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Efficacy of ivermectin against gastrointestinal nematodes of cattle in Denmark evaluated by different methods for analysis of faecal egg count reduction

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ABSTRACT

The efficacy of ivermectin (IVM) against gastrointestinal nematodes in Danish cattle was assessed by faecal egg count reduction test (FECRT). Six cattle farms with history of clinical parasitism and avermectin use were included. On the day of treatment (Day 0), 20 naturally infected calves per farm (total n = 120) were stratified by initial faecal egg counts (FEC) and randomly allocated to a treatment group dosed with 0.2 mg IVM kg^{-1} body weight s.c. (IVM; n = 10) or an untreated control group (CTL; n = 10). Individual FEC were obtained at Day 0 and Day 14 post-treatment and pooled faeces by group were cultured to isolate L3 for detection of *Ostertagia ostertagi* and *Cooperia oncophora* by qPCR. Treatment efficacies were analysed using the recommended WAAVP method and two open-source statistical procedures based on Bayesian modelling: 'eggCounts' and 'Bayescount'. A simulation study evaluated the performance of the different procedures to correctly identify FEC reduction percentages of simulated bovine FEC data representing the observed real data. In the FECRT, reduced IVM efficacy was detected in three farms by all procedures using data from treated animals only, and in one farm according to the procedures including data from treated and untreated cattle. Post-treatment, *O. ostertagi* and *C. oncophora* L3 were detected by qPCR in faeces of treated animals from one and three herds with declared reduced IVM efficacy, respectively. Based on the simulation study, all methods showed a reduced performance when FEC aggregation increased post-treatment and suggested that a treatment group of 10 animals is insufficient for the FECRT in cattle. This is the first report of reduced anthelmintic efficacy in Danish cattle and warrants the implementation of larger surveys. Advantages and caveats regarding the use of Bayesian modelling and the relevance of including untreated cattle in the FECRT are discussed.

1. Introduction

Grazing cattle are continuously exposed to infection with gastrointestinal nematodes (GIN) that can severely impair the health and productivity of pasture-based livestock systems (Corwin, 1997; Shaw et al., 1998; Charlier et al., 2014). In practice, the control of GIN in cattle largely relies on the routine use of anthelmintic drugs, mainly from the macrocyclic lactone (ML) family (Vercruysse and Rew, 2002; Geurden et al., 2015). As a consequence, worm populations resistant to MLs have been selected, and anthelmintic resistance (AR) is now becoming a serious threat to the control of bovine nematodes in several countries (Sutherland and Leathwick, 2011; Gasbarre, 2014;

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Sutherland and Bullen, 2015). Coinciding with the development of AR, concerns regarding the prophylactic use of veterinary drugs and chemical residues in both food and environment have led to stricter regulations on the use of anthelmintics in some nations (Thamsborg et al., 1999). In 1999, Denmark became the first country to introduce prescription-only use of anthelmintics in livestock, requiring a mandatory veterinary diagnosis before treatment in both organic and conventional farms (Anonymous, 1998, 2013). Since 2000, there has been an additional requirement for all prescriptions in production animals to be registered in 'VetStat' – the Danish system for surveillance of the veterinary use of drugs (Stege et al., 2003). Preliminary analyses in VetStat indicate that MLs accounted for ~85% of all anthelmintics prescribed for Danish cattle between 2010 and 2012, with ivermectin (alone or in combination) representing 72% of all ML prescribed (Peña-Espinoza et al., unpublished data). However, and despite the significance of ivermectin for current parasite control strategies in cattle, its field efficacy against GIN has not been investigated in Denmark.

In the absence of quantitative molecular techniques for the detection of ML-resistance, and the high cost of the controlled efficacy test (the current gold standard method for verification of anthelmintic activity; Wood et al., 1995), the only readily available technique for investigating field drug efficacy is the faecal egg count reduction test (FECRT). This technique estimates the efficacy of an anthelmintic to reduce the faecal egg counts (FEC) of infected animals based on measurements pre- and post-treatment, or between treated and untreated individuals. The major advantages of the FECRT are that all drugs can be tested regardless of active compounds or formulation and that it relies on FEC detection methods readily available in most diagnostic laboratories. The current recommendations to conduct and analyse FECRT in cattle derive from guidelines by the World Association for Advancement of Veterinary Parasitology (WAAVP), which were originally developed for detection of AR in sheep nematodes (Coles et al., 1992). However, potential limitations have been highlighted concerning the use of FECRT with bovine nematodes, mainly due to the lower faecal egg excretion of cattle, compared to sheep, and the highly aggregated distribution of FEC in cattle groups (Coles, 2002; Coles et al., 2006; Demeler et al., 2010; El-Abdellati et al., 2010; Sutherland and Leathwick, 2011). These factors may limit the correct analysis of FECRT data and inference of drug efficacy in cattle using the WAAVP guidelines. More recently, Bayesian modelling using Markov chain Monte Carlo (MCMC) methods have been advocated as robust statistical analyses to cope with low and aggregated FEC data (Denwood et al., 2010; Torgerson et al., 2014). These MCMC-based procedures, available as open-source R packages or web-interface software, are being increasingly used to infer drug efficacy and to monitor AR in horse nematodes (Denwood et al., 2010; Fischer et al., 2015) and cattle helminths (Neves et al., 2014; O'Shaughnessy et al., 2014; Geurden et al., 2015; Novobilský and Höglund, 2015; Ramos et al., 2016). However, the performance of these MCMC procedures with the low mean FEC and parasite aggregation levels commonly found in cattle has not yet been evaluated. In addition, sensitive and species-specific tests to detect which GIN species survive treatment are critical for the surveillance of AR and are urgently required for cattle (Coles, 2002; Sutherland and Leathwick, 2011).

The objectives of the present study were: 1) to assess the efficacy of ivermectin (IVM) against GIN in naturally infected Danish cattle by FECRT, and 2) to evaluate the performance of different statistical approaches for estimating drug efficacy using simulated bovine FEC data of similar characteristics to those observed in Danish cattle. In addition, we investigated the prescription patterns of anthelmintics in the study farms in order to examine a possible relationship between previous use of avermectins and IVM efficacy in the FECRT.

