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Benzimidazole resistance survey for *Haemonchus*, *Teladorsagia* and *Trichostrongyulus* in three European countries using pyrosequencing including the development of new assays for *Trichostrongyulus*

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**ABSTRACT**

Resistance to benzimidazoles (BZs) in trichostrongyloid nematodes is a worldwide problem for livestock production, particularly regarding small ruminants. Sensitive and reliable methods are required to assess anthelmintic resistance status. Currently available methods for BZ resistance detection can be divided into three main groups, *in vivo* (e.g. faecal egg count reduction test), *in vitro* (e.g. egg hatch assay) and molecular tests. Three single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin gene of various nematode species correlate with BZ resistance. While PCR-based methods have been reported for the three most economically important nematodes of sheep, namely, *Trichostrongyulus*, *Haemonchus* and *Teladorsagia*, pyrosequencing assays are so far only available for the latter two. Here, the design and evaluation of pyrosequencing assays for isotype-1 and isotype-2 β-tubulin genes of *Trichostrongyulus* are described. PCR fragments carrying the susceptible and corresponding resistant genotypes were combined in defined ratios to evaluate assay sensitivity and linearity. The correlation between the given and the measured allele frequencies of the respective SNPs (codons F167Y, E198A and F200Y) was very high. Pyrosequencing assays for *Haemonchus*, *Teladorsagia* and *Trichostrongyulus* were subsequently used for a BZ resistance survey, carried out in the three European countries, namely Ireland, Italy and Switzerland. Larval cultures obtained from field survey samples in 2012 and 2013 were used for pyrosequencing. The test was applied when the target species represented at least 10% of the sample. *Trichostrongyulus* and *Teladorsagia* were detected in all countries’ samples whereas *Haemonchus* was not detected in samples from Ireland. SNPs in isotype-1 associated with resistance were detected for all three species, with frequencies at codon F200Y far exceeding those at codons F167Y and E198A. Elevated SNP frequencies in isotype-2 of *Trichostrongyulus* were only rarely detected. Farms with BZ resistance-associated SNP frequencies above 10% were most often found in Switzerland followed by Ireland and Italy.

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1. Introduction

Of the main anthelmintic drug classes, the benzimidazoles (BZs) have been most widely used since their release/launch onto the market in the 1960s for helminth control in livestock. BZs represent a class of broad-spectrum anthelmintic substances including several chemical compounds which all share the same mode of action.
action. They are known to inhibit the polymerisation of α- and β-tubulin dimers to microtubules (Sangster et al., 1985), leading to interference with the formation of the cytoskeleton, the mitotic spindle and intracellular transport. This results in the disruption of the worm’s metabolism, and particularly, the inhibition of glucose uptake and intracellular transport leads to death of the worm (Lacey, 1988). Shortly after the introduction of BZs, resistance was detected (Conway, 1964), particularly in parasitic nematodes of small ruminants. The spread of BZ resistance has led to major problems in small ruminant farming industries worldwide (Kaplan, 2004; Howell et al., 2008; Papadopoulos et al., 2012; Torres-Acosta et al., 2012; Falzon et al., 2013). Of the currently available anthelmintics, the mechanism of BZ resistance has been intensively studied in the past decades. First, loss of function mutants (deletions) of the ben-1 gene were identified in an in vitro screening assay using Caenorhabditis elegans. All mutants successfully developing in the presence of the BZ benomyl were lacking functional ben-1 which encodes a β-tubulin in C. elegans (Driscoll et al., 1989). The first single nucleotide polymorphism (SNP) identified in a parasitic nematode correlating with BZ resistance was found in codon F200Y (TTC to TAC) of isotype-1 β-tubulin of Haemonchus contortus, leading to the expression of tyrosine instead of phenylalanine (Kwa et al., 1994, 1995). In vitro, two additional codons, codon F167Y (TTC to TAC) (Prichard, 2001) and codon E198A (GAA to GCA) (Chishti et al., 2007) have also been reported to be associated with BZ resistance. Until now, these three SNPs in the isotype-1 β-tubulin gene have been described for different parasitic nematodes of ruminants, such as H. contortus, Teladorsagia circumcincta, Ostertagia ostertagi and Cooperia oncophora (von Samson-Himmelstjerna et al., 2007) and Haemonchus placei (Chaudhry et al., 2014). Primarily, codon 200 was thought to play the most important role in BZ resistance, while some studies also reported elevated allele frequencies in codon F167Y (TTC to TAC) and E198A (GAA to GCA) (Silvestre and Cabaret, 2002; von Samson-Himmelstjerna et al., 2007; Chaudhry et al., 2015; Redman et al., 2015). Analyses of field populations are still relatively rare, but data obtained from surveys conducted in Eastern Canada and the USA initially suggested that, particularly in H. contortus but also in H. placei codon F200Y (TAC) is widespread and often highly frequent, however changes in allele frequencies at codon 167 are relatively low (Barrere et al., 2013a, 2013b; Chaudhry et al., 2014), and codon E198A (GCA) appears to be rarely involved. Moreover, investigations conducted in Brazil revealed a high level in TAC frequencies at codons F167Y and F200Y in H. contortus from field samples (dos Santos et al., 2014). Data from a recent survey in India and Pakistan confirmed the TAC SNP in codon F200Y as the most prevalent one, but in contrast to the results from previous studies in America and Europe, no mutations were found at codon F167Y (TAC) and only a small number of populations displayed the SNP in codon E198A (GCA) in India (Chaudhry et al., 2015). Microsatellite marker analysis of H. contortus populations from Pakistan suggests that frequent BZ drug treatment does not result in a reduction of overall genetic diversity (Chaudhry et al., 2016). There have been very few molecular genotyping studies performed in cattle parasitic nematodes. SNPs at all three codons have been associated with benzimidazole resistance in trichostrongylid parasite species of small ruminants and have been recently reported in three cattle parasites, H. placei, O. ostertagi and C. oncophora (Njue and Prichard, 2003; Winterrowd et al., 2003; Brasil et al., 2012; Demeler et al., 2013; Chaudhry et al., 2014). This diversity complicates the molecular detection and accordingly requires the examination of all three codons when molecular tests are applied to field samples. To enable effective anthelmintic management programs, regular surveys of drug efficacies on farms are urgently required (Sutherland and Leathwick, 2011; Kaplan and Vidhyashankar, 2012).

