Applied and Environmental Microbiology, Feb. 2011, p. 1135–1139 Vol. 77, No. 3
0099-2240/11/$12.00 doi:10.1128/AEM.01803-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Real-Time PCR Assay for *Clostridium perfringens* in Broiler Chickens in a Challenge Model of Necrotic Enteritis[▽]

Shu-Biao Wu,* Nicholas Rodgers, and Mingan Choct

School of Environmental and Rural Science, University of New England, Armidale, NSW 2351, Australia

Received 29 July 2010/Accepted 29 November 2010

We compared ileal *Clostridium perfringens* quantification results produced by real-time PCR and culture-based methods in broiler chickens in a challenge model of necrotic enteritis. Assessment of the relative standard deviations (RSDs) revealed that the real-time PCR assay generated a smaller standard deviation and thus was more precise than the culture-based method. Linear regression analysis indicated that the bacterial counts of these two methods were highly correlated ($R^2 = 0.845$). We suggest that real-time PCR could be a replacement of the culture method for quantifying *C. perfringens* in the intestinal tracts of broiler chickens.

Clostridium perfringens, a Gram-positive spore-forming and anaerobic nonmotile rod bacterium, is an important pathogenic bacterium in humans and livestock. It can be found in animal and human intestinal tracts and feces and in environmental samples (such as soil and wastewater samples). *C. perfringens* types A, B, C, D, and E produce many different toxins that may be involved in pathogenesis (18). In poultry, this bacterium often causes necrotic enteritis (NE) which leads to losses of over $2 billion each year in the world's broiler industry (5). Bacteria in the gastrointestinal tracts of chickens play an important role in health (15). Therefore, quantification of the major bacterial communities in the chicken gut is essential for monitoring changes in microbial ecology in experiments involving *C. perfringens* challenge. Traditionally, analysis of gastrointestinal communities has depended on bacterial culture-based counting methods or microscopy. The methodologies involved are time-consuming and require researchers to possess substantial microbiological expertise. Furthermore, potential bias is present, since only those bacteria whose physiological and metabolic requirements are reproducible *in vitro* can be cultivated (25).

Molecular approaches have been applied to rapid characterization of bacteria. These approaches include denaturing gradient gel electrophoresis (DGGE) (10, 24), temperature gradient gel electrophoresis (TGGE) (29), conventional PCR (13), and terminal restriction fragment length polymorphism (T-RFLP) (23). These approaches are able to identify relevant bacterial groups, but the drawback of these techniques is that they are not fully quantitative and so cannot act as a standalone alternative method to *in vitro* culture and enumeration. In contrast, real-time PCR can be used to quantify bacteria, as the number of target gene copies can be determined in DNA extracted from samples. Hence, real-time PCR has recently been used to enumerate bacteria in environmental samples (3, 21) and in animal gastrointestinal tracts and feces (6, 7, 9, 26).

This study compared the precision of real-time PCR quantification of *C. perfringens* (PCR targeting the 16S rRNA genes of *C. perfringens*) with the culture-based colony counting method. The analysis of relative standard deviations (RSDs) indicated that real-time PCR quantification was more precise and reproducible than the traditional culture-based method. Therefore, this efficient and cost-effective molecular approach can replace the culture-based counting method for quantification of *C. perfringens* in the intestinal tracts of chickens in a challenge model of NE disease.

Sampling of ileal digesta of the birds. The animal experiment was conducted as described recently (28). Briefly, 1,350 birds were raised for 5 weeks with the birds in each cage assigned to one of nine treatment groups with six birds per treatment (25 birds/cage). On day 9, the birds in groups that would be challenged were given *per os* three *Eimeria* species (Bioproperties Pty Ltd., Glenorie, New South Wales, Australia), and on days 14, 15 and 16, they were inoculated *per os* with approximately 10^8 CFU of a pathogenic strain of *C. perfringens* type A (CSIRO Livestock Industries, Geelong, Victoria, Australia). The experimental design and the treatment acronyms are shown in Table 1. On days 13 and 17, 2 birds were randomly chosen in each cage and sacrificed for sample collections. Approximately 1 g of the ileal digesta was collected for microbial culture, and a section of approximately 3 cm of ileum (including digesta) was taken at the midpoint between Meckel's diverticulum and cecal tonsils per bird for quantitative PCR analysis of *C. perfringens*.

