UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

DESENVOLVIMENTO E VALIDAÇÃO DE UM TESTE DE DIAGNÓSTICO PARA MONITORAMENTO DA RESISTÊNCIA ANTI-HELMÍNTICA EM REBANHOS OVINOS

Yousmel Alemán Gainza

Orientador: Profª. Dra. Ana Carolina de Souza Chagas

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Campus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Medicina Veterinária (área: Medicina Veterinária Preventiva).

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Yousmel Alemán Gainza

Médico Veterinário e Zootecnista

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Câmpus de Jaboticabal

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TÍTULO DA TESE: DESENVOLVIMENTO E VALIDAÇÃO DE TESTE DE DIAGNÓSTICO PARA MONITORAMENTO DA RESISTÊNCIA ANTI-HELMÍNTICA EM REBANHOS OVINOS

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Jaboticabal, 29 de maio de 2020

DADOS CURRICULARES DO AUTOR

Yousmel Alemán Gainza nasceu na cidade de Havana, Cuba, no dia 11 de maio de 1983. Em setembro de 2003 iniciou sua graduação na Faculdade de Veterinária da “Universidad Agraria de la Habana” - UNAH, obtendo o título de Médico Veterinário e Zootecnista em julho de 2008. Em setembro de 2008 ingressou no “Centro Nacional de Sanidad Agropecuaria” – CENSA, onde foi pesquisador assistente no laboratório de parasitologia veterinária até dezembro de 2015. Iniciou os estudos de pós-graduação no Programa de Pós-graduação em Medicina Preventiva Veterinária (Saúde Animal) pela UNAH, obtendo o título de mestre em outubro de 2014. Parte das pesquisas de mestrado foram desenvolvidas no Laboratório de Parasitologia Veterinária na Embrapa Pecuária Sudeste, de agosto de 2013 a maio de 2014, por meio do projeto de cooperação internacional Brasil/Cuba, pela chamada CAPES/MESCUBA: "Diagnóstico da resistência anti-helmíntica e fitofármacos: contribuição ao controle integrado de nematoides gastrintestinais em pequenos ruminantes” (processo n°. 147/12). Foi professor assistente das disciplinas de Fisiologia Animal e Fisiologia Animal Aplicada pela faculdade de Medicina Veterinária da UNAH, no período de janeiro a julho de 2016. Em agosto de 2016 iniciou o doutorado pelo programa de pós-graduação em Medicina Veterinária (Medicina Veterinária Preventiva) pela Universidade Estadual Paulista “Júlio de Mesquita Filho” – campus de Jaboticabal, desenvolvendo a parte experimental de seu projeto na Embrapa Pecuária Sudeste, com bolsa outorgada pela FAPESP (Processo Nº 2016/07132-8).

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estiveram e estão sempre presentes em meu coração.

Dedico

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CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Embrapa

Pecuária Sudeste

CERTIFICADO

PRT Nº 04/2017

Certificamos que o projeto de pesquisa intitulado: **CARACTERIZAÇÃO GENÉTICA E DE RESPOSTAS IMUNES ASSOCIADAS AO FENÓTIPO DE RESISTÊNCIA PARASITÁRIA EM REBANHO OVINO DA RAÇA MORADA NOVA**, registrado com o nº 04/2017 sob a responsabilidade do pesquisador científico Ana Carolina de Souza Chagas, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA EMBRAPA PECUÁRIA SUDESTE.

São Carlos,

Dra. Márcia Cristina de Sena Oliveira

Presidente da Comissão de Ética no Uso de Animais

Embrapa Pecuária Sudeste

Reunião de ___/___/____

Finalidade	Pesquisa Científica
Vigência da autorização	Abril/2017 a março 2019
Espécie/linhagem/raça	Ovinos Morada Nova
Nº de animais	494
Peso/Idade	15-40 Kg
Sexo	174 M e 320 F
Origem	CPPSE

Desenvolvimento e validação de um teste de diagnóstico para monitoramento da resistência anti-helmíntica em rebanhos ovinos

