An automated, multiplex-tandem PCR platform for the diagnosis of gastrointestinal nematode infections in cattle: An Australian-European validation study

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Abstract

Detecting the genera and species of gastrointestinal (GI) nematode infections in faecal samples obtained from cattle requires the incubation of faeces ('larval culture') followed by identification of the third-stage larvae that are harvested after 10-14 days. Substantial research in the development of PCR-based methods for the rapid and specific identification GI nematodes has been conducted for small ruminants, whilst only few such assays have been developed for cattle. In the present paper we describe the development of an automated, robotic PCR platform for the detection and genus and/or species-specific identification of GI nematodes from bovine faecal samples. This test was then validated using samples from different regions of three countries (Australia, Belgium and Scotland). The PCR platform was found to be highly sensitive and specific for the identification of the important GI nematodes in naturally infected cattle (both estimates >90%). The PCR platform can also estimate the percentage of genera or species present in a mixed-species infection, and was found superior to larval culture in terms of speed (1-2 days *versus* 1-2 weeks for culture), sensitivity and specificity. The PCR was simple to use and the operator requires no knowledge or experience to identify the nematodes present, compared to larval culture where even experienced operators can make substantial errors due to considerable overlap in the published characteristics of key species.

Keywords:

Ruminants

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Epidemiology

1. Introduction

A rapidly increasing human population, combined with a warming climate and changes in the temporal and geographical distribution of major animal pathogens, represent major challenges for efficient and sustainable agricultural and livestock production. Underpinning the response to these challenges is the need for increasingly sensitive and rapid diagnostic methods that can detect subtle changes in the epidemiology of common livestock diseases (van Dijk et al., 2008; Skuce et al., 2013; Moustafa, 2015).

Gastrointestinal (GI) nematode infections (Order Strongylida) are common and widespread in domestic livestock, including sheep, goats and cattle, and are of major socioeconomic importance in both developing and developed economies (Charlier et al., 2015). In the developed world, infections with GI nematodes are associated predominantly with lost production, such as decreased weight gain, reduced feed consumption, impaired reproduction and ill-thrift (Hawkins, 1993; Perry and Randolph, 1999). Together, these can significantly reduce the productivity and profitability of farming enterprises (Charlier et al., 2014). Infections with GI nematodes are routinely treated with broad-spectrum anthelmintics, which until recently were derived from only three chemical classes (benzimidazole, levamisole and macrocyclic lactone) (Besier and Love, 2003). Two more classes have recently become available for use in sheep, monepantel (an amino-acetonitrile derivative) and derquantel (paraherquamide), but these are not yet marketed for use in cattle (Kaminsky et al., 2008). Resistance to anthelmintics in GI nematodes of cattle is not yet as severe, nor as prevalent, as it is in small ruminants (Kaplan, 2004; Kaplan and Vidyashankar, 2012), although reports are becoming increasingly common (Kaplan, 2004; Rendell, 2010; Felippelli et al., 2014; Geurden et al., 2015). The development

of new anthelmintics is expensive, and can take decades, whereas resistance can develop more rapidly, often within a few years of the release of a new drug (Kaplan, 2004). Clearly there is a need to preserve the effectiveness of existing anthelmintics, and this can be supported by more frequent monitoring of worm burdens and adoption of management practices that delay the development of resistance (Besier et al. 2010; Larsen, 2014; Leathwick, 2014).

The routine diagnosis of GI nematode infections is traditionally based on counting their eggs in a microscopic chamber using a faecal flotation method ('worm egg counts', MAFF, 1986). However, this provides little information on the infecting species because, apart from *Nematodirus* spp., the eggs of most important GI nematodes are morphologically indistinguishable unless viewed individually at high magnification (Georgi and McCulloch, 1989). This is labour-intensive and expensive and potentially unreliable, since there is substantial overlap between the size of eggs from different species. Consequently, larval culture (LC) is needed to identify which genera of nematodes are predominant in any given sample. This requires incubation of faecal samples for 7-10 days to hatch infective third-stage larvae (L_3), which are then identified using published information on size and morphology of the important nematode genera and species (Levine, 1968; MAFF, 1986, Van Wyk et al., 2004). This approach is both laborious and potentially inaccurate, as there is considerable overlap in the measurements of these genera (Roeber and Kahn, 2014). In addition, the temperature at which faeces are incubated can markedly affect the relative abundance of the infective larvae harvested and counted, and so the results of LC may not reflect the original population of nematode eggs that would ultimately go onto pasture (Berrie et al., 1988; Dobson et al., 1992; Roeber & Kahn 2014). Another limitation of traditional diagnostic methods is that in cattle, worm egg counts are

114 generally lower compared to small ruminants, and so flotation methods that are
115 suitable for sheep and goats are not sensitive enough to accurately monitor worm
116 burdens in adult cattle. This has implications for both the routine monitoring of worm
117 egg counts and testing for anthelmintic resistance using the faecal egg count
118 reduction test (Mes et al., 2001; Levecke et al., 2012).

119 A limited number of PCR or RT-PCR assays have been developed for the diagnosis
120 of GI nematode infections in cattle, but these are either limited in the number of
121 species or genera they detect, or involve time-consuming, manual reactions and
122 visualisation of PCR amplicons during electrophoresis, increasing the potential for
123 cross-contamination between samples. In addition, these do not allow a quantitative
124 or even semi-quantitative estimate of the proportion of the genera or species present
125 (Zarlenga et al., 2001; Höglund et al., 2013). More recently, a deep amplicon
126 sequencing approach has been described that shows very promising results in
127 quantifying the composition of gastrointestinal nematode communities in cattle
128 (Avramenko et al., 2015). However, the high technical requirements of this approach
129 might limit its usefulness to research applications but are less suitable for the routine
130 diagnosis of GI nematode infections in veterinary service laboratories.
131

132 Recently, the use of multiplexed-tandem PCR ('MT-PCR') (Stanley and Szewczuk,
133 2005) has been described for the specific diagnosis of GI nematode infections in
134 small ruminants (Roeber et al., 2012). MT-PCR consists of two amplification phases:
135 (i) a primary 'target enrichment' phase, through a small number of PCR cycles, using
136 multiplexed primer sets, and (ii) a subsequent analytical amplification phase (using a
137 diluted product from the primary amplification as a template), consisting of the
138 targeted amplification, in tandem rather than by multiplex, of each genetic locus

using specific, nested primers. Using this method, the initial amplification phase is limited to 10–15 cycles and so interactions between or among multiplexed primer sets are minimized. This reduces competition or the generation of artefactual products, hence limiting amplification bias which would otherwise prevent downstream quantification (Stanley and Szewczuk, 2005). The primary amplicons are diluted prior to their use as templates in the secondary phase, and so primer carry-over and PCR inhibition are also reduced. Conducting the secondary (analytical) amplification phase in tandem means that this method can be coupled to a single-channel RT-PCR thermocycler, allowing rapid screening of multiple samples in parallel and quantification employing one fluorogenic dye (e.g., SYTO-9) at a reduced cost.

In the present paper we adapt techniques previously developed for small ruminants to the diagnosis of key species and genera of GI nematodes in cattle. The aim was to critically evaluate the performance of this new test using known positive control genomic DNA samples from target nematode species, and compare it with the current routine diagnostic tests, namely faecal egg counts and LC. Further, to examine the utility of MT-PCR as a routine diagnostic test for GI nematode infections in cattle, and its suitability for screening bovine faecal samples in different regions, we deployed this test in three different laboratories in Australia, Belgium and Scotland. Each laboratory analysed faecal samples from naturally infected cattle in their respective region, comparing the results of MT-PCR with their routine diagnostic methods.

