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Comparison of microchamber-based digital PCR and pyrosequencing for quantification of F200Y single nucleotide polymorphism in *Haemonchus contortus* larval cultures

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Summary

Control of gastrointestinal nematode (GIN) infections in small ruminants still relies mainly on using various anthelmintic drugs, of which most popular are benzimidazoles (BZ). An inevitable consequence is development of resistance to BZ, which results from single nucleotide polymorphism (SNP) in β-tubulin isotype 1 gene. Major SNP appears to be thymine into adenine transversion at position 200 (F200Y). It can be detected using various molecular methods, of which pyrosequencing is considered the reference method owing to its proven performance in quantifying benzimidazole resistance-associated SNPs. Recently digital PCR based either on water-in-oil droplet emulsification (droplet digital PCR) or use of microchambers has been introduced. As microchamber-based digital PCR (mdPCR) has not previously been compared with pyrosequencing for quantification of the F200Y SNP in *Haemonchus contortus*, the aim of this study was to determine the agreement between the two methods. The study was carried out on larval cultures obtained from 63 goat herds which were confirmed to contain the third-stage larvae (L3) of *H. contortus* by microscopic examination and the real-time PCR. The mdPCR yielded significantly higher F200Y SNP frequency values than pyrosequencing, with a median difference of 10%. The agreement in quantitative results between both methods was only moderate ($R_c = 0.52$). The agreement assuming 4-category classification (0-25%, 26-50%, 51-75%, and > 75%) was moderate to good. The observed agreement was 82.5% and the chance-corrected agreement expressed using the Gwet's AC_2 unweighted coefficient was 0.789. In conclusion, the mdPCR and pyrosequencing show only moderate agreement in determination of F200Y SNP frequency in larval cultures and the former method yields significantly higher values. The higher F200Y SNP frequencies obtained by mdPCR may influence the interpretation of benzimidazole resistance levels and should therefore be considered when comparing results generated by different molecular methods. Further validation studies are needed to establish the causes of the observed differences and define the role of mdPCR in routine monitoring of benzimidazole resistance.

Keywords: GIN, anthelmintic resistance, digital PCR, pyrosequencing, goats

Gastrointestinal nematode (GIN) infections are one of the major threats for the health and welfare of goats. Among myriad of GINs, *Haemonchus contortus* (so called a barber pole worm) is especially dangerous due to its absolute hematophagy and high prolificacy (5000 eggs laid per female and per day) (10). Despite many strategies of sustainable parasite control, the use of anthelmintics remains the mainstay of routine GIN control in goats (7, 20). The efficacy of this practice has, however, been markedly reduced by widespread resistance to major classes of anthelmintics across Europe (32). This problem is particularly pronounced in relation to one of the most commonly used anthelmintic classes – benzimidazoles (BZ) (32, 36). In Poland, the BZ resistance in GIN population of goats was first described in 2017 (28) and a large scale survey carried out in following years revealed that the BZ-resistance was present in approximately 90% of goat herds (28, 29).

BZ-resistance of GIN populations may be detected using three different approaches. An *in vivo* fecal egg count reduction test (FECRT) quantifies the percentage reduction of eggs excreted by goats in feces after deworming. Two *in vitro* tests – egg hatch test (EHT) and larval development test (LDT) – measure percentage of hatching eggs and developing larvae when exposed to gradually increasing thiabendazole concentrations. Molecular methods detect single nucleotide polymorphism (SNP) in the β-tubulin isotype 1 gene coding target proteins for BZ (23, 24). Several SNPs have so far been linked with BZ-resistance at nucleotide positions 167, 198, and 200 (33). The first SNP discovered and the one most widespread is transversion from thymine (TTC, TTT) to adenine (TAC, TAT) in the codon 200 (F200Y) (23, 24). The percentage of alleles of the β-tubulin isotype-1 gene which contain F200Y SNP can be quantified using several PCR-based techniques such as classic PCR, Sanger sequencing, real-time quantitative PCR (qPCR), PCR-Restriction Fragment Length Polymorphism, deep amplicon sequencing, and pyrosequencing (2, 4, 17, 31, 34, 35, 37, 41). The latter assay based on a non-electrophoretic DNA sequencing method is currently considered a reference standard for quantification of BZ-resistance-associated SNPs (6, 22, 33, 35). Recently digital PCR (dPCR), referred to as the third-generation PCR, has become available in veterinary parasitology for quantification of BZ-resistance-associated SNPs (3, 13).

