Veterinary Microbiology 189 (2016) 46–51

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Determining the optimal number of individual samples to pool for quantification of average herd levels of antimicrobial resistance genes in Danish pig herds using high-throughput qPCR

Julie Clasen[a], Anders Mellerup[a], John Elmerdahl Olsen[b], Øystein Angen[c], Anders Folkesson[a], Tariq Halasa[a], Nils Toft[a], Anna Camilla Birkegård[a,*]

[a] National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark
[b] Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg, Denmark
[c] Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 16 February 2016
Received in revised form 19 April 2016
Accepted 21 April 2016

Keywords:
Pooling
Antimicrobial resistance gene
Pig
High-throughput qPCR
Sample size
Herd level

ABSTRACT

The primary objective of this study was to determine the minimum number of individual fecal samples to pool together in order to obtain a representative sample for herd level quantification of antimicrobial resistance (AMR) genes in a Danish pig herd, using a novel high-throughput qPCR assay. The secondary objective was to assess the agreement between different methods of sample pooling. Quantification of AMR was achieved using a high-throughput qPCR method to quantify the levels of seven AMR genes (*ermB*,*ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O) and *tet*(W)). A large variation in the levels of AMR genes was found between individual samples. As the number of samples in a pool increased, a decrease in sample variation was observed. It was concluded that the optimal pooling size is five samples, as an almost steady state in the variation was observed when pooling this number of samples. Good agreement between different pooling methods was found and the least time-consuming method of pooling, by transferring feces from each individual sample to a tube using a 10 μl inoculation loop and adding 3.5 ml of PBS, approximating a 10% solution, can therefore be used in future studies.

1. Introduction

There is a considerable societal interest in the careful monitoring of AMR levels in humans and animals, and both national surveillance programs such as DANMAP in Denmark (DANMAP, 2014) and European surveillance programs (EFSA, 2015) exist. Surveillance programs most often monitor AMR in one of the three major categories of organisms: animal bacterial pathogens; zoonotic bacteria or commensal bacteria (Franklin et al., 2001). In Denmark, AMR is monitored in *Escherichia coli* and *Enterococcus* spp. (DANMAP, 2014). These are considered good indicator bacteria as they are part of the normal gut flora and constitute a reservoir of resistance genes (Franklin et al., 2001). With more than 400 different bacterial species in the gut, *E. coli* constitutes less than 1% of these (Berg, 1996) and a large variation in the abundance of *E. coli* (Dunlop et al., 1999), the AMR levels might be underestimated using indicator bacteria. DNA-based methods make it possible to investigate total community DNA and quantify the AMR gene levels in complex samples such as porcine feces (Schmidt et al., 2015). Sample pooling has proven beneficial, including screening for presence or absence for a range of diseases (Arnold and Cook, 2009; Rovira et al., 2008; Tavornpanich et al., 2004; Weinstock et al., 2001). Few studies have assessed the value of pooling to quantify the herd level for a disease (Davies et al., 2003; Pedersen et al., 2014; Schmidt et al., 2015). However, they do not establish how many individual samples (IS) would be optimal to pool, which is necessary in order to represent the true herd AMR status. Minimizing the number of individual samples is important, because the sampling process is time-consuming and therefore expensive.

The primary objective of this study was to determine the minimum number of individual fecal samples to pool together in order to obtain a representative sample for herd level

* Corresponding author at: National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg, Denmark.
E-mail addresses: jucl@vet.dtu.dk (J. Clasen), amellerup84@gmail.com (A. Mellerup), jeo@sund.ku.dk (J.E. Olsen), ysan@ssi.dk (Ø. Angen), afol@vet.dtu.dk (A. Folkesson), tahbh@vet.dtu.dk (T. Halasa), ntoft@vet.dtu.dk (N. Toft), acbir@vet.dtu.dk (A.C. Birkegård).

http://dx.doi.org/10.1016/j.vetmic.2016.04.017
0378-1135/

quantification of AMR genes in a Danish pig herd, using a novel high-throughput qPCR assay. The secondary objective was to assess the agreement between different methods of sample pooling.

2. Materials and methods

2.1. Study design

Two studies were conducted. In study one, the minimum number of IS to pool in order to obtain a representative sample for herd level quantification of AMR genes in a Danish pig herd was estimated. In study two, three different pooling methods were compared.

2.2. Sample collection

In study one, 20 IS were collected in November 2014 from a pig herd on Funen, housing 1700 finisher pigs and 1150 weaner pigs. Fecal samples were collected from one section with finisher pigs close to slaughter. The section had eight pens housing 3–18 pigs. Between one and five IS were taken from each pen, depending on the number of pigs in the pen. The samples were collected from the rectum of the pig using disposable plastic gloves, which were changed between samplings. As the sampling did not involve invasive handling of the animals, permission for sampling was not required by Danish law. The samples were stored in plastic containers with tight lids and immediately placed in a Styrofoam box with cooling elements, then transported and stored overnight at 5 °C before pooling.

