Received Date : 24-Feb-2016

Revised Date : 28-Oct-2016

Accepted Date : 28-Oct-2016

Article type : Original Article

Presence of *Clostridium difficile* in pig faecal samples and wild animal species associated to pig farms

S. Andrés-Lasheras[1], R. Bolea[1], R.C. Mainar-Jaime[1], **E. Kuijper[2]**, E. Sevilla[1], I. Martín-B**urriel[3]** and M. Chirino-Trejo[4]

1 Departamento de Patología Animal. Facultad de Veterinaria. Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain
2 Department of Medical Microbiology, Centre of Infectious Diseases, Leiden University Medical Centre, Leiden, The Netherlands
3 Laboratorio de Genética Bioquímica (LAGENBIO). Facultad de Veterinaria. Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain
4 Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

Abbreviated running headline: *Clostridium difficile* from wildlife

Correspondence: R. Bolea, Departamento de Patología Animal. Facultad de Veterinaria. Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), 50013 Zaragoza, Spain. E-mail: rbolea@unizar.es

Abstract

Aims: To determine the presence of *Clostridium difficile* on fattening pig farms in north-eastern Spain.

Methods and Results: Twenty-seven farms were sampled. Pools of pig faecal samples (*n* = 210), samples of intestinal content from common farm pests species (*n* = 95) and environment-related samples (*n* = 93) were collected. Isolates were tested for toxin genes of *C. difficile*, and typed by PCR-ribotyping and toxinotyping. The minimal inhibitory

concentrations of six antimicrobial agents were determined using Etest. Thirty-four isolates were obtained from 12 farms, and 30 (88.2%) had toxin genes. Seven ribotypes were identified. Ribotype 078 and its variant 126 were predominant (52.9%). The same ribotypes were isolated from different animal species on the same farm. None of the isolates were resistant to metronidazole or vancomycin.

Conclusions: *Clostridium difficile* was common within the pig farm environment. Most of the positive samples came from pests species or were pest-related environmental samples.

Significance and Impact of the Study: Pest species were colonized with toxigenic and antimicrobial-resistant *C. difficile* strains of the same ribotypes that are found in humans and pigs. Rodents and pigeons may transmit toxigenic and antimicrobial-resistant *C. difficile* strains that are of the same ribotypes as occurs in humans.

Keywords: *Clostridium difficile*, environment, PCR-Ribotyping, pig, rodents, toxins, wildlife

Introduction

Clostridium difficile is a gram positive anaerobic bacterium. It forms highly resistant spores which can persist in the environment for long periods, facilitating its transmission (Roberts *et al.* 2008). It is considered an opportunistic pathogen for humans and some animal species. Two major toxins, A and B, are responsible for disease. A third toxin, the binary toxin (CDT), is of uncertain significance in the pathogenesis of infection (Stubbs *et al.* 2000).

Human *C. difficile* infection (CDI) has been usually associated with hospitalised elderly individuals (i.e., of nosocomial origin). However, in recent years its epidemiology has changed with modifications in its clinical presentation, the description of new potential risk factors and a significant increase in CDI prevalence in the European and North American populations (Freeman *et al.* 2010). Severe community-acquired *C. difficile* infections (CA-

CDI) are becoming more frequent (Rupnik, 2007), and thus *C. difficile* is now considered a potential emerging community-associated pathogen (Hensgens *et al.* 2012). Many of these epidemiological changes have been attributed to the emergence of the hypervirulent 027 and 078 ribotypes (Goorhuis *et al.* 2008).

Clostridium difficile is ubiquitous in the environment (Freeman *et al.* 2010) and several animal species can become colonized with this bacterium, e.g. companion and food animals, and wild animals such as the coati (*Nasua nasua*) and some carnivores (Silva *et al.* 2014). In addition, contaminated meat, raw vegetables and water may also play an important role as sources of human infection since *C. difficile* strains similar to those found in humans have been identified in most of these foods (Rodriguez-Palacios *et al.* 2013). Also, there is indirect evidence of between-species transmission of some *C. difficile* ribotypes (Knight *et al.* 2015), particularly the 078 ribotype strain (Knetsch *et al.* 2014).

Pests can play a role in the transmission to pigs of different pathogens such as *Campylobacter* spp., *Salmonella* spp., and some viruses and parasites (Fischer *et al.,* 2001; Kijlstra *et al.*, 2008; van de Giessen *et al.,* 2009; Blunt *et al.,* 2011; Nathues *et al.,* 2013; Andrés-Barranco *et al.,* 2014), but little is known about their potential role as a reservoir for *C. difficile.* A recent study showed the isolation of *C. difficile* ribotype 078 from house mice, drain flies, lesser houseflies, yellow mealworms, house sparrows and wild bird faeces from a pig farm in the Netherlands (Burt *et al.* 2012), and ribotypes such as 078, 027, 001 and 017 have been isolated from the colon contents of urban rats (*Rattus novergicus* and *Rattus rattus*) in Canada (Himsworth *et al.* 2014).

The potential emergence of *C. difficile* as a community-associated pathogen and its importance in human disease makes necessary a thorough evaluation of different possible sources of exposure (Debast *et al.,* 2009). The main aim of this study was to investigate the presence of *C. difficile* in pooled faeces in fattening pigs and determine whether some pest

species commonly present in the same pig farms (rats, mice and pigeons) are carriers of *C. difficile*. Further, using phenotypic and molecular characterization of the isolated strains, we investigated their similarity to those commonly found in humans, pigs and other animal species.

Materials and methods

Animals and sample collection

Between April 2010 and June 2012 a convenience sampling of 27 fattening pig farms in an area of North east Spain selected on the basis of farmer collaboration. Samples collected from the pig farms included intestinal contents of common farm pests, i.e. rats (*Rattus* spp.), mice (*Mus musculus*) and pigeons (*Columba livia*), trapped on the farms, and environmental samples, i.e. pooled remnants of pig feed found underneath the outside silos, and rodent and bird faeces identified by experts, collected from locations to which pigs never have access and therefore could not contaminate directly (e.g. window ledges, top of pen walls, etc.). Rodents and pigeons were trapped and killed within the farm pest control programs and submitted to our laboratory. The number of samples collected in each farm was variable, depending upon the observation of rodents, pigeons and the accumulation of bird/rodent faeces on the farm. From each trapped animal the whole intestine was extracted and homogenized under sterile conditions for further microbiological processing.