Quantification of ileal *C. perfringens*. The quantification of ileal *C. perfringens* using the culture-based method followed the protocol described earlier (28). The *C. perfringens* bacteria were cultured and counted on Perfringens tryptose-sulfite-cycloserine and Shahidi-Ferguson Perfringens agar base mixed with egg yolk emulsion and Perfringens selective supplement (Oxoid). *C. perfringens* plates were incubated anaerobically for 48 h at 39°C prior to counting. Bacterial numbers were expressed as $\log_{10}$ CFU/gram of digesta.

Extraction of DNA from ileal content was conducted using

* Corresponding author. Mailing address: School of Environmental and Rural Science, University of New England, Armidale, NSW 2351, Australia. Phone: 61-2-67732238. Fax: 61-2-67733922. E-mail: shubiao.wu@une.edu.au.

▽ Published ahead of print on 10 December 2010.

TABLE 1. Experimental design of the treatments with fish meal feeding, *Eimeria* infection, and *C. perfringens* challenge[a]

Treatment group[b]	Fish meal	*Eimeria*	*C. perfringens*
1 (NFM−)	None	None	None
2 (LFM−)	Low	None	None
3 (HFM−)	High	None	None
4 (NFM/Cp)	None	None	Yes
5 (LFM/Cp)	Low	None	Yes
6 (HFM/Cp)	High	None	Yes
7 (NFM+)	None	Yes	Yes
8 (LFM+)	Low	Yes	Yes
9 (HFM+)	High	Yes	Yes

[a] The fish meal levels were 0, 250, and 500 g per kg of starter diet in the none, low, and high fish meal groups, respectively. On day 9, the birds in treatment groups 7, 8, and 9 were given *per os* a suspension of 5,000 sporulated oocysts of three *Eimeria* species (*Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*) (Bioproperties Pty., Glenorie, New South Wales, Australia) in 1 ml of sterile phosphate-buffered saline (PBS), and the birds in the other treatment groups were given 1 ml of sterile PBS instead of the *Eimeria* suspension. On days 14, 15, and 16, the birds in treatment groups 4 to 9 were inoculated *per os* with 1 ml of *C. perfringens* suspension at a concentration of 10^8 to 10^9 CFU/ml, and the birds in treatment groups 1, 2, and 3 received 1 ml of sterile thioglycolate broth in place of *C. perfringens*.

[b] The treatment group abbreviations shown in parentheses are as follows: NFM−, not fed fish meal and negative for *C. perfringens* and *Eimeria*; LFM−, fed low level of fish meal and negative for *C. perfringens* and *Eimeria*; HFM−, fed high level of fish meal and negative for *C. perfringens* and *Eimeria*; NFM/Cp, not fed fish meal and challenged with *C. perfringens* only; LFM/Cp, fed low level of fish meal and challenged with *C. perfringens* only; HFM/Cp, fed high level of fish meal and challenged with *C. perfringens* only; NFM+, not fed fish meal and challenged by *C. perfringens* and *Eimeria*; LFM+, fed low level of fish meal and challenged by *C. perfringens* and *Eimeria*; HFM+, fed high level of fish meal and challenged by *C. perfringens* and *Eimeria*.

a QIAamp DNA stool kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer with slight modifications. First, 180 to 220 mg frozen digesta was taken from stored samples, and glass beads (300 mg) (0.1 mm; Biospec Products, Bartlesville, OK) were used to disrupt the cells in 400 μl of ASL lysis buffer by shaking the sample on a miniBeadBeater (Biospec Products, Bartlesville, OK) for 30 s. The cells were then lysed after adding 1 ml of ASL lysis buffer, stool particles were removed, and PCR inhibitors in the supernatant were absorbed by the InhibitEX tablet. DNA was precipitated by adding 200 μl of ethanol, captured on the QIAamp spin column, washed by 500 μl of washing buffers AW1 and AW2, and eluted in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]).

