IJP: Drugs and Drug Resistance 8 (2018) 411–419

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

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

Exploring benzimidazole resistance in *Haemonchus contortus* by next generation sequencing and droplet digital PCR

Paulius Baltrušis, Peter Halvarsson, Johan Höglund*

Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health, Section for Parasitology, P.O. Box 7036, Uppsala, Sweden

ARTICLE INFO

Keywords:
Strongyles
ddPCR
NGS-Sequencing
Benzimidazoles
Anthelmintic resistance

ABSTRACT

Anthelmintic resistance in gastrointestinal nematode (GIN) parasites of grazing ruminants is on the rise in countries across the world. *Haemonchus contortus* is one of most frequently encountered drug-resistant GINs in small ruminants. This blood-sucking abomasal nematode contributes to massive treatment costs and poses a serious threat to farm animal health. To prevent the establishment of resistant strains of this parasite, up-to-date molecular techniques need to be proposed which would allow for quick, cheap and accurate identification of individuals infected with resistant worms. The effort has been made in the previous decade, with the development of the pyrosequencing method to detect resistance-predicting alleles. Here we propose a novel droplet digital PCR (ddPCR) assay for rapid and precise identification of *H. contortus* strains as being resistant or susceptible to benzimidazole drugs based on the presence or absence of the most common resistance-conferring mutation F200Y (TAC) in the β tubulin isotype 1 gene. The newly developed ddPCR assay was first optimized and validated utilizing DNA templates from single-worm samples, which were previously sequenced using the next generation PacBio RSII Sequencing (NGS) platform. Subsequent NGS results for faecal larval cultures were then used as a reference to compare the obtained values for fractional abundances of the resistance-determining mutant allele between ddPCR and NGS techniques in each sample. Both methods managed to produce highly similar results and ddPCR proved to be a reliable tool which, when utilized at full capacity, can be used to create a powerful mutation detection and quantification assay.

1. Introduction

The blood-sucking nematode of small ruminants *Haemonchus contortus* has become a thorn in the side for farmers across the world. Even though gastrointestinal nematode (GIN) infections in livestock are usually found to be mixed, *H. contortus* stands out as the most pathogenic and abundant species (Besier et al., 2016). In Australia, prevention costs against nematode parasites amount to the major part of the country's spending in the red meat industries (Lane et al., 2015). In the UK, total annual losses in the sheep sector due to GIN parasites constituted £84 million more than 10 years ago, and have since then likely increased (Nieuwhof and Bishop, 2005). In addition, developing countries, such as India, suffer from immense annual treatment costs which can be considerably detrimental to the countries' economies ($103 mln.) (McLeod, 2004). Apart from gross economic losses, impaired animal health and welfare are also key issues.

To date, three major drug classes are utilized to treat GIN infections in ruminants – benzimidazoles (BZ), levamisole (LEV), and macrocyclic lactones (ML), all of which are broad spectrum anthelmintics which reduce the existing worm burdens and if used persistently decrease pasture contamination and thus prevent the establishment of infective-stage larvae (L3) (Hoste et al., 2011; Sutherland and Leathwick, 2011). However, years of improper drug use have led to the development of anthelminthic resistance (AR) among these worms and somewhat halted the ever-increasing development of the animal production sector worldwide. Even in most European countries, such as the United Kingdom (Hong et al., 1996), Netherlands (Van den Brom et al., 2013), Spain (Requejo-Fernández et al., 1997), Switzerland (Schnyder et al., 2005), Germany (Bauer, 2001), Poland (Mickiewicz et al., 2017), Italy (Cringoli et al., 2007), France (Paraud et al., 2009), Denmark (Peña-Espinoza et al., 2014), Sweden (Höglund et al., 2009) and Norway (Domke et al., 2012), sheep and goat GIN parasite resistance towards benzimidazole anthelmintic drugs has become a common occurrence.

One of the most significant properties of *H. contortus*, is the propensity to develop resistance to anthelminthic drugs, which has been well documented and described by Kotze and Prichard (2016). This in part can be explained by very high gene flow between populations, remarkable within-population genetic diversity (Blouin et al., 1995;

* Corresponding author.
E-mail address: johan.hoglund@slu.se (J. Höglund).

https://doi.org/10.1016/j.ijpddr.2018.09.003
Received 25 June 2018; Received in revised form 14 September 2018; Accepted 16 September 2018
Available online 19 September 2018

Troell et al., 2006) and high effective population size. This increased variability within-populations is especially true for β-tubulin genes, which account for resistance to BZ substance class of anthelmintic drugs (Beech et al., 1994).

To tackle the problem of growing resistance of *H. contortus* towards BZ drugs, alternatives to anthelminthic treatment must be considered and an end to ineffective drug use has to be implemented. To achieve this, a reliable, rapid and reasonably cost-effective screening-diagnostic tool is of paramount importance (Kenyon and Jackson, 2012). The current, gold standard for identifying clinical anthelmintic resistance in flocks of animals is the Faecal Egg Count Reduction Test (FECRT) (Coles et al., 2006). Albeit a well-described and firmly established method, it is lacking in sensitivity (Höglund et al., 2009). Furthermore, a number of elements can dramatically influence the outcome of the results, such as the level of excretion and aggregation of FEC, sample size and dilution factors, making this tool nothing more than a 'rough estimation' which is inefficient, difficult to interpret and reproduce (Levecke et al., 2012). Although a step towards developing and validating molecular tools for the detection, screening and evaluation of resistance towards anthelmintic drugs in parasitic nematodes was taken in the previous decade, mainly in the form of pyrosequencing (Von Samson-Himmelstjerna et al., 2007, 2009; Höglund et al., 2009), the search for novel, 'better' methods continues.