In addition, four randomly selected pens from each fattening unit on each farm were sampled; five individual fresh faecal samples were collected from each pen and pooled. All samples from a given pig farm were collected on the same day. Table 1 shows a summary of the number of samples collected.

Bacterial isolation and molecular characterization

To perform the bacterial isolation, 1 g from each homogenized sample was pre-enriched in 9 ml of BHI (Oxoid, Basingstoke, Hampshire, UK) supplemented with *C. difficile* selective supplement (C.D.M.N. Selective supplement, Oxoid, Basingstoke, Hampshire, UK) during 6 d at 37°C, in an anaerobic atmosphere (2.5 l anaerobic jar. Oxoid, Basingstoke, Hampshire, UK). Then, a 2 ml aliquot of each sample was mixed with ethanol 95% (1 : 1), in order to select potential spore forms. After 1 h of incubation at room temperature, each sample was centrifuged for 15 min at 2500xg. Supernatants were discarded and the pellets were plated on Columbia blood agar (Oxoid, Basingstoke, Hampshire, UK) and cycloserine-cefoxitin agar (CLO agar; bioMérieux, Marcy l'Etoile, France). Plates were incubated at 37°C in anaerobic conditions. After 48 h of incubation, plates were examined for the presence of *C. difficile*. When no *C. difficile* suspect growth was observed, plates were re-incubated for another 72 h. Basic phenotypic bacterial identification was based on colony morphology and its typical odour (horse manure), and cell morphology using the Gram staining (Rousseau *et al.* 2010). Three colonies from primary culture were subcultured and then stored at −80°C for further studies.

Putative isolates were confirmed as *C. difficile* through the detection of the *tpi* housekeeping gene by PCR as previously described (Lemee *et al.* 2004). All *C. difficile* strains were examined for the presence of the genes coding for their two main virulence factors: toxins A and B. Toxin A gene (*tcdA*) was tested by two independent PCRs using two different pairs of primers for the detection of non-repeating and repeating fragments of the gene (NK3/NK2 and NK11/NK9 primers, respectively) (Kato *et al.* 1998). The presence of toxin B (*tcdB* gene) was also tested by PCR following previously reported procedures (Lemee *et al.* 2004). Since *C. difficile* can harbour the CDT toxin, the presence of the *cdtA* and *cdtB* genes was tested by PCR (Stubbs *et al.* 2000). In case of ambiguity of non-toxigenic strains, i.e. absence

of *tcdA* and *tcdB* genes, the Lok3/Lok1 primers were used to for confirmation (Braun *et al.* 1996). A summary of the PCR primers, amplicon sizes, and protocols for each PCR assay are detailed in Table 2.

Clostridium difficile isolates were analysed by PCR-ribotyping based on capillary gel electrophoresis (Fawley *et al.* 2015). Briefly, the intergenic spacer region (ISR) located between *16S* and *23S* rRNA genes was amplified using the primers described by Bidet (1999) and then the PCR products were analysed by capillary gel electrophoresis. Ribotypes were designated using the Leiden-Leeds database. Besides, a PCR-RFLP based toxinotyping protocol was performed in all the toxigenic strains to analyse the variability of toxins A and B (Rupnik, 2010).

To perform all the above amplifications, DNA was extracted from pure cultures grown in blood agar plates using the FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Europe, Vienna, Austria) following the manufacturer's instructions. One microliter of DNA (1 ng μl^{-1}) was used as template for PCR reactions (Table 2). To provide a molecular mass ladder for the amplicons obtained in the PCR assays (Figures 1 and 2), 100 bp DNA Ladder and 1 kb DNA Ladder (Bioron, Ludwigshafen, Germany) were used.

Positive and negative controls were included in each PCR assay. *C. difficile* ATCC 43255 was used as a positive control for *tcdA*, *tcdB* and *tpi* genes. A genome sequenced *C. difficile* strain from our collection (data not shown) was used as positive control for the genes of CDT toxin. Due to the lack of a positive control for non-toxigenic strains (lok1/3 primers), the amplicons from some representative strains in our collection using these primers were sequenced (GenBank accession no. KX378382, KX378383 and KX378384) in order to confirm the sequences and use them as positive controls. To perform the sequencing, PCR amplicons were treated with llustra™ ExoProStar™ 1-Step (GE Healthcare Sciences Europe, Barcelona, Spain) and sequenced by Sanger sequencing method.

Antimicrobial susceptibility testing

Clostridium difficile isolates were tested for susceptibility to clindamycin, erythromycin, metronidazole, moxifloxacin, tetracycline and vancomycin using the Etest (Barbut et al., 2007), according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). Isolates cultured on blood agar plates for 24 h were suspended to a density of 1.0 McFarland and rapidly swabbed in three directions on the plates (Erikstrup *et al.* 2012). All susceptibility tests were performed on pre-reduced *Brucella* Blood Agar (Oxoid, Basingstoke, Hampshire, UK). After applying the Etest strips containing the antibiotics, the plates were incubated in an anaerobic atmosphere (2.5 l anaerobic jar. Oxoid, Basingstoke, Hampshire, UK) for 24 h and 48 h at 37°C. In order to detect metronidazole hetero-resistant subpopulations, metronidazole plates were further incubated up to 5 d (Peláez *et al.* 2008). *Clostridium difficile* ATCC 43255 was used as control strain in each test (Ackermann *et al.* 2005).

Breakpoints for clindamycin, metronidazole and moxifloxacin were those established by the Clinical and Laboratory Standards Institute (CLSI) for anaerobic bacteria in the M100-S24 document (CLSI document M100-S24, 2014).The breakpoint for tetracycline was ≥ 8 μg ml^{-1} (Spigaglia *et al.* 2008). The remaining breakpoints were based on the literature (Álvarez-Pérez *et al.* 2014). Multidrug resistance (MDR) was defined as resistance to three or more different classes of antimicrobial agents (Souli *et al.* 2008).