The dPCR was developed in early 2000s. The method is based on partitioning the PCR mixture which contains DNA material into many thousands of compartments so that the target molecule is randomly distributed among the partitions. Next, individual target-containing partitions are amplified and undergo separate end-point fluorescence analysis. The target concentration is calculated from the fraction of positive and negative partitions using the Poisson distribution. In this manner, dPCR turns quantification based on the analysis of continuous signal typical of qPCR into adding up independent digital (binary i.e. positive and negative) outcomes (5, 30). Two main partitioning systems are currently in use: microchambers, first commercialized in 2006, and water-in-oil droplet emulsification, first commercialized in 2010 (18, 38, 39). The latter type of dPCR is called droplet digital PCR (ddPCR) and has been already introduced into veterinary parasitology and shown to be virtually perfectly consistent with pyrosequencing (4). Compared with conventional PCR-based approaches, microchamber-based dPCR (mdPCR) combines the advantages of absolute quantification with a simplified workflow, high reproducibility, and easy automation, making it a promising tool for routine molecular diagnostics (39). However, despite these practical advantages, its performance for quantification of the F200Y SNP associated with benzimidazole resistance in GIN has not yet been investigated. Therefore, the aim of this study was to evaluate the agreement between microchamber-based digital PCR (mdPCR) and pyrosequencing in the quantification of F200Y SNP frequency in *H. contortus* larval cultures and to assess the potential applicability of mdPCR for molecular detection of benzimidazole resistance.

Material and methods

Study population. The study was carried out on cultures of the third-stage larvae (L3) of *H. contortus* isolated from 63 goat herds. The goat herds participated in the voluntary program of GIN control run by the parasitological laboratory of the Division of Veterinary Epidemiology and Economics at the Warsaw University of Life Sciences – SGGW between 2017 and 2024. The herd was included in the study if the following inclusion criteria were met: (i) the owner provided informed consent for participation in the study and for the use of the results in scientific publications; and (ii) fecal samples were collected from a representative group of goats within the herd or, whenever possible, from all animals, and submitted to the laboratory for analysis.

Fecal samples were collected directly from the rectum or immediately after defecation, by the scientific team during veterinary consultations on goat farms or occasionally by farmers if the herd was distantly located. If collected by the scientific team, fecal samples were vacuum-packed, immediately transported to the laboratory at room temperature and examined the same day. If collected by farmers, fecal samples were packed in sealed bags, delivered to the laboratory at a temperature as close to room temperature as possible, and examined within 24 h after collection. Cooling packs were used only in the summer when air temperature exceeded 30°C. Fecal egg count (FEC) was determined using the modified McMaster technique with an analytical sensitivity (limit of detection) of 50 eggs per gram (epg) according to the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (8, 9).

***Haemonchus contortus* larval cultures.** Larval cultures were prepared for each herd by mixing 5 g of feces col-

lected from each animal into one pool, and incubated in the dark for 7 days at 26°C. After baermannization, 100 L3 from each pool were identified to the genus/species level in the microscopic examination according to van Wyk and Mayhew (42), and the percentage of each nematode genus/species was calculated.

Genomic DNA was extracted from the entire larval sample including various number of L3 belonging to various species of GIN. The concentration of extracted DNA in the sample was measured in the NanoDrop One spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). The NucleoSpin DNA Stool kit (Macherey Nagel, Germany) was used, according to the manufacturer's manual, as this DNA extraction kit has been shown to produce the highest amounts of ITS2 amplicon copies and show the lowest variability (19). The DNA was eluted in 100 µl of the elution buffer and the DNA samples were stored at –20°C until further analyses.