The quantitative real-time PCR assay was conducted by the method of Wise and Siragusa (27). TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) was used. A pair of primers (CPerf165F [5′-CGCATAACGTTGAAAGA TGG-3′] and CPerf269R [5′-CCTTGGTAGGCCGTTACCC-3′]; Invitrogen, Mulgrave, Victoria, Australia) and a dual labeled TaqMan probe (CPerf187F [5′-FAM-TCATCATTCA ACCAAAGGAGCAATCC-TAMRA-3′ where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine]) (Applied Biosystems, Foster City, CA) that targets *C. perfringens* 16S rRNA genes were also used. The DNA extracted from ileal digesta was diluted five times in TE buffer, and then 2 μl of diluted sample was used in PCR amplification in a total volume of 20 μl. PCR was performed in a Rotorgene 6500 real-time PCR machine (Corbett, Sydney, Australia) with three replicates for each sample. A threshold cycle (C_T) average from the replicate samples was used for data analysis. Serial dilutions of a *C. perfringens* DNA with known concentration and in a background of ileal DNA that contained no *C. perfringens* were used to construct a standard curve. The corresponding number of cells was calculated by taking the genome size of *C. perfringens* into consideration (6). Bacterial numbers were expressed as $\log_{10}$ genomic DNA copy number per gram of digesta (wet weight).

All data were analyzed using the statistical package Minitab for Windows 12.1 (Minitab Inc., State College, PA). To assess the correlation between the real-time PCR method and the culture method, simple linear regression of the treatment means produced by these two methods was performed. The precision of different measurements can be evaluated by the comparison of their relative standard deviations. It has been proposed that a higher RSD demonstrates poorer precision of the measurement or *vice versa* (11, 19, 22). To assess the precision of the real-time PCR method compared to the culture-based method, RSDs were calculated according to the following formula: percent RSD $= (\sigma/\bar{x}) \times 100$ where $\bar{x}$ is the mean value and σ is the standard deviation. The RSDs of both methods were compared for each treatment.

Efficiency of the quantitative PCR assay. The sensitivity and amplification efficiency of the real-time PCR assay were tested by amplification of serial dilutions of *C. perfringens* DNA samples. When the threshold cycles were plotted against the $\log_{10}$ values of the initial number of *C. perfringens* DNA copies in the PCR to construct a standard curve for the assay, linearity was observed with the following regression curve: $y = -3.434x + 34.850$, where y is the C_T values and x is the amount of bacteria ($\log_{10}$ value). A significant coefficient of correlation ($R^2 = 1.00$) was attained, and a high amplification efficiency (96%) was achieved. Therefore, it is considered that the assay produced an accurate quantification of *C. perfringens* when the initial amount of *C. perfringens* DNA in the PCR fell in the range of 7.6×10^7 to 7.6×10^{-1} copies.

Comparison of *C. perfringens* values produced by real-time PCR and culture-based methods. As shown in Fig. 1, the changes in the $\log_{10}$ values of CFU/g of *C. perfringens* among the treatments at day 17 briefly showed similar patterns between the real-time PCR and culture-based methods. The regression results showed that the real-time PCR method correlated highly with the results of the culture-based method ($R^2 = 0.845$), and the regression curve had a slope of 1.10 (Fig. 2), indicating the validity of the real-time PCR method for the quantification of *C. perfringens*.