Statistical analysis

Comparison of the prevalence among samples collected directly from animals (i.e. from the intestine of rats, mice and pigeons) was assessed by univariable random-effect logistic analysis. The outcome variable was the presence/absence of *C. difficile*, and the explanatory variable was the source of the sample (intestinal content from rats, mice or pigeons). Since samples belonged to different pig farms, the farm was included in the model as the random

factor. Similar analysis was used to compare the proportion of *C. difficile* positive samples among environmental faecal samples. In this case, and given the difficulty to determine the true origin of the environmental faecal samples (from rats or mice) through simple visual inspection, samples from these species were included into a group called "rodents" (Table 1). Likewise, similar comparisons among *C. difficile* positive samples from different sources were performed for the presence of toxins A and B and the binary toxin. All statistical analyses were performed using STATA software (STATA, StataCorp LP, College Station, TX, USA). Significance was set at $P-$values ≤ 0.05.

Results

Bacterial isolation and molecular characterization

From the 27 farms included in the study a total of 398 samples were collected and tested for the presence of *C. difficile*. The number of samples collected in each farm was very variable. Thus rats were trapped in seven farms (median number of samples 3, range 1−9), mice on nine farms (median 4, range 1−20), and pigeons on two farms (median 8, range 5−11). Rat faeces from the environment were collected on 17 farms (median 1, range 1−6), mouse faeces on eight farms (one sample per farm) and bird faeces on 18 farms (median 1, range 1−5). Pools of pig faecal samples were collected on 27 farms (median 7, range 2−25) and pools of remnants of pig feed found underneath the outside silos on 12 farms (median 1.5, range 1−3).

Results of *C. difficile* isolation are summarized in Table 1. In a total of 34 (8.5%) samples the bacterium was isolated. Positive samples were found in 12 (44.4%) pig farms. There were not significant differences in *C. difficile* prevalence on intestinal content among rats, mice and pigeons (Table 3). However, *C. difficile* was significantly more prevalent in environmental rodent faeces (24.3%) than in other type of environmental samples (Tables 1 and 3).

Thirty (88.2%) out of the 34 isolates had *tcdA* (repeating and non-repeating portions) and *tcdB* genes. No differences were found among different type of samples regarding the proportion of *tcdA* or *tcdB* positive strains (results not shown). From these strains 18 (60%) had also both *cdtA* and *cdtB* genes (*cdtA*/*cdtB* positive strains were isolated from all type of samples analyzed) (Table 4) (Figure 1). Only four (11.8%) *C. difficile* isolates were negative for all toxin genes tested, two of them isolated from rat environmental faeces from different farms, and the other two from the same farm (from mouse intestinal content and pig faeces). These four strains yielded a positive result with Lok3/Lok1 primers confirming their non-toxigenic status. A total of seven different ribotypes were identified. Ribotype 078 was predominant (44.1%) followed by ribotype 005 (26.5%), 126 (8.8%), 010, 012 and 204 (5.9%), and 295 (2.9%) (Table 4). Within toxigenic strains (n = 30) two different toxinotypes were identified (Table 4) (Figure 2). Multiple positive samples were found in four pig farms, and in all of them there were ribotypes shared among different sample types (Table 5).

Antimicrobial susceptibility testing

All 34 isolates were susceptible to vancomycin ($\leq$0.5 μg ml^{-1}) and metronidazole ($\leq$0.125 μg ml^{-1}). Slow growing metronidazole hetero-resistant subpopulations were not detected inside the inhibition zone of the Etest strip. Resistance to clindamycin, erythromycin, moxifloxacin and tetracycline was variable (41.2%, 52.9%, 5.9% and 76.5%, respectively). Multidrug resistance was observed in 12 out of 34 (35.3%) of the isolates, showing all of them resistant to clindamycin, erythromycin and tetracycline. Results are summarized in Table 6.

Discussion

To our knowledge, this is the first report in Spain describing the isolation of *C. difficile* from the pig farm environment. The results of this study suggested that *C. difficile* contamination was well established within the pig farm environment in this area since more than 40% of the farms presented at least one positive sample. The proportion of positive samples within the positive farms ranged from a 4.3% to 29.2%, but considering that the number and type of pest and environmental samples collected was very variable among farms, direct within-farm sample-prevalence comparisons were not possible.

The ribotypes identified in this study included five internationally described ribotypes among which 078 was the most prevalent (44.1%). Ribotype 078 is an emerging cause of CA-CDI (Goorhuis *et al.* 2008). In Spain it is one of the most prevalent ribotypes (Alcalá *et al.* 2015). It has been isolated from dogs (Álvarez-Pérez *et al.* 2015) and, in high numbers, from pigs (Peláez *et al.* 2013), which supports the possible zoonotic transmission of *C. difficile*. In The Netherlands *C. difficile* PCR ribotype 078 has been recognized as an important cause of diarrhoea in piglets, and genetically indistinguishable strains have been identified in human and porcine isolates which presented CDI (Debast *et al.*, 2009). Besides, high intestinal colonization percentages of up to 25% have been found in families and employees living and working on pig farms (Keessen *et al.* 2013a). Ribotypes 012 and 005 have been detected in faecal samples from urban wild rats in Canada (Himsworth *et al.* 2014) and humans in humans from several European countries including Spain, being RT012 the eighth most prevalent ribotype in Europe (Bauer *et al.* 2011; Reil *et al.* 2012; Alcalá *et al.*, 2015). Ribotype 126 is one of the most frequently isolated in humans in Spain (Alcalá *et al.* 2015) and it has many similarities to 078. Indeed, it is considered as a variant of 078 and has been reported in river water and different animal species in other countries (Janezic *et al.* 2012; Janezic *et al*. 2014), which also suggests the possible zoonotic potential of this ribotype. The

non-toxigenic ribotype 010 has been isolated from dogs in Spain (Álvarez-Pérez *et al.* 2015). This ribotype has been also described in several animal species, especially companion animals, as well as in some human samples (Keel *et al*. 2007; Koene *et al*. 2012; Janezic *et al*. 2014), suggesting that it too might have zoonotic potential.

