.953. This high index was probably achievable because *C. perfringens* contains an average of 9.5 highly conserved operons for rRNA genes (http://ribosome.mmg.msu.edu) distributed throughout the chromosome (Garnier et al., 1991; Forsblom et al., 1995; Schalch et al., 1997). This high degree of discrimination demonstrates that this method

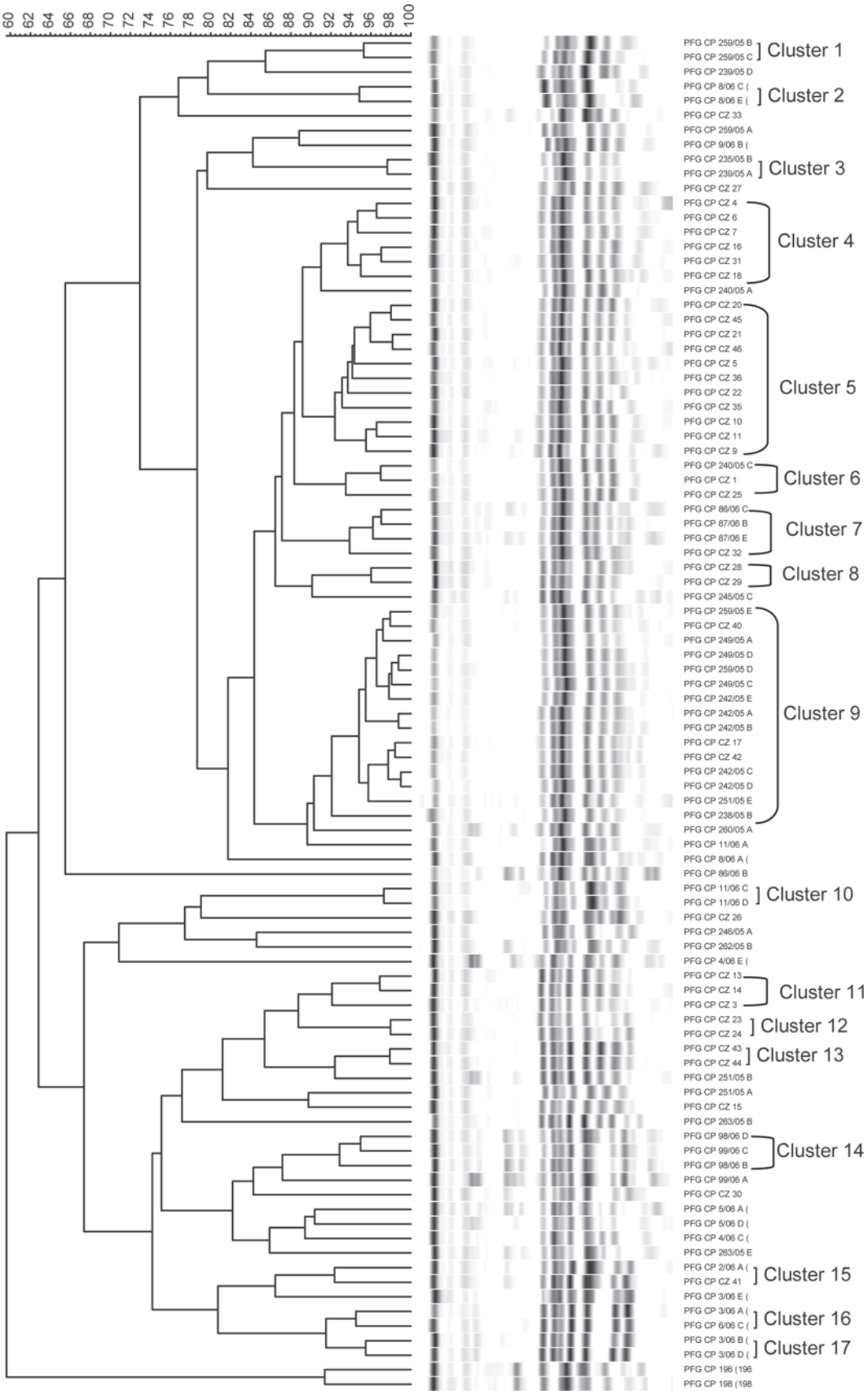

Figure 1. Cluster analysis of ribotyped strains. PFG CP = Italian strain labels; PFG CP CZ = Czech Republic strain labels.

is suitable to differentiate *C. perfringens* strains colonizing healthy birds and may be useful for tracing *C. perfringens* strains capable of causing NE.

Different ribotypes were identified in 57.1 and 76.5% of the Czech Republic and Italian flocks, respectively, in which more than 1 isolate was collected for typing. This result shows that it is necessary to characterize an appropriate number of isolates per each flock to accurately map the whole *C. perfringens* population colonizing that flock. Common ribotypes were identified between strains belonging to a single flock but also among strains belonging to up to 8 different flocks (Table 4). This outcome, as well as the identification for the first time of the same *C. perfringens* ribotypes among strains isolated in the 2 European countries, located about 1,000 km apart, confirms the observation of Craven et al. (2003), who suggested that some *C. perfringens* ribotypes are ubiquitous in poultry production and persist over time. Therefore, permanent collection and typing of *C. perfringens* chicken isolates followed by storage of data obtained within molecular databases might help to find and identify poultry-related *C. perfringens* strains or clones as well as specific strains or clones with the potential to cause avian or human diseases. To actively involve poultry producers in the building of such a database, an automated technique such as that described in this paper might be proposed. In fact, this method eliminates the need for highly skilled laboratory personnel and increases precision and reproducibility from run to run and between laboratories. Moreover, the system facilitates digital data storage and archiving.

In conclusion, this study provides a picture of *C. perfringens* occurrence in broilers reared in 2 different European countries following the withdrawal of antibiotic growth promoters from chicken feed. Moreover, it shows for the first time that specific *C. perfringens* strains are traceable in different European countries. These findings warrant further investigation of the specific ribotypes that might be associated with those *C. perfringens* strains capable of causing NE in poultry, such as the recently described *net*B-positive strains (Keyburn et al., 2008).

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