Risk factor analysis of equine strongyle resistance to anthelmintics

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Abstract

Digestive strongyles are the most problematic endoparasites of equids as a result of their wide distribution and the spread of resistant isolates throughout the world. While abundant literature can be found on the extent of anthelmintic resistance across continents, empirical knowledge about associated risk factors is missing. This study brings together results from anthelmintic efficacy testing and risk factor analysis to provide evidence-based guidelines in the field. It involves 688 horses from 39 French horse farms and riding schools to both estimate egg reduction rate (ERR) after anthelmintic treatment and to interview premise managers about their practices. Risk factors associated with reduced anthelmintic efficacy in equine strongyles have been estimated across drugs or for every drug class separately using a marginal modelling approach. Results demonstrate ivermectin efficacy (96.3% ERR), the inefficacy of fenbendazole (42.8% ERR) and an intermediate profile for pyrantel (90.3% ERR). Risk factor analysis provide robust support to advocate for FEC-based treatment regimens as well as individual determination of anthelmintic dose that contribute to significantly lower drug resistance risk by factors 1.72 and 2.56 respectively. In addition, reduced horse movements should also be recommended to sustain anthelmintic efficacy (odd ratio=0.42 [0.26;0.69]) while the horse-to-worker ratio may be an indicator of better efficacy. On the contrary, outdoor-breeding systems, grazing systems with little rotation and manure spreading significantly increased the risk of reduced drug efficacy by factors 1.71 to 7.56. This is the first empirical risk factor analysis for anthelmintic resistance in equids, whose findings should guide the implementation of more sustained strongyle management in the field.

Keywords: horse, nematode, anthelmintic resistance, strongyle, odd ratio

1. Introduction

The diversity of helminth species infecting horses is large, and differences in life cycles, epidemiology, pathogenicity and drug susceptibility make it increasingly challenging to define good sustainable parasite control programs. Strongyles remain a major concern. They can be classified into two sub-families, namely Strongylinae (large strongyles) and Cyathostominae known as small strongyles or cyathostomins (Lichtenfels et al., 2008). The large strongyle *Strongylus vulgaris* is associated with high mortality rate as a result of verminous arteritis it can cause during its life cycle (Nielsen et al., 2016). This species has been successfully maintained under low prevalence levels by anthelmintics, despite recent reports of putative re-emergence associated with reduced frequency of anthelmintic treatments (Nielsen et al., 2016; Nielsen et al., 2012). On the contrary, cyathostomins have been a growing concern in the field (Matthews, 2014; Peregrine et al., 2014). This group of nematodes encompasses 50 known species (Lichtenfels et al., 2008), with a ubiquitous distribution throughout geo-climatic conditions (Sallé, 2015) and infecting both young and adult horses (Corning, 2009). Their life cycle is direct and usually involves encystment of infective larvae into the caeco-colic mucosa of their hosts (Corning, 2009). In heavily infected horses, *en-mass* emergence of these encysted larvae can lead to critical clinical state characterized by a loss of weight, colic, diarrhoea, protein-losing enteropathy and eventually death of the animal (Love et al., 1999; Murphy and Love, 1997).

Use of anti-infectious drugs put pathogen populations under selection pressure that can ultimately lead to the emergence of resistant or multi-drug resistant populations (Kennedy and Read, 2017). Over the past decades, small strongyle populations, as other livestock-infecting parasitic nematodes (Kaplan and Vidyashankar, 2012), have demonstrated a gradual increase in their resistance to available anthelmintics in every part of the world (Matthews, 2014; Peregrine et al., 2014). Under the French setting (Traversa et al., 2012) like in other

European (Relf et al., 2014; Traversa et al., 2007; Traversa et al., 2009) or American countries (Canever et al., 2013; Lyons et al., 2008; Molento et al., 2008; Slocombe and de Gannes, 2006), resistant strongyle populations have been reported for every available class of anthelmintics, namely benzimidazoles, tetrahydropyrimidines or macrocyclic lactones.

Even if these results shed lights on how widespread the resistance is, critical assessment of associated risk factors and species composition of resistant parasitic populations is still lacking (Nielsen, 2012), hence preventing the implementation of clear guidelines in the field. There have been a limited number of reports focusing on factors associated with prevalence of strongyle infection in horses in Germany (Fritzen et al., 2010; Hinney et al., 2011a) or the impact of faeces removal on prevalence in UK (Corbett et al., 2014). However, available knowledge gathered so far has usually considered separately drenching practices (Hinney et al., 2011b; Lendal et al., 1998; Lind et al., 2007; O'Meara and Mulcahy, 2002; Relf et al., 2012) and estimation of anthelmintic efficacy if any (Relf et al., 2014; Tzelos et al., 2017). As a consequence, a knowledge gap about putative risk factors and their impacts remains (Nielsen, 2012).

This study results of a large-scale survey involving 688 horses from 39 French horse farms and riding schools that have been submitted to both an anthelmintic efficacy test and a questionnaire interview about their practices. Risk factors associated with reduced anthelmintic efficacy in equine strongyles have been estimated across drugs or for every drug class separately. The objective of this study was to bring together anthelmintic efficacy testing and risk factor analysis to provide evidence-based guidelines in the field.

2. Materials and methods

2.1. Farm and riding school sampling

Our study aimed to evaluate drug efficacy for three drug classes and if possible, to have a control group. Following the WAAVP guidelines (Coles et al., 2006), this is equivalent to identify four groups of at least five individuals with faecal egg count (**FEC**) above 150 eggs/g. Therefore, stud farms with at least 20 births per year were selected from the French Horse and Riding Institute (IFCE) database. This condition brought the focus onto four French regions, *i.e.* Normandy, Loire Valley, Aquitaine and Burgundy.

Two additional criteria were defined to increase the chance of finding horses with sufficient excretion load to undertake FEC reduction test. First, premises with less than 40 horses were discarded as FEC is usually over-dispersed and focusing on less individuals would not have permitted to build treatment groups. Second, last anthelmintic treatment should have been done three months earlier that corresponds to post-moxidectin treatment egg reappearance period. Flyers explaining the purpose of the project were then sent to pre-selected farms before a phone call was made to each manager to make sure that their premises fulfilled requested criteria (at least 40 horses not drenched in the last three months) and to confirm their willingness to participate. In the end, 19 stud farms were enrolled, *i.e.* five in Normandy, four in Loire Valley, four in Burgundy and six in Aquitaine. For each of these, matching riding schools located within each stud farm area were subsequently identified and enrolled for anthelmintic efficacy test, with an extra-riding school enrolled in Aquitaine, providing a set of 20 riding schools. This set of matching riding schools was used to investigate putative differences between stud farms and riding schools.