Most of the positive samples came from pest species or from environmental faecal samples from them (73.5%). In five (41.7%) of the 12 positive farms *C. difficile* was isolated directly from the intestinal contents of rats and mice, and in six (28.6%) farms the pathogen was isolated also from environmental rodent faeces (Table 1). The odds of finding *C. difficile* from environmental rodent faeces was 10 times higher than from pig faecal samples though it was not possible to determine whether positive environmental rodent faeces belonged to true carriers of *C. difficile* or were simply contaminated once in the environment. Indeed, *C. difficile* is a ubiquitous bacterium (Freeman *et al.* 2010) that has been detected in airborne samples from *C. difficile* positive pig farms (Keessen *et al.* 2011). In any case, given the high percentage of farms (five of 12) with rodents presenting *C. difficile* in their intestinal content, these findings suggest that rodents can transmit toxigenic *C. difficile* on these farms (Table 4).

Despite the thorough sampling of pig faeces, only two farms (7.4%) were found positive. Thus, when estimated from fattening pigs, the *C. difficile* farm prevalence was lower than that observed in other countries (Weese *et al*. 2011; Keessen *et al*. 2013a). A total of seven positive samples were detected on these farms, yielding an overall prevalence of 3.3%, but this result was biased by the fact that six of them were detected on the same farm. The high prevalence of *C. difficile* observed in environmental samples and the low prevalence in pig faecal samples would support the idea that fattening pigs are not as susceptible to *C. difficile* colonization as piglets (Songer and Anderson, 2006). The fact that the farm with the highest prevalence in pig faecal samples (ID 26, Table 5) also had the highest prevalence in

environmental samples (50%), would suggest that fattening pigs may require of higher levels of exposure to become colonized. Interestingly, in this farm, four samples from environmental rat faeces and three from mouse intestinal content were positive, and they belonged to ribotypes 078 and 005, the same ribotypes isolated from the pig faecal samples. These results indicated the high level of *C. difficile* contamination in this particular farm environment, and the likely circulation of these potential zoonotic ribotypes among animal species. The similarity of PCR-ribotypes between rodent and pig samples was also observed in one other farm (Table 5) supporting the potential for between-species transmission of *C. difficile*, but more detailed molecular typing data is necessary to confirm this hypothesis.

Pigeons were captured on only two pig farms. *Clostridium difficile* was isolated from two pigeons on one of these, on which rats had been also found positive (Table 5, farm ID 2). In the other farm neither the pigeons nor other types of samples collected (pigs and bird environmental faeces) tested positive. These results were in line with those from a previous study that found *C. difficile* in faeces from wild birds (house sparrows) trapped in or close to pig farms (Burt *et al.* 2012). Interestingly, other bird species have also found positive for ribotypes which are commonly found in farm animals and humans (Bandelj *et al.*, 2014). As observed in other infections such as salmonellosis (Andrés *et al.* 2013), wild birds in close contact with pig farms may be at higher risk of harbouring the pathogens present in the farm, especially if the farm environment appears to be highly contaminated. These results differed from those from other European countries, including Spain, in which *C. difficile* was not found in wild birds or was isolated from only a few samples (Alderete, 2011; Bandelj *et al.* 2011; Bandelj *et al.* 2014).However, in these studies most of the sampled birds were not related to pig farming. It seems that birds may be acting mostly as indicators of high *C. difficile* environmental contamination levels. In general, the pig farm environments we examined seemed to have conditions for the between-species *C. difficile* transmission, as

pigeons on one farm harboured ribotypes 005 and 078, the latter also being present in three of the five positive rats from that farm (Table 5).

Vancomycin and metronidazole are the two first line antibiotics used for treatments for CDI in humans. Previous studies on human strains of *C. difficile* have found reduced susceptibility to both, or strains resistant especially to metronidazole (Peláez *et al.* 2008). In addition, metronidazole hetero-resistant *C. difficile* strains have been isolated in a swine study conducted in the same geographic area (Peláez *et al.* 2013). In our study, however, all the strains were fully susceptible to vancomycin and metronidazole, and no metronidazole hetero-resistant isolates were detected, which agreed with a previous European prospective study on *C. difficile* infections in humans (Barbut *et al.* 2007). The differences observed may lie in part in the origin of the *C. difficile* strains. While we analyzed a large proportion of samples from wild animal species, all their samples belonged to pigs, which were more likely to have been exposed to antibiotics.

Only two (6.9%) *C. difficile* strains, both belonging to ribotype 078 and isolated from the intestinal content of a mouse and a pigeon from different farms, were found resistant to moxifloxacin. Prevalence of this type of resistance varied between 0 and 34.7% for ribotype 078 strains isolated from pigs in Spain (Álvarez-Pérez *et al*. 2013; Peláez *et al*. 2013). Resistance to this class of antimicrobials is common in strains of *C. difficile* isolated from human and animal samples, which has been attributed to new selective pressure resulting from its increasing use in humans and animals (Keessen *et al.* 2013b). The presence of this type of resistance in *C. difficile* strains isolated from wild species would suggest pigs were the likely source of infection.

Resistance of *C. difficile* to clindamycin, erythromycin and tetracycline varies widely (Gerding 2004; Keessen *et al*. 2013b). Our results showed that these types of resistance were quite frequent among the isolates tested (41.2%, 52.9% and 76.5%, respectively), and that

MDR to these antibiotics was also quite common (12 out 34; 35.3%). The MDR strains were distributed among all described ribotypes except for ribotypes 126 and 295 (Table 4). Four out of 12 MDR isolates were non-toxigenic, which agrees with a previous study that showed that non-toxigenic ribotypes were highly resistant to clindamycin and erythromycin (Álvarez-Pérez *et al.* 2015), highlighting the importance of these and similar strains may have as a source for antimicrobial resistance traits to other bacteria.