The presence of DNA of *H. contortus* was confirmed in collected samples using the real-time PCR. The assay was performed using the HaeCon dtec-qPCR Monodose Kit with internal control (Genetic PCR Solutions, Spain) according to the manufacturer's instructions. Each Target Species MONODOSE qPCR tube contained a dehydrated reaction mix including specific primers and probe, DNA polymerase, dNTPs, and buffer. For each reaction, 6 µL of template DNA and 14 µL of RNase-free water were added to reconstitute the mix to a final volume of 20 µL. Reactions were performed on a LightCycler 480 II system (Roche, Switzerland). Thermal cycling consisted of an initial activation step at 95°C for 2 min, followed by 40 amplification cycles comprising denaturation at 95°C for 5 s, and hybridization/extension with fluorescence data collection at 60°C for 20 s. Each run included a negative control (no-template control), a positive control, and a sample inhibition control to ensure assay sensitivity, specificity, and the absence of PCR inhibitors in the tested samples. The real-time PCR result was considered positive if the threshold cycle (Ct) was < 35.

Only larval cultures confirmed to contain L3 of *H. contortus* by both microscopic examination and the real-time PCR were included in the study.

Quantification of F200Y single nucleotide polymorphism. Two molecular methods were used for determination of the percentage of alleles of the β-tubulin isotype-1 gene which contained F200Y SNP – mdPCR and pyrosequencing. Each sample was analyzed once by each molecular method, and no replicate analyses were performed.

The mdPCR assay used allele-specific hydrolysis probes designed to distinguish between wild-type and mutant alleles and was optimized for DNA extracted from larval cultures. Two probe mixes were designed: one for the wild-type allele (Hc-β-tub-200WT), which included the forward primer 5'-TCGTGGAACCCTACAATGCT-3', the reverse primer 5'-TCAAAGTGCGGAAGCAGATA-3', and a FAM-labeled probe 5'-AACACCGATGAAACATTCTGTATTGAC-3'; and one for the mutant allele (Hc-β-tub-200 MT), using the same primers and a HEX-labeled probe 5'-AACACCGATGAAACATACTGTATTGAC-3'. Reactions were carried out using the QIAcuity Probe PCR Kit (QIAGEN) in Nanoplate 8.5K 24-well plates according to the manufacturer's instructions. An 8.5K in the name means that each sample is broken into 8500 partitions of volume of 1.41 nL. Each 12 µL reaction included the QIAcuity Probe PCR Master Mix, allele-specific primers and probes at final concentrations of 0.8 µM for each primer and 0.4 µM for each probe, template DNA, and RNase-free water. Reaction mixes were assembled in a standard PCR plate, template DNA was added, and the reactions were transferred to the nanoplate, sealed, and processed on the QIAcuity Digital PCR system. Thermal cycling was performed with an initial denaturation at 95°C for 2 min, followed by 45 cycles consisting of denaturation at 95°C for 20 s and annealing/extension at 60°C for 45 s. Fluorescence detection was performed automatically by the QIAcuity instrument after amplification. Imaging was carried out with exposure times of 500 ms for both the FAM and HEX channels. No-template controls (NTCs), as well as wild-type-only and mutant-only controls, were included in each run to confirm assay specificity. The wild-type control consisted of DNA extracted from L3 larvae of the anthelmintic-susceptible ISE strain of *H. contortus* (HCS; Moredun nomenclature Mhco3), whereas the mutant control consisted of DNA extracted from L3 larvae of the White River strain (HCR; Moredun nomenclature Mhco4), resistant to benzimidazoles, closantel, rafoxanide, and ivermectin. Both control isolates were kindly provided by Prof. Marián Várady (Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 040 01 Košice, Slovakia). Allele detection was based on the number of fluorescent-positive partitions observed in each channel, with the FAM signal corresponding to the wild-type allele and HEX to the mutant allele. Data were analyzed using the QIAcuity Software Suite (QIAGEN, Germany), and thresholds were manually adjusted to ensure clear separation between positive and negative partitions.

