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Assessment of the *F200Y* mutation frequency in the β tubulin gene of *Haemonchus contortus* following the exposure to a discriminating concentration of thiabendazole in the egg hatch test

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ABSTRACT

The ruminant livestock production sector is under threat due to the infections with gastrointestinal nematode parasites and the subsequent development of anthelmintic resistance. One of most common and pathogenic species in small ruminants is *Haemonchus contortus*. The ability to control the infections with this and other gastrointestinal nematodes relies heavily on the use of anthelmintic drugs. Although resistance to all major classes of anthelmintics has been shown in *H. contortus*, the precise mechanism of resistance acquisition is only known for benzimidazoles. *F200Y* (TAC) is a common point mutation in the isotype 1 β tubulin gene which is associated with an effective increase in the resistance towards benzimidazole drugs. Here, we show the utility of using this mutation as a marker in a droplet digital PCR assay to track how two *H. contortus* laboratory strains, characterized by different resistance levels, change with respect to this mutation, when subjected to increasing concentrations of thiabendazole. Additionally, we wanted to investigate whether exposure to a discriminating dose of thiabendazole in the egg hatch test resulted in the death of all *H. contortus* eggs with a susceptible genotype. We found the *MHco5* strain to maintain an overall higher frequency of the *F200Y* mutation (80–100%) over all drug concentrations, whilst a steady, gradual increase from around 30%–60% was observed in the case of the *MHco4* strain. This is further supported by the dose-response curves, displaying a much higher tolerance of the *MHco5* strain (LD_{50} = 0.38 μg/ml) in comparison to the *MHco4* strain (LD_{50} = 0.07 μg/ml) to the effects of thiabendazole. All things considered, we show that the *F200Y* mutation is still a viable and reliable marker for the detection and surveillance of benzimidazole drug resistance in *H. contortus* in Europe.

1. Introduction

Infections with gastrointestinal nematodes (GINs) in small ruminants, pose an array of problems to the industry, mainly in the form of stunted growth reducing yields and the increasing costs of treatment. One of the most pathogenic and well-studied GIN species in sheep and goats is the abomasal nematode *Haemonchus contortus* (Emery et al., 2016).

One particular feature of this species is its capacity to rapidly develop resistance to drugs across relatively short periods of time (Coles et al., 2005; de Albuquerque et al., 2017). To date, resistant field isolates to all three most commonly used anthelmintic drug classes, i.e. macrocyclic lactones, imidazothiazoles/tetrahydropyrimidines and benzimidazoles (BZ), are found to be widely distributed across most the world: in Europe (Rose et al., 2015), Australia (Playford et al., 2014), South Africa (Tsotetsi et al., 2013), Asia (Han et al., 2017), the Americas (Barrere et al., 2013; Jaeger and Carvalho-Costa, 2017).

Thus, the growing importance of anthelmintic resistance has led to an increased need for reliable and standardised methods of detection of worm populations with reduced anthelmintic susceptibility in routine field diagnostics. Although options range from various *in vitro* (Coles et al., 2006) to molecular assays (von Samson-Himmeltjerna et al., 2009;

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Roeber et al., 2012), *in vivo* tests, such as the faecal egg count reduction test, have been used as the golden standard method for the detection of anthelmintic resistance for many decades. The egg hatch test (EHT) and larval development test are the two most frequently used *in vitro* methods for the purpose of resistance detection. In the case of both of these tests, sensitivity can be substantially enhanced by using a discriminating dose (Čudeková et al., 2010). However, it is not known whether the fraction of the surviving first stage larvae (L1), which hatched at the discriminating dose in the EHT, are only of the resistant genotype.

Soon after the introduction of the broad-spectrum anthelmintic drugs, such as BZs, subsequent emergence of resistance was observed (Kotze and Prichard, 2016). Thus, research into the mechanisms of resistance towards the drugs of this class were initiated. As a result, three single nucleotide polymorphisms, SNPs, in the isotype 1 β tubulin gene, leading to an altered amino acid composition of the mature protein in *H. contortus* were identified and linked with increased resistance towards otherwise lethal effects of the drug (Kwa et al., 1994; Silvestre and Cabaret, 2002; Ghisi et al., 2007).