2. Materials and Methods

2.1 Faecal sample procurement and worm egg counts

Three laboratories participated in this study. Collection of faecal samples from cattle and subsequent microscopic and molecular testing were carried out by the Mackinnon group at the University of Melbourne (Werribee, Australia), the Laboratory of Parasitology at Ghent University (Merelbeke, Belgium) and Moredun Research Institute (Edinburgh, Scotland). In Australia, between November 2014 and January 2016 a total of 144 fresh faecal samples were collected from cattle from different properties and climatic zones. 115 of these samples were collected from 7 different properties in Victoria (VIC) (characterised as a winter-dominant rainfall zone) and 29 samples were collected from 3 different properties in central and northern New South Wales (NSW) (characterised by summer-dominant rainfall). Samples were also collected from farms in Belgium (n=23) and Scotland (n=35). Where available, farm details (age of the animals, size of the mob and herd, co-grazing with sheep, drench history, etc.) were also collected. At each laboratory, 3-4 grams of faeces were used and faecal egg counts performed according to the local routine laboratory protocol (i.e. modified McMaster method (MAFF, 1986) in Australia and Belgium, and a modified salt-flotation method as described by Christie & Jackson (1982) at Moredun.

2.2 Larval culture and speciation of third-stage larvae (L_3)

Larval cultures (LC) were carried out in Australia (n=58) and Belgium (n=15). In brief, 20-30 grams of fresh faecal material were mixed with vermiculite (Exfoliators Aust, Pty. Ltd., Geelong, Australia) until a well-aerated moist mixture resulted. For the incubation of LC the laboratories in Australia and Belgium used different incubation protocols based on their standard practices. In Australia, the LC were incubated at 22 °C for ten days for all samples originating from a winter rainfall environment (n=29), which is dominated primarily by species of *O. ostertagi* and *C. oncophora*.

188 Samples from Australia's summer rainfall environment (n=29) were incubated at 24
189 °C to achieve a better representation of *Haemonchus* spp. in these culture samples.
190 In Belgium, all samples were incubated at 25 °C for 12 days. Optimal humidity,
191 temperature and aeration of the cultures were checked regularly during incubation.
192 On the 11th day (12^{th} day in Belgium) the containers were exposed to light to induce
193 phototropism in the third-stage larvae (L_3), causing them to surface and climb the
194 walls of the container. The container walls were washed using tap water and the L_3
195 were extracted and stored at 4 °C until speciation. In Belgium, L3's were collected
196 after 12 days by the Baermann technique and when present 200 larvae were
197 identified in-house according to their routinely employed protocol (Borgsteede and
198 Hendriks, 1974; MAFF, 1986, Van Wyk et al., 2004). In Australia, L_3 from the faecal
199 cultures were sent to the Veterinary Health Research (VHR) laboratory, Armidale for
200 independent identification.

201 2.3 Egg harvest, genomic DNA extraction and PCR

202 The steps required for sample preparation, DNA extraction and MT-PCR setup were
203 carried out in each location according to the same procedure. Four grams of faeces
204 were suspended in 50 ml of saturated saline in a conical centrifuge tube (Biologix
205 Group Ltd. KS) and centrifuged at 1000 g for 2 min. The supernatant (5 ml) was
206 decanted into a fresh tube and the volume was increased to 50 ml by adding water.
207 The suspension was centrifuged at 2000 g for 5 min. The supernatant was discarded
208 without disturbing the faecal pellet in the bottom of the tube. The pellet was
209 harvested into a 2 ml Eppendorf tube, and stored at -20°C, or was directly used for
210 DNA extraction. The pellet was spun at 1000 g for 1 min and a 250 µl aliquot of the
211 precipitate harvested for DNA extraction using a commercial kit following the
212 manufacturer's instructions (PowerSoil® DNA Isolation Kit, Mobio Laboratories Inc.,

213 USA). For a subset of samples (Australia, n=58, Belgium, n=15), a similar procedure
214 was used to extract DNA from 250 µl of each LC.

215 2.4 Robotic reaction setup and MT-PCR assays

216

217 MT-PCR was performed using the Easy-Plex system (AusDiagnostics Pty. Ltd.,
218 Australia), consisting of a Rotor-Gene 6000 real-time PCR thermocycler (Qiagen),
219 and a Gene-Plex CAS1212 liquid handling robot (AusDiagnostics Pty. Ltd.). Two MT-
220 PCR test panels were designed, one for Australia (Cat.: 38094) and one for Europe
221 (Cat.: 38095), based on the importance of different nematode species in the two
222 locations. The Australian version contained an assay for *Bunostomum phlebotomum*
223 and the European version contained an assay for *Dictyocaulus viviparus*. Otherwise,
224 the remaining target nematode assays were identical for both panel versions.
225 Specific primers (AusDiagnostics Pty. Ltd.) were designed to the internal regions of
226 the nuclear ribosomal DNA sequences of *Haemonchus contortus* and *H. placei*,
227 *Ostertagia ostertagi*, *Trichostrongylus* spp., *Oesophagostomum radiatum*, *Cooperia*
228 *oncophora*, *Bunostomum phlebotomum* (AU) and *Dictyocaulus viviparus* (EU) in
229 order to produce amplicons of 100–200 bp (depending on species) in the second
230 phase of MT-PCR. Additionally, a pan-nematode assay was developed specific to
231 the nuclear ribosomal DNA sequences of nematodes and included as a positive
232 control. The following sequences were used as positive controls during the design of
233 the genus and/or species-specific assays: GenBank accession nos. *Ostertagia*
234 KX929994.1, *Haemonchus* KC415119.1, *Trichostrongylus* KU891930.1, *O. radiatum*
235 KP150505.1, *Bunostomum* FJ616999.1, *Cooperia* spp. KC998737.1, *Dictycaulus*
236 KM359413.1. The discs, containing lyophilized primer sets, are available through
237 AusDiagnostics Pty. Ltd. In the primary amplification, 5 µl of genomic DNA

representing each sample (n = 8, plus one no-template (negative) control) was loaded into 0.2 ml PCR strips and placed into a 24-well thermocycling block within the Easy-Plex robotic platform. Following the loading of each sample and the initiation of the 'Cattle Parasites Assay', the "low sensitivity" setting (10 cycles in Step 1 PCR) was selected and the remainder of the set-up process and analysis was directed by the program 'Easy-Plex Assay Setup' (AusDiagnostics Pty. Ltd.), with all of the remaining steps of the MT-PCR procedure being semi-automated (Stanley and Szewczuk, 2005). A sample was recorded as test-positive using the 'auto-call function' of the Easy-Plex software, if the amplicon produced a single melting curve which was within 1.5 °C of the expected melting temperature, the height of the peak was higher than 0.2 dF/dT and the peak width was ≤ 3.8 °C (Aus-Diagnostics Pty. Ltd.). Cycle threshold (Ct) values were recorded for each test-positive sample, and quantitative values for each parasite in each sample were determined using an automated comparison with Ct data determined for an internal spike-control (tube containing 10,000 copies of a synthesized oligonucleotide template and a specific primer set) for each sample tested (Stanley and Szewczuk, 2005). Percentage results for the individual parasites in every sample were calculated by the 'Easy-Plex Analysis' software (AusDiagnostics Pty. Ltd.).