Pyrosequencing was performed using a PyroMark PCR Kit (QIAGEN, Germany). Each 25 µl reaction consisted of 12.5 µl PyroMark PCR Master Mix (QIAGEN, Germany), 0.2 mM biotinylated primer, 0.4 mM non-biotinylated primer, 2.5 µl CoralLoad Concentrate, and 5 µl of genomic DNA extracted from pooled larval cultures. Cycling conditions were: initial denaturation and polymerase-activation step at 95°C for 15 min, followed by 40 cycles at 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min, and a final elongation step at 72°C for 10 min. β-tubulin isotype-1 was amplified using the forward primer HcPy2PCR for (5'-GACGCATTCACTTGGAGGAG-3') and a biotinylated reverse primer HcPy2PCR Rev (5'Biotin-CATAGGTTGGATTTGTGAGTT-3'). Gel electrophoresis was used to verify template DNA amplification, with 5 µl of the PCR product running on a 2% agarose gel. The assays were carried out according to the manufacturer's protocol using PyroMark Q48 Autoprep (QIAGEN, Germany), PyroMark Q48 Advanced reagents (QIAGEN, Germany) and sequencing primer HcPyIso1Seq198_200 (5'-GGTAGAGAACACCGATG-3'). Next, based on the relative peak heights for the different alleles on the pyrograms, a percent quantification for the frequency of each SNP was determined.

Statistical analysis. Numerical variables (the percentage of *H. contortus* L3 in the larval culture, Ct of the real-time

PCR, and frequency of F200Y SNP by mdPCR and pyrosequencing) were examined for normality of distribution using the normal probability Q-Q plots and the Shapiro-Wilk W test. As normality assumption was violated in the case of all numerical variables ($p < 0.001$), they were summarized as the median, interquartile range (IQR), and range. The agreement in frequency of F200Y SNP between mdPCR and pyrosequencing was determined at two levels: i) agreement between quantitative results (exact frequencies) was examined using the concordance correlation coefficient (R_c,) (26, 43); moreover, quantitative results were compared between assays with Wilcoxon's signed-rank test and the median difference with 95% confidence interval (CI 95%) calculated according to the exact method (1) was calculated; the 95% Bland-Altman limits of agreement (LoA) were estimated to determine the expected magnitude of disagreement between individual tested samples; ii) chance-corrected agreement between arbitrary categorical classification of frequency of F200Y SNP into 0%-25%, 26%-50%, 51%-75%, and > 75% was examined using the Gwet's AC_2 coefficient (unweighted and weighted using linear and quadratic function) (16, 40). Chance-corrected agreement was classified as poor to fair if the AC_2 coefficient was < 0.40, moderate to good if the AC_2 coefficient was 0.40-0.75, and very good if the AC_2 coefficient was > 0.75 (14). The correlation between difference in frequency of F200Y SNP and selected numerical variables was examined using Spearman's rank-order correlation coefficient (R_s) with CI 95% calculated according to the method based on Fisher's z-transformation (1). The CI 95% for proportions were calculated using Wilson's score method (1). All p-values were two-sided, and the significance level (α) was set at 0.05. Statistical analysis was performed in TIBCO Statistical 13.3 (TIBCO Software Inc., Palo Alto, CA).

Results and discussion

The percentage of *H. contortus* L3 in the larval culture ranged from 1% to 100% with the median of 63% (IQR: 23-91%). The real-time PCR confirmed the presence of *H. contortus* DNA in all 63 larval cultures with Ct between 9 and 34 (median Ct: 14; IQR: 12-18).

The mdPCR assay yielded significantly higher F200Y SNP frequency values than pyrosequencing. Although frequency of F200Y SNP ranged from 0% to 100% in both methods, it was significantly higher in mdPCR assay (median: 100%, IQR: 94-100%) than in pyrosequencing (median: 86%, IQR: 74-92%; $p < 0.001$) (Fig. 1). Frequency of F200Y SNP equaled 100% in 45/63 samples (71.4%) according to mdPCR while in only 1/63 samples (1.6%) according to pyrosequencing.