2.2. Horse sampling and anthelmintic resistance tests

A first round of faecal sampling was made one week before drenching, to select for individuals with a minimum excretion level of 150 eggs per g (epg) as recommended by the World Association for the Advancement of Veterinary Parasitology, WAAVP (Coles et al., 2006). Faecal material was stored at 4°C before being processed for faecal egg counting within 24h. Based on individual FEC measured, three treatment groups, *i.e*. fenbendazole (**FBZ**), pyrantel (**PYR**), or ivermectin (**IVM**), balanced for FEC were built. In case additional individuals were available, they were allocated to a control group. On day 0, each horse was weighed using a girth tape and orally administered an anthelmintic dose following manufacturer's requirements.

Faecal material was subsequently taken from each horse 14 days after treatment. Ivermectin-treated individuals were also sampled 30 days after drenching to detect reduced egg-reappearance period. This short time interval was chosen as an optimum between being able to identify suspicious situations and minimising disturbance with premise activities (horse sales or movements).

2.3. Processing of faecal material

FEC were measured by sampling 5g of faecal material for each individual horse, subsequently diluted and thoroughly mixed into 75 mL of a NaCl solution (density of 1.18). Prepared solution was loaded on a McMaster slide and strongyle eggs were counted with a sensitivity of 15 epg.

2.4. Questionnaire survey and variable definition

A questionnaire, built upon previous published surveys (Fritzen et al., 2010; Maddox et al., 2012), was used to interview each manager as part of larger survey on antibiotic and anthelmintic resistance. The anthelmintic-associated questions grouped into four categories, addressing husbandry, available pasture and management, horse health management, and drenching strategy.

For statistical inference, a few variable levels were redefined to avoid redundancy and to provide the analysis with more statistical power. Therefore, one farm that did not apply systematic drenching upon horse arrival was considered as not drenching any horse upon arrival at all. Rotation between pastures was recoded as occurring either more (frequent) or less (rare) than every 3 months or never.

In addition, pasture strategies either involved own private pastures dedicated to horses or alternative strategies that included co- grazing with cattle, or access to pastures shared between several breeders. Available pasture surface was considered as a three-level variable, *i.e.* less than 30 ha, more than 80 ha or in-between.

Two types of managers were distinguished between the ones who tried to manage health problem themselves before calling their practitioner or others, calling as soon as possible.

Anthelmintic provider was considered as a two-level factor contrasting cases where veterinarians delivered the drug or not. Reasons grounding drenching programs were the same across farms, *i.e.* driven by horse well-being and growth, and were not included further in the risk factor analysis.

2.5. Statistical analyses

2.5.1. Egg Reduction Rate (ERR) and bootstrapping procedure

Sample size to estimate reduced ERR prevalence was determined using EpiTools web-server (Humphry et al., 2004).

When a control group was available, ERR values was computed at farm level by averaging treatment group FEC following (Dash et al., 1988):

$$ERR = 1 - \left(\frac{\text{mean FEC control, before treatment}}{\text{mean FEC control, after treatment}} \times \frac{\text{mean FEC treated, after treatment}}{\text{mean FEC treated, before treatment}}\right) \times 100,$$

hereafter referred to as **Method 1**.

Otherwise, ERR has been computed following (Coles et al., 1992):

$$ERR = \left(1 - \frac{\text{mean FEC treated, after treatment}}{\text{mean FEC treated, before treatment}}\right) \times 100,$$

hereafter denoted **Method 2**.

For both methods, associated 95% percentile confidence intervals was determined for the region, premise type and drug class and their respective intersections following a block bootstrap approach. Drug class ERR confidence intervals at the farm level were not estimated as too few individuals were available within each treatment group preventing robust inference of estimate variability (Chernick, 1999). This approach takes into account the correlation among observations from the same individual (before and after treatment). For both ERR computation methods, blocks of FECs from the same horse were sampled with replacement from the observed data collected before and after treatment (within region, premise type and drug class), and were used to compute an ERR estimate using equation (1) or (2) accordingly. In that case of method 1, the time-matched control group is used to account for variation in FEC between the two sampling time-points independent of treatment. Therefore, blocks of individual FEC before and after treatment were sampled with replacement from horses belonging to the treated or the control group within a farm.

In both cases, computation was performed 10,000 times to yield the empirical distribution of the ERR from which 2.5 and 97.5% percentiles were sampled to derive the 95% confidence interval.

2.5.2. Variable selection procedure

The aim of this study was to quantify risk factors associated with reduced anthelmintic efficacy, hence associating measured drug efficacy with management practices. This requires to select the set of variables that would maximize total variance explained, while avoiding likelihood inflation through over-parameterization. Therefore, for every model construction, a forward-backward procedure was implemented on a full generalized linear model including every variable built from the questionnaire and premise type and regions. To overcome model convergence issues, variables showing too little or no variation across farms (control serology or coproscopy upon horse arrival, faeces removal, access to pasture, horse weight estimation, veterinarian specialization, number of veterinary practices considered for diagnostic) were discarded. Additionally, the total number of veterinary drugs found on-site or the frequency of health register updates were not considered further as determinants of anthelmintic efficacy. This lead to 21 variables on the top of premise type and regions.

2.5.3. Marginal modelling of drug efficacy

A marginal modelling approach of individual horse egg count reduction rate (ERR) was applied as outlined elsewhere (Walker et al., 2014) and implemented in R (R Core Team, 2016) with the geepack package (Højsgaard S., 2006). In that framework, individual egg counts measured at a given time, before or after treatment, are assumed to be Poisson distributed and thus modelled with a log-linear regression model.

This model includes environmental variables (listed in Supplementary table 4 for each specified model) interacting with a binary variable coding for the treatment, *i.e.* taking value of 1 after treatment or 0 before treatment. This variable accounts for the treatment-mediated change in egg count reduction, hence estimating ERRs, while the fitted interactions estimate the contribution of considered environmental conditions to ERRs (Crellen et al., 2016). Exponentiated estimates therefore provide the relative risk of increased (relative risk above one) / decreased (relative risk smaller than one) ERRs associated with a given environmental variable. Any variable whose relative risk confidence interval does not include one is declared as significantly impacting on the ERRs. A first model aimed to quantify universal risk factors, *i.e.* considered environmental effects across drug class. Treatment group was thus fitted into the model. Drug class-specific analyses based on individual treatment group data taken separately were subsequently implemented for fenbendazole and pyrantel, to identify drug-specific factor involved in reduced efficacy. The lack of variation in ivermectin ERR did not permit to investigate further ivermectin-specific risk factors.

3. Results

3.1. Observations from questionnaire surveys

Detailed answers from questionnaire surveys are provided as supplementary data 1.

3.1.1. Premise structure and organisation

Considered premises had a mean herd size of 70 horses, ranging from 21 to 250 individuals. This variation was mostly driven by the type of premise considered (p=0.008), as stud farms comprised more horses than riding schools (average herd size of 88.3 and 52.2 respectively) whatever region. Horses were generally housed (figure 1A) in individual boxes (n=31/39) and a few premises had an outdoor-only breeding system (n=7/39). Noticeably staff team did not strongly scale with herd size (r=0.33, p=0.04), especially in stud farms where workers were in charge of 13 horses more than in riding schools ($p<10^{-4}$).