RESUMO – O teste de desenvolvimento larvar (TDL) pode ser uma ferramenta de diagnóstico da resistência anti-helmíntica de rebanhos, permitindo seu monitoramento e ajustes de manejo. O objetivo deste estudo foi desenvolver e validar um teste de diagnóstico *in vitro* da resistência de *Haemonchus contortus* a anti-helmínticos comerciais e validar os resultados a campo. A eficácia do tiabendazol (TBZ), levamisol (LEV), ivermectina-monossacárida (IVM-M), monepantel (MPT) e Zolvix® (ZLV) foi avaliada no TDL em isolados de *H. contortus* susceptível (Echevarria1991-HcEc91) e resistente (Botucatu-HcBot), em placas de cultura de 24 e 96 poços. Testes complementares foram posteriormente realizados com a ivermectina aglicona (IVM-A). Para validação do TDL, realizou-se o teste de redução da contagem de ovos nas fezes (TRCOF) em cinco rebanhos ovinos com anti-helmínticos comerciais dos mesmos grupos químicos (grupos de 7 animais com OPG ≥ 200). TDL e coproculturas também foram realizadas com as amostras dos rebanhos (HcRebanhos). *H. contortus* foi o parasita majoritário em todas as fazendas (média de 74%). O fator de resistência (FR) definido para os isolados foi superior a 3, indicando que o teste foi capaz de diferenciar HcEc91 e HcBot para TBZ, LEV, MPT, ZLV, IVM-A, e de maneira menos consistente para IVM-M. A similaridade das curvas dose-resposta entre as placas e interação placa*concentração (R^2 de 98,4 a 99,0% para HcEc91/HcBot e de 99,4 a 99,0% para HcRebanhos), bem como baixas diferenças na eficácia média (0,02 a 2,26% para HcEc91/HcBot e 0,02 a 4,90% para HcRebanhos) para todos os anti-helmínticos, exceto para MPT, indicaram concordância confiável do TDL em ambas as placas, tanto para a avaliação de HcEc91/HcBot quanto para HcRebanhos. No último caso, a detecção de resistência pelo ZLV foi mais clara e mais estável do que pelo MPT. A adaptação do teste para placas de 96 poços resultou em economia de pelo menos 51.9%. Por meio do TRCOF, detectou-se resistência a todos os grupos químicos em todos os rebanhos, exceto para ZLV (40% resistentes e 20% suspeitos de resistência). Os resultados do TDL nos rebanhos indicaram resistência a TBZ (100%), LEV (80%), ZLV (20%), IVM-M (0%) e IVM-A (100%). Foi obtida concordância total quanto aos resultados de ambos os testes para TBZ e IVM-A (k=1,00). Já para

LEV e ZLV, apesar de haver divergência de resultados em dois rebanhos, esta não foi estatisticamente diferente para ambas as drogas (P= 0,077 e P= 0,197, respectivamente), obtendo-se concordância substancial (k= 0,8 e 0,6, respectivamente). Os dados do presente estudo indicam que os resultados dos dois testes são comparáveis, validando, portanto, esse teste como uma opção para o diagnóstico da resistência anti-helmíntica para produtores de pequenos ruminantes.

Palavras-chave: diagnóstico, RESISTA-*Test*, resistência anti-helmíntica, nematoides gastrintestinais, ovinos.

Development and validation of a diagnostic test for anthelmintic resistance monitoring in sheep flocks

ABSTRACT – The larval development test (LDT) can be a diagnostic tool for anthelmintic resistance in flocks, allowing monitoring and management adjustments. The aim of this study was to develop and validate an *in vitro* diagnostic test of *Haemonchus contortus* resistance to commercial anthelmintics and to validate the results at the farm level. The efficacy of thiabendazole (TBZ), levamisole (LEV), ivermectin-monosaccharide (IVM-M), monepantel (MPT) and Zolvix® (ZLV) were evaluated in LDT in susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) isolates, in 24 and 96 well culture plates. Complementary tests were subsequently performed with ivermectin aglycone (IVM-A). To validate the LDT, the fecal egg count reduction test (FECRT) was performed in five sheep flocks with commercial anthelmintics from the same chemical groups (groups of 7 animals with FEC ≥ 200). LDT and fecal cultures were also carried out with the samples of the flocks (HcFlocks). *H. contortus* was the major parasite on all farms (average of 74%). The resistance factor (RF) defined for the isolates was greater than 3, indicating that the test was able to differentiate HcEc91 and HcBot for TBZ, LEV, MPT, ZLV, IVM-A, and in a less consistent way for IVM-M. The similarity of the dose-response curves between the plates and plate*concentration interaction (R^2 from 98.4 to 99.0% for HcEc91/HcBot and from 99.4 to 99.0% for HcFlocks), as well as low differences in efficacy mean (0.02 to 2.26% for HcEc91/HcBot and 0.02 to 4.90% for HcFlocks) for all anthelmintics, except for MPT, indicated a reliable agreement of the LDT in both plates, both for the evaluation of HcEc91/HcBot as for HcFlocks. In the latter case, resistance detection by ZLV was clearer and more stable than by MPT. The adaptation of the test to 96-well plates resulted in savings of at least 51.9%. Through FECRT, resistance to all chemical groups was detected in all flocks, except for ZLV (40% resistant and 20% suspected of resistance). LDT results in flocks indicated resistance to TBZ (100%), LEV (80%), ZLV (20%), IVM-M (0%) and IVM-A (100%). Total agreement was obtained regarding the results of both tests for TBZ and IVM-A (k = 1.00). For LEV and ZLV, although there was a divergence of results in two flocks, this was not statistically different for both drugs (P = 0.077 and P = 0.197, respectively),