Rodents are well known for their ability to act as reservoirs or to transmit a large number of infectious agents such as *Salmonella* and *Staphylococcus aureus* (Kijlstra *et al.*, 2008; van de Giessen *et al.,* 2009; Andrés-Barranco *et al.,* 2014), among which *C. difficile* could be included. This study suggests that rodents and pigeons may play a role in the transmission of toxigenic antimicrobial-resistant *C. difficile*, thus favouring the maintenance and spread of the contamination within the farm environment through their faeces. Inter-species transmission could be possible since the same ribotypes were isolated from different animal species within the same pig farm environment but more detailed molecular typing data is necessary to demonstrate this. Some of the observed ribotypes isolated from rodents and pigeons have also been found in human isolates of *C. difficile* (Koene *et al.,* 2012). However, further studies on the genetic relationship between farm environment and human *C. difficile* strains are required to help to better understand the epidemiology of this pathogen and its role in CA-CDI.

Acknowledgments

We very much appreciate the collaboration of the pig farmers. This study was possible in part thanks to the help of the National Institute for Agricultural and Food Research and Technology through its project no. FAU2008-16. We also thank Anabel Sánchez Bellido and

Sara Andrés-Barranco for their technical assistance, and Dr. Lydden Polley, Professor Emeritus (WCVM, Canada), for the English grammar review.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Ackermann, G., Thomalla, S., Ackermann, F., Schaumann, R., Rodloff, A.C. and Ruf, B.R. (2005) Prevalence and characteristics of bacteria and host factors in an outbreak situation of antibiotic-associated diarrhoea. *J Med Microbiol* **54**, 149–153.

Alcalá, L., Reigadas, E., Marín, M., Martín, A., Catalán, P. and Bouza, E. (2015) Impact of clinical awareness and diagnostic tests on the underdiagnosis of *Clostridium difficile* infection. *Eur J Clin Microbiol Infect Dis* **34**, 1515–25.

Alderete, A. P. (2011) *Clostridium difficile*. Prevalencia e importancia ecológica en animales domésticos y fauna salvaje. Thesis, Universidad Complutense de Madrid, Spain.

Álvarez-Pérez, S., Blanco, J.L., Martínez-Nevado, E., Peláez, T., Harmanus, C., Kuijper, E. and García, M.E. (2014) Shedding of *Clostridium difficile* PCR ribotype 078 by zoo animals, and report of an unstable metronidazole-resistant isolate from a zebra foal (*Equus quagga burchellii*). *Vet Microbiol* **169**, 218–222.

Álvarez-Pérez, S., Blanco, J.L., Peláez, T., Astorga, R.J., Harmanus, C., Kuijper, E. and García, M.E. (2013) High prevalence of the epidemic *Clostridium difficile* PCR ribotype 078 in Iberian free-range pigs. *Res Vet Sci* **95**, 358–361.

Álvarez-Pérez, S., Blanco, J.L., Peláez, T., Lanzarot, M.P., Harmanus, C., Kuijper, E. and García, M.E. (2015) Faecal shedding of antimicrobial-resistant *Clostridium difficile* strains by dogs. *J Small Anim Pract* **56**, 190–5.

Andrés, S., Vico, J.P., Garrido, V., Grilló, M.J., Samper, S., Gavín, P., Herrera-León, S. and Mainar-Jaime, R.C. (2013) Epidemiology of subclinical salmonellosis in wild birds from an area of high prevalence of pig salmonellosis: phenotypic and genetic profiles of *Salmonella* isolates. *Zoonoses Public Health* **60**, 355–365.

Andrés-Barranco, S., Vico, J.P., Garrido, V., Samper, S., Herrera-Leon, S., de Frutos, C. and Mainar-Jaime, R.C. (2014) Role of wild bird and rodents in the epidemiology of subclinical salmonellosis in finishing pigs. *Foodborne Pathog Dis* **11**, 689–697.

Bandelj, P., Trilar, T., Blagus, R., Ocepek, M., Rousseau, J., Weese, J.S. and Vengust, M. (2014) Prevalence and molecular characterization of *Clostridium difficile* isolated from European Barn Swallows (*Hirundo rustica*) during migration. *BMC Vet Res* **10**, 40.

Bandelj, P., Trilar, T., Racnik, J., Zadravec, M., Pirš, T., Avbersek, J., Micunovic, J., Ocepek, M. and Vengust, M. (2011) Zero prevalence of *Clostridium difficile* in wild passerine birds in Europe. *FEMS Microbiol Lett* **321**, 183–5.

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E., Poxton, I., Ackermann, G., Bouza, E., Balmelli, C., Drudy, D., Ladas, H., Nagy, E., Pituch, H., Wullt, M., Yücesoy, M. and Rupnik, M. (2007) Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin Microbiol Infect* **13**, 1048–1057.

Bauer, M.P., Notermans, D.W., van Benthem, B.H., Brazier, J.S., Wilcox, M.H., Rupnik, M., Monnet, D.L., van Dissel, J.T. and Kuijper, E.J. (2011) *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet* **377**, 63–73.

Bidet, P., Barbut, F., Lalande, V., Burghoffer, B. and Petit, J.C. (1999) Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett* **175**, 261–266.

Blunt, R., McOrist, S., McKillen, J., McNair, I., Jiang, T. and Mellits, K. (2011) House fly vector for porcine circovirus 2b on commercial pig farms. *Vet Microbiol* **149**, 452–455.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M. and Von Eichel-Streiber, C. (1996) Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* **181**, 29–38.

Burt, S.A., Siemeling, L., Kuijper, E.J. and Lipman, L.J.A. (2012) Vermin on pig farms are vectors for *Clostridium difficile* PCR ribotypes 078 and 045. *Vet Microbiol* **160**, 256–258.

CLSI (2014). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement.* CLSI document M100-S24. Wayne, PA: Clinical and Laboratory Standards Institute.

Debast, S.B., van Leengoed, L.A.M.G., Goorhuis, A., Harmanus, C., Kuijper, E.J. and Bergwerff, A.A. (2009) *Clostridium difficile* PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. *Environ Microbiol* **11**, 505–511.

Erikstrup, L.T., Danielsen, T.K.L., Hall, V., Olsen, K.E.P., Kristensen, B., Kahlmeter, G., Fuursted, K. and Justesen, U.S. (2012) Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates. *Clin Microbiol Infect* **18**, 266–272.

Fawley, W.N., Knetsch, C.W., MacCannell, D.R., Harmanus, C., Du, T., Mulvey, M.R., Paulick, A., Anderson, L., Kuijper, E.J. and Wilcox, M.H. (2015) Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *Clostridium difficile*. *PLoS One* **10**, e0118150.