The first ever discovered and, thus, studied for the longest time, point mutation *F200Y* (TTC→TAC), has been reported to be the commonly encountered mutation type, conferring resistance to BZ drugs in *H. contortus*, found in European sheep (Gallidis et al., 2012; Redman et al., 2015; Ramünke et al., 2016; Baltrušis et al., 2018; Sallé et al., 2019). Furthermore, the increased frequencies of this mutation has been shown to be associated with the increased resistance status of the isolate (Čudeková et al., 2010; Yilmaz et al., 2017).

With the recent advancements in diagnostic technologies, method platforms such as droplet digital (dd)PCR prove to be advantageous in creating rapid and reliable screening assays for mutation detection and quantification (Uchiyama et al., 2016; Srisutham et al., 2017; Luo and Li, 2018). Here, we utilized the previously developed ddPCR assay for *F200Y* mutation detection (Baltrušis et al., 2018), to estimate the frequencies of the mutated (*F200Y* mutation possessing) β tubulin allele in the L1 (after the eggs had been hatched under gradually increasing thiabendazole (TBZ) concentrations) of two laboratory maintained strains of *H. contortus*, *MHco4* and *MHco5*, exhibiting distinct resistance statuses. Both strains have been previously shown to not be polymorphic for the 198 codon and only the *MHco4* strain displayed low levels of the polymorphism in the 167 codon (7.2%) (von Samson-Himmeltjerna et al., 2009). Thus, the aim of this experimental study was to evaluate if the L1 of *H. contortus*, which hatched at doses higher than the discriminating (0.1 μg/ml) in the EHT possessed only the resistant genotype (homozygous for *F200Y*) and overall describe the changes in the frequency of the mutant allele, for two differently resistant strains.

2. Materials and methods

2.1. Parasite strains and egg hatch test

Two laboratory-maintained strains that have been passaged for multiple generations in naive 5–6-month-old lambs (two per isolate), which were housed individually. The lambs were infected orally with approximately 5000 third stage *H. contortus* larvae and EHTs were performed 4–6 weeks later. The *MHco5* strain is a previously described inbred, resistant strain (Roos et al., 2004). The *MHco4* strain corresponds to a multi-resistant (ivermectin, BZs, rafoxanide, closantel) field isolate, retrieved from South Africa (Van Wyk et al., 1999) and displaying low to moderate level of BZ-drug resistance (Čudeková et al., 2010).

The EHTs were carried out as follows: *H. contortus* eggs were isolated from freshly collected fecal samples from experimentally infected sheep. Eggs were collected by sieving through three stacked sieves with apertures of 250, 100 and 25 μm, respectively. The material retained on the 25 μm sieve was washed with water, sedimented and made to float with saturated sodium chloride (Coles et al., 1992). The EHTs, for the determination of dose-response relationships between the concentration of TBZ (μg/ml) and the inhibited hatching of two *H. contortus* strains (*MHco4* and *MHco5*), were subsequently performed according to Coles et al. (2006), using 5 different drug concentrations (0.05, 0.1, 0.3, 0.5 and 1.0 μg/ml of TBZ in DMSO) and a negative control (containing 10 μL of DMSO). The EHTs were carried out independently, in duplicate fashion. Eggs and hatched larvae were counted using an inverted microscope to determine LD_{50} values.

To obtain larvae for molecular analysis, egg suspension (100 eggs/ml) was carefully transferred onto wells on the top of the 25 μm migration sieves used in larval migration inhibition tests described by Demeler et al. (2010). Four concentrations of the drug (0.05, 0.1, 0.3 and 0.5 μg/ml of TBZ in DMSO) were added to 24-well plates containing the egg suspensions. Two wells in each plate, containing 10 μL of DMSO instead of TBZ, were used as negative controls. The plates were incubated at 27 °C for 48 h, then the migration sieves with unhatched eggs were removed and larvae from each concentration were collected into tubes. Approximately 300 larvae for each concentration were collected and stored at −20 °C in 70% ethanol. The test was carried out in triplicates.

2.2. DNA extraction

Genomic DNA was extracted from approximately 300 hatched L1 per studied concentration using the NucleoSpin tissue kit (*Macherey Nagel*, Germany), following the manufacturers guidelines.