The currently available BZ resistance detection methods can be grouped into three categories: i) in vivo methods, mainly represented by the faecal egg count reduction test (FECRT) or the controlled efficacy test; ii) in vitro methods, particularly the egg hatch assay (EHA) and the larval development assays (LDA) and iii) molecular tools. The FE CRT is labour- as well as cost-intensive and can only provide reliable results once the resistant portion of the population has exceeded at least 25% (Martin et al., 1989). However, advanced statistical analysis methods (Torgerson et al., 2014), in combination with the use of more sensitive coproscopical methods such as (Mini-)FLOTAC (Barda et al., 2013), might improve the power of the FE CRT. The EHA is relatively simple and inexpensive but requires fresh faecal samples, as the inhibition of hatching is the main determinant. The LDA is very sensitive but more labour-intensive than the EHA and also takes at least a week to provide results. Molecular tests include conventional PCR (Silvestre and Humbert, 2000; Njue and Prichard, 2003; Winterrowd et al., 2003), real-time PCR (Alvarez-Sanchez et al., 2005; Walsh et al., 2007) as well as pyrosequencing (Höglund et al., 2009; von Samson-Himmelstjerna et al., 2009; Demeler et al., 2013) and are all based on the detection of SNPs in the three above-mentioned codons in the isotype-1 β-tubulin gene.

In vitro, two additional codons, codons F167Y (TTC to TAC) (Prichard, 2001) and codon E198A (GAA to GCA) (Chishti et al., 2007) have also been reported to be associated with BZ resistance. Until now, these three SNPs in the isotype-1 β-tubulin gene have been described for different parasitic nematodes of ruminants, such as H. contortus, Teladorsagia circumcincta, Ostertagia ostertagi and Cooperia oncophora (von Samson-Himmelstjerna et al., 2007) and Haemonchus placei (Chaudhry et al., 2014). Primarily, codon 200 was thought to play the most important role in BZ resistance, while some studies also reported elevated allele frequencies in codon F167Y (TTC to TAC) and E198A (GAA to GCA) (Silvestre and Cabaret, 2002; von Samson-Himmelstjerna et al., 2007; Chaudhry et al., 2015; Redman et al., 2015). Analyses of field populations are still relatively rare, but data obtained from surveys conducted in Eastern Canada and the USA initially suggested that, particularly in H. contortus but also in H. placei codon F200Y (TAC) is widespread and often highly frequent, however changes in allele frequencies at codon 167 are relatively low (Barrere et al., 2013a, 2013b; Chaudhry et al., 2014), and codon E198A (GCA) appears to be rarely involved. Moreover, investigations conducted in Brazil revealed a high level in TAC frequencies at codons F167Y and F200Y in H. contortus from field samples (dos Santos et al., 2014). Data from a recent survey in India and Pakistan confirmed the TAC SNP in codon F200Y as the most prevalent one, but in contrast to the results from previous studies in America and Europe, no mutations were found at codon F167Y (TAC) and only a small number of populations displayed the SNP in codon E198A (GCA) in India (Chaudhry et al., 2015). Microsatellite marker analysis of H. contortus populations from Pakistan suggests that frequent BZ drug treatment does not result in a reduction of overall genetic diversity (Chaudhry et al., 2016). There have been very few molecular genotyping studies performed in cattle parasitic nematodes. SNPs at all three codons have been associated with benzimidazole resistance in trichostrongylid parasite species of small ruminants and have been recently reported in three cattle parasites, H. placei, O. ostertagi and C. oncophora (Njue and Prichard, 2003; Winterrowd et al., 2003; Brasil et al., 2012; Demeler et al., 2013; Chaudhry et al., 2014). This diversity complicates the molecular detection and accordingly requires the examination of all three codons when molecular tests are applied to field samples. To enable effective anthelmintic management programs, regular surveys of drug efficacies on farms are urgently required (Sutherland and Leathwick, 2011; Kaplan and Vidhyashankar, 2012).
were checked periodically and moistened if necessary (van Wyk et al., 2004). Third stage larvae (L3) were recovered and identified using the morphological keys proposed by van Wyk et al. (2004). When a coproculture had 100 or fewer L3, all were identified; when more than 100 larvae were present, only the first 100 were identified. It is worth noting that *Teladorsagia* and *Trichostrongylus* larvae were difficult to differentiate based on sheath extension length alone. To further refine their differentiation, additional morphological features were required based on the presence of an inflexion at the cranial extremity of *Teladorsagia* larvae (Roebert et al., 2013).