2.5 Assessment of assay performance (analytical specificity, sensitivity, repeatability and reproducibility)

To estimate the analytical sensitivity of the cattle nematode 8-plex panels, monospecific eggs of *H. placei* were obtained from the VHR laboratory, Armidale, New South Wales. Eggs originated from experimentally infected cattle, housed indoors and under controlled conditions. Eggs were isolated from faecal matter by salt flotation and samples containing defined numbers of eggs were prepared

representing 5, 10, 50, 100, 200, 500, 750 and 1000 eggs, respectively. DNA from these egg samples was isolated using the Powersoil DNA extraction kit (MoBio) according to the manufacturer's recommendations. Additionally, to determine the minimum detection limit, the "5 eggs" sample was further diluted to prepare theoretical dilutions of 2.5, 1.25, 0.62, 0.3 and 0.12 eggs. Every sample was run on 5 separate occasions in the MT-PCR. MT-PCR experiments were carried out using 5 µl of undiluted (5-1000 eggs) or diluted (0.12-2.5 eggs) DNA samples. Subsequently, another experiment was carried out using the same egg numbers (5-1000) spiked into four grams of helminth-free faecal samples. For every sample, three replicates were produced and were further processed as described above. Specificity of the cattle nematode 8-plex panels (AU and EU version) was assessed based on the testing of genomic DNA samples from morphologically identified adult male worms of target nematode species, including *H. placei*, *O. ostertagi*, *C. oncophora*, *Oe. radiatum*, *T. colubriformis*, *T. vitrinus*, *T. axei*, *T. rugatus* and *D. viviparus* as well as a wide range of closely related nematode species such as *H. contortus*, *Teladorsagia circumcincta*, *Chabertia ovina*, *Oesophagostomum venulosum*, *Nematodirus spathiger* and other parasites known to occur in the bovine digestive tract, such as *Fasciola hepatica*, *Cryptosporidium parvum* and *Giardia duodenalis*. For the assessment of the *B. phlebotomum* assay, a synthetic sequence was used because no genomic DNA sample could be obtained for this species. Additionally, a number of (n=12) helminth-naïve faecal samples from previously treated cattle were tested as known negative controls.

To estimate repeatability (i.e. same samples tested within the same laboratory) and reproducibility (i.e. same samples tested at different laboratories) of results, 16 faecal samples from naturally infected cattle were used. Of these samples, a subset

288 (n = 8) originated from Scotland, UK and were first tested at the Moredun Research
289 Institute and then re-tested in the same laboratory the following day. An aliquot of
290 each sample was sent via courier to the Laboratory of Parasitology, Ghent
291 University, to be tested one more time under the same conditions but by a different
292 operator in a different laboratory. The other subset (n = 8) samples originated from
293 Germany and was first tested at Ghent University, re-tested the following day and an
294 aliquot sent to Moredun, UK.

295 Because MT-PCR is considered more sensitive and specific for the diagnosis of GI
296 nematode infections than the LC method, this fell into the well-understood context of
297 evaluation of diagnostic sensitivity and specificity of assays in the absence of a 'gold-
298 standard'. Consequently, a Bayesian latent-class analysis (including the Australia
299 data only), was employed as recommended by the World Organisation for Animal
300 Health in such settings (OIE, 2014), to estimate diagnostic sensitivity and specificity
301 of the individual MT-PCR assays. The latent class approach also allows estimation
302 of the diagnostic sensitivity and specificity of comparison tests.

303 2.6 Statistical analysis

304 To estimate the analytical sensitivity of the MT-PCR and examine the correlation of
305 MT-PCR results (gene copy number) with the number of monospecific *H. placei* eggs
306 the following analysis was carried out using the SPSS® software (v.22.0, IBM™
307 Corp, Armonk, NY). Spearman's correlations were used to investigate the
308 relationship between the MT-PCR results (gene copy number) for the *Haemonchus*
309 spp. assay, as well as for the pan-nematode assay, with the number of *H. placei*
310 eggs in the presence and absence of faecal matter. To investigate the repeatability
311 and reproducibility of the MT-PCR, the Bland-Altman test (Bland & Altman, 1986)

was used (SPSS) to determine the agreement between the replicates for 16 samples (8 from Germany and 8 from Scotland) that were tested and re-tested on consecutive days and in different laboratories. To determine the agreement between the percentage results for the different species profiles as generated by the MT-PCR (Easy-Plex Analysis software) versus the percentage results as determined by LC method 58 samples from Australia (29 from VIC and 29 from NSW) and 15 samples from Belgium were used.

To determine the agreement of positive versus negative results between both techniques (i.e. MT-PCR versus LC) pivot tables were created in the Microsoft Excel 2010 software package and the level of agreement as well as Kappa values were determined using an established approach (Conraths and Schares, 2006) and using the WinEpiscope online tool (http://www.winepi.net/). Kappa values were adjusted for bias and prevalence (PABAK) according to Byrt et al. (1993). Interpretation of Kappa was done according to the definitions of Landis and Koch (1977). The percentage results as determined by MT-PCR as well as by LC were plotted in a stacked column chart using Microsoft Excel 2010. To further compare the percentage results determined for the different species/genera and with the different test methods concordance correlation coefficients were determined (SPSS). Because the LC method provides an imperfect gold-standard for comparison of performance for the MT-PCR results were further assessed by Bayesian latent-class analysis. The latter two statistical approaches were only applied to the Australian dataset because of the information available for these samples and better defined sampling localities.

3. Results

336 3.1 Assay performance – Analytical sensitivity, specificity, repeatability and
337 reproducibility

338 Positive MT-PCR results were obtained from every sample and each replicate
339 containing 5 - 1000 *H. placei* eggs for assays of *Haemonchus* spp. and pan-
340 nematode (Table 1), and there was no inhibition evident in any of the samples as
341 indicated by the amplification of the spike control. Amplification in the pan-nematode
342 assay was more efficient resulting in higher gene copy numbers recorded for every
343 sample and replicate. An almost perfect linear correlation between egg numbers and
344 observed MT-PCR gene copy numbers of rs= 1.0 ($P<0.001$) and rs= 0.995
345 ($P<0.001$) was determined for the *Haemonchus* spp. and pan-nematode assay,
346 respectively. Samples further diluted to contain 0.12-2.5 eggs also produced positive
347 results for every sample and for both assays but not for every replicate (Table 1). For
348 the faecal samples spiked with known quantities of eggs, the lowest detectable
349 quantity of eggs was 5 eggs (in 2/3 replicates) and the highest detectable quantity
350 was 1000 eggs, for both, the *Haemonchus* spp. and pan-nematode assay, and was
351 consistently achieved for all three replicates (data not shown). Correlation between
352 egg numbers and MT-PCR gene copy numbers was rs = 1.0 ($P<0.001$) for both
353 assays.

354 Testing of positive control genomic DNA samples from adult nematodes yielded
355 specific results for every assay in the panel (Table 2). The *Haemonchus* spp. assay
356 produced positive results for both species, *H. placei* and *H. contortus*, and the
357 *Trichostrongylus* spp. assay produced positive results for *T. vitrinus*, *T. colubriformis*,
358 *T. rugatus* and *T. axei*. The pan-nematode assay yielded positive results for every
359 nematode DNA sample, including DNA samples of all six target nematode species
360 and genera, as well as for the non-target nematode samples tested in this

experiment including species of *Teladorsagia circumcincta*, *Chabertia ovina*, *Oesophagostomum venulosum* and *Nematodirus spathiger* (but not for any of the trematode (i.e., *Fasciola hepatica*) or protozoan parasites (i.e., *Giardia* and *Cryptosporidium*)). Due to the unavailability of a genomic DNA sample for *B. phlebotomum* specificity testing for this species was based on a synthetic sequence.

With the exception of one sample in which minute amounts of nematode DNA were detected by the pan-nematode assay, no specific amplification was observed in any of the negative control samples and amplification of the internal spike control indicated the absence of inhibition of the MT-PCR (Table 2). The replicate testing of 16 samples in two different laboratories demonstrated high levels of repeatability and reproducibility for all samples tested and across all of the target nematode species (Table 3). There were only three samples (1UK, 7 UK and 5 Ger) in which minute amounts of either *O. ostertagi* DNA (samples 1 UK and 5 Ger) or *Trichostrongylus* DNA (sample 7UK) were detected in one of the replicates but not in the others. Bland-Altman agreement tests showed that in the three MT-PCR replicates tested for each of the 16 samples there was a significant agreement of P>0.06, P>0.11, P>0.126, P>0.25, and P>0.33 for species/genera of *O. ostertagi*, *Haemonchus* spp., *Trichostrongylus* spp., *Oe. radiatum* and *C. oncophora*, respectively. *D. viviparus* was not detected in any of the tested European samples.