2. Materials and methods

2.1. Selection of farms

Cattle farms (~50) with a history of clinical parasitism were contacted through local veterinarians across Denmark during spring 2013 and 2014. The farmers were offered free FEC analyses and evaluation of anthelmintic efficacy by FECRT. Farms were selected based on the following criteria: herd size ≥20 first-season grazing (FSG) calves with ≥4 weeks of grazing (before the initial screening) and not treated with anthelmintics within 8 weeks prior to sampling. In addition, the availability of a cattle crush or barn was required for the handling of animals. A total of 19 farms (8 in 2013 and 11 in 2014) that fulfilled these criteria accepted the invitation. Individual faecal samples were collected from 20 FSG calves in each farm between mid-June and early September of 2013 and 2014 for analysis of FEC (initial screening). Due to a low number of farms with mean FEC >150 strongyle eggs per g (epg) of faeces (as recommended by Coles et al., 1992), farms with a mean FEC ≥ 75 epg were selected for the FECRT. Of the six farms finally included in the study, one herd was a conventional beef farm (farm #1), three were organic dairy farms (#2, #4 and #6), one was an organic beef farm (#5) and one was a conventional dairy farm (#3). In Denmark, organic cattle farms should by law provide access to pasture from 15 April until 1 November (Anonymous, 2016), while conventional farms do not have to comply with this rule. The cattle breeds in the investigated farms were Danish Holstein crossbreeds (#1 and #5), Danish Holstein (#2, #3, and #6) and Danish Jersey (#4). All the selected farms were located in the Jutland Peninsula and the FECRT was conducted within one to four weeks after the initial screening.

2.2. Faecal egg count reduction test (FECRT)

The FECRT was performed to test the efficacy of IVM based on WAAVP recommendations (Coles et al., 1992). Pre- and post-treatment faecal samples from treated and untreated animals were included, and a total of 120 FSG calves were enrolled in the FECRT studies. On the day of treatment (Day 0), 20 FSG animals from each farm were stratified by FEC (based on the initial screening) and randomly allocated to a treatment group (IVM; n = 10) or an untreated control group (CTL; n = 10) of similar (initial) mean FEC. Due to a limited number of animals available in farms #4 and #6 at the start of the FECRT, the CTL groups at these properties consisted of nine calves. Oral formulations of IVM are not registered for use in cattle in Denmark, thus injectable IVM was used. At Day 0, individual body weights (BW) were estimated in the IVM group using a girth tape for cattle (Rondo combi®, Kruuse, Denmark), and the calves in the treatment group were injected with the recommended dose of IVM (0.2 mg kg^{-1} BW s.c., Ivomec® 10 mg/mL, Merial Norden A/S). A comparison of BW estimations between girth tape and electronic scale in a group of 30 FSG calves (BW range = 84–172 kg) was performed prior to the study and demonstrated a very high correlation between the methods (Pearson's correlation = 0.98). Faecal samples were collected rectally from all animals on Day 0 and 14 days post-treatment (Day 14). Immediately after collection, the faecal samples were vacuum packed (Freshield Touch, CSE Co, Gyeonggi-do, Korea) to create anaerobic conditions and transported to the laboratory in a cooling box. On all farms, animals in the IVM and CTL groups grazed together on the same pastures until Day 14, when all control calves

were treated with the recommended dose of injectable IVM as described above.

2.3. Parasitological analyses

Upon arrival at the laboratory, faecal samples were refrigerated at 5 °C until analysis. Individual FEC were determined using an accredited, modified McMaster technique with a sensitivity of 5 epg (Henriksen and Aagard, 1975). At Day 0 and Day 14, pooled larval cultures were prepared from the IVM and CTL groups by mixing 10 g of faeces from each animal of the same group into a pool, which was then cultured according to Roepstorff and Nansen (1998). After 14 days of incubation at 20 °C, nematode L3 were recovered by Baermannisation and stored at 12 °C. A small number of L3 were harvested in the post-treatment larval cultures from farms #1, #2, #5 and #6 (<40 larvae per group). All pooled L3 were used for molecular detection of *Ostertagia ostertagi* and *Cooperia oncophora* by real-time quantitative PCR (qPCR).

2.4. Species-specific identification of nematode larvae by qPCR

Molecular detection of *O. ostertagi* and *C. oncophora* in the pooled L3 suspensions was performed using the qPCR method described by Höglund et al. (2013), with modifications. Briefly, all L3 pooled per group were concentrated by centrifugation and transferred into a 2 mL cryotube. Larvae (in 200 µl of tap water) were mixed with 1 mL buffer ATL (QIAGEN, Germany) and 600 µl of 0.5 mm Zirconia beads (BioSpec Products, USA) and homogenised by bead-beating for 1 min at 6.5 m/s (FastPrep®-24, MP Biomedicals, USA). Subsequently, the suspension was digested at 56 °C for 60 min using 20 µl of Proteinase K (20 mg/mL, QIAGEN, Germany) following manufacturer's instructions. Genomic DNA was extracted from the digested larval homogenate by QIAamp® DNA Mini Kit (QIAGEN, Germany). For qPCR analyses, primers and probes targeting the second internal transcribed spacer (ITS-2) of the ribosomal DNA of *O. ostertagi* and *C. oncophora* were used (Höglund et al., 2013). ITS-2 copies of both nematode species were quantified by correlating cycle threshold (Ct) values to a standard curve with 2×10^7, 2×10^6, 10^5, 10^4 and 10^3 molecules μl^{-1} of stock plasmid DNA. The plasmid DNA was made from a pCR® 2.1 vector (Thermo-Fischer Scientific, USA) that comprised ITS-2 sequences of *C. oncophora* (GenBank® accession no. AB245040.2, position 651-729) and *O. ostertagi* (GenBank® accession no. AB245021.2, position 1036-1126) (Höglund et al., 2013). The reactions were run in a Rotor-Gene Q RG-6000® (QIAGEN, Germany) in total volumes of 25 µl using 2 µl DNA as a template. The PCR mix contained 0.65 U Taq2000® polymerase (Agilent Technologies, USA), 0.3 µM forward and reverse primers, 0.2 µM probe, 200 µM dNTP and 5.5 mM $MgCl_2$. Rotor-Gene Q® series software (QIAGEN, Germany) was used to determine Ct values for each run. The cycling conditions were 95 °C for 10 min and 50 cycles of amplification (95 °C, 15 s, 62 °C, 60 s). All samples and standards were carried out in technical duplicates, with exception of the standard curves which were carried out in triplicates. Sensitivity and specificity of the qPCR method was 97.2% (95% confidence interval [CI] = 83.8–100%) and 83.3% (95% CI = 36.4–99%), respectively, based on analyses of spiked larval samples (n = 42) containing known numbers of mixed or pure *O. ostertagi* and/or *C. oncophora* L3 (unpublished results).