The agreement in quantitative results between both methods was only moderate ($R_c = 0.52$; CI 95%: 0.46, 0.57) (Fig. 2a). The median difference in frequency of F200Y SNP between mdPCR assay and pyrosequencing was 10% (CI 95%: 7%, 13%). The IQR was from 3% to 18%; however, the most extreme differences in frequency of F200Y SNP were –98% (1 larval culture negative in mdPCR, 98% F200Y frequency in pyrosequencing) and > 90% (4 larval cultures with 100% F200Y frequency in mdPCR and < 10% in pyrosequencing).

The difference in frequency of F200Y SNP between mdPCR assay and pyrosequencing was not correlated either with the percentage of *H. contortus* L3 in the larval culture ($R_s = 0.14$; CI 95%: –0.11, 0.37; $p = 0.275$) or the Ct of real-time PCR for *H. contortus* ($R_s = –0.03$;CI 95%: –0.28, 0.22; $p = 0.803$).

The agreement between mdPCR assay and pyrosequencing assuming 4-category classification of F200Y SNP frequency was moderate to good. The observed agreement was 82.5% (CI 95%: 71.4%, 90.0%; 52/63 L3 cultures) and the chance-corrected agreement expressed using the Gwet's AC_2 coefficient was 0.789 (CI 95%: 0.673, 0.906) when unweighted, 0.772 (CI 95%: 0.635, 0.910) in the case of linear weighting, and 0.792 (CI 95%: 0.660, 0.925) for quadratic weighting.

Bland-Altman analysis further demonstrated substantial variability between the two methods. The Bland-AltmanLoAranged from –34% (CI 95%: –44%, –23%) to 63% (CI 95%: 52%, 73%) (Fig. 2b); however, due to very asymmetric distribution of the differences between mdPCR and pyrosequencing this estimation was of limited value. In fact, the range had to span from –39% (2.5th percentile) to 94% (97.5th percentile) to cover 95% of the observed differences.

Our study showed limited agreement between mdPCR and pyrosequencing in terms of F200Y SNP quantification. Compared with pyrosequencing, mdPCR

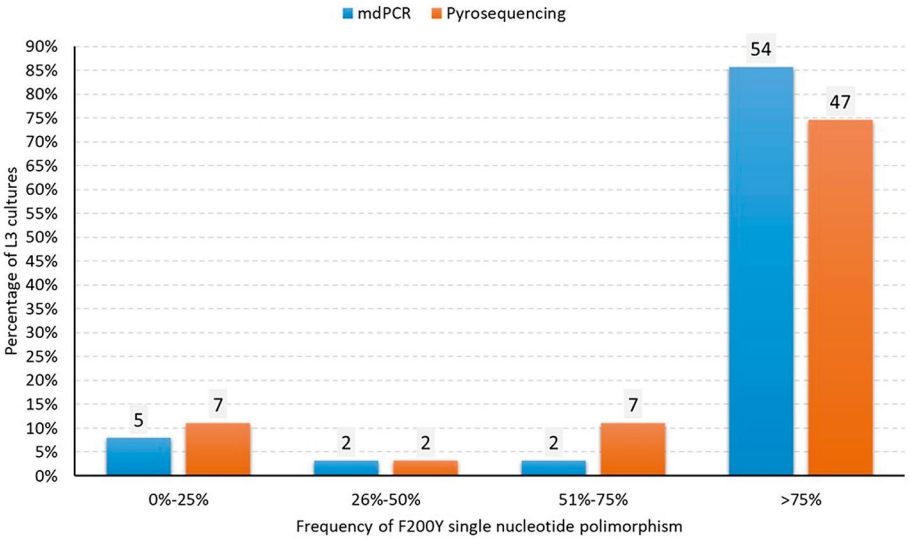

Fig. 1. Frequency distribution of the percentage of F200Y-mutated alleles in the third-stage larvae cultures according to the microchamber-based digital PCR (mdPCR) and pyrosequencing