3.1.2. Horse movements on site

Horse movement occurred in half of the premises (n=21/39) at least once a month while seven premises were rarely hosting horses from other places (figure 1A). For these introductions, no serology, no coproscopy and no anthelmintic efficacy test was performed in any of the 39 premises while anthelmintic drenching upon arrival was implemented in 11 riding schools and seven stud farms (figure 1A). Only four managers reported seeking advice from their veterinarians to manage these movements.

3.1.3. Pasture availability and management

In every considered premise, horses had access to permanent pastures whose surface ranged from 5 to 425 ha (most of which falling below 30 ha, figure 1B). This surface was similar between regions but higher in stud farms, *i.e.* 107.1 ha compared to 20.4 ha on average in riding schools. Rotation between pastures was implemented in 29 premises at least once a year (figure 1B) and driven by grass growth and horses could generally (n=27/39) graze permanently (figure 1B). Three premises had access pastures shared with other breeders, and mixed grazing of horses with cattle was implemented in seven premises, while others used their own pastures dedicated for horses only (figure 1B). Faeces removal was never implemented but in one premise, while manure spreading was performed in one third (n=10/39) of the surveyed premises (figure 1B).

3.1.4. Health management and interactions with veterinarians

About two-third of the premises relied on specialized equine practitioners (n=24/39), who were often called after managers had already attempted to manage health problem themselves (n=28/39; figure 1C). Half of the premises (n=20/39) were consulting several practices to cross-validate advice or benefit from several skills or both.

Yearly veterinary expenses by individual horse varied from less than 100 € (n=15), between 100 and 200 € (n=14) or more than 200 € (figure 1C). Mandatory on-site health register was variably used (figure 1C), *i.e.* 20 managers fulfilled it regularly (systematically or on a regular basis), while 19 rarely did it (never or doing it from time to time). The number of veterinary drugs found within on-site pharmacy greatly varied from null to 15, with two-third of premises having 5 drugs or less (figure 1C) and slight trend of more medications found in horse riding schools (Kruskal-Wallis test, p=0.07).

3.1.5. Drenching strategy for intestinal nematodes

Anthelmintic dosing was usually based on a visual weight estimation (n=27/39) that could be combined with girth tape (n=9/39) but more rarely relying on a scale (n=2/39). Grouped-based drenching was implemented in 11 premises (figure 1D). Time of drenching was registered most of the time (n=31 premises; figure 1D). Drenching frequency evenly occurred twice (n=13), thrice (n=11) or four times (n=14) a year (figure 1D), and drenching programs were generally alternating between drug classes (figure 1D). Noticeably, a limited fraction of premises (n=6) reported off-license use of anthelmintics (figure 1D). These involved ivermectin (n=3), doramectin (n=2) or praziquantel (n=2) licensed for ruminants and were found in premises seeking advice from their veterinarians (5 out of 6 premises).

Anthelmintics were bought from veterinarians in 62% of cases (figure 1D), while three and 16 managers reported buying from the internet or their pharmacist, respectively. For this latter case, three managers only reported the need of their veterinarian's prescription.

FEC-based drenching regimen was implemented in 14 premises (figure 1D).

Despite stud farm managers were not more independent in the management of health-related issues than riding school managers (p=0.12), they relied more on their veterinarian's advice for drenching in comparison to their counterparts ($p=2x10^{-4}$).

3.2. Results of anthelmintic efficacy tests

A total of 688 horses from 39 premises were sampled at least once during this experiment. This design provided enough resolution to detect prevalence as low as 1%, with precision of 0.05 and assuming FEC sensitivity of 70% and specificity of 90%.

Out of these, 601 horses excreting more than 150 epg before treatment were enrolled for the anthelmintic resistance test (Table 1, supplementary Tables 2 and 3). Control groups were available in 24 out of the 39 retained farms (Table 1). Average FEC before treatment was 912 ± 762 epg (supplementary Table 2).

Estimated ERRs and associated variation have been reported in Table 1 while farm-level estimated ERR have been attached as supplementary data 3. ERRs of the two implemented methods show highly consistent results for ivermectin and fenbendazole (Pearson's correlation coefficient of 100 and 82%, respectively). This correlation however dropped to 65% for pyrantel.

Estimated ERRs demonstrated the almost generalized inefficacy of fenbendazole with an average ERRs of 46.2% (sd=33.5%) or 42.8% (sd = 33.4%) for method 1 and 2, respectively and confidence intervals not including 100% efficacy in Burgundy and Aquitaine (Table 1). Nevertheless, two riding schools and one stud farm located in Normandy exhibited ERRs of at least 90% (supplementary data 3).

Observed trends for ivermectin were the exact opposite of these, as the mean estimated ERRs were 98.1% (sd: 8.6%) and 96.3% (sd: 14.5%) according to methods 1 and 2, respectively (Table 1, supplementary data 3). Seven horses from three riding schools and three stud farms exhibited ERRs lower than 90% after ivermectin treatment, resulting in bigger confidence intervals in Aquitaine and Normandy (Table 1). Egg reappearance was investigated 30 days after ivermectin drenching in 157 horses. Nine horses encountered in Aquitaine (n=5), Burgundy (n=3) and Loire Valley excreted eggs (mean FEC of 14.6 epg), three of which displaying egg excretion levels above 50% of their initial excretion before treatment.

Pyrantel exhibited an intermediate profile in comparison to the two other drugs as average ERRs were close to the 90% threshold, *i.e.* 92.5% (sd: 15.4%) and 90.3% (sd: 19.6%) for methods 1 and 2, respectively.

3.3. Risk factors associated with anthelmintic efficacy

Questionnaire data were combined with individual FEC measures to investigate what management practices underpinned the measured anthelmintic efficacy. Relative risks of reduced egg reduction rate (ERR) associated with management practices were estimated across drug categories, any relative risk above 1 signing increased egg count after treatment and thus better ERR. Drug-specific risk factors were subsequently estimated considering observations from each treatment group independently.

3.3.1. Risk-factors across anthelmintic drug class

A first analysis investigated universal factors associated with drug efficacy, measured by ERR, that would be true across anthelmintic drugs and would not depend on drug mode of action. Relative risks associated with the retained variables have been plotted on figure 2.

As expected from the estimated ERRs, PYR and IVM were less at risk of reduced ERRs than FBZ considered as the reference level (figure 2). Premises in Aquitaine region had slightly more risk of drug resistance (OR=1.68, p=0.07) contrasting Loire Valley where ERRs were generally lower (OR=0.59, p=0.08), but the premise type was not retained as a variable contributed to ERR (figure 2).