Fischer, O., Mátlová, L., Dvorská, L., Švástová, P., Bartl, J., Melichárek, I., Weston, R.T. and Pavlík, I. (2001) Diptera as vectors of mycobacterial infections in cattle and pigs. *Med Vet Entomol* **15**, 208–211.

Freeman, J., Bauer, M.P., Baines, S.D., Corver, J., Fawley, W.N., Goorhuis, B., Kuijper, E.J. and Wilcox, M.H. (2010) The changing epidemiology of *Clostridium difficile* infections. *Clin Microbiol Rev* **23**, 529–549.

Gerding, D.N. (2004) Clindamycin, cephalosporins, fluoroquinolones, and *Clostridium difficile*–associated diarrhea: this is an antimicrobial resistance problem. *Clin Infect Dis* **38**, 646–648.

Goorhuis, A., Bakker, D., Corver, J., Debast, S.B., Harmanus, C., Notermans, D.W., Bergwerff, A.A, Dekker, F.W. and Kuijper, E.J. (2008) Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis* **47**, 1162–1170.

Hensgens, M.P.M., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G.J., De Boer, E., Lipman, L.J.A. and Kuijper, E.J. (2012) *Clostridium difficile* infection in the community: a zoonotic disease? *Clin Microbiol Infect* **18**, 635–645.

Himsworth, C.G., Patrick, D.M., Mak, S., Jardine, C.M., Tang, P. and Scott Weese, J. (2014) Carriage of *Clostridium difficile* by wild urban Norway rats (*Rattus norvegicus*) and black rats (*Rattus rattus*). *Appl Environ Microbiol* **80,** 1299–1305.

Janezic, S., Ocepek, M., Zidaric, V. and Rupnik, M. (2012) *Clostridium difficile* genotypes

other than ribotype 078 that are prevalent among human, animal and environmental isolates. *BMC Microbiol* **12**, 48.

Janezic, S., Zidaric, V., Pardon, B., Indra, A., Kokotovic, B., Blanco, J.L., Seyboldt, C., Diaz, C.R., Poxton, I.R., Perreten, V., Drigo, I., Jiraskova, A., Ocepek, M., Weese, J.S., Songer, J.G., Wilcox, M.H. and Rupnik, M. (2014) International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. *BMC Microbiol* **14**, 173.

Kato, H., Kato, N., Watanabe, K., Iwai, N., Nakamura, H., Yamamoto, T., Suzuki, K., Kim, S.M., Chong, Y. and Wasito, E.B. (1998) Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* **36**, 2178–2182.

Keel, K., Brazier, J.S., Post, K.W., Weese, S. and Songer, J.G. (2007) Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J Clin Microbiol* **45**, 1963–1964.

Keessen, E.C., Donswijk, C.J., Hol, S.P., Hermanus, C., Kuijper, E.J. and Lipman, L.J.A. (2011) Aerial dissemination of *Clostridium difficile* on a pig farm and its environment. *Environ Res* **111**, 1027–1032.

Keessen, E.C., Harmanus, C., Dohmen, W., Kuijper, E.J. and Lipman, L.J. (2013a) *Clostridium difficile* infection associated with pig farms. *Emerg Infect Dis* **19**, 1032–1034.

Keessen, E.C., Hensgens, M.P., Spigaglia, P., Barbanti, F., Sanders, I.M., Kuijper, E.J. and Lipman, L.J. (2013b) Antimicrobial susceptibility profiles of human and piglet *Clostridium difficile* PCR-ribotype 078. *Antimicrob Resist Infect Control* **2**, 14.

Kijlstra, A., Meerburg, B., Cornelissen, J., De Craeye, S., Vereijken, P. and Jongert, E. (2008) The role of rodents and shrews in the transmission of *Toxoplasma gondii* to pigs. *Vet Parasitol* **156**, 183–190.

Knetsch, C.W., Connor, T.R., Mutreja, A., van Dorp, S.M., Sanders, I.M., Browne, H.P., Harris, D., Lipman, L., Keessen, E.C. and Corver, J. (2014) Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. *Eurosurveillance* **19**, 1–12.

Knight, D.R., Elliott, B., Chang, B.J., Perkins, T.T. and Riley, T.V (2015) Diversity and evolution in the genome of *Clostridium difficile*. *Clin Microbiol Rev* **28**, 721–741.

Koene, M.G.J., Mevius, D., Wagenaar, J.A., Harmanus, C., Hensgens, M.P.M., Meetsma, A. M., Putirulan, F.F., van Bergen, M.A.P. and Kuijper, E.J. (2012) *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. *Clin Microbiol Infect* **18**, 778–784.

Lemee, L., Dhalluin, A., Testelin, S., Mattrat, M.A., Maillard, K., Lemeland, J.F. and Pons, J.L. (2004) Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile*. *J Clin Microbiol* **42**, 5710–5714.

Nathues, C., Grüning, P., Fruth, A., Verspohl, J., Blaha, T., Kreienbrock, L. and Merle, R. (2013) *Campylobacter* spp., *Yersinia enterocolitica*, and *Salmonella enterica* and their simultaneous occurrence in German fattening pig herds and their environment. *J Food Prot* **76**, 1704–1711.

Peláez, T., Alcalá, L., Blanco, J.L., Álvarez-Pérez, S., Marín, M., Martín-López, A., Catalán, P., Reigadas, E., García, M.E. and Bouza, E. (2013) Characterization of swine isolates of *Clostridium difficile* in Spain: a potential source of epidemic multidrug resistant strains? *Anaerobe* **22**, 45–49.

Peláez, T., Cercenado, E., Alcalá, L., Marín, M., Martín-López, A., Martínez-Alarcón, J., Catalán, P., Sánchez-Somolinos, M. and Bouza, E. (2008) Metronidazole resistance in *Clostridium difficile* is heterogeneous. *J Clin Microbiol* **46**, 3028–3032.

Reil, M., Hensgens, M.P.M., Kuijper, E.J., Jakobiak, T., Gruber, H., Kist, M. and Borgmann, S. (2012) Seasonality of *Clostridium difficile* infections in Southern Germany. *Epidemiol Infect* **140**, 1787–93.