2.5. Use of anthelmintics in the selected farms between 2002 and 2012

Recordings of all anthelmintics prescribed between 2002 and 2012 in the six farms selected for the FECRT were extracted from the VetStat database (Stege et al., 2003). In Denmark, every veterinary drug prescribed for production animals is dispensed to farmers by official pharmacies or by veterinary practitioners. The dispensing pharmacy or veterinarian must register in VetStat the total amount of a specific drug sold to the farmer, the farm identity, the animal species and the age group which received the prescribed treatment. In VetStat, cattle are divided into three age groups: 1) calves <12 months old (heifer and bull calves); 2) young cattle ≥ 12 months old (heifers until first calving and steers until slaughter) and 3) adult cattle (cows after first calving). For the study of anthelmintic use, data retrieved included the name and active compound of the prescribed anthelmintic, the formulation and total amount (in total mL or g) of the drug prescribed, and the targeted cattle age group. However, the exact number of treatments actually performed in each prescription is not recorded in VetStat. We therefore estimated the number of treatments for each prescription in the 'targeted' group (i.e. calves, young cattle or adult cattle) within each farm using the total amount of a prescribed drug (exact data from VetStat), the recommended dose of a given anthelmintic (considering its formulation) and a defined BW for each cattle age group. The defined BW for each age group was estimated based on data from the Danish Cattle Association (Danmap, 2012) and considering common anthelmintic treatment practices in each group as: 200 kg BW for calves, 450 kg BW for young cattle and 620 kg BW for adult cattle. Due to this defined BWs and the likely variation in the actual amount of active compound used for different animals following each prescription, the calculated number of treatments is only a proxy of the real number of animals treated. In addition, it was assumed that anthelmintics delivered to farmers were used within a month, which may not always have been the case; however, this potential bias is presumably consistent across the different drugs and age groups. The number of animals per age group in each farm at the prescription date was retrieved from the Central Husbandry Register (Ministry of Environment and Food of Denmark, http://chr.fvst.dk, accessed on 15 March 2016). The efficacy of previously used anthelmintics had not been tested in any of the six farms prior to the study.

2.6. Estimation of treatment efficacy

The efficacy of IVM in the FECRT was analysed by calculating the arithmetic mean FEC reduction percentage (FECR%) with 95% CI using the recommended WAAVP method and two procedures using Bayesian MCMC methods:

2.6.1. WAAVP

Following recommended WAAVP guidelines (Coles et al., 1992) as: *i)* FECR% (*With* CTL) = $100 \times (1-[T_2/C_2])$, where T_2 and C_2 are the arithmetic mean FEC of the IVM and CTL group at Day 14, respectively, and *ii)* FECR% (*No* CTL) = $100 \times (1-[T_2/T_1])$, where T_1 and T_2 are the arithmetic mean FEC of the IVM group at Day 0 and 14, correspondingly. The calculations of FECR% and 95% CI with the WAAVP method were performed according to Coles et al. (1992) in Microsoft Excel® 2010.

2.6.2. EggCounts

Using the Bayesian MCMC procedure implemented in 'eggCounts' (version 1.1–1) described by Torgerson et al. (2014). The analyses were performed via the freely available web interface of the procedure (available at: http://shiny.math.uzh.ch/user/furrer/shinyas/shiny-eggCounts/; visited on 01 September 2016). This software is also available as an R package (Wang et al., 2016). The eggCounts procedure uses MCMC to fit a model using a gamma-Poisson distribution for pre- and post-treatment FEC data, thus accounting for aggregation in FEC data and the Poisson errors of the

egg counting process, to generate the FECR% with 95% CI (Torgerson et al., 2014). The procedure uses a single prior for the over-dispersion parameter (aggregation). Two model options are available using the eggCounts web interface: *i)* the unpaired model, which models pre- and post-treatment data as independent gamma-Poisson distributions, with a scaled mean and common aggregation parameters, and *ii)* the paired model, which fits a gamma-Poisson distribution to the pre-treatment data only and scales the means of the Poisson processes from each individual by the same constant to model the mean of the Poisson processes representing the post-treatment data. Therefore in the paired model, pre- and post-treatment mean FEC come from the same Poisson distribution (i.e. this assumes that the degree of FEC aggregation does not change post-treatment relative to the pre-treatment). Both models are available with or without a zero-inflation component. For comparison, we used both paired and unpaired models to analyse our FECRT data, with the default moderately informative prior distributions as described by Paul et al. (2014) and Wang et al. (2016), without zero-inflation. A correction factor of 5 epg was applied to the data (to obtain the number of eggs counted) and FEC data from only the IVM group at Day 0 and 14 were used (*No* CTL), as the software currently does not incorporate the FEC variation of a separated CTL group.

2.6.3. Modified Bayescount

Using a model based on that implemented by the Bayesian MCMC 'Bayescount' procedure described by Denwood et al. (2010) and Geurden et al. (2015). The Bayescount paired model describes the pre-treatment FEC data as a compound gamma-gamma-Poisson distribution, with the first gamma distribution reflecting the variation between animals and the second gamma-Poisson (negative binomial) distribution describing the variation in observed FEC that would be expected with repeated samples from the same animal. The post-treatment FEC data is modelled as a separate gamma-Poisson (negative binomial) distribution based on the estimated mean for that animal, and scaled by the FECR%. The procedure provides the 95% CI of the FECR%, while accounting for the FEC aggregation between individuals and for the Poisson errors of the egg counting method. The model is also able to separate the between- and within-animal FEC variation, and allows for changes in the FEC aggregation of post-treatment data relative to the corresponding pre-treatment observation from the same animal. This increases the uncertainty in the estimates compared with assuming that FEC aggregation is identical at Day 0 and 14, but allows for potential differences in drug efficacy between animals to result in a higher FEC aggregation post-treatment. Further description of the method can be found in the appendix of Geurden et al. (2015). Furthermore, for the present study the Bayescount paired model was modified to incorporate the FEC data from the initial screening in each farm and to model the data from all six herds simultaneously, therefore allowing for pooling of one or more variance parameters between farms. As a result, the procedure allows inference to be made on three FEC variability (aggregation) estimates (k) separately: *i)* the expected variability between the unobserved true mean of the animals (between-animal pre-treatment k); *ii)* the expected variability between pre-treatment samples from the same animal (within-animal pre-treatment k) and *iii)* the expected variability between post-treatment samples from the same animal (within-animal post-treatment k, which also captures variation in the true efficacy between animals). The mean FEC from each time point was modelled independently in each farm, so that no pooling of mean FEC or FECR % parameters took place between herds. Minimally informative DuMouchel priors (Denwood, 2016) were used for the mean FEC and various k parameters, and a Beta(1,1) prior was used for the FECR% and 95% CI estimates. Using this modified Bayescount model, the efficacy of IVM in the FECRT studies was evaluated considering *i)* the FEC variation in the IVM and CTL groups between Day 0 and 14 (*With* CTL), and *ii)* the FEC variation only in the IVM group between Day 0 and 14 (*No* CTL). All FEC data were transformed to the number of eggs counted (FEC divided by 5) for analysis. All modelling was performed in JAGS (Plummer, 2003) using the 'runjags' package (Denwood, 2016) in R version 3.2.2 (R Core Team, 2015), with convergence assessed both visually and using the Gelman-Rubin statistic. Full model code can be obtained from the corresponding author.