3.2 MT-PCR assessment in relation to traditional diagnostic methods – Faecal egg counts and larval culture method

In Australia, a total of 144 faecal samples were tested, of which 133 were positive by microscopy. MT-PCR detected a total of 140 samples to be positive for either one or

more target nematode species. There were 8 microscopy negative samples which were positive by MT-PCR for one or more target nematode species and 1 microscopy positive sample (count of 15 eggs per gram) which was MT-PCR 'false-negative'. In Scotland, 35 faecal samples were tested, of which 33 were positive by microscopy and 34 were positive by MT-PCR. MT-PCR detected 2 additional positive samples and there was 1 microscopy positive sample (count of 13 eggs per gram) that was MT-PCR 'false-negative'. In Belgium, 23 faecal samples were tested, of which 16 were positive by microscopy and 21 were positive by MT-PCR. The amplification of the internal spike control in the 2 MT-PCR 'false-negative' (1 from Australia and 1 from Scotland) samples showed that negative results were not due to inhibition of the MT-PCR. A representative number of MT-PCR products (n = 43) for each species were subject to DNA sequencing which confirmed the produced amplicons to be 100% specific.

To compare the results of MT-PCR and LC method, each Australian and Belgium sample was tested in three different ways: MT-PCR eggs (MT-PCR $_{(e)}$), MT-PCR larvae (MT-PCR $_{(l)}$) and LC. There was a varying level of agreement between the results of MT-PCR (eggs and/or larvae) and the LC technique for the different species detected (Table 4).

In Australia (including samples from Victoria and New South Wales), the agreement between positive *versus* negative results between the MT-PCR$_{(e)}$ and LC method was highest for *Haemonchus* spp., *O. ostertagi* and *C. oncophora*, with a calculated 96.60% agreement. Agreement between MT-PCR$_{(e)}$ and LC for both *Trichostrongylus* spp. and *Oe. radiatum* was lower (86.20% and 75.90%, respectively). Adjusting Kappa for prevalence and bias (PABAK) resulted in an improved Kappa of 0.931 ('almost perfect agreement') for *Haemonchus* spp., *C.*

410 *oncophora* and *Ostertagia*, a Kappa of 0.724 ('substantial agreement') for species of
411 *Trichostrongylus* and 0.517 ('moderate agreement') for *Oesophagostomum* (Table
412 4).

413 In Belgium, the agreement between MT-PCR$_{(e)}$ and LC was highest for *Haemonchus*
414 spp., *C. oncophora* and *Trichostrongylus* spp., with a calculated 100% agreement,
415 Kappa 1.000 ('perfect agreement') for all genera/species. Agreement between MT-
416 PCR$_{(e)}$ and LC for *O. ostertagi* was 93.30%, and it was lowest for *Oe. radiatum*
417 (73.30% agreement). Adjustment of Kappa for prevalence and bias (PABAK)
418 resulted in improved Kappa of 0.867 ('almost perfect agreement') for *O. ostertagi*
419 and a Kappa of 0.467 ('moderate agreement') for *Oe. radiatum*. In the Belgian
420 dataset, there were 4 samples in which the LC method detected *Oe. radiatum* but
421 this could not be confirmed by either the MT-PCR$_{(e)}$ or MT-PCR$_{(l)}$ method on the
422 same samples, suggesting that in these samples long-tailed larvae were
423 *Nematodirus* rather than *Oe. radiatum*. Assuming that in all 4 samples a
424 misidentification of *Nematodirus* for *Oe. radiatum* had occurred during morphological
425 identification the agreement was re-calculated as 100%, Kappa 1.000 ('perfect
426 agreement').

427 Overall, the comparison of MT-PCR$_{(l)}$ versus LC resulted in lower agreement
428 compared to the agreement as determined between the results of MT-PCR$_{(e)}$ versus
429 LC (Table 4). In Australia (including samples from VIC and NSW), the agreement
430 between positive *versus* negative results between the MT-PCR$_{(l)}$ and LC method
431 were similar to those of MT-PCR$_{(e)}$ versus LC. Highest agreements were calculated
432 for *Haemonchus* spp. and *O. ostertagi*, with 96.60% agreement and Kappas of 0.931
433 ('almost perfect agreement'), followed by *C. oncophora* with a 94.80% agreement.
434 Agreement between the two diagnostic methods was lower for *Oe. radiatum*, with

435 74.10% agreement, and lowest for *Trichostrongylus* spp. with 63.80% agreement.
436 Adjusting Kappa for prevalence and bias resulted in improved Kappa values for *C.*
437 *oncophora*, 0.897 ('almost perfect agreement'), *Oe. radiatum*, 0.483 ('moderate
438 agreement'), and for *Trichostrongylus* spp., 0.276 ('fair agreement').

439 In Belgium, the agreement between MT-PCR$_{(l)}$ and LC was highest for *O.ostertagi*
440 with 100% agreement, Kappa 1.000 ('almost perfect agreement'), followed by
441 *Haemonchus* spp. and *C. oncophora* with 93.30% agreement, for both species.
442 Agreement between MT-PCR$_{(l)}$ and LC was lower for *Trichostrongylus* spp., with
443 86.70% agreement, and lowest for *Oe. radiatum* with 66.70% agreement. Adjusting
444 Kappa for prevalence and bias resulted in improved Kappa values for *Haemonchus*,
445 0.867 ('almost perfect agreement'), *Trichostrongylus* spp., 0.733 ('substantial
446 agreement'), and for *Oe. radiatum*, 0.333 ('fair agreement') (Table 4). Similar to the
447 comparison between MT-PCR$_{(e)}$ and LC, making the assumption that in 4 samples
448 *Nematodirus* was misidentified for *Oe. radiatum*, the agreement was recalculated as
449 93.30% agreement, Kappa 0.867 ('almost perfect agreement').
450
451 Neither *B. phlebotomum* (AU panel) nor *D. viviparus* (EU panel) were detected in
452 any of the samples tested or by any of the three methods employed during this
453 study, thus the agreement was 100% (data not shown).

454 The Bayesian estimates of diagnostic sensitivity and specificity for the three different
455 diagnostic methods (i.e. MT-PCR$_{(e)}$, MT-PCR$_{(l)}$ and LC) showed that both MT-PCR
456 methods (i.e. eggs and larvae) achieved high diagnostic performance of >90%
457 diagnostic sensitivity and specificity across the target nematode species. Results
458 obtained by the MT-PCR$_{(l)}$ method showed a slightly lower sensitivity of 76-86% for
459 species of *Trichostrongylus* and *Oe. radiatum*. The Bayesian estimates of diagnostic

460 performance for the LC method showed that this method generally achieved similar
461 sensitivities in the detection of the target nematode species, but was inferior in terms
462 of specificity (Table 5).

463 Comparing the proportions of different nematode species for each test sample and
464 between the three different diagnostic methods used (i.e. MT-PCR$_{(e)}$, MT-PCR$_{(l)}$ and
465 LC) showed that, in most samples, *C. oncophora* and/or *O. ostertagi* were the
466 dominant species, whereas *Trichostrongylus* spp. and *Oe. radiatum* had a lower
467 contribution to mixed infections and were generally less prevalent in the samples
468 tested (see Figure 1; see supplementary data for full dataset). *Haemonchus*
469 infections were limited to samples from NSW and were the main contributing species
470 in 6 of the 29 samples tested from that region, with the results consistent by all three
471 test methods.