Premises with higher horse-to-worker ratios were less prone to reduced ERRs (OR=0.36 [0.19;0.67] and 0.53 [0.33;0.86] for intermediate and highest horse-to-worker ratios respectively). Outdoor-only breeding systems were associated with reduced ERRs (figure 2) and premises with rare horse movement were less at risk of drug resistance (OR=0.42 [0.26;0.69]) in contrast to premises with occasional horse movements (OR=2.82 [1.54;5.17]).

Pasture management had a major impact on ERRs, especially premises performing manure spreading (OR=1.88 [1.03;3.48]) were more at risk of reduced ERRs. In addition, closed pasture systems, *i.e.* with little or no rotation between pastures (OR= 7.56 [3.30;17.27] and 2.48 [1.53;5.17] respectively) or premises with pastures dedicated to horses only (OR=1.71 [1.09;2.70]), were more at risk of displaying reduced ERRs (figure 2).

On the contrary premises with highest veterinary expenses per horse (OR=0.53 [0.31;0.91]) or managers more independent in horse health management (OR=0.53 [0.31;0.89]) were significantly less at risk of reduced ERRs (figure 2). Similarly, drenching strategy relying on

FEC or estimating drenching dose on an individual basis respectively diminished the risk of decreased ERRs by factors 1.72 and 2.56 (figure 2). Surprisingly, premises seeking advice from their practitioners for the design of their drenching program also displayed reduced drug efficacy (OR=3.36 [2.00;5.64], figure 2).

3.3.2. Drug-specific risk factors

As each of the tested anthelmintic drug have different modes of action, we examined the contribution of management practice to their respective ERRs by applying the marginal modelling approach to each subset of FBZ- and PYR-treated individuals (figure 3). Although this strategy dampens the power of the analysis as drug-specific estimates were inflated in comparison to the across-drug analysis (figure 3), it gives insight into putative drug-specific risk factors.

Despite this, most of the retained variables did not differ from the across-drug analysis and underlined the importance of pasture management on drug efficacy (figure 3). Both FBZ and PYR-ERRs were reduced by pasture systems with rarest pasture rotations and risk of sub-optimal ERRs was found in premises seeking advice from their veterinarians in both cases (figure 3). As for the across-drug analysis, ERR had less chance of being reduced in premises with higher horse-to-worker ratio although this was not significant for FBZ ($p>0.06$ for both tested ratios, figure 3).

However, while FBZ resistance had not geographical pattern (variable not retained by the variable selection procedure, figure 3), Aquitaine displayed the highest risk of reduced PYR-efficacy (OR=7.02 [1.96;25.2]). In addition, seasonal grazing increased the risk of FBZ-resistance (OR=1.75 [1.13;2.72]) but dampened it for PYR (OR=0.14 [0.06;0.35]), whereas three-treatment drenching programs had a beneficial effect on FBZ efficacy (OR=0.55

[0.33;0.93], figure 3) but increased the risk of PYR resistance (OR=30.54 [9.77;95.5], figure 3) in comparison to two-treatment programs.

Premises with managers more independent for health management issues also had less risk of FBZ-ERR (OR=0.46 [0.29;0.75]) whereas premises with the lowest veterinary expenses per horse had increased risk of reduced FBZ ERR (OR=2.26 [1.23;4.15]). Noticeably, most extreme veterinary expenses were associated with lower risk of PYR-resistance ($p<10^{-4}$ in both cases).

4. Discussion

Current knowledge about anthelmintic resistance in equine strongyles is usually scattered across drug efficacy reports and questionnaire surveys about parasite management (Nielsen, 2012). This leaves a major knowledge gap in the critical assessment of factors underpinning anthelmintic resistance in equids. Our study aims to fill in this gap with the first report of an association between anthelmintic ERRs and management practices in horses.

The drug efficacy landscape in the present study remains similar to what has been reported in a previous study in France (Traversa et al., 2012) and what has been described in other countries (Matthews, 2014). As a summary, FBZ cannot be used for the management of small strongyles any more, in contrast to IVM whose efficacy remains above 95% and the intermediate pattern for PYR whose average ERRs is centred on the minimal efficacy level, *i.e.* 90% reduction of egg excretion.

However, two original findings tend to depart from this general pattern. First, a few premises (3/39) still harbour FBZ-susceptible strongyle populations as reported in a previous study (1/18) conducted in France (Traversa et al., 2012). Although knowledge of implemented management practices was available in the latter study, it was not possible to identify obvious consistent factor that would explain this sustained FBZ efficacy. In-depth investigation of practices and analysis of parasitic community structure with a nemabiome approach (Avramenko et al., 2015) may help better understanding this feature and confirm this FBZ-susceptibility by interrogating beta-tubulin sequences and allelic frequencies (Lake et al., 2009). Second, measured ERP tend to show that IVM efficacy may not be sustained at its current level in the near future despite the short time interval monitored. Indeed, larger ERR confidence intervals were encountered in Normandy and Aquitaine, suggestive of a higher variability in ERR. In addition, original ERP was 9 weeks for ivermectin (Boersema et al., 1996) but indications of shortened ERP have been collected in various countries, *e.g.*

Germany (von Samson-Himmelstjerna et al., 2007), the UK (Daniels and Proudman, 2016), Belgium, the Netherlands and Italy (Geurden et al., 2014) and had never been reported in France. In this study, ERP remains more qualitative than truly quantitative as the short time-interval considered will underestimate real ERP. This reduced time interval had been considered to minimize interferences of our design with premise activities and to ensure that most of the treated horses would still be available for sampling. Despite this, a few horses had already been sold or sent to other premises for training.

Beyond the crude estimation of drug efficacy, this study aimed to identify major determinants underpinning egg reduction rate, and to estimate their respective relative contributions to provide evidence-based recommendations in the field.

While the overall resistance pattern between drugs remains similar to current knowledge, it is worth noticing that significant regional variations were found. Especially, Loire Valley was generally less at risk of resistance whereas Aquitaine premises were particularly at risk both across drugs and for pyrantel resistance. This should encourage practitioners to implement more drug efficacy tests in this latter region. That difference may also result from more sustained practices but contribution of climatic conditions to maintain larger refugium (van Wyk, 2001) than in Aquitaine (hot and dry summer), hence dampening selection for drug resistance.

Pasture-related variables were also significant contributors to the variation in drug efficacy measured by egg reduction rate. Reduced anthelmintic efficacy were found in premises with closed pasture system, *i.e.* private pastures dedicated to horses only, with little or no rotation between pastures, and with outdoor breeding system. It is probable that such setting favours parasite development by a constant renewal of generations with dense host populations. Once a more resistant population arises it will have high chance to be maintained and be selected for as no dilution (into other host species for instance) will be permitted. In addition, a decrease of egg reduction rate was over-represented in premises implementing manure spreading. This in combination with the other factors certainly helps scattering resistant populations across pasture surface and increases the chance of horse exposure to these. However, it seems that premises with a grazing surface larger than 80 ha are prone to harbour more pyrantel-resistant parasite populations. Pasture surface may hence not contribute enough to minimize resistant parasite replication.