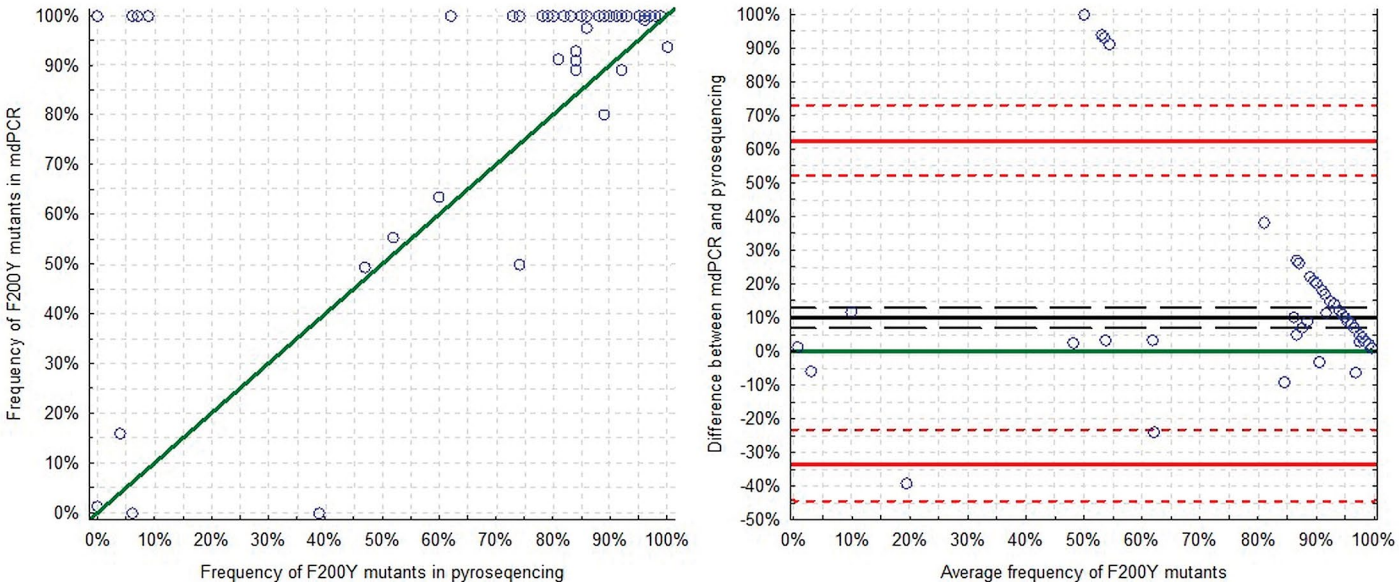

Fig. 2. Agreement between the frequency of F200Y-mutated alleles detected using microchamber-based digital PCR (mdPCR) and pyrosequencing. a) The line of equality plot: green solid line is the line of equality. b) The Bland-Altman limit of agreement plot: green solid line is the line of equality, black solid line is the median difference with 95% confidence intervals (dashed black lines), and red solid lines are limits of agreement with 95% confidence intervals (dashed red lines).

yielded higher F200Y SNP allele frequency values, by the median value of 10% (CI 95%: 7%, 13%), with very wide LoA (approx. from –40% to 60%).