### 2.2. Extraction of DNA

DNA was extracted only from those samples in which one of the target species was present at a proportion of at least 10%, according to morphological identification. For extraction of DNA, the NucleoSpin Tissue 8 kit (Macherey-Nagel, Düren, Germany) was used, following the manufacturer’s instructions.

### 2.3. Pyrosequencing assays for determination of β-tubulin allele frequencies in *H. contortus* and *T. circumcincta*

Pyrosequencing assays targeting codons F167Y, E198A and F200Y of the isotype-1 β-tubulin gene in *H. contortus* and *T. circumcincta*, respectively, were already available (von Samson-Himmelstjerna et al., 2009; Skuce et al., 2010) and were employed in the present study. The respective primer sets are detailed in Table S1. For the analysis of all three SNPs in *T. circumcincta* as well as the SNP in codon 167 in *H. contortus*, PCR reactions were conducted using Novataq Hot Start PCR Mastermix (Mercck). Each 50 μl reaction contained 25 μl mastermix buffer, final MgCl2 concentration of 1.5 mM, 0.2 mM biotinylated primer, 0.4 mM of the non-biotinylated primer and 4 μl of template DNA, in this case, genomic DNA extracted from pools of L3 from the study farms in the respective countries. Amplification of template DNA was verified by gel electrophoresis, 5 μl PCR product run on a 2% agarose gel. All pyrosequencing reactions were carried out using 45 μl PCR product and followed the protocols as described previously (von Samson-Himmelstjerna et al., 2009; Skuce et al., 2010).

The primers used for *H. contortus* pyrosequencing assays for codons 198 and 200 were previously described by von Samson-Himmelstjerna et al. (2009). PCRs and a modified protocol of the pyrosequencing reaction were conducted as recently reported by Ademola et al. (2015). Only the sequencing primer for codon 198 was used to quantify SNP frequencies in both codons E198A and F200Y. Nucleotides were dispensed as provided in Table S2. Pyrosequencing reactions always contained 45 μl of the initial PCR reaction.

### 2.4. Development of pyrosequencing assays for *T. colubriformis*

Pyrosequencing assays for codons F167Y, E198A and F200Y of the isotype-1 and -2 β-tubulin genes of *T. colubriformis* were not available and so were developed specifically for the present study. For isotype-1, three independent assays (based on the same PCR) were developed, while for isotype-2, one assay for codon 167, and a combined assay for codons 198 and 200 were designed based on an alignment of the published *T. colubriformis* sequences for isotype-1 (HQ116825) and isotype-2 (L23506) β-tubulin genes. Initially, a B2 susceptible laboratory isolate was used to obtain the respective fragments of the susceptible alleles in the β-tubulin isotype-2 gene. Controls encoding the potentially resistance-associated alleles (isotype-2) or encoding both alleles in isotype-1 were obtained by custom DNA synthesis (ShineGene Molecular Biotech, Shanghai, China).

Plasmid DNAs containing either the susceptibility- or resistance-associated allele in the target codons were mixed in 17 different ratios (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, 50:50, 40:60, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, 0:100). PCRs were carried out using Phusion® Hot-Start II High Fidelity DNA polymerase (Thermo Scientific, St. Leon-Rot, Germany) as follows: 50 μl 1 × Phusion HF buffer, 200 μM dNTPs (Thermo Scientific), 250 nM of each primer, 1 μM biotin primer and 5–50 ng genomic DNA or 1 ng plasmid mixture. Primer sequences and corresponding annealing temperatures are listed in the supplemental data (Table S1). Cycling conditions were: initial denaturation at 98 °C for 30 s, 40 cycles with 98 °C for 10 s, a primer pair-specific annealing temperature for 30 s and elongation at 72 °C for 30 s followed by a final elongation at 72 °C for 10 min. Pyrosequencing assays were performed on a PyroMark Q24 instrument as previously described in Demeler et al. (2013) using 22–45 μl of the PCR product. Nucleotides were dispensed as provided in Table S2.

### 2.5. Statistical analyses

Samples were considered to show an elevated frequency of an allele conferring a susceptible phenotype if this allele represented 10% of the pyrosequencing signal. This threshold was chosen since there is a technical background of 1–5% (rarely up to 9%) in samples that contain only plasmid DNA representing a single allele. In order to obtain a conservative estimate of the prevalence of B2 resistance, this threshold ensured that no false positive samples with low SNP frequency were considered positive. To evaluate the performance of the newly developed *T. colubriformis* assays, measured allele frequencies were plotted against given frequencies of plasmid mixtures used as templates in GraphPad Prism 5.02. Linear regressions with 95% confidence bands were calculated. For comparison of SNP frequencies at the three codons and between countries, either the Mann Whitney U Test (for comparison between two groups) or the Kruskal-Wallis test, followed by the Dunn’s Multiple Comparison test, were performed.