Roberts, K., Smith, C.F., Snelling, A.M., Kerr, K.G., Banfield, K.R., Sleigh, P.A. and Beggs, C.B. (2008) Aerial dissemination of *Clostridium difficile* spores. *BMC Infect Dis* **8**, 7.

Rodriguez-Palacios, A., Borgmann, S., Kline, T.R. and LeJeune, J.T. (2013) *Clostridium difficile* in foods and animals: history and measures to reduce exposure. *Anim Health Res Rev* **14**, 11–29.

Rousseau, C., Poilane, I., Diakite, F., Feghoul, L., Cruaud, P. and Collignon, A. (2010) Comparaison de trois milieux pour la culture de *Clostridium difficile*: intérêt des milieux favorisant la germination des spores?. *Pathol Biol* **58**, 58–61.

Rupnik, M. (2007) Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? *Clin Microbiol Infect* **13**, 457–459.

Rupnik, M. (2010) *Clostridium difficile* toxinotyping. *Methods Mol Biol* **646**, 67–76.

Silva, R.O.S., Ribeiro de Almeida, L., Junior, C.A.O., de Magalhães Soares, D.F., Pereira, P.L.L., Rupnik, M. and Lobato, F.C.F. (2014) Carriage of *Clostridium difficile* in free-living South American coati (*Nasua nasua*) in Brazil. *Anaerobe* **30**, 99–101.

Songer, J.G. and Anderson, M.A. (2006) *Clostridium difficile*: an important pathogen of food animals. *Anaerobe* **12**, 1–4.

Souli, M., Galani, I. and Giamarellou, H. (2008) Emergence of extensively drug-resistant and pandrug-resistant Gram-negative bacilli in Europe. *Eurosurveillance* **13**, 1–11.

Spigaglia, P., Barbanti, F. and Mastrantonio, P. (2008) Tetracycline resistance gene *tet*(W) in the pathogenic bacterium *Clostridium difficile*. *Antimicrob Agents Chemother* **52**, 770–773.

Stubbs, S., Rupnik, M., Gibert, M., Brazier, J., Duerden, B. and Popoff, M. (2000) Production of actin-speci ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* **186**, 307–312.

van de Giessen, A.W., van Santen-Verheuvel, M.G., Hengeveld, P.D., Bosch, T., Broens, E.M. and Reusken, C.B. (2009) Occurrence of methicillin-resistant *Staphylococcus aureus* in rats living on pig farms. *Prev Vet Med* **91**, 270–273.

Weese, J.S., Rousseau, J., Deckert, A., Gow, S. and Reid-Smith, R.J. (2011) *Clostridium difficile* and methicillin-resistant *Staphylococcus aureus* shedding by slaughter-age pigs. *BMC Vet Res* **7**, 41.

Table 1 Type and number of samples analyzed, number of farms, and number and percentage of positive farms and samples

Type of sample	Origin*	No. of samples	No. of positive samples (%)	No. of farms	No. of positive farms (%)
Intestinal	Rat	26	5 (19.2)	7	1 (14.3)
	Mouse	53	7 (13.2)	9	4 (44.4)
	Pigeon	16	2 (12.5)	2	1 (50)
Environmental	Rodents	37	9 (24.3)	21	6 (28.6)
	PPF	210	7 (3.3)	27	2 (7.4)
	BF	37	2 (5.4)	18	2 (11.1)
	PFF	19	2 (10.5)	12	2 (16.7)
Total		398	34 (8.5)	27	12 (44.4)

*Rodent environmental faeces (rat and mouse); PPF, pool of pig faecal samples (pen floor); BF, bird environmental faeces: PFF, pool of pig feed.

Table 2 List of oligonucleotide primers, PCR protocols and amplicons for each assay used in this study

Gene	Primer sequences (5'–3') and PCR protocols	Amplicon size (bp)	Reference
tpi	F – AAAGAAGCTACTAAGGGTACAAA R – CATAATATTGGGTCTATTCCTAC 95ºC, 3 min; [95ºC, 30 s; 55ºC, 30 s; 72ºC, 30 s] x40; 72ºC, 5 min	230	Lemee e*t al.*, 2004
*tcdA**	F – TGATGCTAATAATGAATCTAAAATGGTAAC R – CCACCAGCTGCAGCCATA 95ºC, 5 min; [95ºC, 30 s; 62ºC, 30 s; 72ºC, 1 min] x40; 72ºC, 10 min	700 or 1266	Kato *et al.*, 1998
tcdA†	F – GGAAGAAAAGAACTTCTGGCTCACTCAGGT R – CCCAATAGAAGATTCAATATTAAGCTT 95ºC, 5 min; [95ºC, 30 s; 55ºC, 30 s; 74ºC, 30 s] x35; 74ºC, 5 min	252	Kato *et al.*, 1998
tcdB	F – GGAAAAGAGAATGGTTTTATTAA R – ATCTTTAGTTATAACTTTGACATCTTT 95ºC, 3 min; [95ºC, 30 s; 55ºC, 30 s; 72ºC, 30 s] x40; 72ºC, 5 min	160	Lemee *et al.*, 2004
cdtA	F – TGAACCTGGAAAAGGTGATG R – AGGATTATTTACTGGACCATTTG 94ºC, 5 min; [94ºC, 30 s; 55ºC, 30 s; 72ºC, 30 s] x35; 72ºC, 5 min	375	Stubbs *et al.*, 2000
cdtB	F – CTTAATGCAAGTAAATACTGAG R – AACGGATCTCTTGCTTCAGTC 94ºC, 5 min; [94ºC, 45 s; 52ºC, 1 min; 72ºC, 1 min] x35; 72ºC, 10 min	510	Stubbs *et al.*, 2000
NTS‡	F – AAAATATACTGCACATCTGTATAC R – TTTACCAGAAAAAGTAGCTTTAA 95ºC, 5 min; [95ºC, 1 min; 55ºC, 1 min; 72ºC, 1 min] x35; 72ºC, 10 min	700	Braun *et al.*, 1996

*Repeating sequence, primers NK11/NK9**;** †Non-repeating sequence, primers NK3/NK2; ‡Non-toxigenic strains, primers lok3/lok1.