The fit of all MCMC models to the FECRT data was assessed using the Deviance Information Criterion (DIC) (Spiegelhalter et al., 2002). For the modified Bayescount procedure, DIC was used to compare the fit of models with between- and within-animal k parameters estimated separately or pooled between farms. For the eggCounts procedure, DIC was obtained using JAGS models with identical formulations to those used by eggCounts (Wang et al., 2016) to compare the fit of the paired vs. unpaired models.

2.7. Interpretation of treatment efficacy

Results with the methods described in Section 2.6 were used to estimate the efficacy of IVM treatments based on the obtained FECR % and lower 95% CI as recommended by Coles et al. (1992), as well as the upper 95% CI as suggested by Lyndal-Murphy et al. (2014), from which we categorised three conditions:

i) Efficacious treatment, when mean FECR% and upper CI $\geq$ 95% and lower CI $\geq$ 90%;
ii) Reduced efficacy, when mean FECR% and upper CI < 95% and lower CI < 90%;
iii) Inconclusive, when none of the above conditions were met.

2.8. Simulation study

A simulation study was carried out to compare the performance of the methods outlined in Section 2.6. For this study, we analysed the FECR% between Day 0 and 14 in simulated treatment groups (without untreated animals) with different levels of FEC aggregation. Datasets were simulated based on hierarchical gamma-gamma-Poisson distributions with parameter estimates obtained from the modified Bayescount procedure as described in Section 2.6 and applied to the real FECRT data. Based on the fit of these models, we assumed that pre-treatment k is fixed between farms (i.e. not herd dependent), but that post-treatment k varies (independently) between herds. As a result, a total pre-treatment $k = 0.8$ (divided into between-animal pre-treatment $k = 1.3$ and within-animal pre-treatment $k = 4.1$) were used for all simulations. Three different total post-treatment k parameters were simulated: $k = 0.8$ (no change of FEC aggregation between Day 0 and 14), $k = 0.3$ (moderately increased FEC aggregation between Day 0 and Day 14) and $k = 0.1$ (substantially increased FEC aggregation between Day 0 and Day 14). Pre-treatment (Day 0) FEC datasets were simulated with sample sizes of n = 10, 20, 30 and 40 and a mean FEC of 34 eggs counted (equal to 170 epg using a FEC method with a detection limit of 5 epg), using the same paired model as described for the modified Bayescount procedure. Post-treatment (Day 14) data were simulated using a FECR% of 85% or 97%. This procedure was repeated 500 times for each combination of the (two) simulated FECR%, (three) total post-treatment k levels and (four) sample sizes described (i.e. representing the results of 500 different treatment groups for each set of parameters). Each of the simulated datasets were then analysed using *i)* the WAAVP procedure, *ii)* the

paired and unpaired eggCounts procedures implemented using the eggCounts package version 1.1–1 (Torgerson et al., 2014), and *iii)* the standard (unmodified) Bayescount paired model procedure using equivalent (moderately informative) priors to those used by eggCounts in order to facilitate comparison. The mean FECR% and 95% CI obtained for a given simulated dataset using each procedure were recorded, and the performance of the procedures was investigated by studying the probability of the simulated FECR% being included in the 95% CI (i.e. coverage probability of the 95% CI) along with the uncertainty of the 95% CI (i.e. the relative size of the 95% CI) provided by each procedure. All data simulation and analysis procedures were performed in R version 3.2.2 (R Core Team, 2015).

Table 1
Faecal egg counts (FEC) of 20 first-season grazing calves for each of the 19 Danish farms sampled during initial screening in the 2013 and 2014 grazing seasons. A total of 380 first-season grazers were examined. Data are presented as arithmetic mean FEC, range (in egg per gram of faeces, epg) and the date of sampling. Six farms (farms #1–6) were included in the subsequent faecal egg count reduction tests (FECRT).

Farm	Mean FEC (epg)	Range (epg)	Date of sampling[b]
1	438	85–975	16 June 2013
2	101	5–205	24 June 2013
3	184	5–730	20 August 2013
4	273	20–960	27 June 2014
5	75	10–225	09 July 2014
6	75	15–215	14 August 2014
7	8	0–40	02 July 2013
8	37	0–135	04 July 2013
9	44	0–135	23 July 2013
10	3	0–30	05 August 2013
11	17	0–80	14 August 2013
12	17	0–60	24 June 2014
13	221[a]	5–1165	02 July 2014
14	18	0–55	22 July 2014
15	8	0–40	23 July 2014
16	6	0–25	15 August 2014
17	3	0–20	25 August 2014
18	2	0–10	26 August 2014
19	11	0–40	07 September 2014

[a] Despite high FEC this farmer did not want to participate in the FECRT.
[b] Turn-out of grazing cattle in Denmark is usually around late April/early May.

3. Results

3.1. Initial screening of farms

Faecal egg count data observed in groups of 20 FSG calves from the 19 farms initially screened are summarised in Table 1. A total of 380 animals were sampled during mid-June and early September of 2013 and 2014. Strongyle eggs were detected on all properties but very low egg excretion levels (mean FEC $\leq$ 44 epg) were seen in 12 farms (63%). All six farms selected for the FECRT had an initial arithmetic mean FEC $\geq$75 epg.