2.3. Droplet digital PCR

Droplet digital PCR was run on extracted DNA samples using a previously described set up for the identification of the fractional abundance of the *F200Y* mutation in the isotype 1 β tubulin gene (i.e. mutant allele frequency) (Baltrušis et al., 2018). In short, sample reactions were assembled in 96-well plates (final volume 22 μL), following the guidelines issued by the manufacturer (BioRad). Droplets were generated and dispensed into a new 96-well plate using an automated droplet generator (QX200, BioRad). The new plate was heat sealed and transferred into a thermal cycler (MyCycler™ Thermal Cycler). The PCR conditions were as follows: a single cycle of 95 °C for 10 min, 40 cycles of 94 °C for 30 s and then 58 °C for 1 min, followed by a single cycle of 98 °C for 10 min to deactivate the enzyme. After the amplification step, the plate containing the droplets was loaded into the droplet reader (QX200, BioRad) and further analyzed using QuantaSoft (v1.April 7, 0917) software, which generates DNA copy measurements, allele frequencies and error bars based on Poisson statistics (refer to the Applications Guide http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). The output from QuantaSoft was then visualized using the *ggplot2* package (v3.2.1) for R software (v3.6.2).

2.4. Statistical analysis and genotype frequency calculations

To determine LD_{50} in the EHT the data was analyzed by a logistic regression model (Dobson et al., 1987). The frequency of the mutation *F200Y* data for the two strains was analyzed using the R software (v.3.6.2) and package *stats* (v.3.6.2). Command *aov* was utilized to conduct a one-way ANOVA and *TukeyHSD* (Tukey Honest Significant Differences) to estimate the probabilities of differences between sample groups. The *F200Y* mutation frequency values for the *MHco4* strain were *logit* transformed to improve the normality of the data, as determined by inspecting quantile-quantile plots (data not shown). Genotype frequency data was calculated for each strain (MHco4 and MHco5) of *H. contortus* L1, hatched unexposed to the effects of TBZ. Mean mutation *F200Y* frequencies, obtained from three biological replicates, were used to obtain genotype frequencies (SS – both susceptible alleles, SR – susceptible and mutated alleles and RR – both alleles mutated), assuming Hardy-Weinberg equilibrium. Subsequent results were visualized using

the *ggplot2* package (v3.2.1) for R software (v.3.6.2).

3. Results and discussion

By examining the L1 of two *H. contortus* strains – *MHco4* and *MHco5*, hatched under gradually increasing concentrations of TBZ (0–0.5 μg/ml), stark overall differences in egg hatching (Fig. 1) and the *F200Y* mutation frequencies between the strains were observed (Fig. 2).

The dose-response relationships between the concentration of TBZ and the inhibited hatching of the L1 of *H. contortus* strains (*MHco4* and *MHco5*) in the EHT are shown in Fig. 1 (A, B). The *MHco5* strain demonstrated markedly higher resistance towards the effects of TBZ (LD_{50} = 0.38 μg/ml), in comparison to the *MHco4* strain (LD_{50} = 0.07 μg/ml). Having assumed that larvae hatched unexposed to selection by TBZ would be in Hardy-Weinberg equilibrium, we calculated the genotype frequencies for each strain using mutation *F200Y* frequency measurements (Fig. 1 C and D). The obtained genotype frequencies help corroborate the LD_{50} values and patterns observed in dose-response relationship curves for both strains. Thus, the higher the frequency of the SS (susceptible-susceptible) and likely SR (susceptible-mutated) genotypes, the more immediate the effects of the TBZ as well as lower LD_{50} values are seen. In addition, since, on average, 32% (*MHco4*) and 90% (*MHco5*) of resistant alleles were observed in pooled individual L1 (Fig. 2), hatched at the discriminating dose, the use of the LD_{50} criterion (0.1 μg/ml TBZ) as a threshold for determining resistance in the EHT (Coles et al., 1992) was found to be inappropriate in this study and may lead to false-negative observations.

An approximate two-fold increase in the frequency of the β tubulin allele carrying *F200Y* mutation in the *MHco4* strain is observed when comparing the control and the 0.5 μg/ml concentration sample groups (p = 0.001) (Fig. 2; on the left-hand side). While no statistically significant difference was found between the groups of the *MHco5* strain, regardless of whether the data was *logit, log* or non-transformed (Fig. 2; on the right-hand side). The frequency of the mutation *F200Y* allele in this strain fluctuated between 80% and 100% but, overall, stayed overwhelmingly high, irrespective of the drug concentration the eggs were subjected to.