Frequency of horse movements was also a major determinant of drug efficacy as premises with rare movements had less chance of reduced drug efficacy than premises with monthly movements. Animal movement is a major determinant of gene flow between strongylid populations and certainly contributes to the spreading of drug resistance alleles across sites (Blouin et al., 1995). Despite the apparent benefit to minimal horse movements, premises with monthly movements had less risk of reduced efficacy that premises with occasional movements. While solving this incongruence is difficult with current data, monthly movements may be quicker hence reducing the chance for contaminating pastures. In addition, the implementation of drenching or quarantine or both upon arrival did not contribute enough to drug efficacy to be retained by the variable selection procedure. While such quarantine may prevent the diffusion of drug resistant populations, the beneficial justification of this strategy hence remains unresolved by our data. A lack of statistical power cannot be ruled out as only 39 farms were available for this survey.

Interestingly, higher drenching frequency, *i.e.* one treatment every 3 or 4 months, significantly increased the risk of pyrantel resistance which was neither the case for lower treatment pressure (one every 6 to 12 months) advocating for reduced treatment pressure.

While these factors should be considered in the field to guide the implementation of thorough monitoring of parasite resistance, other factors favouring sustained anthelmintic efficacy were identified. Noticeably, FEC-based drenching programs and determination of drug dose on an individual basis were significantly associated with higher drug efficacy across drugs, especially for fenbendazole. Both practices have long been advocated for in ruminant and equine systems as a sustainable parasite management practices, as the former is thought to reduce selection pressure (Kenyon et al., 2009) and the latter is thought to prevent under dosing whose impact on drug resistance development relies on many parameters (Silvestre et al., 2001; Smith et al., 1999). Our findings provided strong evidence to promote their enforcement in the field.

In addition, risk for drug resistance decreased with higher horse-to-worker ratios which may indicate an overall better management in these premises, corroborated by the better ERR measured in premises where managers were more independent in managing health problems. Overall, these indicators of more specialized or experimented managers could constitute good predictors for implementing drug resistance testing.

Importantly, veterinary expenses were a significant driver of drug efficacy, premises spending more having less chance of reduced efficacy across drugs, whereas premises with less than 100€ per horse per year had more risk of drug resistance, especially for fenbendazole that is the cheapest anthelmintic available. This association may hence reveal the more frequent use of fenbendazole in premises with lower expenses. Also, four out of 6 premises reporting off-licence use of anthelmintics did not spend more than 200€ per horse per year. This indication of collinearity between the two variables may explain why off-license use of drugs was not retained by the variable selection procedure. While off-license use of anthelmintics is certainly driven by financial interests, *i.e.* the buying of cheaper ruminant-dedicated products, it practice may lead to sub-optimal drug concentrations that usually favour the emergence of resistant populations (Day and Read, 2016) and could explain the trend between expenses and resistance risk. However, off-license uses generally involved doramectin. Although cross-resistance between ivermectin and doramectin could be involved as already described in ovine parasitic nematodes (Martinez-Valladares et al., 2012), the impact of doramectin use on other drug efficacy is less probable as current knowledge of molecular bases of drug resistance leaves only minor room for overlap between fenbendazole, pyrantel and macrocyclic lactone resistance (Kotze et al., 2014).

Surprisingly, off-license use of drugs was generally found in premises seeking advice from their veterinarians for parasite management. Our findings also suggest that premises relying on their veterinarians for their drenching regimen design exhibited reduced drug efficacy. These two findings need cautious interpretation and the latter association may be the consequence of identified problematic parasite control by managers who ultimately ask their veterinarians for advice. Recent study in the UK suggests that practitioners may provide useful advice on drug use (Easton et al., 2016) and thus reinforce their role in sustainable parasite control. However, a sub-optimal awareness of veterinarians in integrated management of strongyles in France cannot entirely be ruled-out (Sallé and Cabaret, 2015).

Conclusions

This study reports the first risk estimation analysis between management practices and drug efficacy in equine strongyles. While drug resistance prevalence remains in agreement with previous surveys from France and other countries, *i.e.* a generalized failure of fenbendazole, a decreasing efficacy of pyrantel and reasonably high efficacy of ivermectin despite evidence of reduced egg reappearance period. Most importantly, we have quantified the relative risks and benefits associated with equine farms management practices. These estimations provide robust support to advocate for FEC-based treatment regimens as well as individual determination of anthelmintic dose. In addition, horse movements were a significant contributor of drug resistance suggesting that better control and management of these movements should be recommended. The horse-to-worker ratio may also be an indicator of better efficacy. On the contrary, outdoor-breeding systems, grazing systems with little rotation and manure spreading increased the risk of reduced drug efficacy.

These first insights into determinants of drug efficacy only focused on environmental factors, putting aside intrinsic worm characteristics, like species composition, that should be investigated further. Recent advances in parasite metagenomics would help addressing this question.

Figure 1. Environmental variables and respective level frequency observed from questionnaire data

Retained variables for association with infection status and egg reduction rate and their respective level frequencies have been plotted for questions related to premise major features (A), pasture description and management (B), horse health management (C) and parasite management strategies (D). For each variable, levels and their cumulative occurrence in questionnaire data have been represented with a different colour.

Figure 2. Risk factors associated with drug efficacy across drug class

For each of the retained management practice, the relative risk of reduced (below 1) or increased (higher than 1) Egg Reduction Rate (ERR) is plotted. Each dot stands for the mean odd ratio associated to the considered variable, relative to a reference level specific to each variable. Associated 95% confidence intervals are represented as horizontal lines. The vertical grey line stands for an odd ratio of 1, *i.e.* no risk difference. Any variable whose confidence interval does not cross the vertical line is significantly affecting the measured ERR. Colours have been chosen to distinguish between variables relative to premise description (light green), horse movements (green), pasture management (blue), health management (pink) and parasite management (red).

Figure 3. Risk-factors associated with drug-specific efficacy rate

A drug-specific risk analysis has been performed to identify risk specifically impacting on fenbendazole (A) and pyrantel (B) Egg Reduction Rate (ERR). Figure interpretation is the same as for figure 2.