Table 3 Results of the random-effect logistic regression analysis for the association between type of samples and isolation of *Clostridium difficile*

Factor	Logistic regression parameters				
	β	Standard Error (β)	*P*−value	Odds Ratio (OR)	95% CI (OR)
Intestinal content samples					
Pigeon				1	
Mouse	1.51	1.02	0.14	4.5	0.61, 33.8
Rat	0.57	0.96	0.55	1.7	0.27, 11.6
Environmental samples					
Pool of pig faecal samples				1	
Feed on floor	1.12	0.89	0.21	3.1	0.53, 17.9
Bird faecal samples	0.62	0.86	0.47	1.9	0.34, 10.1
Rodent faecal samples*	2.3	0.59	<0.001	10	3.14, 31.9
Coefficient	−4.08	0.62	<0.001		

*It includes environmental faeces from rats and mice.

Table 4 Ribotype, toxinotype and MDR identified in *Clostridium difficile* strains

Type of sample	Ribotype identified (no. isolates)	Toxinotype identified	Number of MDR strains* (%)
Rat intestinal content (RC)	005 (3)	0	1 (33.3)
	126 (2)‡	V	-
Rat environmental faeces (RF)	012 (1)	0	1 (100)
	078 (4)‡	V	1 (25)
	126 (1)‡	V	-
	204 (2)†	-	2
	295 (1)	0	-
			Total 44.4%
Mouse intestinal content (MC)	005 (1)	0	1 (100)
	010 (1)†	-	1 (100)
	012 (1)	0	-
	078 (4)‡	V	-
			Total 28.6%
Mouse environmental faeces (MF)	-	-	-
Pigeon intestinal content (PC)	005 (1)	0	-
	078 (1)‡	V	-
Pool of pig faecal samples (pen floor) (PPF)	005 (3)	0	3 (100)
	010 (1)†	-	1 (100)
	078 (3)‡	V	-
			Total 57.1%

Pool of pig feed (PFF)	078 (2)‡	V	-
Bird environmental faeces (BF)	005 (1)	0	1 (100)
	078 (1)‡	V	-
			Total 50%

*Multidrug resistance (MDR): presence of antimicrobial resistance to ≥3 different classes of antimicrobial agents. †Non-toxigenic strains. ‡*cdtA*/*cdtB* positive strains.

Table 5 Ribotype and toxinotype identified in the isolates from those farms where *Clostridium difficile* was isolated from different type of samples

Farm ID	Type of sample*	Number of isolates	Ribotype identified	Toxinotype identified
2	RC	5	005 (3/5)	0
			126 (2/5)	V
	PC	2	005	0
			078	V
4	MC	1	012	0
	PFF	1	078	V
	BF	1	078	V
20	MC	1	010†	-
	PPF	1	010†	-
26	RF	4	078	V
	MC	3	005 (1/3)	0
			078 (2/3)	V
	PPF	6	005 (3/6)	0
			078 (3/6)	V

*RC, rat intestinal content; RF, rat environmental faeces; MC, mouse intestinal content; PC, pigeon intestinal content; PPF, pool of pig faecal samples (pen floor); PFF, pool of pig feed, BF, bird environmental faeces. †Non-toxigenic strains.

Table 6 *In vitro* activity of six antimicrobials against the *Clostridium difficile* isolates

Antimicrobial agent	Range (μg ml^{-1})	Breakpoint* (μg ml^{-1})	Number resistant isolates (%)
Clindamycin	0'016−256	≥8†	14 (41.2)
Erythromycin	0'016−256	≥8	18 (52.9)
Metronidazole	0'016−256	≥32†	0
Moxifloxacin	0'02−32	≥8†	2 (5.9)
Tetracycline	0'016−256	≥8	26 (76.5)
Vancomycin	0'016−256	≥32	0

*The breakpoints for resistance established by the Clinical and Laboratory Standards Institute (CLSI) for anaerobic bacteria are those marked by †. The breakpoint for tetracycline was ≥8 μg ml^{-1} (Spigaglia *et al.* 2008). The remaining breakpoints were based on the literature (Álvarez-Pérez *et al.* 2014).

List of figure legends

Figure 1 PCR agarose gel (1.5%) images from *Clostridium difficile* field isolates and *Clostridium difficile* ATCC 43255 (positive control). (a) *tpi* gene, 230 bp: lanes 2−5 field isolates, lane 6 ATCC, lane 7 negative control. (b) *tcdA* gene (non-repeating sequence), 252 bp: lanes 2−5 field isolates, lane 6 ATCC, lane 7 negative control. (c) *cdtA* gene, 375 bp: lanes 2, 5 and 6 field isolates, lane 3 negative control, lane 4 ATCC. (d) *tcdB* gene, 160 bp: lanes 2, 5 and 6 field isolates, lane 3 negative control, lane 4 ATCC. (e) *tcdA* gene (repeating sequence), 1266 bp: lane 2 negative control, lane 3 ATCC, lanes 4−7 field isolates. (f) *cdtB* gene, 510 bp: lanes 2 and 3 field isolates, lane 4 ATCC, lane 5 negative control. In all cases

100 bp molecular mass was used (arrows point 500 bp fragment) except in gel (e) in which 1kbp marker was utilized (arrow points 1 kbp fragment).

Figure 2 Toxinotyping patterns of different *Clostridium difficile* field strains and *Clostridium difficile* ATCC 43255 (toxinotype 0) as control in 1% agarose gel. (a) A3 fragment unrestricted, 3.1 kbp: lanes 2−4 field isolates, lane 5 ATCC. (b) A3 fragment restricted with *Eco*RI: lanes 2−4 field isolates, lane 5 ATCC. (c) B1 fragment unrestricted, 3.1 kbp: lanes 2−4 field isolates, lane 5 ATCC. (d) B1 fragment restricted with *Hinc*II/*Acc*I: lanes 1 and 2 field isolate, lanes 3 and 4 ATCC. In all cases 1 kbp molecular mass was used (upper arrows point to 3 kbp fragment and lower arrows point to 1 kbp fragment).