### 3. Results

#### 3.1. Pyrosequencing assay evaluation for *T. colubriformis*

The isotype-1 and -2 β-tubulin genes of *T. colubriformis* were both evaluated. Pyrosequencing assays were successfully developed for all three codons, F167Y, E198A and F200Y, and in both isotypes. Plasmid DNA containing cDNA inserts encoding the alleles associated with susceptibility and resistance, respectively, were obtained and mixed to obtain defined allele frequencies. Correlations obtained between calculated and observed frequencies for the SNP determination were excellent, with $R^2 > 0.95$ for all 6 SNPs (Fig. 1).

Cross-reactivity was excluded by performance of species-specific assays (*Trichostrongylus*) with the respective heterologous controls (*Haemonchus*, *Teladorsagia* and *Cooperia*).

#### 3.2. Field samples

In total, field samples from 120 sheep farms were included from the year 2012 and 94 from 2013. The respective number of farms sampled in each country and the final number of samples included in the pyrosequencing analyses are provided in Table 1. *T. circumcincta* and *T. colubriformis* were present in all three
countries. In some samples, the percentage of the target species was lower than 10% and, accordingly, these were excluded from the respective pyrosequencing survey. This was particularly the case for *H. contortus* in samples from Ireland, where the total prevalence was very low and the number of L3s was always below 10%. Therefore, no samples from Ireland were analysed for the presence of SNPs in *H. contortus*. However, for some samples, the amount of DNA was not sufficient to conduct all pyrosequencing assays (Table 1).

### 3.3. Pyrosequencing results

#### 3.3.1. Isotype-1 β-tubulin

In codons F167Y and E198A, elevated frequencies (≥10%) of the resistance-associated alleles were only rarely observed (Fig. 2). In 2012, codon F167Y T→C frequency was only found to be elevated above background in three samples for *Haemonchus* (10%, 10% and 91%) and one sample for *Teladorsagia* (23%), all from Switzerland. In the following year, for both, *Haemonchus* and *Teladorsagia*, one sample was found with elevated frequencies (41.5% and 32%, respectively) in Switzerland. Additionally, values above 10% were observed for four samples for *Trichostrongylus* (13–14%) from all three countries. For codon E198A, only two farms in Switzerland displayed elevated GCA values in assays targeting *Haemonchus* (11 and 15%) in 2012. In 2013, only one *Trichostrongylus* sample from

![Fig. 1](image)

**Fig. 1.** Regression analysis of pyrosequencing assays for *Trichostrongylus* isotype 1 (A) and isotype 2 (B) β-tubulin assays for codons F167Y (TTC to TAC), E198A (GAA to GCA) and F200Y (TTC to TAC). Artificial mixtures of plasmids containing the target sequences as templates were mixed and analysed by pyrosequencing. Observed frequencies (mean of 3–5 replicates ± SEM) were plotted against calculated input frequencies. Regression plots (including linear equation and Pearson correlation coefficient) with 95% confidence bands are shown.

| Species | 2012       | 2013       |
|---------|------------|------------|
|         | Switzerland | Italy      | Ireland | Switzerland | Italy | Ireland |
| *Haemonchus* |           |            |          |            |       |         |
| 167     | 31/63      | 8/39       | 0/18     | 30/56      | 11/26 | 0/15    |
| 198/200 | 18         | 7          | nd       | 30         | 11    | nd      |
| *Teladorsagia* | 44/63 | 36/39 | 7/18 | 31/56 | 20/26 | 11/15 |
| 167     | 43         | 36         | 6        | 31         | 19    | 11      |
| 198/200 | 44         | 35         | 6        | 31         | 20    | 11      |
| *Trichostrongylus* | 49/63 | 31/38 | 9/18 | 35/56 | 19/26 | 12/15 |
| 167     | 27         | 23         | 3        | 22         | 14    | 4       |
| 198 Iso1 | 27         | 23         | 3        | 21         | 14    | 4       |
| 200 Iso1 | 27         | 23         | 3        | 21         | 14    | 4       |
| 167 Iso2 | nd         | nd         | nd       | 33         | 19    | 12      |
| 198 Iso2 | nd         | nd         | nd       | 33         | 19    | 12      |
| 200 Iso2 | nd         | nd         | nd       | 33         | 19    | 12      |

Table 1

| Total number of farms sampled per country and year. The number of farms positive for each species as well as the number of pyrosequencing assays performed are provided.
|---|---|---|---|---|---|
| 2012 | 2013 |
| Switzerland | Italy | Ireland | Switzerland | Italy | Ireland |
| *Haemonchus* | | | | | | |
| 167 | 31 | 8 | nd | 29 | 10 | nd |
| 198/200 | 18 | 7 | nd | 30 | 11 | nd |
| *Teladorsagia* | | | | | | |
| 167 | 43 | 36 | 6 | 31 | 19 | 11 |
| 198/200 | 44 | 35 | 6 | 31 | 20 | 11 |
| *Trichostrongylus* | | | | | | |
| 167 | 27 | 23 | 3 | 22 | 17 | 4 |
| 198 Iso1 | 27 | 23 | 3 | 21 | 14 | 4 |
| 200 Iso1 | 27 | 23 | 3 | 21 | 14 | 4 |
| 167 Iso2 | nd | nd | nd | 33 | 19 | 12 |
| 198 Iso2 | nd | nd | nd | 33 | 19 | 12 |
| 200 Iso2 | nd | nd | nd | 33 | 19 | 12 |

nd, not done.