To assess the precision of the real-time PCR method compared to the culture-based method, the RSDs of both methods were calculated, and the values are shown in Table 2. For all treatments, the RSDs of real-time PCR analysis were smaller than those of the culture-based method, suggesting that the real-time PCR was more precise than the culture-based method. For example, the RSD of the culture-based method was as high as 10.8 times that of the real-time PCR method in the high-fish-meal-fed birds without *C. perfringens* and *Eimeria* infections (HFM−). The closest RSDs between these two methods were observed in the low-fish-meal-fed birds with *C. perfringens* infections (LFM/Cp), and interestingly, the RSD of the culture-based method in this group was the smallest among the RSDs of all the treatments measured by culture-based method. When the data for day 17 from both methods were

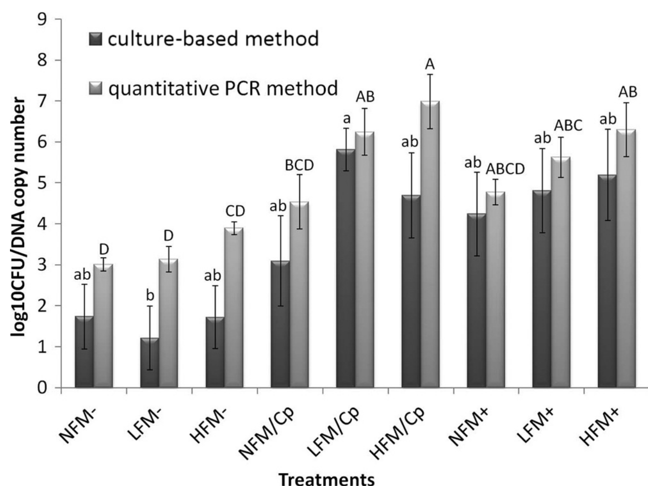

FIG. 1. *C. perfringens* counts in response to experimental treatments on day 17 measured by culture and real-time PCR. The changes in the $\log_{10}$ values of CFU/g of *C. perfringens* in the treatment groups briefly showed similar patterns by real-time PCR and the culture-based method. Data are expressed as means ± standard errors (SE) (error bars) ($n = 6$). Treatment group abbreviations: NFM−, not fed fish meal and negative for *C. perfringens* and *Eimeria*; LFM−, fed low level of fish meal and negative for *C. perfringens* and *Eimeria*; HFM−, fed high level of fish meal and negative for *C. perfringens* and *Eimeria*; NFM/Cp, not fed fish meal and challenged with *C. perfringens* only; LFM/Cp, fed low level of fish meal and challenged with *C. perfringens* only; HFM/Cp, fed high level of fish meal and challenged with *C. perfringens* only; NFM+, not fed fish meal and challenged by *C. perfringens* and *Eimeria*; LFM+, fed low level of fish meal and challenged by *C. perfringens* and *Eimeria*; HFM+, fed high level of fish meal and challenged by *C. perfringens* and *Eimeria*. Bars with different letters above them correspond to values that are significantly different for culture (lowercase) and real-time PCR (uppercase) methods.

compared, it was apparent that the higher the level of fish meal in the diet, the greater the counts of *C. perfringens* produced by real-time PCR in all the groups with the same *Eimeria* and/or *C. perfringens* challenge treatments. However, this trend was observed only in the birds infected with both *C. perfringens* and *Eimeria* but not in other two groups for culture-based counts (Fig. 1).

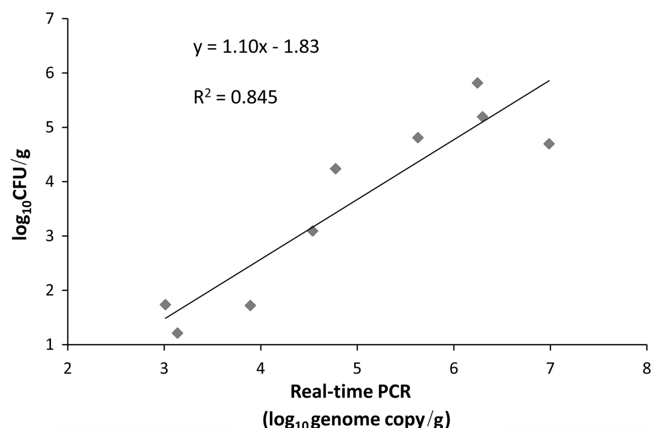

FIG. 2. Simple linear regression of $\log_{10}$ DNA versus $\log_{10}$ CFU of *C. perfringens* in the birds subjected to different treatments. Data are means for six birds for each treatment group. The results for the real-time PCR method showed a high correlation with the results for the culture-based method ($\log_{10}$ CFU/g) ($R^2 = 0.845$), and the regression curve had a slope of 1.10, indicating the validity of the real-time PCR method for the quantification of *C. perfringens*.