On the other hand, for the *MHco4* strain (Fig. 2), statistically significant increases in the *F200Y* mutation frequency were observed between the 0.5 μg/ml concentration and both – 0.05 μg/ml and 0.1 μg/ml sample-groups (p = 0.001 and p = 0.003 respectively), except for 0.3 μg/ml group (p = 0.16). The 0.3 μg/ml sample group showed a significant difference in terms the mutant allele frequency when compared to the 0.05 μg/ml group (p = 0.04) and an indication of difference when compared to the control group (p = 0.06). However, the effect of the drug does not produce pronounced effects at concentrations 0.05 and 0.1 μg/ml as no statistically significant differences in frequencies were found between these and the control samples (p = 0.99 and p = 0.97, respectively). Even though the data were *logit* transformed, the raw values generate almost the exact same results, however, the significance between the sample 0.3 μg/ml and the control groups increases (p = 0.055). It is noteworthy that the third replicate in the 0.1 μg/ml concentration group was an outlier. If it was to be removed, statistically significant differences would be observed between all sample groups

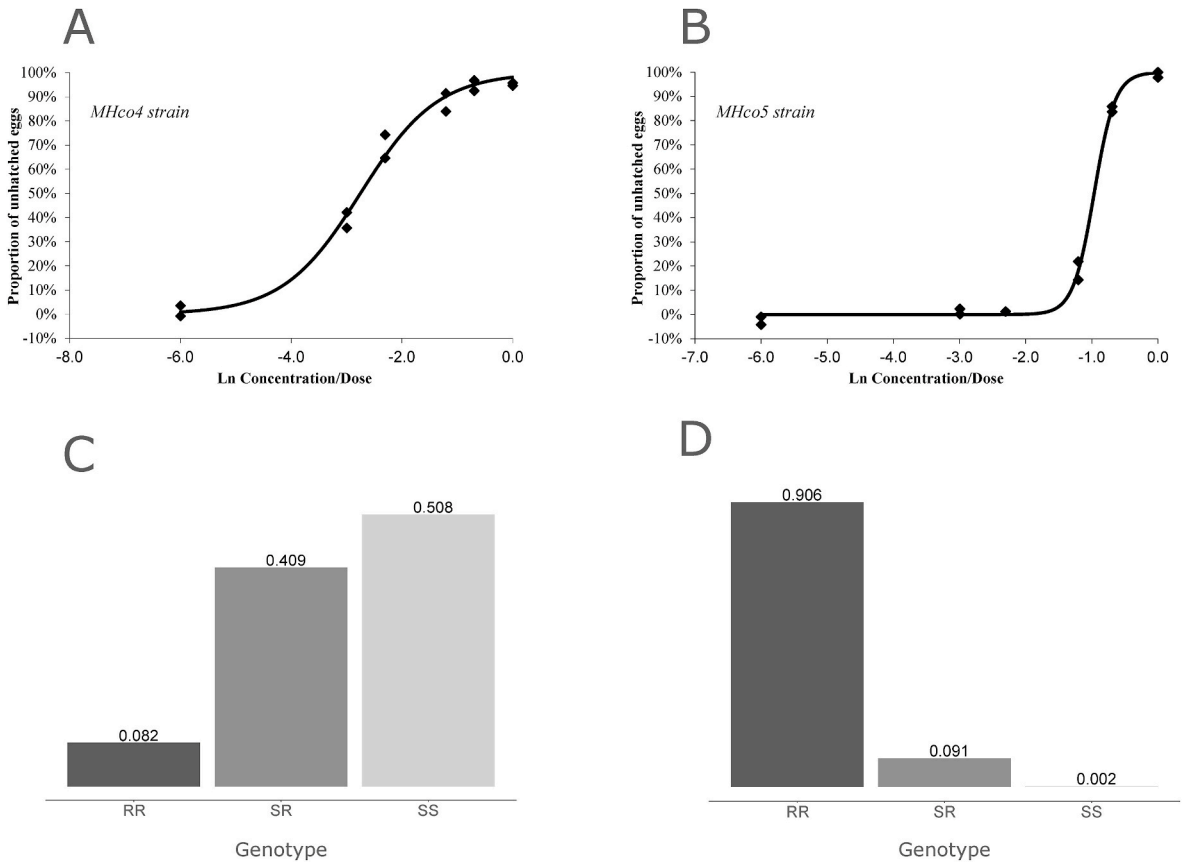

Fig. 1. Dose-response relationship of thiabendazole (TBZ) against two strains of *Haemonchus contortus* in the egg hatch test (EHT) after 48 h of incubation at 27 °C (A, B) and genotype frequencies (SS – susceptible, RS – heterozygous and RR – resistant) for mutation *F200Y* in the β-tubulin gene, obtained