In study two, five IS from five different pig herds (i.e. 25 IS), were collected in January 2015 at an abattoir in Jutland in the lairage, just prior to slaughter. The samples were collected and transported as done in study one but pooled the same day as the sampling.

2.3. Pooling of samples

In study one, a 10% Phosphate Buffered Saline (PBS) solution was made twice for each of the 20 IS by transferring 1 g of feces to 9 ml of PBS. Pooled samples (PS) were then made from the first dilution of the 20 IS by mixing 0.5 ml of the diluted samples. A total of 48 pools were made: 11 pools of two IS, 11 pools of three IS, 11 pools of four IS, six pools of five IS, four pools of ten IS, four pools of 15 IS and one pool of 20 IS. The pools with the same number of IS were made of different IS, but for the pools of two, three and four IS, two duplicates of pools were made resulting in six pairs of identical pools. This was done to validate the consistency of the pooling method. All samples (IS and PS) were stored at −20 °C until DNA extraction.

In study two, three pooling methods were used. Pooling method 1: the same method as for study one. Pooling method 2: feces from each IS were transferred to a tube using a 10 μl inoculation loop. The PS was weighed and the amount of PBS required for a 10% solution was calculated and added. Pooling method 3: using a 10 μl inoculation loop, feces from each IS were transferred to a tube and a fixed amount of PBS was added, approximating a 10% solution. The amount of PBS used in pooling method 3 was calculated as the mean of the amount of PBS used for the samples in pooling method 2. The PS were stored at −20 °C before DNA extraction. The time spent making ten samples was measured for each method.

2.4. Quantification of AMR genes using real-time qPCR

Total DNA was extracted from the 20 IS and PS, using the Maxwell® 16 LEV Blood DNA Kit (Promega), details can be obtained by the corresponding author. Samples were diluted to 40 ng/μl in nuclease-free water (Qiagen, Hilden, Germany) and stored at −20 °C until further processing. Seven AMR genes, *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O) and *tet*(W), were included in the array. Primers and probes have been validated by Schmidt et al. (2015). 16S rDNA primers and probes were included in the assay as a reference gene; here were used as forward primer: TGGAGCATGTGGTTTAATTCGA, as reverse primer: TGCGGGACTTAACCCAACA and as probe: CCTTTGACAACTCTAGAGATAGAGCCTTCCC, all synthesized by DNA Technology A/S (Aarhus Denmark). qPCR amplifications for the quantification of the included genes were performed with the Fluidigm HD Biomark system. The PCR protocol was as follows: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 60 s at 59 °C for extension and annealing, where the fluorescence was measured after each cycle. Standard curves for the qPCR was generated from 10-fold and 2-fold serial dilutions of a fecal samples containing target DNA for each primer set for determination of efficiency, i.e. limit of detection (LOD) and limit of quantification (LOQ).

2.5. Data analysis

Raw quantification cycle (C_q) values generated by the qPCR were exported from the Fluidigm Real-Time PCR Analysis Software, version 4.2.1 (Fluidigm, 2014) to R (R Core Team, 2015). Samples with technical replicates with a discrepancy exceeding 0.5, C_q values exceeding primer-specific LODs or with non-detects of one or both of the technical replicates were excluded. The mean of C_q values for technical replicates were calculated, then corrected with the IPCs included in all runs, along with an efficiency calibration (Ståhlberg et al., 2013) based on standard curves generated. Relative quantification (RQ) was then determined for each of the samples using a modified Livak-method (Eq. (1))(Livak and Schmittgen, 2001):

$$RQ = 2^{-\left(C_{q,AMRgene} - C_{q,reference\ gene}\right)} \quad (1)$$

Table 1
Primer efficiency, R^2, dynamic range, LOD and LOQ for qPCR assays.

Gene	Efficiency	R^2	Dynamic Range	LOD (C_q value)	LOQ (C_q value)
ermB	98.0%	0.9896	9-fold	23	23
ermF	94.5%	0.9739	7-fold	24	24
sulI	100.0%	0.9510	7-fold	26	26
sulII	102.7%	0.9698	7-fold	23	23
tet(M)	108.2%	0.9547	4-fold	25	25
tet(O)	94.9%	0.9900	10-fold	23	23
tet(W)	90.9%	0.9929	12-fold	24	24
16S rDNA	94.2%	0.9953	9-fold	24	18

LOD: limit of detection, LOQ: limit of quantification. R^2: determinant coefficient. C_q: Cycle of quantification.