Even though pyrosequencing is only one of molecular techniques available for quantification of SNPs and historically was not the first laboratory method used for this purpose, it is currently the mainstay of molecular BZ resistance diagnostics. Therefore, it usually serves as a reference standard with which other methods are compared in order to estimate their accuracy and precision (6, 22, 33, 35). The only so far published comparison of dPCR technique (precisely – ddPCR) with pyrosequencing has shown almost perfect concordance ($R_c = 0.987$) (4). However, here it is worth noting that, while belonging to the same family of technologies, there are key differences between the ddPCR used by Baltrušis et al. (3, 4), and the work carried out in the current study. This relates primarily to the method and format of the partitioning process using oil-based nanodroplet formation in the case of ddPCR, rather than the commercial microplate assay format used in the current study, albeit with the same primers as described by Baltrušis et al (3, 4). It is furthermore, worth noting that Baltrušis et al. (4) used an in-house developed protocol (3) for both the studies carried out in relation to the use of dPCR for the detection of the F200Y SNPs. Moreover, the same authors compared the performance of ddPCR for F200Y in *H. contortus* with the next-generation sequencing (NGS), also with good results indicating high agreement (3). In this context, while we only achieved moderate agreement of mdPCR with pyrosequencing, this is not unexpected due to the novelty of the technique, and the necessity for ongoing optimization. It is, nonetheless, critical that studies such as these are carried out to validate new technologies for the detection of anthelmintic resistance (21).

Frequency of F200Y SNP in the mdPCR assay is calculated as the proportion from the sum of the estimated number of copies of F200Y SNP-bearing alleles and the estimated number of copies of wild-type alleles. The numbers are estimated using the Poisson distribution (12, 30) and directly depend on the number of partitions in which PCR reaction occurred. In our results, the estimated number of copies of wild-type alleles was relatively low. If this had resulted from some technical issues which hindered PCR reaction (low affinity of primers, inhibition, etc.), the frequency of F200Y SNP would have been falsely overrated. Approximately 70% of tested samples had 100% frequency of F200Y SNP according to mdPCR which means that not a single copy of wild-type allele was detected. This may represent an overestimation on the part of mdPCR; however, it may also be possible that this represents a highly homozygous population, with frequencies of the F200Y allele exceeding 90% in many herds. There is, however, a potential precedent for this as recent work has demonstrated a high herd level prevalence for the F200Y SNP in Polish goat herds (29), and recently carried out mixed amplicon metabarcoding revealed a relative abundance of > 95% F200Y in many of the herds examined (own data unpublished).

However, due to the lack of both standardized comparisons of different dPCR methods (15), and a lack overall of detailed allele frequency data for BZ resistance SNPs in many countries, including Poland, substantial further work is necessary to determine if these results represent a true improvement in sensitivity of the mdPCR, or technical limitations of pyrosequencing.

On the other hand, dPCR appears to be more resistant to various inhibitors than qPCR (5, 11). Moreover, determination of the proportion of SNP-bearing alleles is performed in pyrosequencing by visual comparison of relative peak heights for different mutated and wild-type alleles (35) and may be suspected of limited precision. Therefore, purely quantificational methods such as dPCR (both ddPCR and mdPCR) could be expected to outperform pyrosequencing.

Summing it all up, drawing any final conclusions regarding the advantage of any of the two F200Y SNP quantification methods from our results is impossible because the agreement analysis yields no grounds for evaluation of accuracy. The only undeniable observation is that the agreement between the two F200Y SNP quantification methods was only moderate. It should prompt further studies investigating accuracy of currently available methods for quantification of F200Y SNP frequency.

The mdPCR and pyrosequencing show only moderate agreement in determination of F200Y SNP frequency in larval cultures. The mdPCR yields significantly higher values of F200Y SNP frequency than pyrosequencing. The level of agreement does not seem to be associated with the percentage of *H. contortus* L3 in the larval culture. Although mdPCR represents a promising tool for molecular detection of benzimidazole resistance, the observed differences between the methods indicate that the two techniques should not be used interchangeably for routine diagnostics without further validation. Further studies are warranted to clarify the source of the observed discrepancies.

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Ethics declarations: All procedures were in line with Polish law regulations. The study was carried out in accordance with the standards recommended by the EU Directive 2010/63/EU for animal experiments and Good Laboratory Practice and The Act of the Polish Parliament of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes (Journal of Laws 2015, item 266). According to Polish legal regulations included above no formal ethics consent was required for this study except for the informed consent of participants.

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