TABLE 2. Comparison of relative standard deviations for culture-based and quantitative PCR methods for bacterial ileal contents of birds in different treatment groups on day 17[a]

Treatment group[b]	RSD (%)[c]		Ratio of culture RSD to qPCR RSD
	Cp-culture	Cp-qPCR	
1 (NFM−)	111.4	13.0	8.5
2 (LFM−)	158.1	24.2	6.5
3 (HFM−)	109.9	10.1	10.8
4 (NFM/Cp)	87.4	35.8	2.4
5 (LFM/Cp)	21.7	22.2	1.0
6 (HFM/Cp)	54.4	23.1	2.4
7 (NFM+)	58.7	16.1	3.7
8 (LFM+)	52.6	21.5	2.4
9 (HFM+)	52.5	25.8	2.0

[a] For all the treatments, the relative standard deviations (RSDs) for real-time PCR analysis were smaller than the RSDs for the culture-based method, suggesting that the real-time PCR was more precise than the culture-based method. qPCR, quantitative PCR.

[b] The treatment group abbreviations shown in parentheses are as follows: NFM−, not fed fish meal and negative for *C. perfringens* and *Eimeria*; LFM−, fed low level of fish meal and negative for *C. perfringens* and *Eimeria*; HFM−, fed high level of fish meal and negative for *C. perfringens* and *Eimeria*; NFM/Cp, not fed fish meal and challenged with *C. perfringens* only; LFM/Cp, fed low level of fish meal and challenged with *C. perfringens* only; HFM/Cp, fed high level of fish meal and challenged with *C. perfringens* only; NFM+, not fed fish meal and challenged by *C. perfringens* and *Eimeria*; LFM+, fed low level of fish meal and challenged by *C. perfringens* and *Eimeria*; HFM+, fed high level of fish meal and challenged by *C. perfringens* and *Eimeria*.

[c] Abbreviations: Cp-culture, *C. perfringens* culture; Cp-qPCR, *C. perfringens* quantitative PCR.

High precision of real-time PCR quantification of *C. perfringens*. By using the optimized infection-producing necrotic enteritis challenge model (28), we have determined that although both culture-based and real-time PCR methods produced comparable results for broiler ileal *C. perfringens* counts across nine treatment groups, the precision of real-time PCR methods was substantially better than that of the culture-based method in all treatment groups with only one exception (one group). In addition, the real-time PCR method has been proven to be a reliable, efficient, and cost-effective method in contrast to the tedious and costly culture-based method. Therefore, it is suggested that quantitative real-time PCR could be a good method to replace the traditional culture-based method for the enumeration of *C. perfringens* in the intestinal tracts of broiler chickens.

Enumeration of intestinal bacteria using the culture-based method has its disadvantage, as the processes are extremely laborious (4, 26). Alternative methods including DGGE (10, 24), TGGE (29), conventional PCR (13), and T-RFLP (23) have been applied to analyze bacteria in the environment and animal gastrointestinal tract; however, these approaches are not fully quantitative. Therefore, a quantitative real-time PCR assay has been developed as an alternative to bacterial culture-based counting methods (14, 17, 20, 27). Real-time PCR assays have been established to quantify *C. perfringens* in chicken gastrointestinal tract or other animal feces by targeting 16S rRNA genes (16, 27), chaperonin 60 gene (6), or genes encoding alpha-toxin (1, 2, 8). The latter assays targeted virulent *C. perfringens* strains rather than the total amount of the species