3.2. FECRT

At Day 0, IVM groups in farms #5 and #6 had a very low mean FEC ($\leq$55 epg). In farm #6, three calves in the IVM group could not be sampled at Day 14 and were excluded from the analyses. In the model fitting analyses, the eggCounts unpaired and the modified Bayescount with pooled pre-treatment *k* between farms (herd dependent) and separate post-treatment *k* between farms (herd independent) had the lowest DIC values (data not shown), offering the best fit for the FECRT data, and were therefore selected for the final FECR% analyses.

Results of the FECR% analyses using the WAAVP method and the selected eggCounts and modified Bayescount procedures are presented in Table 2. The modified Bayescount procedure also provided inference on the FEC aggregation estimates (*k*). The pre-treatment *k* was modelled as fixed between farms, with an estimate of 0.8 (95% CI 0.6–0.9) for all six herds. In contrast, the post-treatment *k* was allowed to vary between farms and there was evidence for a decrease in post-treatment *k* (i.e. increased FEC aggregation) in Farms #3, #4 and #6 (Table 2).

The WAAVP and the modified Bayescount procedures including the FEC of treated and untreated animals (*With CTL*) declared reduced IVM efficacy in farm #2, whereas only the modified Bayescount stated reduced drug efficacy in farm #1 (Table 2; *With CTL*). Furthermore, the WAAVP and modified Bayescount procedures (*With CTL*) indicated efficacious treatment in farm #5, while all other results were inconclusive. While in analyses including the

Table 2
Faecal egg count reduction test in calves naturally infected with gastrointestinal nematodes in six Danish cattle farms. A total of 115 first-season grazers were sampled on the day of treatment (Day 0) and 14 days post-treatment (Day 14). The calves were treated with ivermectin (IVM, 0.2 mg kg^{-1} body weight s.c.) or left untreated (CTL). Treatment efficacies were calculated including the variation of faecal egg counts (FEC) in treated and control groups (*With* CTL) or in treated groups only (*No CTL*). Total post-treatment FEC aggregation estimates (*k*) for each farm are shown according to the modified Bayescount.

	Farm #1 (Beef, conv.)		Farm #2 (Dairy, org.)		Farm #3 (Dairy, conv.)		Farm #4 (Dairy, org.)		Farm #5 (Beef, org.)		Farm #6 (Dairy, org.)	
Group	IVM	CTL	IVM	CTL	IVM	CTL	IVM	CTL	IVM	CTL	IVM	CTL
n	10	10	10	10	10	10	10	9	10	10	7	9
FEC Day 0	333	325	76	69	173	209	354	351	55	39	31	77
FEC Day 14	46	311	14	55	15	286	51	562	4	98	4	126
k	0.7 (0.4–0.9)		0.7 (0.1–1.0)		0.3 (0.1–0.6)		0.3 (0.2–0.5)		1.0 (0.6–1.4)		0.2 (0.1–0.4)	
With CTL	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]
WAAVP[a]	85^{I}	[67–95]	75^{R}	[27–92]	95^{I}	[56–99]	91^{I}	[74–97]	96^{E}	[92–99]	97^{I}	[65–99]
Bayescount[b]	87^{R}	[80–94]	78^{R}	[61–92]	93^{I}	[70–99]	91^{I}	[73–99]	96^{E}	[92–99]	90^{I}	[52–99]
No CTL	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]	FECR%	[CI]
WAAVP[a]	86^{R}	[66–94]	82^{R}	[47–94]	92^{I}	[30–99]	86^{R}	[67–94]	94^{I}	[87–97]	83^{I}	[−50–98]
Bayescount[b]	87^{R}	[81–93]	83^{R}	[72–92]	90^{I}	[62–98]	81^{R}	[50–94]	92^{I}	[84–98]	81^{I}	[25–99]
eggCounts[c]	84^{R}	[55–94]	80^{R}	[50–91]	86^{I}	[21–98]	83^{R}	[47–94]	93^{I}	[82–97]	83^{I}	[22–97]

FEC = arithmetic mean faecal egg count; *k* = total post-treatment FEC aggregation estimate; FECR% = FEC reduction percentage; CI = 95% confidence interval.
^{E}Efficacious; ^{R}Reduced efficacy; ^{I}Inconclusive; conv. = conventional; org. = organic.
[a] Coles et al. (1992).
[b] Modified Bayescount (paired model with pooled between-animal and within-animal pre-treatment *k*).
[c] eggCounts unpaired model (Torgerson et al., 2014).

FEC fluctuations in the treated groups only (*No CTL*), the WAAVP, modified Bayescount and eggCounts procedures unanimously indicated reduced IVM efficacy in farms #1, #2 and #4. All methods (*No CTL*) yielded inconclusive results in farms #3, #5 and #6 (Table 2; *No CTL*).

3.3. Real-time qPCR for detection of O. ostertagi and C. oncophora

Proportions of ITS-2 copies of *O. ostertagi* and *C. oncophora* detected in pre- and post-treatment pooled larval cultures in the FECRT are presented in Table 3. At Day 0, *O. ostertagi* and *C. oncophora* ITS-2 copies were detected in both groups from all farms, with the exception of the CTL group in farm #2, where only *C. oncophora* was identified. Post-treatment, *O. ostertagi* ITS-2 copies were detected only in IVM groups of farms #3 and #4, while *C. oncophora* ITS-2 copies were identified in treated groups from all six farms. Amplification efficiencies of the standard curves were 96% and 100% for *C. oncophora* and *O. ostertagi*, respectively, with correlation coefficients (R^2) of 0.99 for both species.

3.4. Anthelmintic prescriptions in the farms selected for FECRT

The anthelmintic prescriptions between 2002 and 2012 in the six farms selected for FECRT and the estimated number of animals treated are shown in Supplementary Table 1. Prescriptions with anthelmintics other than MLs were rarely observed (only in farm #2 with albendazole and farm #3 with levamisole). In calves from all six farms, IVM was the most commonly used ML (87.2% of all prescriptions), followed by moxidectin (12.8%). Similarly, IVM was the predominant ML used in young cattle (84.7% of all prescriptions), followed by moxidectin (14.7%). In adult cattle, IVM constituted 61.5% of all prescriptions in farms #2, #3, #4 and #6, followed by eprinomectin (23.8%) and moxidectin (12.5%). No adult cattle were recorded in farms #1 and #5 during the period. Topical (pour-on) drugs were the predominant formulations used in all six farms (98.6%, 95.1% and 88.4% of all prescribed treatments in calves, young and adult cattle, respectively). In general, treatments were prescribed at irregular intervals in all farms throughout the decade preceding the FECRT, and 61% of all prescriptions were estimated to have been targeted to less than 50% of the animals in a given age group. Nevertheless, evidence suggesting whole-group treatments was observed at all farms, particularly farm #1 (the only conventional beef herd in the study) and farm #6 (organic dairy farm). There were no marked differences in the prescription patterns between beef and dairy or conventional and organic farms.