472 Interestingly, for samples collected from VIC, which were incubated at 22 °C, *C.*
473 *oncophora* showed a higher proportion by MT-PCR$_{(e)}$ whilst the results of MT-PCR$_{(l)}$
474 and LC for the same samples consistently showed a higher proportion of *O. ostertagi*
475 and less for *C. oncophora* (Figure 2). This trend was absent in samples from NSW,
476 which were incubated at 24 °C, and those from Belgium which were incubated at 25
477 °C. In these samples, a similar proportion of the two species was determined by all
478 three methods indicating that these temperatures provided more suitable conditions
479 for the development of these species ().

480 The concordance correlation coefficients for the percentage results as determined by
481 the two different MT-PCR approaches and in comparison to LC showed that
482 agreements for percentages were generally better for samples from NSW, compared

to VIC. There was also a higher agreement between the results of MT-PCR$_{(l)}$ versus LC compared to the agreement between MT-PCR$_{(e)}$ versus LC.

MT-PCR testing of faecal samples (MT-PCR$_{(e)}$) from Australia (VIC, n = 115; NSW, n = 29) and Europe (Belgium, n = 23; Scotland, n = 35) showed that the species *O. ostertagi* was most prevalent in all samples tested (71-97% prevalence) followed by *C. oncophora* (52-86% prevalence) (Table 7). *Haemonchus* spp. were most prevalent (83%) in samples from NSW, Australia, of low prevalence in samples from Scotland (17%) and Belgium (4%), and absent from VIC, Australia. *Trichostrongylus* spp. were most prevalent (72%) in samples from NSW, and of moderate prevalence in samples from VIC, Belgium and Scotland (22-54%). *Oe. radiatum* was of moderate prevalence in samples from NSW, VIC and Belgium (13-31%), and absent in samples from Scotland. No sample was positive for either *B. phlebotomum* (AU panel) or for *D. viviparus* (EU panel).

4. Discussion

This study demonstrated the MT-PCR platform, together with the developed cattle parasites kits, to be an advanced method for the specific diagnosis of GI nematode infections in cattle. The testing of samples spiked with known quantities of monospecific *H. placei* eggs showed sensitive and linear amplification of samples ranging between 0.125 eggs to a 1000 eggs. The *Haemonchus* spp. assay was slightly less sensitive in the range of 0.125-5 eggs, which resulted in some of the replicates not being detected, whereas the pan-nematode assay detected all 5 replicates, also at a range of 0.125-5 eggs. Even though, only *H. placei* was available for the assessment of analytical specificity, and variations may occur based on the number of DNA target copies in different species, we don't think that these

potential variations would significantly impact the result as long as results are used for semi-quantitation (i.e. to determine the proportion of different nematode species from a sample) rather than trying to quantify the precise number of eggs for each species. Furthermore, during the testing of genomic DNA samples of different trichostrongylid nematode species and at known concentrations we achieved similar amplification for the different species.

The testing of positive and negative control DNA samples, including samples from adult worms and negative faecal samples, as well as the sequencing of MT-PCR products, demonstrated each of the developed assays to be specific for its relevant target and there were no signs of cross-reactivity. Repeatability and reproducibility of results was high for 16 samples that were tested and retested within and between different laboratories (Scotland and Belgium) and operators.

MT-PCR detected more positive faecal samples than microscopy, and there were only two samples for which the MT-PCR returned ‘false negative’ results. In both of these samples the estimated number of nematode eggs was very low (13-15 EPG) and amplification of the spike control indicated that negative results were not due to inhibition of the PCR. Based on our previous experiences with GI nematodes of sheep (Roeber et al., 2012), we know that occasionally, in very low egg count samples, nematode eggs can get lost during the sample preparation procedure (i.e. the transfer of sample material between different tubes) and lead to negative MT-PCR results.

The comparison of MT-PCR results with LC for 58 samples from Australia and 15 samples from Belgium showed a high agreement between the results of both methods (62.10 – 100%, depending on the species and sample type (i.e. eggs or

larvae)). Interestingly, a higher level of agreement was observed between the results of MT-PCR$_{(e)}$ *versus* LC rather than MT-PCR$_{(l)}$ *versus* LC, which was unexpected but most likely relates to technical issues and/or sample bias (i.e. different subsamples were taken to carry out the different test methods). Furthermore, the volume of sample material used differs significantly between these diagnostic methods (i.e. 250 microliter of concentrated eggs for DNA extraction and subsequent MT-PCR as opposed to 20-30 g of faecal matter used in LC method). One factor that probably contributed to these results is that LC samples were not concentrated as much as the eggs (i.e. 250µl of LC sample was used for DNA isolation but not all larvae in a sample were spun down to create a larval pellet as was done for the eggs). Perhaps this led to a poorer representation of species in the MT-PCR larvae samples because not all larvae were used for DNA isolation. Generally, highest agreements between the results of MT-PCR and LC were determined for the species of *O. ostertagi, Haemonchus* spp. and *C. oncophora* (89.70 - 100%), whereas a lower agreement was observed for *Trichostrongylus* spp. and *Oe. radiatum* (62.10 – 96.60%). This is probably related to the fact that the former species are easier to distinguish from one another in LC based on the morphological characteristics of the L_3, whereas the larvae of *Trichostrongylus* spp. can be more easily confused with those of either *Ostertagia* or *Cooperia* spp., and *Oe. radiatum*, can be confused with *Nematodirus* larvae, as appears to have happened here. This would explain the lower level of agreement for these species (vanWyk et al., 2004). Indeed, in 4 samples from Belgium in which *Oe. radiatum* larvae were recorded in LC, no DNA of this species could be detected by either MT-PCR of eggs or larvae from the same sample, suggesting that larvae were misidentified and were in fact *Nematodirus*. Assuming that *Oe. radiatum* was misidentified in these 4 samples improved the

calculated agreement for this assay from 73.3%, Kappa 0.250 to 100%, Kappa 1.000.

An interesting finding was that for samples incubated at 22 °C, the proportions of *O. ostertagi* and *C. oncophora* differed within the same sample depending on which sample template (eggs or larvae) was tested in the MT-PCR. Lower proportions of *O. ostertagia* were detected in MT-PCR$_{(e)}$, whereas higher proportions were detected in MT-PCR$_{(l)}$ and LC. These results suggest that an incubation temperature of 22°C (VIC samples) leads to preferential development of *Ostertagia* over *Cooperia*, causing skewed estimates in the LC. This trend was not apparent in samples that were incubated at either 24 °C (NSW samples) or 25 °C (Belgium samples), with similar proportions of each species determined by the three different diagnostic methods (i.e. MT-PCR$_{(e)}$, MT-PCR$_{(l)}$, LC). However, the same samples were not compared at different incubation temperatures, and so this finding only provides an indication of the effect of different incubation temperatures on the developmental rate of different species (i.e. it was a coincidental finding rather than a planned component of this study).

The MT-PCR platform also allows the specific identification of adult nematodes and/or larvae from DNA, but our findings suggest that fresh eggs are the best sample for subsequent MT-PCR analysis for several reasons. Firstly, a molecular diagnosis from eggs does not involve incubation of eggs/larvae beforehand, reduces the potential for preferential development of certain species during culture (e.g. *Ostertagia* over *Cooperia*), and thus provides a less biased result. The recovery of nematode eggs from faecal samples is also easily integrated into the routine faecal egg count procedure of a typical parasitology laboratory, and so does not require additional time, materials or labour for the incubation of nematode larvae. Some

studies (Drag et al., 2016) suggest that it may be advantageous to allow the development of larvae because ITS-2 copy numbers in larvae may be less variable than in eggs. However, we have generally found that this is not a problem if results obtained from nematode eggs are used for semi-quantification (i.e. to determine the proportion of different species in a sample rather than quantify the number of eggs of each species).