* Number of positive farms per total number of farms.
* Number of successful pyrosequencing assays performed.
Italy had an elevated E198A GCA value (16.5%). In contrast, frequencies of TAC codons associated with BZ resistance at codon F200Y were considerably higher than frequencies in the other two investigated codons in all three countries (Fig. 2) but there were also remarkable differences between the countries. For *Haemonchus*, TAC frequencies were significantly higher in both years in Switzerland than in Italy (p < 0.0003). For *Teladorsagia*, elevated TAC frequencies in codon 200 was most frequently observed in Ireland, followed by Switzerland and Italy. All differences were significant (p < 0.05) with the exception being between Ireland and Switzerland in 2012. For *Trichostrongylus*, frequencies were significantly higher in Switzerland compared to Italy (p < 0.0001). Comparative statistics were not performed for *Trichostrongylus* samples from Ireland since the total number of samples was very low (2–4 farms).

In the following, only those farms for which data for all three codons were available were included. Using a cut-off value of 10% for an elevated SNP frequency, all farms in Switzerland had elevated values for either TAC in codon F200Y alone or TAC in codon F200Y in combination with one of the other resistance-associated codons (Fig. 3) for *Haemonchus*. In contrast, the majority of farms in Italy (71.4%) did not show any elevated frequencies of resistance-associated codons. The only elevated frequencies were found in codon F200Y.

For *Teladorsagia*, an elevated frequency above background was only observed for TAC in codon F200Y on 80% (2012) and 100% (2013) of the investigated farms in Ireland (Fig. 4). In Switzerland, only approximately 15% of the farms displayed no elevated frequencies, while the majority showed an elevation of TAC in codon 200 and only one farm was found with an elevated frequency of TAC in both, codons F167Y and F200Y. In Italy, again the majority of farms did not show any increase (80% in 2012; 65% in 2013) and those with elevated frequencies were only displayed at codon 200.

For *Trichostrongylus*, the situation was slightly different between the two years (Fig. 5). In 2012, one farm in Switzerland showed no elevation of any resistance-associated alleles while, in 2013, on all farms an elevated frequency of TAC in codon F200Y was found. In Ireland, all farms sampled in 2012 displayed the TAC in codon F200Y. In 2013, only half of the farms were found with this SNP while the other half had additionally elevated frequencies of TAC at

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**Fig. 2.** Frequency of single nucleotide polymorphisms associated with benzimidazole resistance in isotype 1 β-tubulin in codons F167Y (TAC), E198A (GCA) and F200Y (TAC) in 2012 (A) and 2013 (B). Values represent means of two pyrosequencing assays per farm. The data are shown separately according to country (CH, Switzerland; IT, Italy; IRE, Ireland) and parasite (Hae, *Haemonchus* spp., Tel, *Teladorsagia* spp., Tri, *Trichostrongylus* spp.). The black line represents the overall mean.
codon F167Y. However, it needs to be mentioned that the total number of farms in Ireland tested by pyrosequencing was much lower than in the two other countries. In Italy, in 2012, eight farms and in 2013, nine farms showed an elevation of TAC frequency at codon F200Y while no-resistance-associated alleles were detected in codons F167Y or E198A. In 2012, the remaining 15 farms were negative for any resistance-associated alleles. In 2013, one farm had an elevated frequency of GCA in codon E198A and four farms were negative for resistance-associated SNPs.

On 39 farms a total of 48 parasite/farm combinations were investigated in both years (data available for both years for a certain parasite on a particular farm). In 77.1% of the combinations, the obtained results were qualitatively very similar to almost identical between the years. In 14.6% the respective resistance associated SNP increased in 2013 compared to 2012. On four farms (8.3%) the frequency of the resistance associated allele decreased in 2013 compared to 2012.

3.3.2. Isotype-2 β-tubulin

SNP frequencies in isotype-2 β-tubulin were only measured in *Trichostrongylus*. In comparison to isotype-1, the picture is significantly different. In general, the majority of farms tested showed no or only moderate elevation of F167Y, E198A and F200Y alleles (Fig. 6). However, a few farms showed frequencies of approximately 60%. These five farms were split across the three countries and elevated SNP frequencies were observed at all three codons (TAC in codons F167200 and F200Y and GCA in codon E198A). Additionally, a tendency for higher prevalence of elevated frequencies of these SNPs was observed in Ireland.