Table 3
Proportion (percentage) of the second internal transcriber space (ITS-2) copy numbers of *Ostertagia ostertagi* and *Cooperia oncophora* detected by real-time quantitative PCR in pooled L3 cultured from faeces of naturally infected calves. The calves were treated with the recommended dose of injectable ivermectin (IVM) or left untreated (CTL). The samples were collected on the day of treatment (Day 0) and 14 days post-treatment (Day 14).

Farm	Group	Day 0		Day 14	
		O.o.	*C.o.*	*O.o.*	*C.o.*
#1	IVM	42	58	0	100
	CTL	39	61	57	43
#2	IVM	79	21	0	100
	CTL	0	100	2	98
#3	IVM	45	55	85	15
	CTL	78	22	74	26
#4	IVM	8	92	0.5	99.5
	CTL	17	83	6	94
#5	IVM	42	58	0	100
	CTL	84	16	0	100
#6	IVM	3	97	0	100
	CTL	6	94	0	100

O.o. = *Ostertagia ostertagi*; *C.o.* = *Cooperia oncophora*.

3.5. Simulation study

The performance of the different procedures used to estimate drug efficacy in the FECRT is presented in Table 4. The coverage probability of the 95% CI describes the ability of each procedure to correctly include the simulated FECR% in the generated CI under varying degrees of FEC aggregation (simulated total post-treatment *k*). The target coverage of the 95% CI is expected to be close to 95%. Whereas the uncertainty of the 95% CI is defined as the average difference between the upper and lower 95% CI generated by the procedure with each dataset, with a smaller uncertainty representing (on average) a narrower 95% CI, and therefore the dataset is less likely to be classified as inconclusive. The WAAVP method had good coverage for most parameter sets, although this was lower for the datasets with very low post-treatment *k*, regardless of sample size and FECR% (Table 4). The eggCounts paired model had consistently lower coverage probabilities compared with the unpaired model, at all levels of post-treatment *k*, sample size and FECR% within the conditions simulated. The unpaired eggCounts procedure had good coverage for most datasets and reduced uncertainty compared to the Bayescount procedure, which reflects the assumption that FEC aggregation in post-treatment data is the same as that in the pre-treatment data. However, where this assumption was strongly violated by high post-treatment aggregation (post-treatment $k = 0.1$), then coverage of the unpaired eggCounts method dropped to 76% for the highest sample size. The 95% CI provided by the Bayescount procedure had good coverage for all parameter sets, although generally higher uncertainty than the other procedures for the 97% simulated FECR%.

Coverage and uncertainty of the 95% CI generated by all procedures increased or decreased, respectively, following the increase in group size in both simulated FECR% scenarios (Table 4). Based on results with the WAAVP method (which generally gives a good balance between coverage and uncertainty), and applying the classification criteria described in Section 2.7, the minimum sample size that is required before 80% of datasets with a FECR = 97% can be correctly classified as efficacious treatment is n = 20 for moderately increased FEC aggregation (pre and post-treatment $k = 0.8$ and 0.3, respectively) or n = 40 for extremely increased FEC aggregation (pre and post-treatment $k = 0.8$ and = 0.1, correspondingly; data not shown). Similarly, to give an 80% chance of correctly classifying datasets with a FECR = 85% as reduced efficacy, a minimum of n = 20 is required for no increase in post-treatment aggregation, or n = 30 for moderately increased aggregation. With extreme increases in aggregation, even a sample size of n = 40 may be expected to give inconclusive results approximately 50% of the time (data not shown).

4. Discussion

In the FECRT, reduced IVM efficacy was detected in three farms by all analyses based on the FEC of treated cattle only, and in one farm according to both procedures including the FEC from treated and untreated animals. Post-treatment, *O. ostertagi* and *C. oncophora* L3 were identified in faeces of treated animals in one and three farms with declared reduced IVM efficacy, respectively. This is the first report of reduced anthelmintic efficacy in Danish cattle.

During the initial screening prior to the FECRT, FSG calves with mean FEC <100 epg were detected in most farms. Similar observations were reported in untreated FSG calves from Belgium, Germany (El-Abdellati et al., 2010) and France (Geurden et al., 2015). At

Table 4
Coverage probabilities and uncertainty of 95% confidence intervals (CI) provided by the WAAVP, eggCounts (paired and unpaired model) and Bayescount (standard paired model) procedures with datasets of simulated FECR = 85% or 97% and with varying group sizes (n) and total post-treatment aggregation estimate (k). Results are presented as the percentage of iterations in which the simulated FECR% was correctly included within the 95% CI provided by the procedure. A total of 500 iterations were performed for each combination of true FECR% and k. See text for further details on the simulated parameters.

		Coverage 95% CI				Uncertainty 95% CI			
FECR% = 85%		WAAVP[a]	eggCounts[b]		Bayescount[c]	WAAVP[a]	eggCounts[b]		Bayescount[c]
n	k		paired	unpaired			paired	unpaired	
10	0.1	82	23	80	95	65	8	45	74
	0.3	95	40	96	99	49	9	45	51
	0.8	98	63	99	100	34	9	36	32
20	0.1	88	26	82	97	45	6	34	60
	0.3	94	39	94	95	31	6	27	30
	0.8	100	64	99	99	23	6	22	17
30	0.1	90	22	84	96	40	5	29	50
	0.3	96	44	95	95	25	5	21	20
	0.8	100	67	100	99	18	5	17	13
40	0.1	91	22	81	97	34	4	23	40
	0.3	97	39	94	96	22	4	18	17
	0.8	100	64	99	97	16	4	15	10
		Coverage 95% CI				Uncertainty 95% CI			
FECR% = 97%		WAAVP[a]	eggCounts[b]		Bayescount[c]	WAAVP[a]	eggCounts[b]		Bayescount[c]
n	k		paired	unpaired			paired	unpaired	
10	0.1	84	49	85	99	15	4	14	68
	0.3	93	67	93	100	10	4	11	38
	0.8	98	85	99	100	9	4	10	18
20	0.1	87	46	79	97	11	3	7	43
	0.3	96	74	96	99	7	3	6	11
	0.8	98	83	99	99	5	3	5	5
30	0.1	89	43	78	95	8	2	5	25
	0.3	96	68	94	97	5	2	4	6
	0.8	99	83	99	98	4	2	4	4
40	0.1	90	44	76	95	7	2	4	15
	0.3	97	70	94	95	5	2	4	4
	0.8	98	82	98	97	4	2	3	3

[a] Coles et al. (1992).
[b] Torgerson et al. (2014).
[c] Geurden et al. (2015); FECR% = Simulated faecal egg count reduction; n = simulated group size; k = simulated total post-treatment FEC aggregation estimate.