The estimation of nematode egg numbers by MT-PCR is still a challenge. We used known quantities of monospecific eggs of *H. placei* in our experiment, and were able to demonstrate a significant linear correlation between egg numbers and determined gene copy number values. However, such correlations are less consistent when field samples of unknown composition are tested (data not shown). Therefore, we suggest using the MT-PCR in conjunction with faecal egg counts. This allows an initial examination of the faecal sample for the presence of eggs, and their stage of development, as well as providing a quantitative result that can help estimating the worm burden (e.g. 200 EPG). MT-PCR is then used for the specific identification of different species in this pool of eggs, and to estimate their proportions in mixed infections. During the preparation of worm egg counts, nematode eggs are already separated from faecal matter and so it is relatively easy to harvest the supernatant (containing the eggs) and use it for subsequent MT-PCR analysis. Based on the results presented here, and from our previous experience with the sheep parasite test (Roeber et al., 2012), we have found that this approach has advantages in that it is robust and not prone to PCR inhibition. It is also easy to integrate into the routine of a diagnostic laboratory, and provides a faster, more specific and less biased result than LC. However, egg counts in adult cattle are of less diagnostic value than for sheep because they usually don't correlate as well with the worm burden (McKenna,

1981). Consequently, the direct extraction of nematode DNA from faeces (i.e. without previous egg counts) could be considered as a routine diagnostic approach for adult cattle.

5. Conclusion

In conclusion, MT-PCR together with the developed cattle parasites kits has been demonstrated to be an effective and advanced tool for the specific diagnosis of GI nematode infections in cattle. MT-PCR was far superior to the traditional LC, both in terms of sensitivity and specificity as well as the time, labour and expertise it requires. The assay panels used performed well when tested on a diverse range of samples from different countries and/or regions, and also with different parasite life-cycle stages (i.e. DNA extracted from adult worms, larvae or eggs). This gives the platform great versatility and it could be used for a wide range of applications. These could include epidemiological studies, to monitor spatial and temporal changes in the prevalence of a variety of parasites in different countries, testing cattle before transport from or introduction onto a farm to avoid the spread of certain species (e.g. *Haemonchus*), or to estimate populations of specific infective larvae on pasture. In addition, by modifying the primers, additional test kits could be developed to screen for anthelmintic resistance markers in nematode or trematode populations, should any such markers become available. However, more research is needed to develop such assays and validate them for each application. In terms of costs for testing, following to an initial investment for the required technology (*Easy-Plex* system), we estimate that the testing of samples can be achieved at a costs that is slightly higher than that of LC but which is offset by the reduced labour and time requirements of the MT-PCR platform.

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Conflict of interest statement

All authors declare that there is no conflict of interest. FR is an employee of AusDiagnostics and DH is an employee of Merial.

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Table 1

Analytical sensitivity of the multiplexed-tandem (MT)-PCR, based on the testing of monospecific samples of *H. placei* ranging from 0.125 – 1000 eggs. Each sample was tested on 5 separate occasions with the *Haemonchus* spp. and pan-nematode assays. Shown are the number of replicates tested, their mean gene copy number, mean cycle-threshold (Ct) values and standard deviations for each set of replicates.

	Haemonchus assay			Pan-nematode assay		
Eggs	**Number of replicates detected**	**Mean expression (mean Ct)**	**SD (Ct SD)**	**Number of replicates detected**	**Mean expression (mean Ct)**	**SD (Ct SD)**
0.125	3/5	400.70 (25.10)	13 (0.13)	5/5	596.00 (24.7)	67.26 (0.12)
0.3	2/5	538.50 (24.83)	104.5 (0.13)	5/5	758.20 (24.35)	101.10 (0.15)
0.6	5/5	716.40 (24.48)	222.87 (0.42)	5/5	1366.40 (23.43)	191.20 9 (0.31)
1.25	5/5	760.20 (24.27)	473.90 (0.67)	5/5	2726.80 (22.09)	349.12 (0.25)
2.5	3/5	2513.30 (22.36)	708.80 (0.68)	3/5	6303.33 (20.88)	469.40 (0.20)
5	5/5	2537.60 (22.73)	2150.90 (1.54)	5/5	8319.00 (20.53)	4021.50 (0.78)
10	5/5	2660.20 (22.85)	2669.30 (2.12)	5/5	7932.20 (20.65)	5205.75 (1.40)
50	5/5	25573.00 (18.82)	30868.20 (19.7)	5/5	102147.40 (17.06)	91619.60 (1.70)
100	5/5	113872.00 (16.38)	39500.80 (0.80)	5/5	258865.40 (15.14)	107655.36 (0.98)
200	5/5	140232.00 (16.32)	25354.70 (0.61)	5/5	305002.80 (15.23)	147650.94 (1.11)
500	5/5	332932.00 (14.76)	121173 (0.82)	5/5	665115.00 (13.70)	265139.53 (0.93)
750	5/5	508015.00 (14.24)	113771 (0.4)	5/5	1162591.80 (12.94)	177752.20 (0.37)
1000	5/5	709605.00 (13.58)	208337 (0.57)	5/5	1662657.60 (12.22)	232995.23 (0.22)

771 Table 2

772 Analytical specificity based on the testing of genomic DNA from morphologically identified, male reference specimens and negative
773 control faecal samples (n=12). Every sample was tested with the specific multiplexed-tandem (MT)-PCR assays for genera/species
774 of *O. ostertagi*, *Haemonchus* spp., *Trichostrongylus* spp., *Oe. radiatum*, *B. phlebotomum* (AU), *D. viviparus* (EU) and the pan-
775 nematode assay (as positive control). In every sample tested, inhibition and efficiency of the MT-PCR was tested by the inclusion of
776 a spike control.

Sample	**MT-PCR**								
	Ostertagia	***Haemonchus***	***Trichostrongylus***	***Oesophagostomum***	***Bunostomum* (AU)**	***Dictyocaulus* (EU)**	***Cooperia***	**Pan-nematode**	**Spike control**
Ostertagia ostertagi	positive							positive	positive
Haemonchus placei		positive						positive	positive
Oesophagostomum radiatum				positive				positive	positive
Cooperia oncophora							positive	positive	positive
Trichostrongylus rugatus			positive					positive	positive
T. colubriformis			positive					positive	positive
T.axei			positive					positive	positive
T.vitrinus			positive					positive	positive
*Bunostomum phlebotomum**					positive				positive
Haemonchus contortus		positive						positive	positive
Teladorsagia circumcicnta								positive	positive
Chabertia ovina								positive	positive
Oesophagostomum venulosum								positive	positive
Dictyocaulus viviparus						positive		positive	positive
Nematodirus spathiger								positive	positive
Fasciola hepatica									positive
Giardia duodenalis									positive
Cryptosporidium parvum									positive
Helminth negative faeces (n=12)								1/12*	positive

777

Table 3

Repeatability and reproducibility testing of the multiplexed-tandem (MT)-PCR based on 16 samples collected in the different countries. Each sample was tested twice within the same laboratory (repeatability estimate) and then again tested at another laboratory by a different operator (reproducibility estimate). Shown are the determined gene copy number values for each sample and replicate tested and the proportion for each species in percent (%). Highlighted are the three samples in which some variation occurred between the results of replicate testing.