Droplet Digital PCR (ddPCR) is a recent modification of the third generation digital PCR. It boasts high accuracy, versatility and removes the need for standards or references in comparison to qPCR (Hindson et al., 2011). In this study, the ddPCR method was employed to create a universal protocol for the detection and quantification of the transversion occurring at the 200th codon (P200) (T**T**C→T**A**C) in the β-tubulin isotype 1 gene, resulting in the acquisition of AR to BZ drugs in different strains of *H. contortus*. For this purpose, mixed ovine faecal larval culture samples containing *H. contortus*, derived from Swedish farms pre- and post-treatment with a BZ drug (albendazole), were subjected to molecular characterization of the β-tubulin isotype 1 gene locus, first by utilizing next generation PacBio RSII Sequencing (NGS) to obtain a reference read of the samples contents' and then by the optimized ddPCR, developed by the authors. The fractional abundance (FA) values for both alleles (wild-type and P200 mutant) were compared between the two methods to determine if the newly developed protocol is reliable and precise. In addition to this, adult *H. contortus* single-worm samples, collected from 13 different countries were subjected to the same NGS of the β-tubulin isotype 1 gene locus and were further used not only in the initial development of the ddPCR protocol but also in the construction a Bayesian phylogenetic tree based on the retrieved exon sequences.

2. Material and methods

2.1. Sources of DNA

Genomic DNA was extracted from two different sources: 1) individual adult *H. contortus* worms and 2) mixed faecal larval cultures. The adult worms were previously isolated, bio-banked samples from naturally infected sheep (Troell et al., 2006), and were initially used in the ddPCR method development and optimisation stage. During the method validation stage, DNA samples, derived from a mixture of strongyles and extracted from faecal larval cultures collected from 13 sheep farms pre- and/or post-treatment around southern Sweden, were used (Supplementary Table 1) (Note: farms K-M only had a single pre- or post-treatment sample available). DNA extractions were performed according to the instructions of the supplier on thawed samples using NucleoSpin Tissue kit (Macherey Nagel).

2.2. DNA library creation and sequencing

Genomic DNA samples from *H. contortus* single-worms and faecal larval cultures of sheep were used to amplify a partial β-tubulin isotype 1 encoding gene sequence harbouring the three most common mutation sites (at codon positions (P)200, (P)198 and (P)167), changes in which confer resistance to BZ drugs. The original primer sequences were described by Redman et al. (2015). Upon confirmation of successful amplification of a partial gene product of approximately 922 base-pairs (bp) in size (using a randomly selected, readily available genomic *H. contortus* DNA template) by conventional temperature gradient PCR, unique tags were developed to be used in the library creation step (Supplementary Table 2).

The conditions for the thermal cycling reactions were selected according to the manufacturer's guidelines (Thermo Scientific DreamTaq Green DNA Polymerase #EP0712) – a single cycle of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 1 min. An additional one cycle of final extension at 72 °C for 10 min was included at the very end of the reaction. The final volume of samples was 15 μL. After the amplification step, samples were run on a 1% agarose gel and visualized by a BioRad Gel Doc™ system.

Amplified adult-worm DNA samples were pooled together in categories, according to the signal strengths of visualized bands on agarose gels (larval culture samples did not undergo the initial pooling step, but were subjected to the subsequent clean-up). These were then further subjected to a clean-up step using the AMPure XP magnetic beads, following the instructions detailed by the manufacturer (Beckman Coulter Inc.). The pooled pure partial β-tubulin locus sequences present in adult-worm and larval culture DNA samples were subjected to DNA quantification. Qubit dsDNA HS Assay Kit was employed to determine DNA concentrations in each sample, strictly following the guidelines issued by the manufacturer (Life Technologies). After this step, the remaining samples were further joined together (single-worm and larval culture samples were pooled separately) in equal concentrations forming five distinct batches and stored at −20 °C before sequencing. Pooled amplicon DNA samples were sent for sequencing to Uppsala Genome Center, Science for Life Laboratory, Dept. of Immunology, Genetics and Pathology, Uppsala University, BMC, Box 815, SE-752 37 UPPSALA and further sequenced using the PacBio RSII Technology Platform.

2.3. NGS data analysis

Sequencing data for both single-worms and larval culture pools was analysed using jMHC software (version 1.6.1624) (Stuglik et al., 2011). FASTA files for each sample pool were imported and samples were demultiplexed using unique nucleotide tag combinations. Sequencing errors were filtered out using jMHC and only sequences that were found in at least 3 reads were considered. The output file for single adult worms was analysed by manually enforcing strict criteria to filter out artefacts and obtain well-characterized DNA sequence variant(s) in each sample in three steps: (1) Sequences in samples were removed if they had less than three reads, (2) Assuming only up to two allele variants should be present in each single-worm sample, a sequence, whose coverage (i.e. number of reads) was lower than 50% of the highest coverage possessing sequence in that particular sample, was removed, (3) Samples containing multiple sequences with two or more possibilities of inferring binary allele combinations after steps (1) and (2) were removed. Sequence variants found in larval culture sample pools were also manually processed using these two criteria: (1) A sequence in a particular isolate sample was filtered out if its coverage was less than 3 reads, (2) Sequences present only in a single isolate sample and having a coverage of 3 or less were removed.

2.4. Phylogenetic analysis of partial β-tubulin sequences found in single-worm samples

The β-tubulin isotype 1 locus DNA sequences found in single-worm