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Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the *chaperonin*-60 target

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Abstract

We used qPCR and the target gene *chaperonin*-60 (*cpn*60) to enumerate *Clostridium perfringens* genomes in DNA extracts from contents of the chicken gastrointestinal tract with the aim of optimizing this methodology to enumerate any bacterium of interest. To determine the most accurate protocols for determining target species abundance, we compared various DNA extraction methods in combination with four methods for producing standard curves. Factors affecting accuracy included the co-purification of PCR inhibitors and/or fluorescence quenchers and the yield of target DNA in the extract. Anion exchange chromatography of the spiked test samples enabled accurate enumeration of *C. perfringens* using a standard curve comprised of a plasmid containing a fragment of *C. perfringens cpn*60. We used qPCR to enumerate *C. perfringens* and other intestinal bacteria in ileum and cecum samples from chickens that had been challenged with *C. perfringens* and compared the results with viable counts on corresponding selective agars. We conclude that qPCR-based molecular enumeration of target species in the gastrointestinal tract is feasible, but care must be taken in order to mitigate the effects of confounding factors that can affect the apparent cell count.

Keywords: *Chaperonin*-60; cpn60; Quantitative PCR; Antibiotics; Molecular enumeration

1. Introduction

The gastrointestinal tract of animals harbors a large, complex, and dynamic microbial community, the composition of which has a strong impact on host health and nutrition (Xu and Gordon, 2003). In

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animals of agricultural importance, the prophylactic application of antibiotics to swine and poultry has a positive impact on animal health and production performance, presumably due to effects on the host microflora (Cromwell, 2002; Gaskins et al., 2002; Hardy, 2002). However, concerns about the potential for the selection and spread of antimicrobial resistance genes to human pathogens has led to a search for alternatives to antibiotics (Reid and Friendship, 2002) and in some countries a moratorium on their prophylactic use (Stein, 2002).

Such concerns have led to an increased interest in the structure and function of the gastrointestinal microbial communities of agricultural animals. Most of the current body of knowledge concerning the composition of gut microflora in animals has come from culture-based studies, in which the growth conditions of microorganisms are reproduced in the laboratory and colonies are enumerated and identified by biochemical methods. Although it remains the standard for analysis of gut community composition, the limitations of this approach are well known and include the inability to culture many of the resident species and the fact that culture-based analysis is expensive, tedious, and time-consuming. These factors have spurred the development of culture-independent methodologies for examining the composition of the gut microflora (O'Sullivan, 2000; Vaughan et al., 2000; Gong et al., 2002; Hill et al., 2002).

Many culture-independent methods rely on the amplification of specific, universal target molecules using PCR and the sequencing or electrophoretic analysis of the amplified products. The *cpn*60 gene, which encodes the 60 kDa chaperonin (also known as GroEL or Hsp60), has been established as a useful target for molecular phylogenetics (Jian et al., 2001) and the characterization of complex microbial communities (Hill et al., 2002, 2005). In addition, *cpn*60 sequences have been used to discriminate between even closely related bacterial isolates by hybridization or sequence analysis (Goh et al., 1997a,b, 2000; Brousseau et al., 2001).

The utility of *cpn*60-based methods is enhanced by the fact that an approximately 555-bp segment of the gene corresponding to nucleotides 274–828 of the *Escherichia coli cpn*60 sequence (the *cpn*60 universal target, or *cpn*60 UT) can be amplified from virtually any genome using universal, degenerate PCR primers (Goh et al., 1996) and by the availability of a large reference database of chaperonin sequences (Hill et al., 2004). These factors combine to make *cpn*60 an excellent target for the development of species-specific PCR primers, enabling the design of primers that specifically amplify species of interest from a mixed population (Hill et al., 2005). Since conventional PCR amplification can be considered as a present/absent test only, real-time quantitative PCR (qPCR) has seen increasing application for enumerating specific organisms in a very wide range of clinical and environmental samples (Hristova et al., 2001; Stults et al., 2001; Huijsdens et al., 2002; Matsuki et al., 2002; Stubner, 2002). Each type of environmental sample to be assayed presents unique challenges with respect to PCR-based quantification, and there is a general lack of consensus about how best to approach the quantitative aspect of the assay. For example, qPCR assays have been described using relative quantification of target vs. total bacteria (Yoshida et al., 2003), while many assays rely on quantification using a calibration curve with a range of different standards reported (Lyons et al., 2000; Tajima et al., 2001; Bach et al., 2002).

Our objective was to determine the most accurate and convenient method to enumerate any bacterial species for which *cpn*60 UT sequence information is available within intestinal populations using the *cpn*60 molecular target and qPCR. As a model system for the development of this methodology, we chose the Gram-positive anaerobe *Clostridium perfringens* since it is an important pathogen in the poultry industry (Williams et al., 2003) and it is relatively easy to culture and selectively enumerate. To determine if *cpn*60 sequences can be used to detect other species from the gut microflora, we also developed *cpn*60-specific qPCR assays for five other bacterial species found in the gastrointestinal tract. The procedures described here may be generally applicable for the molecular enumeration of any bacterial target from templates derived from the gastrointestinal microbiota.

2. Materials and methods

2.1. DNA extraction

DNA was extracted from each digesta sample or pure culture of bacteria using a modified version of