			MT-PCR gene copy number (%)						
Sample	Egg count	Replicate/Site of testing	O. ostertagi	Haemonchus spp.	Trichostrongylus spp.	Oe. radiatum	D. viviparous	C. oncophora	Pan-nematode
1UK	72	1 Moredun	0	12458 (80)	1578 (10)	0	0	1470 (9)	26942 (+)
		2 Moredun	132 (1)	17358 (79)	2693 (12)	0	0	1914 (9)	28831 (+)
		3 Gent	0	13963 (76)	3044 (17)	0	0	1376 (7)	34391 (+)
2UK	45	1 Moredun	0	0	793 (65)	0	0	420 (35)	3823 (+)
		2 Moredun	0	0	1020 (64)	0	0	573 (36)	4263 (+)
		3 Gent	0	0	1256 (71)	0	0	505 (29)	3473 (+)
3 UK	9	1 Moredun	0	0	0	0	0	181 (100)	188 (+)
		2 Moredun	0	0	0	0	0	121 (100)	123 (+)
		3 Gent	0	0	0	0	0	152 (100)	175 (+)
4 UK	15	1 Moredun	1197 (100)	0	0	0	0	0	866 (+)
		2 Moredun	908 (100)	0	0	0	0	0	623 (+)
		3 Gent	613 (100)	0	0	0	0	0	686 (+)
5 UK	9	1 Moredun	775 (100)	0	0	0	0	0	890 (+)
		2 Moredun	1014 (100)	0	0	0	0	0	855 (+)
		3 Gent	787 (100)	0	0	0	0	0	821 (+)
6 UK	18	1 Moredun	0	3957 (43)	4453 (49)	0	0	739 (8)	16171 (+)
		2 Moredun	0	3766 (40)	4656 (50)	0	0	975 (10)	16644 (+)
		3 Gent	0	2254 (32)	4175 (60)	0	0	572 (8)	18156 (+)
7 UK	10	1 Moredun	806 (100)	0	0	0	0	0	859 (+)
		2 Moredun	882 (100)	0	0	0	0	0	744 (+)
		3 Gent	945 (86)	0	156 (14)	0	0	0	869 (+)
8 UK	18	1 Moredun	0	12195 (91)	1240 (9)	0	0	0	20790 (+)
		2 Moredun	0	15955 (91)	1607 (9)	0	0	0	19968 (+)
		3 Gent	0	11040 (86)	1728 (14)	0	0	0	21701 (+)
1 Ger	0	1 Gent	8770 (64)	0	5037 (36)	0	0	0	22252 (+)
		2 Gent	4682 (58)	0	3425 (42)	0	0	0	16014 (+)
		3 Moredun	8209 (68)	0	3929 (32)	0	0	0	19944 (+)
2 Ger	150	1 Gent	24705 (6)	0	2226 (<1)	4575 (1)	0	416956 (93)	683160 (+)
		2 Gent	18317 (6)	0	1684 (1)	2579 (1)	0	269786 (92)	480958 (+)
		3 Moredun	35678 (9)	0	1741 (<1)	3687 (1)	0	377075 (90)	697938 (+)
3 Ger	0	1 Gent	17434 (88)	0	1780 (9)	504 (3)	0	150 (1)	24356 (+)
		2 Gent	14483 (89)	0	1267 (8)	384 (2)	0	128 (1)	18697 (+)
		3 Moredun	22375 (92)	0	1384 (6)	619 (3)	0	196 (1)	20708 (+)
4 Ger	0	1 Gent	6414 (77)	0	1862 (23)	0	0	0	11812 (+)
		2 Gent	4041 (76)	0	1270 (24)	0	0	0	7148 (+)
		3 Moredun	5643 (81)	0	1367 (19)	0	0	0	7189 (+)
5 Ger	0	1 Gent	149 (100)	0	0	0	0	0	129 (+)
		2 Gent	0	0	0	0	0	0	0
		3 Moredun	0	0	0	0	0	0	0
6 Ger	0	1 Gent	0	0	0	0	0	0	0
		2 Gent	0	0	0	0	0	0	0
		3 Moredun	0	0	0	0	0	0	0
7 Ger	0	1 Gent	0	0	0	0	0	0	0
		2 Gent	0	0	0	0	0	0	0
		3 Moredun	0	0	0	0	0	0	0
8 Ger	0	1 Gent	0	0	3673 (100)	0	0	0	8872 (+)
		2 Gent	0	0	3087 (100)	0	0	0	8502 (+)
		3 Moredun	0	0	3282 (100)	0	0	0	10503 (+)

Table 4

Assessment of agreement (%) between the results of multiplexed-tandem (MT)-PCR eggs versus larval culture (LC), and MT-PCR larvae (larvae) versus LC technique. Also shown are the calculated Kappa values and the numbers of samples tested positive and negative by each method and for each locality (Victoria and New South Wales) as well as for the entire dataset.

Tests: Larval culture/MT-PCR		Victoria (°C 22)								New South Wales (°C 24)								Australia (combined)						
Species	**n=**	**+,+**	**+,-**	**-,+**	**-,-**	**Agreement (%)**	**Kappa**	**PABAK**	**n=**	**+,+**	**+,-**	**-,+**	**-,-**	**Agreement**	**Kappa**	**PABAK**	**n=**	**+,+**	**+,-**	**-,+**	**-,-**	**Agreement**	**Kappa**	**PABAK**
Ostertagia (MT-PCR $_{(e)}$)	29	29	0	0	0	100.00	-1.000	1.000	29	26	2	0	1	93.10	0.473	0.862	58	55	2	0	1	96.60	0.487	0.931
Ostertagia (MT-PCR$_{(l)}$)	29	29	0	0	0	100.00	-1.000	1.000	29	26	2	0	1	93.10	0.473	0.862	58	55	2	0	1	96.60	0.487	0.931
Haemonchus (MT-PCR $_{(e)}$)	29	0	0	0	29	100.00	-1.000	1.000	29	23	1	1	4	93.10	0.758	0.862	58	23	1	1	33	96.60	0.929	0.931
Haemonchus (MT-PCR $_{(l)}$)	29	0	0	0	29	100.00	-1.000	1.000	29	22	2	0	5	93.10	0.790	0.862	58	22	0	2	34	96.60	0.929	0.931
Trichostrongylus (MT-PCR $_{(e)}$)	29	28	1	0	0	96.60	0.000	0.931	29	20	1	6	2	75.90	0.251	0.517	58	48	2	6	2	86.20	0.266	0.724
Trichostrongylus (MT-PCR $_{(l)}$)	29	18	0	10	1	65.50	0.110	0.310	29	16	1	10	2	62.10	0.121	0.241	58	34	1	20	3	63.80	0.119	0.276
Oe. radiatum (MT-PCR$_{(e)}$)	29	23	6	0	0	79.30	0.000	0.586	29	6	3	5	15	72.40	0.393	0.448	58	29	9	5	15	75.90	0.490	0.517
Oe. radiatum (MT-PCR$_{(l)}$)	29	17	2	6	4	72.40	0.326	0.448	29	6	2	5	16	75.90	0.459	0.517	58	23	4	11	20	74.10	0.489	0.483
C. oncophora (MT-PCR $_{(e)}$)	29	27	2	0	0	93.10	0.000	0.862	29	25	0	0	4	100.00	1.000	1.000	58	52	2	0	4	96.60	0.782	0.931
C. oncophora (MT-PCR $_{(l)}$)	29	26	1	2	0	89.70	-0.048	0.793	29	25	0	0	4	100.00	1.000	1.000	58	51	2	1	4	94.80	0.699	0.897
		Belgium (°C 28)																						
Ostertagia (MT-PCR $_{(e)}$)	15	14	0	1	0	93.30	0.000	0.867																
Ostertagia (MT-PCR $_{(l)}$)	15	15	0	0	0	100.00	-1.000	1.000																
Haemonchus (MT-PCR $_{(e)}$)	15	1	0	0	14	100.00	1.000	1.000																
Haemonchus (MT-PCR $_{(l)}$)	15	14	1	0	13	93.30	0.634	0.867																
Trichostrongylus (MT-PCR $_{(e)}$)	15	0	0	0	15	100.00	-1.000	1.000																
Trichostrongylus (MT-PCR $_{(l)}$)	15	0	2	0	13	86.70	0.000	0.733																
Oe. radiatum (MT-PCR $_{(e)}$)	15	1	0	4	10	73.30	0.250	0.467																
Oe. radiatum (MT-PCR $_{(l)}$)	15	1	1	4	9	66.70	0.118	0.333																
Oe. radiatum (MT-PCR $_{(e)}$) corrected	15	1	0	0	14	100.00	1.000	1.000																
Oe. radiatum (MT-PCR $_{(l)}$) corrected	15	1	1	0	13	93.30	0.634	0.867																
C. oncophora (MT-PCR $_{(e)}$)	15	10	0	0	5	100.00	1.000	1.000																
C. oncophora (MT-PCR $_{(l)}$)	15	10	1	0	4	93.30	0.842	0.867																