Table 1. Average Egg Reduction Rate estimated across premise type and regions

	Region		Ivermectin			Pyrantel			Fenbendazole		
			RS*	SF*	Across region	RS	SF	Across region	RS	SF	Across region
Method 1: FECRT = 100 x (1 - CTL0/CTL14*FEC14/FEC0)	Aquitaine	N	3	3	6	3	3	6	3	3	6
		mean	100	99.9	99.9	75.3	86.7	81	18.8	63	40.9
		sd	0	0.1	0.1	39.5	16.4	27.8	32.5	41.1	41.1
		CI	[100-100]	[91.8-100]	[97.6-100]	[39.4-100]	[41.3-100]	[0-100]	[0-87]	[0-98.9]	[0-98.3]
	Burgundy	N	3	4	7	3	4	7	3	4	7
		mean	99.8	89.5	93.9	99.2	96.3	97.6	31.2	55.9	45.3
		sd	0.3	21.1	15.9	1.4	4.3	3.5	27.9	26	27.8
		CI	[99.4-100]	[0-100]	[0-100]	[100-100]	[83.6-100]	[51.4-100]	[0-77.5]	[0-96.9]	[0-97.1]
	Loire Valley	N	3	3	6	3	3	6	3	3	6
		mean	100	100	100	97.2	93.3	95.2	32.5	68.2	50.4
		sd	0	0	0	2.6	9.3	6.5	29.6	9.6	27.8
		CI	[100-100]	[100-100]	[100-100]	[85.3-100]	[50-100]	[19.6-100]	[0-78.6]	[0-100]	[0-100]
	Normandy	N	2	3	5	2	3	5	2	3	5
		mean	99	99.9	99.5	90.8	99.3	95.9	45.9	50.8	48.9
		sd	1.4	0.2	0.9	12.3	1	7.7	64.9	46.9	46.5
			[82.2-100]	[95-100]	[0-100]	[80.3-100]	[92.8-100]	[60.3-100]	[0-100]	[0-100]	[0-100]
	Across premise type	N	11	13	24	11	13	24	11	13	24
		mean	99.8	96.7	98.1	90.6	94.1	92.5	30.8	59.2	46.2
		sd	0.6	11.7	8.6	20.8	9.3	15.4	32.5	29.6	33.5
		CI	[98.8-100]	[95.2-100]	[95-100]	[39.4-100]	[58.9-100]	[41.3-100]	[0-92.3]	[0-100]	[0-100]

Table 1 continued

	Region				Ivermectin			Pyrantel			Fenbendazole
Method 2: FECRT = 100 x (1 - FEC14/FEC0)	Aquitaine	N	7	6	13	7	6	13	7	6	13
		mean	100	98.7	99.4	93.8	73.4	84.4	13.9	51.6	31.3
		sd	0	2.9	2	6.9	36.2	26.1	14.7	41.8	34.9
		CI	[100-100]	[70.8-100]	[79.2-100]	[0-100]	[0-100]	[0-100]	[0-79.6]	[0-98.7]	[0-98.5]
	Burgundy	N	4	4	8	4	4	8	4	4	8
		mean	99.6	88.1	93.8	98.9	95.6	97.3	22.3	42.5	32.4
		sd	0.5	23.8	16.8	1.5	4.8	3.7	30.2	37	33.1
		CI	[96.3-100]	[0-100]	[0-100]	[91.4-100]	[78.6-100]	[78.6-100]	[0-88.2]	[0-92.4]	[0-92.4]
	Loire Valley	N	4	4	8	4	4	8	4	4	8
		mean	100	79.5	89.8	96.5	74.1	85.3	36.1	60.9	48.5
		sd	0	41	29	2.4	33.8	25.2	15.2	15.5	19.4
		CI	[100-100]	[0-100]	[0-100]	[87.7-100]	[26.7-100]	[26.7-100]	[0-78.6]	[0-100]	[0-100]
	Normandy	N	5	5	10	5	5	10	5	5	10
		mean	99.4	99.9	99.6	97.5	95.5	96.5	83.4	39.7	61.6
		sd	1.3	0.2	0.9	3.2	8.9	6.4	18.2	36.2	35.5
		CI	[60-100]	[99.2-100]	[99.2-100]	[85.1-100]	[38.7-100]	[85.1-100]	[0-100]	[0-100]	[0-100]
	Across premise type	N	20	19	39	20	19	39	20	19	39
		mean	99.8	92.7	96.3	96.3	84	90.3	37.4	48.5	42.8
		sd	0.7	21.1	15	4.8	26.5	19.6	33.6	33.3	33.5
		CI	[96.3-100]	[0-100]	[79.2-100]	[52.6-100]	[38.7-100]	[39.7-100]	[0-100]	[0-100]	[0-100]

Drug-specific average Egg Reduction Rates (mean) and standard deviations (sd) measured 14 days after treatment have been collated for each drug and region of interest for the two egg reduction rate calculation methods used. Cross-sectional confidence intervals N indicates the number of premises available, while RS and SF stand for riding-school and stud farm respectively.

Supplementary Data 1. Retained variables and data distribution across premises

Supplementary Data 2. Average faecal egg count by premise type and region before anthelmintic treatment

Supplementary Data 3. Farm-level egg reduction rates with associated confidence intervals

Supplementary Data 4. Estimated relative risks associated with every retained environmental variable with associated confidence intervals