for each strain (MHco4 – C, MHco5 - D) by examining unexposed, hatched L1. Larvae, hatched under normal conditions (48 h, 27 °C) and unexposed to the effects of TBZ, from both strains, were tested (in biological triplicates) for mutation *F200Y* frequencies using the custom ddPCR set-up (Baltrušis et al., 2018) and mean allele (wild-type/susceptible and mutated/*F200Y* mutation having) frequencies were used to determine genotype frequencies, assuming Hardy-Weinberg equilibrium. In Fig. 1C and D, X axes denote the genotype, whilst the fraction of each genotype (total = 1) in both strains is displayed above the respective bars.

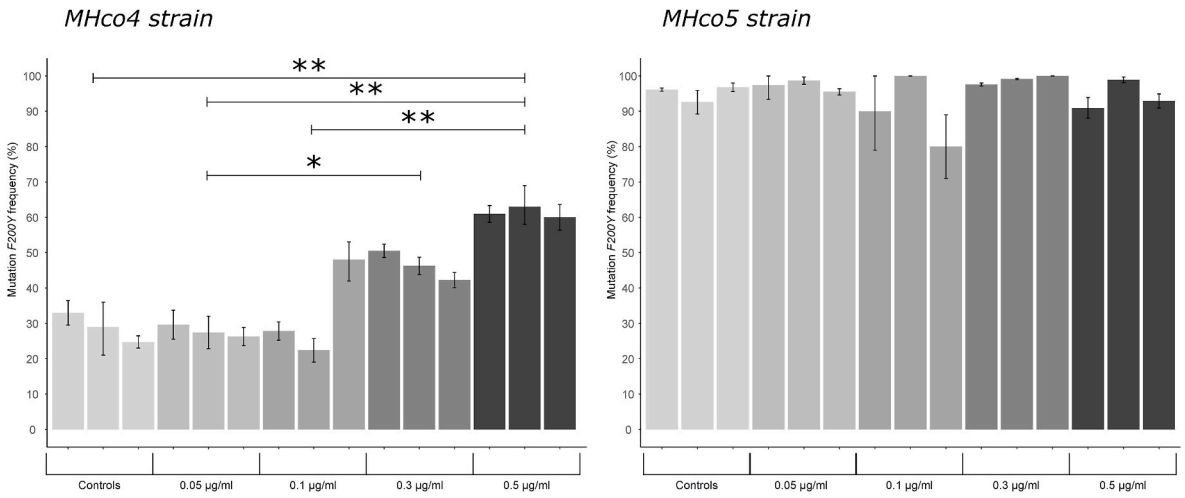

Fig. 2. The frequency of the mutation *F200Y* across different drug concentrations (0–0.5 µg/ml) in two *H. contortus* strains (*MHco4* on the left, *MHco5* on the right). Statistically significant differences between the groups for the *MHco4* strain are shown as either ** ($p \leq 0.01$) or * ($p \leq 0.05$). Samples were run in biological triplicates. Error bars represent 95% Poisson confidence interval values. "Controls" refer to the *F200Y* mutation frequencies obtained when larvae were hatched unexposed to the effects of TBZ.

and 0.5 µg/ml ($p < 0.001$ for C, $p < 0.001$ for 0.05, $p < 0.001$ for 0.1, $p = 0.002$ for 0.3), as well as 0.3 µg/ml ($p < 0.001$ for C, $p < 0.001$ for 0.05, $p < 0.001$ for 0.1) drug concentration groups, but not between the control, 0.05 and 0.1 µg/ml groups ($p = 0.99$, $p = 0.88$ and $p = 0.7$).