4. Discussion

Infection with gastrointestinal nematodes is one of the most important economic constraints on the sheep industry in Europe and possibly worldwide (Sutherland et al., 2010; Miller et al., 2012; Mavrot et al., 2015). One of the aims of the EU-GLOWORM project was to update current knowledge of prevalence of the most important gastrointestinal nematodes, namely, *Haemonchus*, *Teladorsagia* and *Trichostrongylus*, through harmonised sampling and diagnostic procedures in key regions in Europe (Rinaldi et al., 2015c). Overall, it was shown that, in all three pilot countries, Italy, Ireland and Switzerland, prevalences of gastrointestinal nematodes were generally very high, ranging between 91 and 100% (Rinaldi et al., 2015b). However, prevalence of *Haemonchus* differed considerably between countries, with 77% in Switzerland, 73% in Italy and only 4% in Ireland. These results are in agreement with recent studies in southern Italy, showing high prevalences of *H. contortus* (based on coprocultures), often associated with other...
Prevalence of SNPs and combinations of SNPs on the farm level are shown with 95% confidence intervals below the figures. CH, Switzerland; IT, Italy; IRE, Ireland.

Fig. 4. Pie charts showing the prevalence of farms with a frequency >10% of single nucleotide polymorphism (SNPs) in isotype 1 β-tubulin of Teladorsagia spp. associated with benzimidazole resistance. Only farms with complete datasets for all three SNPs were included in the analysis (numbers of farms are shown at the right bottom of the pie charts).

Table 1. Prevalence of SNPs and combinations of SNPs in the populations investigated for the year 2012 and 2013 in different countries

| SNP in codon | 2012       | 2013       |
|--------------|------------|------------|
| Codon 198:   | Switzerland | Italy      | Ireland    |
| 0% (0-4.2%)  | 0% (0-9.9%) | 0% (0-9.9%) | 0% (0-4.3%) |
| Codon 167:   | Switzerland | Italy      | Ireland    |
| 0% (0-6.2%)  | 0% (0-9.9%) | 0% (0-9.9%) | 0% (0-4.3%) |
| Codon 200:   | Switzerland | Italy      | Ireland    |
| 83.7% (70.0-91.9%) | 20.0% (10.0-35.9%) | 80% (37.6-66.4%) | 0% (0-4.3%) |
| Codon 198/200: | Switzerland | Italy      | Ireland    |
| 0% (0-9.9%)  | 0% (0-9.9%) | 0% (0-9.9%) | 0% (0-4.3%) |
| No codon:    | Switzerland | Italy      | Ireland    |
| 14.0% (5.9-26.8%) | 80.0% (64.1-90.0%) | 20% (3.8-62.4%) | 0% (0-4.3%) |

Table 2. Prevalence of SNPs and combinations of SNPs in the populations investigated for the year 2012 and 2013 in different countries

| SNP in codon | 2012       | 2013       |
|--------------|------------|------------|
| Codon 198:   | Switzerland | Italy      | Ireland    |
| 0% (0-8.2%)  | 0% (0-9.9%) | 0% (0-9.9%) | 0% (0-4.3%) |
| Codon 167:   | Switzerland | Italy      | Ireland    |
| 0% (0-6.2%)  | 0% (0-9.9%) | 0% (0-9.9%) | 0% (0-4.3%) |
| Codon 200:   | Switzerland | Italy      | Ireland    |
| 83.7% (70.0-91.9%) | 20.0% (10.0-35.9%) | 80% (37.6-66.4%) | 0% (0-4.3%) |
| Codon 198/200: | Switzerland | Italy      | Ireland    |
| 0% (0-9.9%)  | 0% (0-9.9%) | 0% (0-9.9%) | 0% (0-4.3%) |
| No codon:    | Switzerland | Italy      | Ireland    |
| 14.0% (5.9-26.8%) | 80.0% (64.1-90.0%) | 20% (3.8-62.4%) | 0% (0-4.3%) |

The results show clearly that, in all populations investigated, codon F200Y in the β-tubulin gene isotype-1 is the most common SNP under selection, while elevated allele frequencies associated with BZ resistance above the 10% threshold were only rarely observed for the other codons, F167Y and E198A. This is in accordance with data published previously. Most studies described in the literature, reporting either results of field studies or the use of laboratory isolates, have been performed with Haemonchus. Comparing laboratory isolates from various geographical origins, von Samson-Himmelstjerna et al. (2009) confirmed the importance of codon 200 relative to codon 167. However, codon 198 was not included at that time. A high prevalence of SNPs at codon 200 has been reported from Pakistan (Chaudhry et al., 2016), where no SNPs were obtained in codon 198 but in codon 167 some SNPs were found in Haemonchus. Chaudhry et al. (2015) published data obtained from a survey in India where again F200Y was the most common mutation, E198A was found in one third of the farms but no SNPs were obtained in codon F167Y. In comparison, a similar number of farms in the present study displayed slightly elevated TAC/GCA frequencies in codons F167Y and E198A, respectively. However, TAC in codon 200 was by far the most abundant change observed in Haemonchus. A field study investigating the presence of BZ resistance in...
Haemonchus in cattle in the US using pyrosequencing, revealed that one out of ten farms was identified as harbouring H. contortus and the remaining nine were all H. placei populations (Chaudhry et al., 2014). While in H. contortus, 91% of analysed individual worms carried BZ resistance associated TAC SNPs at codon F200Y, the frequencies observed in H. placei were much lower (between 1.6 and 9.4%). In the only study from Africa using pyrosequencing, Ademola et al. (2015) identified only BZ susceptible genotypes in H. placei from cattle in Nigeria.