in the samples. Although culture-based counting results have been compared with quantitative real-time PCR data in these studies, a conclusion on whether real-time PCR achieved higher precision was not reached. The current study applied the RSDs of *C. perfringens* counts to assess the precision of the results obtained by both methods. It was revealed that real-time PCR enumerations of ileal *C. perfringens* produced less variation, and thus higher precision and repeatability, than the culture-based method. The reliability of real-time PCR was also reflected by the consistent increase in *C. perfringens* levels detected by this approach in response to increasing levels of fish meal in the diet in all groups, whereas the culture-based data showed only the trends in the *Eimeria* and/or *C. perfringens* challenge groups in this study. The low accuracy and precision of the culture-based method can be attributed to the following. (i) The minimal detectable level of bacteria is higher by the culture-based method, leading to inaccurate counts in replicate samples with small amounts of bacteria and thus higher variation. (ii) The culture-based method is a laborious procedure, which can introduce systematic errors. (iii) Other human errors can be introduced, due to multiple operators required for the culture-based method. In contrast, the real-time PCR method has high sensitivity, as it detects as little as a few copies of the DNA and thus a single bacterial cell. In addition, due to its simplicity, there are fewer chances where systematic or human errors can be introduced. Therefore, the possible between-sample variations that may occur in a culture-based method are largely eliminated in the real-time PCR procedure.

The reliability of real-time PCR enumeration of *C. perfringens* can be assessed by linear regression analysis against culture-based counting. In an assay of *C. perfringens* in dog feces, a poor correlation between real-time PCR and agar plate results was found ($R^2 = 0.0016$); however, other reports have demonstrated that the real-time PCR quantification results generally show high correlations with the culture-based counting (R^2 values range from 0.386 to 0.990) (6, 16, 27). In this study, a similar result was achieved by linear regression analysis of the results from these two methods ($R^2 = 0.845$). Although the real-time PCR assay resulted in a lower number of *C. perfringens* than plate counts of bacteria from the intestines of broiler chickens by using the alpha-toxin-encoding gene *plc* as the PCR target (1), overestimations of the number of bacteria have been suggested in a few other reports in which the *cpn-60* gene (6, 12) or 16S rRNA genes (16, 27) were targeted. Our results demonstrated overestimation by real-time PCR compared to culture-based counting, as the culture-based method resulted in an average of approximately 1.3 log units less *C. perfringens* per gram of digesta (wet weight) (data not shown). This result was largely attributed to the inclusion of DNA from dead or nonculturable cells (16, 27). On the other hand, our data revealed that substantially lower counts of the colonies following agar culture of the samples with less *C. perfringens*, such as in the unchallenged groups of this study, may also be responsible for this dissociation.

This study was funded by The Australian Poultry CRC (project 09-22).

We acknowledge Shuyu Song, Mark Porter, and Gary Taylor for their technical assistance, Rob Moore and Anthony Keyburn of CSIRO Livestock Industries, Geelong, Victoria, Australia, for providing a *C. perfringens* strain for the challenge experiment, and Rima Youil, Cloduraldo Villaflor, and Wayne Woods of Bioproperties Pty Ltd., Glenorie New South Wales, Australia, for providing *Eimeria* spp.