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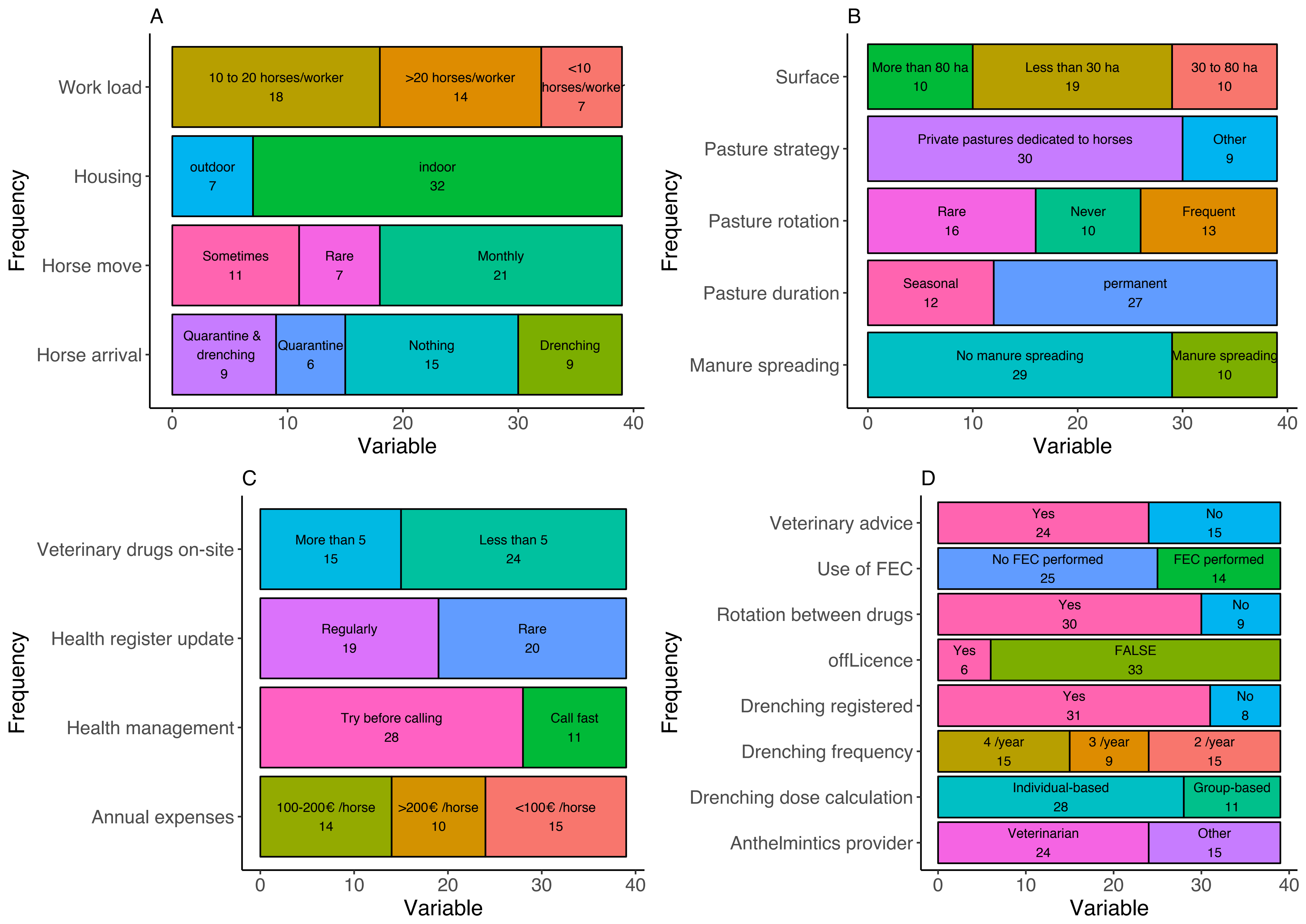
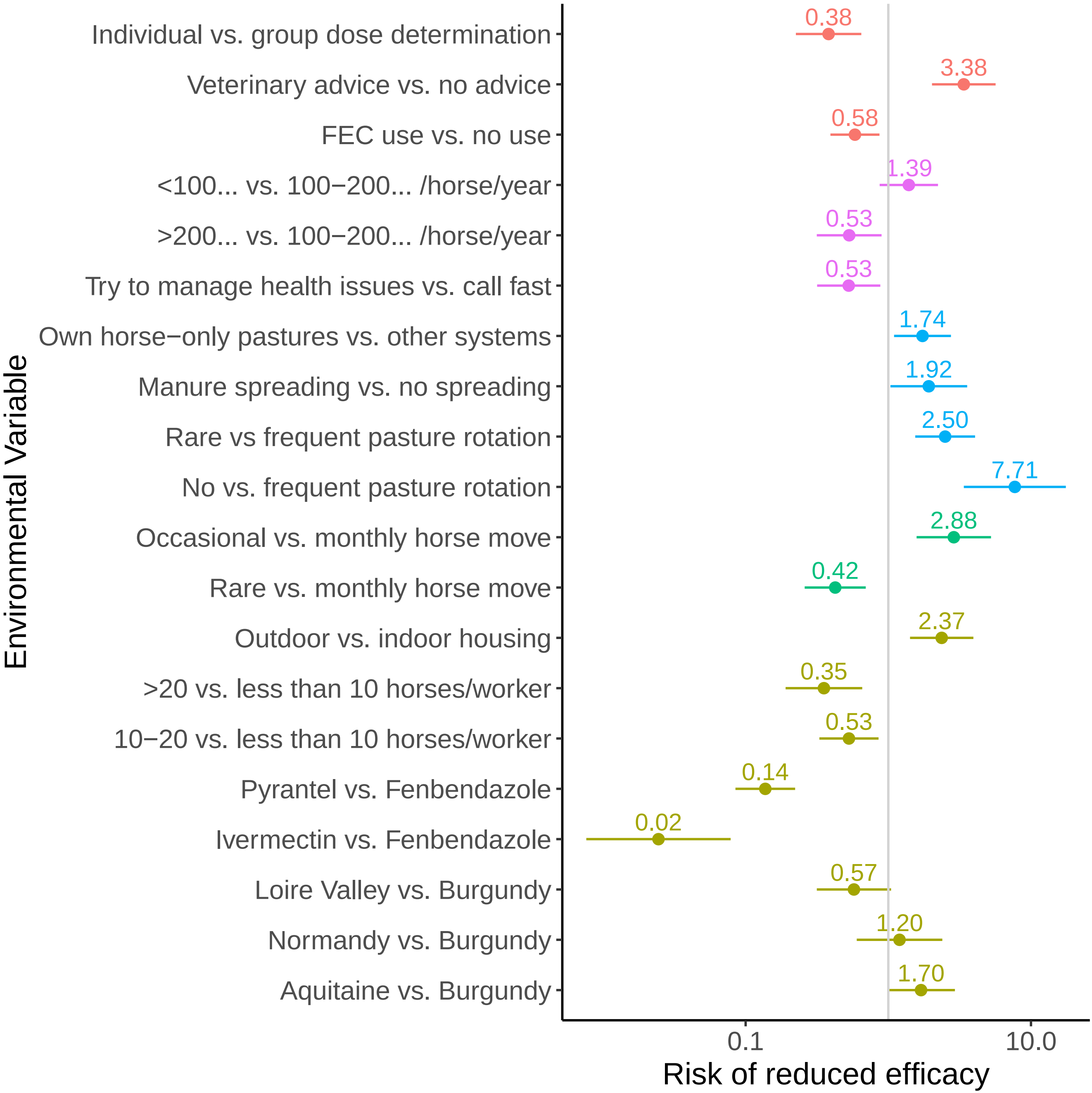
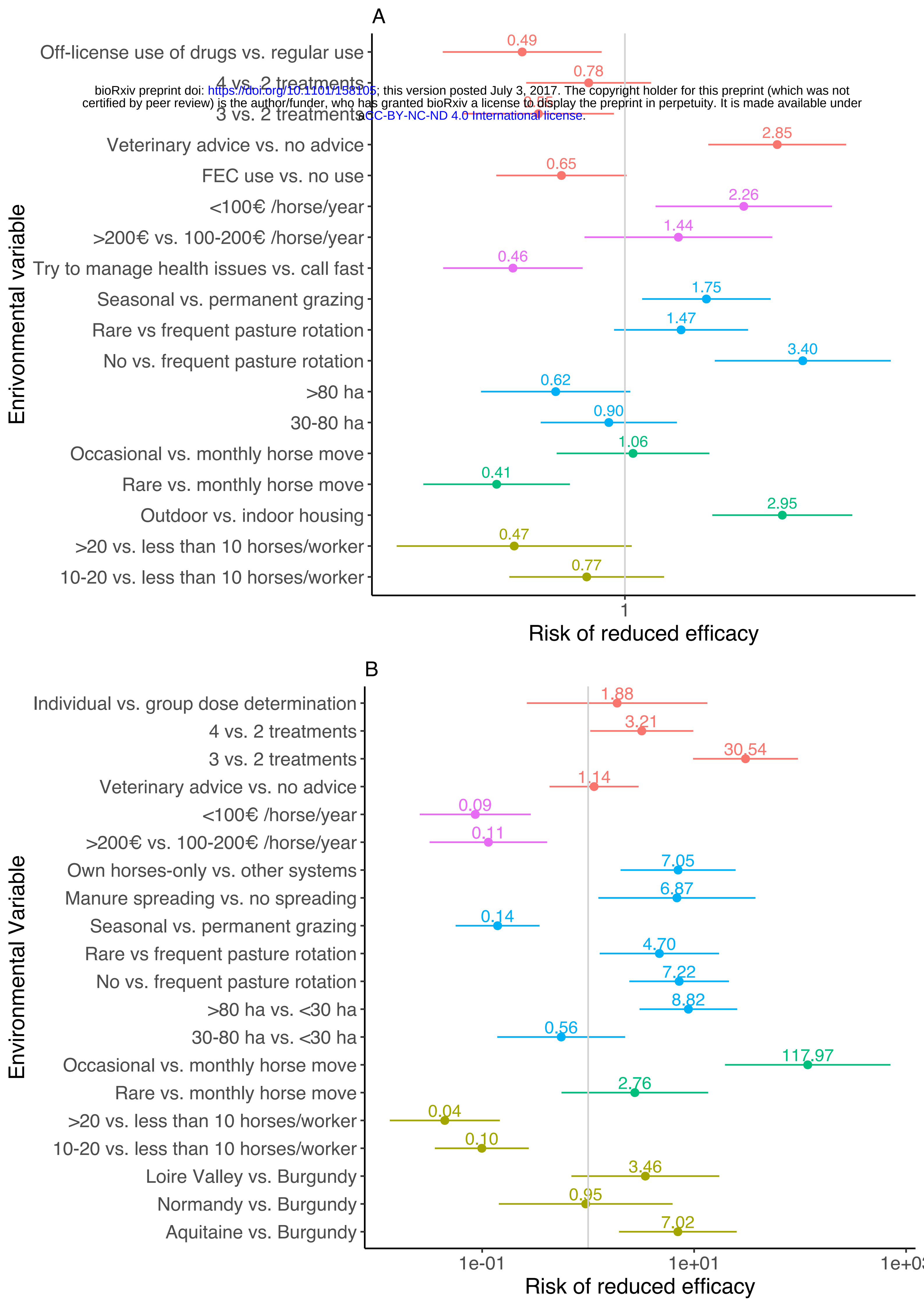