For Teladorsagia, there are even fewer data on BZ-resistant genotypes. PCR-based investigations have almost exclusively been reported for codon F200Y (Elard et al., 1999) and those studies were mainly performed on one or two selected isolates. In one study, codons E198A and F200Y were investigated in field populations in Mexico by PCR, but only TAC SNPs at codon F200Y were found (Liebano-Hernandez et al., 2013). Using pyrosequencing, one initial investigation of a resistant T. circumcincta isolate in Scotland (Skuce et al., 2010), confirmed the importance of the SNP at codon 200. Only one other field study investigating the occurrence of resistance-associated SNPs at all three codons by pyrosequencing has been reported from Spain (Martinez-Valladares et al., 2012), but no resistance-associated SNPs were found. Recently, a study investigating all three resistance-associated codons in H. contortus and T. circumcincta populations from sheep farms in the UK revealed that, for Teladorsagia, again the most abundant SNP was TAC at codon F200Y but, in contrast to Haemonchus, a considerable number of populations also displayed a GCA SNP at codon E198A and only a very low frequency of TCA at codon F167Y was found and only on one farm (Redman et al., 2015).

Fig. 5. Pie charts showing the prevalence of farms with a frequency >10% of single nucleotide polymorphism (SNPs) in isotype 1 β-tubulin of Trichostrongylus spp, associated with benzimidazole resistance. Only farms with complete datasets for all three SNPs were included in the analysis (numbers of farms are shown at the right bottom of the pie charts). Prevalence of SNPs and combinations of SNPs on the farm level are shown with 95% confidence intervals below the figures. CH, Switzerland; IT, Italy; IRE, Ireland.

Fig. 6. Frequency of single nucleotide polymorphisms associated with benzimidazole resistance in isotype 2 β-tubulin in codons F167Y (TTC to TAC), E198A (GAA to GCA) and F200Y (TTC to TAC) in 2013. Values represent means of two pyrosequencing assays per farm. The data are shown separately according to country (CH, Switzerland; IT, Italy; IRE, Ireland) and parasite (Hae, Haemonchus spp., Tel, Teladorsagia spp., Tri, Trichostrongylus spp.). The black line represents the overall mean.
For *Trichostrongylus*, data regarding the occurrence of SNPs at all three resistance-associated codons are not available. Only one real time PCR-based investigation, targeting codon F200Y in *Trichosrongylus vitrinus*, has been reported (Alvarez-Sanchez et al., 2005). To the authors’ best knowledge, the current study is the first report using pyrosequencing analysis and comparing frequencies of SNPs at all three codons, F167Y, E198A and F200Y in *Trichostrongylus*. Even though codon F200Y was found to be the most important codon under selection in *Haemonchus* and *Teladorsagia* in the three investigated countries, the different results obtained for *Trichostrongylus* show that, generally, the other codons should not be neglected.

The data obtained from sheep parasitic nematodes is somewhat different to those obtained for the cattle parasitic nematodes *Cooperia* and *Ostertagia*. Recent analyses showed no clear preference for any of the three SNPs (Demeler et al., 2013; AlGusbi et al., 2014), although it is noteworthy that, in total, a much lower number of populations/isolates has so far been analysed in cattle parasitic nematodes compared to sheep.

The relevance of SNPs in isotype-2 still remains largely unknown. Initially, deletions in the isotype-2 β-tubulin gene, as detected by Southern Blot, have been implicated in a very high BZ resistance phenotype (Kwa et al., 1993). In subsequent studies reporting β-tubulin SNP frequencies, isotype-2 has been largely ignored. von Samson-Himmelstjerna et al. (2009) found only minimal changes in BZ resistance-associated SNPs in *H. contortus* laboratory isolates. However, due to its high expression level, Saunders et al. (2013) recently suggested to further investigate potential roles of strongly isotype-2 in BZ resistance. The fact that elevated SNP frequencies in isotype-2 β-tubulin were observed in some field populations in the current study reinforces the view that, even thought it does not appear to play a major role, isotype-2 should not be completely ignored in future anthelmintic resistance research.