REFERENCES

1. **Abildgaard, L., O. Hojberg, A. Schramm, K. M. Balle, and R. M. Engberg.** 2010. The effect of feeding a commercial essential oil product on *Clostridium perfringens* numbers in the intestine of broiler chickens measured by real-time PCR targeting the α-toxin-encoding gene (*plc*). Anim. Feed Sci. Technol. **157:**181–189.
2. **Albini, S., et al.** 2008. Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. Vet. Microbiol. **127:**179–185.
3. **Bonjoch, X., F. Lucena, and A. R. Blanch.** 2009. The persistence of bifidobacteria populations in a river measured by molecular and culture techniques. J. Appl. Microbiol. **107:**1178–1185.
4. **Dahiya, J., D. Hoehler, A. van Kessel, and M. Drew.** 2007. Effect of different dietary methionine sources on intestinal microbial populations in broiler chickens. Poult. Sci. **86:**2358–2366.
5. **Dahiya, J. P., D. C. Wilkie, A. G. van Kessel, and M. D. Drew.** 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed Sci. Technol. **129:**60–88.
6. **Dumonceaux, T. J., et al.** 2006. Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. J. Microbiol. Methods **64:**46–62.
7. **Fu, C. J., J. N. Carter, Y. Li, J. H. Porter, and M. S. Kerley.** 2006. Comparison of agar plate and real-time PCR on enumeration of *Lactobacillus*, *Clostridium perfringens* and total anaerobic bacteria in dog faeces. Lett. Appl. Microbiol. **42:**490–494.
8. **Fukushima, H., K. Katsube, Y. Hata, R. Kishi, and S. Fujiwara.** 2007. Rapid separation and concentration of food-borne pathogens in food samples prior to quantification by viable-cell counting and real-time PCR. Appl. Environ. Microbiol. **73:**92–100.
9. **Gurjar, A. A., N. V. Hegde, B. C. Love, and B. M. Jayarao.** 2008. Real-time multiplex PCR assay for rapid detection and toxin typing of *Clostridium perfringens* toxin producing strains in feces of dairy cattle. Mol. Cell Probes **22:**90–95.
10. **Hume, M., et al.** 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poult. Sci. **82:**1100–1107.
11. **Jacangelo, J., and N. Seith.** 2001. Investigation of criteria for GWUDI determination. American Water Works Association, Denver, CO.
12. **Karpowicz, E., A. Novinscak, F. Barlocher, and M. Filion.** 2010. qPCR quantification and genetic characterization of *Clostridium perfringens* populations in biosolids composted for 2 years. J. Appl. Microbiol. **108:**571–581.
13. **Kato, N., et al.** 1993. Identification of enterotoxin-producing *Clostridium perfringens* by the polymerase chain reaction. J. Jpn. Assoc. Infect. Dis. **67:**724–729.
14. **Kimura, B., S. Kawasaki, H. Nakano, and T. Fujii.** 2001. Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. Appl. Environ. Microbiol. **67:**206–216.
15. **Lu, J., et al.** 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. **69:**6816–6824.
16. **Matsuda, K., H. Tsuji, T. Asahara, Y. Kado, and K. Nomoto.** 2007. Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. Appl. Environ. Microbiol. **73:**32–39.
17. **Matsuki, T., et al.** 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. **68:**5445–5451.
18. **McDonel, J. L.** 1980. *Clostridium perfringens* toxins (type A, B, C, D, E). Pharmacol. Ther. **10:**617–655.
19. **Peters, F., and H. Maurer.** 2005. Bioanalytical method validation and its implications for forensic and clinical toxicology—a review, p. 1–9. *In* P. Bièvre and H. Günzler (ed.), Validation in chemical measurement. Springer, Berlin, Germany.
20. **Rinttila, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva.** 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J. Appl. Microbiol. **97:**1166–1177.
21. **Shannon, K. E., D. Y. Lee, J. T. Trevors, and L. A. Beaudette.** 2007. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci. Total Environ. **382:**121–129.
22. **Taylor, J.** 1987. Quality assurance of chemical measurements. Lewis Publishers, Boca Raton, FL.
23. **Torok, V. A., K. Ophel-Keller, M. Loo, and R. J. Hughes.** 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. Appl. Environ. Microbiol. **74:**783–791.
24. **van der Wielen, P. W., D. A. Keuzenkamp, L. J. Lipman, F. van Knapen, and S. Biesterveld.** 2002. Spatial and temporal variation of the intestinal bacterial

community in commercially raised broiler chickens during growth. Microb. Ecol. **44:**286–293.
25. **Walter, J., et al.** 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. **66:**297–303.
26. **Wise, M. G., and G. R. Siragusa.** 2007. Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. J. Appl. Microbiol. **102:**1138–1149.
27. **Wise, M. G., and G. R. Siragusa.** 2005. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. Appl. Environ. Microbiol. **71:**3911–3916.
28. **Wu, S.-B., N. Rodgers, and M. Choct.** 2010. Optimised necrotic enteritis model producing clinical and subclinical infection of *Clostridium perfringens* in broiler chickens. Avian Dis. **54:**1058–1065.
29. **Zhu, X., T. Zhong, Y. Pandya, and R. Joerger.** 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microbiol. **68:**124–137.