These results clearly indicate that the two *H. contortus* strains used in this study possess different frequencies of the resistant allele in populations to start with. The fractional abundance of the mutated allele in the control samples (Fig. 2) was between 25 and 33% for the *MHco4* strain and 93–97% for the *MHco5* strain. These results agree well with egg hatching at the discriminating concentration of TBZ (0.1 µg/ml) and support the previously drawn conclusions by (Čudeková et al., 2010), regarding good phenotype-genotype agreement for BZ resistance among different *H. contortus* isolates. It is also plausible to conclude that *in vitro* selection with TBZ in the EHT significantly increased the frequency of the mutation *F200Y* in the *MHco4*-strain, whereas the frequency of the mutation remained unchanged in the *MHco5*-strain, as it naturally (without any selection) already contains a high percentage of the resistance-encoding allele. The statement made by Coles et al. (2006), indicating that the concentration of 0.1 µg/ml of TBZ in the EHT would prevent the hatching of 99% of susceptible eggs was not confirmed in our study. This is based on the observation that the surviving pool of individual L1 of the MHco4 strain hatched at 0.5 µg/ml concentration of TBZ (5 times more than the discriminating dose) and retained around 30% of the susceptible allele. However, subsequent investigation is needed to experimentally confirm whether these, high drug concentration (>0.1 µg/ml) surviving L1, are homozygous or heterozygous (or both) for the susceptible allele. What is more, it would be beneficial to further estimate the impact of the mutation F167Y, previously only described at low frequency (i.e. 7.2%) in the *MHco4* strain (von Samson-Himmeltjerna et al., 2009) on the final allele quantifications. Finally, other resistance mechanisms, such as P-glycoprotein mediated, increased drug efflux (Kerboeuf et al., 2003) could also contribute to the BZ-resistance phenotype in the examined strains. Nevertheless, the clear associations between the dose-responses in the EHT, initial genotype frequencies and changes in the *F200Y* mutation frequencies do suggest that these, alternative mechanisms, if at all present, have only a minor significance in the MHco4 and MHco5 strains.

Taken together, these results seem to indicate the opposite of what's been recently established by (Yilmaz et al., 2017). Contrary to their study, but in agreement with von Samson-Himmeltjerna et al. (2009), here, *MHco5* strain seemed to display persistently higher overall mutation frequency levels among sample groups in comparison to the *MHco4* strain, which, on the other hand, was more responsive to the increase in the concentration of TBZ. This agrees well with the findings from the dose response curves, displaying increased relative resistance ($LD_{50} = 0.38$ µg/ml) to low TBZ concentrations during hatching in the case of the *MHco5* strain and a more linear decline in the population of the hatched *MHco4* strain larvae ($LD_{50} = 0.07$ µg/ml). These findings further compound the association between increased resistance to BZ drugs and the higher proportion of individuals possessing the homozygous genotype for the *F200Y* mutation in the population. Although the *MHco4* strain used in this study originated from the same source as the one described by (Yilmaz et al., 2017), it could be argued that the observed differences in the frequencies of the *F200Y* mutation between the strains are related to the conditions under which they were maintained. The *MHco4* strain described in our study has been maintained at the Institute of Parasitology in Kosice, Slovakia for more than decade without anthelmintic selection during passaging. Furthermore, in the study by (Čudeková et al., 2010), the authors described an average 46.5% frequency for the mutation *F200Y* among the *MHco4* strain isolates. This goes to show the significance of the conditions under which the strain is maintained to the genetic make-up of the community, especially when considering how polymorphic *H. contortus* is (Brasil et al., 2012; Yin et al., 2013; Sallé et al., 2019).

In conclusion, we displayed the capacity to utilize the most prevalent and well-studied *F200Y* mutation as the main marker for the estimation and evaluation of the degree of BZ drug resistance and change over distinct drug concentrations in two strains of *H. contortus* using the sensitive and precise ddPCR method. Furthermore, this study confirms the hatching of eggs with a susceptible or partially susceptible genotype at the discriminating dose during the EHT. However, further research is warranted to determine the degree to which *F200Y* mutation possessing heterozygotes exhibit BZ resistance and which secondary resistance mechanisms, e.g. increased drug metabolism (Yilmaz et al., 2017) or efflux (Kerboeuf et al., 2003), could contribute to the resistance phenotype.

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Declaration of competing interest

The authors of this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter discussed in this work.

CRediT authorship contribution statement

Paulius Baltrušis: Data curation, Formal analysis, Methodology, Visualization, Writing - original draft. **Michaela Komáromyová:** Writing - review & editing, Methodology. **Marián Várady:** Writing - review & editing, Supervision, Resources, Project administration. **Georg von Samson-Himmelstjerna:** Writing - review & editing, Conceptualization. **Johan Höglund:** Writing - review & editing, Conceptualization, Funding acquisition, Supervision.

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