In the current study, there were differences in prevalence of BZ resistance alleles between the countries and the genera. For *Haemonchus*, only Italy and Switzerland were investigated since prevalences and larval counts were too low in Ireland. In Switzerland, every farm showed a considerable elevation in resistance-associated TAC frequency at codon F200Y, while the situation in Italy, with 2 out of 7 farms (28.6%), is still moderate. A similar picture was presented for *Teladorsagia* in the current study where, in Switzerland and Ireland, elevated SNP frequencies were found on the majority of farms and in Italy on only a few farms. In contrast, a BZ resistance study performed in Spain (Martinez-Valladares et al., 2012) did not reveal any SNP’s present at any of the three codons. For *Trichostrongylus*, all farms in Switzerland were observed to have a significant elevation of TAC frequency in codon 200 above background. Even though a much lower number of farms was investigated in Ireland, the mean frequency of the resistance-associated codons was also high, with no farms classified as genotypically BZ susceptible. As seen for the two other genera, Italy again showed the least problem of the three countries with only a moderate elevation of TAC frequency in codon F200Y in 2012 (34%) but an almost doubling of farms with this allele in 2013 (64%). Although not enough farms were analysed in both years to draw valid conclusions this data suggests that there is currently selection on codon F200Y in *Trichostrongylus*. The current findings highlight the importance of investigating the BZ resistance situation for this specific parasite.

The prevalence of anthelmintic resistance is much harder to determine than parasite presence, since samples need to be taken more than once, at specific intervals and to be analysed by highly sensitive methods. Accordingly, it remains difficult, if not impossible, to compare the results obtained in the current survey with previous field data from the same regions. Most previously reported studies investigated only a limited number of farms and concentrated mainly on *Haemonchus*. One reason for this limitation might be that, often, studies have been performed in combination with FECRTs and/or in vitro assays (EHA, LDA), which are laborious procedures. However, BZ resistance in sheep flocks in all of the sampled countries have been previously reported (Hertzberg et al., 2000; Traversa et al., 2007; Good et al., 2012; McMahon et al., 2013; Geurden et al., 2014; Rinaldi et al., 2014; Rose et al., 2015). Particularly in Switzerland it has been assumed that import of ruminants from South Africa carrying resistant nematoded populations led to the spread of anthelmintic resistance (Artho et al., 2007). The relatively low prevalence of anthelmintic resistance in Italy is in agreement with previously reported studies (Rinaldi et al., 2014; Rose et al., 2015), where low treatment frequencies and particularly the absence of anthelmintic treatment during periods of droughts were determined as the major explanatory factors. Field studies, including FECRT, in vitro assays and molecular tests, revealed an overall good agreement between results obtained from in vivo/in vitro tests and pyrosequencing (Höglund et al., 2009; Martinez-Valladares et al., 2012; Barrere et al., 2013a, 2013b).

In order to increase the number of farms investigated, the current study aimed to only use pyrosequencing for the assessment of BZ resistance status. It is the first large-scale survey investigating three genera and all three codons associated with BZ resistance on a molecular basis, with samples obtained from a harmonised spatial sampling approach in three different countries. Even though samples were taken in two consecutive years, only limited comparison between years was possible since a) not from every farm larval cultures were available in both years and b) larval cultures revealed different percentages of the respective genera so comparative data were only available for 39 farms and a total of 48 combinations of farm and parasite genus (data available for a particular farm and a particular parasite for both 2012 and 2013). The vast majority of these combinations did not show any difference in frequency of resistance associated alleles between the two years. For a small percentage of comparisons (same parasite and farm) (14.6%), an increase in frequency of any resistance associated allele was observed from 2012 to 2013. The opposite was found in 8.3% (4 comparisons for the same parasite on the same farm), where the frequency of a resistance associated allele obtained in 2013 were lower than in 2012. This can putatively be explained by sampling of different animals, grazing of animals on different pastures in both years or addition of animals purchased from other flocks. However, the results of this survey still show that BZ resistance is a serious issue across all three genera in Switzerland, with BZ resistance-associated SNP frequencies reaching significantly high values. For Ireland, a similar picture was observed. For the two genera investigated, namely, *Teladorsagia* and *Trichostrongylus*, high SNP frequencies were detected on the majority of farms. The present study also confirms that BZ resistance is rare in sheep in southern Italy, as shown in FECRT-based field surveys (Rinaldi et al., 2014).

5. Conclusion

BZ resistance studies using FECRTs usually have the limitation that mixed infections with different parasite genera are used to calculate one FECR over all trichostrongyloids species. At best, larval cultures or PCRs were used to identify those species surviving treatment. In contrast, genus-specific pyrosequencing assays offer the advantage of obtaining detailed information for each investigated genus. In addition, it appears that detection of BZ resistance alleles using pyrosequencing is more sensitive than FECRTs or even EHAs, even for mono-specific infections. The use of pooled pyrosequencing assays in the current study enabled the inclusion of a
high number of farms. One of the most important constraints in regard to frequent use of pyrosequencing for routine anthelmintic resistance diagnostics is the fact that it is still limited to the few respectively equipped laboratories (due to the high cost of the pyrosequencer). However, once the technology is more widely available, the cost per sample is considerably less expensive than a FE CRT, since it does not require (repeated) farm visits and analyses of numerous individual faecal samples and is additionally independent of previously applied treatments. In comparison to in vitro assays, genotyping/pyrosequencing does not require the availability of fresh faeces containing undeveloped eggs, DNA can be extracted directly from the faecal sample and/or stored for later analysis.

Overall, the results here confirm previous findings of BZ resistance in the three investigated countries. Additionally, it also highlights the importance of inclusion of Teladorsagia spp and Trichostrongylus spp.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ipjiddr.2016.10.002.

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