Day 0 of the FECRT, three farms had a mean FEC <150 epg (farms #4, #5 and #6), which was a consequence of our initial selection of farms with mean FEC $\geq$ 75 epg. Although the use of a FEC method with high sensitivity (5 epg in the present study) reduces the diagnostic uncertainty in samples with low FEC (El-Abdellati et al., 2010; Levecke et al., 2011), a low mean FEC pre-treatment will likely affect the outcome of a FECRT, particularly when group size $\leq$ 10 and FEC are highly aggregated (Levecke et al., 2012). Therefore, the reduced drug efficacy detected in Danish farms with initial mean FEC <150 epg should preferably be confirmed by new FECRTs including cattle with higher egg excretion.

The qPCR method applied in the present study was able to detect ITS-2 copies of *O. ostertagi* and *C. oncophora* in pooled larval cultures from naturally infected cattle, as earlier reported by Areskog et al. (2013). In agreement with previous European reports (Demeler et al., 2009; Geurden et al., 2015), *C. oncophora* was the predominant species surviving IVM treatment in all farms with reduced drug efficacy. This result was expected as *C. oncophora* is the dose-limiting GIN for IVM (Egerton et al., 1981). The more pathogenic *O. ostertagi* was detected in IVM groups from all six farms at Day 0 and from two farms at Day 14. However, reduced efficacy by all methods (*No CTL*) was only detected in one of these farms (#4), therefore the possible presence of an IVM-resistant *O. ostertagi* in this herd should be further confirmed. The absence of *O. ostertagi* L3 in post-treatment cultures from most farms reflects the expected high efficacy of injectable IVM against this abomasal species (Egerton et al., 1981; Lifschitz et al., 2000). Nevertheless, the examination of pooled L3, the low FEC of samples and the poor yield of L3 following culture may have been masking the true ratio of the species surviving treatment. Individual larval cultures could have increased the sensitivity of the test and resulted in a different outcome, however, this was not possible within the timeframe of the study. A practical limitation for the routine use of infective larvae for species detection is the time required to culture L3 and the well-known variability in developmental requirements of different nematodes in larval cultures (Roeber and Kahn, 2014). As alternative, other nematode stages could be used for species-specific detection by molecular techniques (Harmon et al., 2006), and recent studies from our group have explored this by effectively quantifying ITS-2 copies in eggs and first-stage larvae of *O. ostertagi* (Drag et al., 2016).

In the FECRT, methods of calculation that included the FEC of untreated animals resulted in a higher number of inconclusive results, in comparison with methods excluding the CTL group. The inclusion of untreated animals in a FECRT has been recommended to detect changes in the FEC of a herd not related with the treatment, and for the correction of such fluctuations in the estimation of drug efficacy (Coles et al., 1992; Lyndal-Murphy et al., 2014). However, the inclusion of a control group is based on the assumption that treated and untreated animals with comparable FEC share similar worm burdens, which may not be always the case in cattle (Michel, 1967, 1969). Moreover, the density-dependent control of fecundity in some bovine nematodes, such as in *O. ostertagi*, may reduce the FEC in untreated animals due to newly acquired infections and could increase the egg excretion of female worms in treated groups due to lower worm burden and

competition (Dobson et al., 2012). In practice, when few animals are available for a FECRT, the inclusion of a control group will limit the size of the treatment group and consequently increase uncertainty of the estimated efficacy (Denwood et al., 2010). The low number of FSG calves with positive FEC that could be included in our study suggests that in these circumstances, the inclusion of untreated cattle in the FECRT should not be recommended. Instead, it may be advisable to include more animals in the treatment group in order to increase the certainty of the calculated FECR% (Denwood et al., 2010; Levecke et al., 2012), a similar conclusion derived from our simulation study.

The simulation study showed the difficulties of analysing FECRT data, even with sophisticated methods such as that implemented in eggCounts and Bayescount. The poor coverage of 95% CI produced by the paired eggCounts procedure suggests (based on our simulations) that the eggCounts unpaired model should be preferred, even when analysing paired data such as that simulated here. Both eggCounts models were also tested with the use of the zero-inflation option, but results were qualitatively similar to those without zero-inflation (data shown). The relatively simple WAAVP procedure showed a better coverage probability than most of the other procedures, but does have the limitation of not providing a 95% CI when post-treatment FEC are zero (Denwood et al., 2010; Torgerson et al., 2014; Geurden et al., 2015). Only the Bayescount procedure was able to consistently identify the simulated FECR% within the 95% CI produced, although this comes at the cost of increased uncertainty. The results also suggest that a treatment group of $n = 10$ is inadequate for cattle, and also support the observations of Gill et al. (1986) that the minimum required sample size depends strongly on the degree of aggregation that is assumed. Given these complications, new statistical methods are needed to quickly determine the prospective study power of a given animal group size, mean FEC and aggregation parameters. However, despite the limited sample size available in the FECRT studies, we were able to modify the paired Bayescount model to make use of the available data from the initial screening and use of pooled variance parameters to bolster the inference of the model. This produced substantially reduced 95% CI relative to the independently modelled datasets using the same procedure (data not shown), and is therefore highly recommended as a way to maximise the utility of MCMC for these types of data. A second benefit of this approach is that the variability parameters themselves may give useful information; in this case, there is a strong suggestion that the change in FEC aggregation post-treatment is more substantial in some farms than others. If this is due to variation in efficacy between animals, then a large post-treatment change in variability could itself indicate the early signs of developing AR. It is also possible to incorporate moderately informative priors into models fit using Bayesian MCMC as a way of maximising the inference from the data. Informative priors were explored as part of the modified Bayescount model presented, but ultimately did not provide any more information than the minimally informative priors. Moderately informative priors are used by eggCounts, and these are certainly valid in the context given by Paul et al. (2014), but care should be taken when using these models for different datasets to ensure that the priors are appropriate in the situation at hand. If the use of these methods is attempted without some understanding of the theory and application of MCMC, then the potential for erroneous inference is extremely large due to either selection of inappropriate model formulations (including prior selection) or errors introduced by poor convergence and/or high autocorrelation (Brooks and Roberts, 1998; Kass et al., 1998; Toft et al., 2007). We therefore strongly recommend that users without the necessary statistical experience seek assistance with implementation and interpretation of both the eggCounts and Bayescount procedures, although when correctly applied these methods can be used to maximise the information available from the data by incorporating additional data sources and using techniques such as partial pooling. There is also substantial scope for the development of a procedure that is simpler in application and interpretation than MCMC, but until such method is available, our results support the continued use of the WAAVP method to analyse FECRT in cattle where mean post-treatment FEC are greater than zero and statistical expertise is not available.