794 Table 5

795 Bayesian estimates of diagnostic sensitivity and specificity of the three different
796 diagnostic tests, MT-PCR eggs (MT-$PCR_{(e)}$), MT-PCR larvae (MT-$PCR_{(l)}$) and Larval
797 culture (LC). Shown is the estimated diagnostic performance for five nematode
798 species/genera in beef cattle from Victoria and New South Wales, Australia, 2015.

Nematode species	Parameter	Test	Posterior distribution median (95% PI)
O. ostertagia	Prevalence NSW samples	—	0.907 (0.821, 0.961)
	Prevalence VIC samples	—	0.949 (0.873, 0.985)
	Diagnostic sensitivity	MT-$PCR_{(e)}$	0.951 (0.897, 0.982)
		MT-$PCR_{(l)}$	0.984 (0.932, 0.999)
		LC	0.943 (0.877, 0.983)
	Diagnostic specificity	MT-$PCR_{(e)}$	0.949 (0.837, 0.993)
		MT-$PCR_{(l)}$	0.972 (0.882, 0.998)
		LC	0.760 (0.440, 0.954)
Haemonchus spp.	Prevalence NSW samples	—	0.819 (0.670, 0.924)
	Prevalence VIC samples	—	0.074 (0.019, 0.183)
	Diagnostic sensitivity	MT-$PCR_{(e)}$	0.911 (0.825, 0.965)
		MT-$PCR_{(l)}$	0.939 (0.852, 0.984)
		LC	0.929 (0.789, 0.988)
	Diagnostic specificity	MT-$PCR_{(e)}$	0.964 (0.907, 0.993)
		MT-$PCR_{(l)}$	0.984 (0.934, 0.999)
		LC	0.937 (0.865, 0.978)
Trichostrongylus spp.	Prevalence NSW samples	—	0.873 (0.756, 0.957)
	Prevalence VIC samples	—	0.962 (0.892, 0.993)
	Diagnostic sensitivity	MT-$PCR_{(e)}$	0.924 (0.851, 0.972)
		MT-$PCR_{(l)}$	0.763 (0.66, 0.851)
		LC	0.929 (0.842, 0.981)
	Diagnostic specificity	MT-$PCR_{(e)}$	0.946 (0.823, 0.993)
		MT-$PCR_{(l)}$	0.965 (0.864, 0.997)
		LC	0.398 (0.129, 0.752)
Oe. radiatum	Prevalence NSW samples	—	0.255 (0.103, 0.469)
	Prevalence VIC samples	—	0.653 (0.453, 0.812)
	Diagnostic sensitivity	MT-$PCR_{(e)}$	0.917 (0.818, 0.974)
		MT-$PCR_{(l)}$	0.869 (0.754, 0.982)
		LC	0.813 (0.665, 0.928)
	Diagnostic specificity	MT-$PCR_{(e)}$	0.842 (0.712, 0.943)
		MT-$PCR_{(l)}$	0.941 (0.856, 0.992)
		LC	0.748 (0.542, 0.912)
C. oncophora	Prevalence NSW samples	—	0.838 (0.687, 0.938)
	Prevalence VIC samples	—	0.919 (0.838, 0.968)
	Diagnostic sensitivity	MT-$PCR_{(e)}$	0.952 (0.895, 0.984)
		MT-$PCR_{(l)}$	0.971 (0.917, 0.995)
		LC	0.927 (0.849, 0.974)
	Diagnostic specificity	MT-$PCR_{(e)}$	0.957 (0.871, 0.993)
		MT-$PCR_{(l)}$	0.974 (0.891, 0.998)
		LC	0.793 (0.496, 0.961)

799

800

Table 6

Comparison of the percentage results between MT-PCR (eggs and larvae) and LC methods as determined for the different target nematode species. Shown are the concordance correlation coefficients calculated for samples from New South Wales (NSW) and Victoria (VIC) as well as for the methods of MT-PCR(e) and MT-PCR(l).

Species/genus	Test method		Concordance by state	
	MT-PCR	LC	NSW	VIC
Ostertagia	MT-PCR$_{(e)}$	LC	0.47	0.19
	MT-PCR$_{(l)}$	LC	0.72	0.66
Haemonchus	MT-PCR$_{(e)}$	LC	0.73	N/A
	MT-PCR$_{(l)}$	LC	0.89	N/A
Trichostrongylus	MT-PCR$_{(e)}$	LC	0.62	0.31
	MT-PCR$_{(l)}$	LC	0.57	0.09
Oe. radiatum	MT-PCR$_{(e)}$	LC	0.43	0.16
	MT-PCR$_{(l)}$	LC	0.72	0.43
C. oncophora	MT-PCR$_{(e)}$	LC	0.81	0.24
	MT-PCR$_{(l)}$	LC	0.81	0.51

Fig. 1 Shown are the percentage results for the different nematode species detected in samples from Belgium, New South Wales and Victoria. Every sample was tested by three different diagnostic methods (MT-PCR eggs, MT-PCR larvae and Larval culture).

820 Fig. 2. Boxplots showing the percentage results for species of *O.ostertagi* and *C.*
821 *oncophora* as determined by the three different diagnostic methods (MT-$PCR_{(e)}$, MT-
822 $PCR_{(l)}$ and LC) and for samples tested from Belgium, New South Wales (NSW) and
823 Victoria (VIC), Australia.

Table 7

Numbers of samples tested in the different countries (Australia, Belgium, Scotland) and the numbers of samples analysed by faecal egg counts (EPG) and larval culture (LC). Also shown are the numbers of positives as determined by faecal egg counts and by specific MT-PCR testing as well as the numbers of MT-PCR positives for each species and their relative prevalence (%).

		Australia		**Belgium**	**Scotland**
Numbers of samples tested		144		23	35
Victoria		115	(7 farms)		
New South Wales		29	(3 farms)		
Egg count positive		133		16	33
Number of larval cultures		58		15	Nil
Victoria		29			
New South Wales		29			
MT-PCR positives (prevalence %)	Victoria	NSW	Total		
	111 (97)	29 (100)	140 (97)	21 (91)	34 (97)
Monospecific infections	11 (10)	0 (0)	11 (8)	7 (30)	8 (23)
Mixed infections	100 (87)	29 (100)	129 (90)	14 (61)	26 (74)
O. ostertagi	111 (97)	28 (97)	139 (97)	19 (82)	25 (71)
Haemonchus spp.	0 (0)	24 (83)	24 (17)	1 (4)	6 (17)
Trichostrongylus spp.	40 (35)	21 (72)	61 (42)	5 (22)	19 (54)
Oe. radiatum	30 (26)	9 (31)	39 (27)	3 (13)	0 (0)
C. oncophora	99 (86)	25 (86)	124 (86)	12 (52)	25 (71)
B. phlebotomum (AU)	0 (0)	0 (0)	0 (0)	N/A	N/A
D. viviparus (EU)	N/A	N/A	N/A	0 (0)	0 (0)

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