A
Work load
Housing
Horse move
Horse arrival
10 to 20 horses/worker
18
>20 horses/worker
14
<10 horses/worker
7
outdoor
7
indoor
32
Sometimes
11
Rare
7
Monthly
21
Quarantine & drenching
9
Quarantine
6
Nothing
15
Drenching
9
Frequency
0
10
20
30
40
Variable
B
Surface
Pasture strategy
Pasture rotation
Pasture duration
Manure spreading
More than 80 ha
10
Less than 30 ha
19
30 to 80 ha
10
Private pastures dedicated to horses
30
Other
9
Rare
16
Never
10
Frequent
13
Seasonal
12
permanent
27
No manure spreading
29
Manure spreading
10
Frequency
0
10
20
30
40
Variable
C
Veterinary drugs on-site
Health register update
Health management
Annual expenses
More than 5
15
Less than 5
24
Regularly
19
Rare
20
Try before calling
28
Call fast
11
100-200€ /horse
14
>200€ /horse
10
<100€ /horse
15
Frequency
0
10
20
30
40
Variable
D
Veterinary advice
Use of FEC
Rotation between drugs
offLicence
Drenching registered
Drenching frequency
Drenching dose calculation
Anthelmintics provider
Yes
24
No
15
No FEC performed
25
FEC performed
14
Yes
30
No
9
Yes
6
FALSE
33
Yes
31
No
8
4 /year
15
3 /year
9
2 /year
15
Individual-based
28
Group-based
11
Veterinarian
24
Other
15
Frequency
0
10
20
30
40
Variable


Individual vs. group dose determination
Veterinary advice vs. no advice
FEC use vs. no use
<100... vs. 100−200... /horse/year
>200... vs. 100−200... /horse/year
Try to manage health issues vs. call fast
Own horse−only pastures vs. other systems
Manure spreading vs. no spreading
Rare vs frequent pasture rotation
No vs. frequent pasture rotation
Occasional vs. monthly horse move
Rare vs. monthly horse move
Outdoor vs. indoor housing
>20 vs. less than 10 horses/worker
10−20 vs. less than 10 horses/worker
Pyrantel vs. Fenbendazole
Ivermectin vs. Fenbendazole
Loire Valley vs. Burgundy
Normandy vs. Burgundy
Aquitaine vs. Burgundy
0.38
3.38
0.58
1.39
0.53
0.53
1.74
1.92
2.50
7.71
2.88
0.42
2.37
0.35
0.53
0.14
0.02
0.57
1.20
1.70
Environmental Variable
0.1
10.0
Risk of reduced efficacy


A
Off-license use of drugs vs. regular use
4 vs. 2 treatments
3 vs. 2 treatments
Veterinary advice vs. no advice
FEC use vs. no use
<100€ /horse/year
>200€ vs. 100-200€ /horse/year
Try to manage health issues vs. call fast
Seasonal vs. permanent grazing
Rare vs frequent pasture rotation
No vs. frequent pasture rotation
>80 ha
30-80 ha
Occasional vs. monthly horse move
Rare vs. monthly horse move
Outdoor vs. indoor housing
>20 vs. less than 10 horses/worker
10-20 vs. less than 10 horses/worker
0.49
0.78
2.85
0.65
2.26
1.44
0.46
1.75
1.47
3.40
0.62
0.90
1.06
0.41
2.95
0.47
0.77
Enrivonmental variable
1
Risk of reduced efficacy
B
Individual vs. group dose determination
4 vs. 2 treatments
3 vs. 2 treatments
Veterinary advice vs. no advice
<100€ /horse/year
>200€ vs. 100-200€ /horse/year
Own horses-only vs. other systems
Manure spreading vs. no spreading
Seasonal vs. permanent grazing
Rare vs frequent pasture rotation
No vs. frequent pasture rotation
>80 ha vs. <30 ha
30-80 ha vs. <30 ha
Occasional vs. monthly horse move
Rare vs. monthly horse move
>20 vs. less than 10 horses/worker
10-20 vs. less than 10 horses/worker
Loire Valley vs. Burgundy
Normandy vs. Burgundy
Aquitaine vs. Burgundy
1.88
3.21
30.54
1.14
0.09
0.11
7.05
6.87
0.14
4.70
7.22
8.82
0.56
117.97
2.76
0.04
0.10
3.46
0.95
7.02
Environmental Variable
1e-01
1e+01
Risk of reduced efficacy