At the recommended dose of 0.2 mg kg^{-1} BW, injectable IVM is expected to reduce susceptible *O. ostertagi* adults and L4 stages by $\geq$ 99% and susceptible *C. oncophora* adults and L4 stages by $\geq$ 97% (Egerton et al., 1981). However, the current WAAVP guidelines suggests that any treatment with a FECR >90% in cattle should not be considered a case of drug resistance (Coles et al., 1992). In practice, most studies evaluating anthelmintic efficacy in cattle declare AR when FECR <95% and lower 95% CI < 90, as proposed for sheep. Nevertheless, it has been suggested that this criteria is biased towards declaration of AR when there is none, particularly if the mean FECR% is between 90–95% and the CI is wide (Lyndal-Murphy et al., 2014). In the present study, we included the upper 95% CI in the interpretation of the FECRT to increase the certainty of detecting true cases of IVM inefficacy. A similar interpretation for FECRT studies in cattle has been reported in recent investigations by Geurden et al. (2015) and Ramos et al. (2016). However, the effect of including the upper 95% CI in the interpretation for estimating drug efficacy using a FECRT, and how this correlates with an actual resistant phenotype confirmed by controlled efficacy tests, warrant further investigation. It is also important to note that a reduced FECR% may not necessarily be caused by AR. A lower-than-expected *in vivo* efficacy, or varying drug response between animals, could be the result of under dosing (e.g. due to inaccurate estimation of BW) and/or altered drug pharmacokinetics and pharmacodynamics in different animals (e.g. due to nutrition-related variations in fat reserves that may affect the persistent efficacy of ML, erratic absorption of drugs from the site of injection and/or interactions with other co-administered drugs) (González Canga et al., 2008; El-Abdellati et al., 2010; Areskog et al., 2012, 2014; De Graef et al., 2013). These factors can impair the correct estimation of drug efficacy and detection of AR, particularly in the dose-limiting species *C. oncophora*. Recently, *C. oncophora* populations that were declared resistant to the recommended dose of injectable IVM by FECRT in two Swedish cattle farms (with FECR% [upper CI] = 78% [97%] and 79% [98%] in each farm; Demeler et al., 2009) were declared IVM-susceptible when tested in calves under controlled conditions (Areskog et al., 2014). Therefore, the presence of IVM-resistant nematodes suggested by our FECRT in three farms, as well as the AR status in the farms with inconclusive results and low initial FEC, should be confirmed by controlled efficacy test.

The use of anthelmintics in the farms included in our FECRT was investigated to potentially detect trends in drug use and the extent of treatments with avermectins. Data was retrieved from the VetStat database and used to estimate the number of animals treated with a given anthelmintic at each prescription. However, the actual number of cattle treated at each investigated prescription is unknown and our analysis aimed only to offer a rough estimate of the anthelmintic use in these farms. Furthermore, VetStat does not register whether adult cattle are lactating or not at the time of treatment, and therefore the prescription of drugs not allowed for treatment of animals in lactation (e.g. IVM, levamisole) recorded in some of the studied farms deserves further investigation. Based on the data retrieved from VetStat, most of the anthelmintics

prescribed in the six farms between 2002 and 2012 were avermectins, mostly topical IVM products. A similar reliance on avermectins has been preliminarily detected in the entire Danish cattle population in the period 2010–2014, constituting ~80% of all treatments – of which 79% were IVM, mainly in topical formulations (Peña-Espinoza et al., unpublished data). The irregular prescription of anthelmintics observed in the study farms correlates with the prescription-only regulations in Denmark, illustrated by the treatment of single animals or selected groups of animals in the herds. However, prescription patterns suggesting whole-group treatments in some farms indicate that these may be recommended by veterinarians under certain conditions (e.g. during outbreaks of dictyocaulosis), and the effect of this practice on the selection for AR needs further investigation. All treatments against GIN in Denmark should be based on a clinical and/or laboratory examination, and preventive/strategic anthelmintic treatments without such diagnosis are illegal. Organic farms are further encouraged to apply other means of parasite control than use of anthelmintics; however, due to limited knowledge of alternative and effective parasite control methods, most farms (whether organic or not) still rely on anthelmintic drugs. Therefore, and considering the relevance of IVM and other anthelmintics for nematode control in Danish cattle, the true extent of AR in bovine nematodes in Denmark needs to be assessed in larger surveys. Until then, producers and veterinarians should be aware of potentially ineffective treatments against GIN in cattle, while reducing the reliance on anthelmintics by including other parasite control strategies with documented efficacy, such as grazing management and feeding with bioactive forages (Nansen et al., 1987; Peña-Espinoza et al., 2016).

In conclusion, reduced IVM efficacy was detected by all methods for analysis of FECRT data excluding untreated controls in three of six Danish cattle farms investigated. *Cooperia oncophora* was the main species surviving IVM treatment in three farms with confirmed reduced drug efficacy, while *O. ostertagi* was also identified post-treatment by qPCR in one farm with reduced IVM efficacy. Nevertheless, the presence of IVM-resistant nematode strains suggested by the FECRT should preferably be confirmed by controlled efficacy test. The reduced efficacy of IVM detected in this study and the widespread use of ML drugs in Danish cattle suggest that farmers and their advisors should be aware of potentially ineffective treatments and larger surveys are warranted to describe the true extent of the problem. However, further validation of the design and analysis of the FECRT in cattle are urgently needed before such surveys can be implemented in cattle farms.

Conflicts of interests

The authors declare that they have no conflicts of interests.

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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.ijpddr.2016.10.004.

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