with substantial agreement (k = 0.8 and 0.6, respectively). The data from the present study indicate that the results of the two tests are comparable, thus validating this test as an option for the diagnosis of resistance for small ruminant farmers.

Keywords: diagnosis, RESISTA-*Test*, anthelmintic resistance, gastrointestinal nematodes, sheep.

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1 INTRODUÇÃO

O Brasil possui rebanho ovino de aproximadamente 18 milhões de cabeças. (ANUALPEC 2018). Os pequenos ruminantes desempenham importante papel na agricultura familiar e possuem potencial para os mercados emergentes. Em comparação aos bovinos, os ovinos e caprinos são amplamente adaptados a diferentes condições climáticas, além de consumirem menor quantidade de alimentos em razão do menor tamanho corporal (Silanikove, 2000; Markos, 2006). Esses fatores permitem fácil integração dos pequenos ruminantes a diversos sistemas de produção.

O principal problema sanitário na criação de pequenos ruminantes no Brasil é causado pelos nematoides gastrintestinais (NGI). Os animais sofrem redução no ganho de peso, diminuição na taxa de fertilidade, levando a gastos com medicamentos e aumento na mortalidade, causando significativas perdas econômicas (Sczesny-Moraes et al., 2010; Nova et al., 2014). Parasitas como *Haemonchus* e *Trichostrongylus* acarretam milhões em perdas econômicas a cada ano à cadeia de produção ovina. Em levantamento realizado na região sul do Rio Grande do Sul, as perdas econômicas geradas por esses parasitas foram estimadas em R$ 2.016.000/ano (Oliveira et al., 2017). Como forma de minimizar os prejuízos, o controle de parasitas tem sido amplamente realizado por meio de anti-helmínticos, mas a excessiva dependência nessas substâncias tem levado ao desenvolvimento de resistência anti-helmíntica (RA) múltipla e, portanto, essa abordagem não tem sido considerada sustentável para o controle de NGI (Sutherland e Leathwick, 2011; Van Wyk e Reynecke, 2011).

A resistência dos NGI aos anti-helmínticos em animais de produção continua a aumentar globalmente em gravidade. Houve muitos avanços na pesquisa de resistência nos últimos 50 anos, incluindo diagnósticos, descobertas fisiológicas e genéticas importantes, além do desenvolvimento de modelos e ferramentas matemáticas preditivas para ajudar os agricultores a gerenciar a resistência (Kotze et al., 2014a). Entretanto, uma questão fundamental que ainda requer resolução é o uso de técnicas de diagnóstico que estimam a presença de resistência anti-helmíntica em rebanhos ovinos.

O estabelecimento de medidas racionais para prevenir e controlar NGI em pequenos ruminantes é um grande desafio, especialmente em regiões tropicais, onde a espécie predominante é *Haemonchus contortus*, causador de intensa anemia (Amarante et al., 2004). O conhecimento precoce do status de resistência nas propriedades é importante para a manutenção da atividade dos grupos químicos de anti-helmínticos que ainda são eficazes. Portanto, a validação de técnicas para monitorar e controlar a RA em rebanhos é extremamente importante, pois conduz ao manejo sanitário mais racional e permite a orientação de ações futuras para o controle parasitário em cada propriedade.

Desta forma, o presente estudo teve por objetivo desenvolver um teste de diagnóstico *in vitro* do status de resistência de *H. contortus* a anti-helmínticos comerciais e validar os resultados a campo.

1.2 REVISÃO DE LITERATURA

1.2.1 Aspectos epidemiológicos dos nematoides gastrintestinais no Brasil

Várias espécies de NGI já foram identificadas em ovinos no Brasil. A diversidade de espécies que parasitam os animais é influenciada pela frequência de tratamentos anti-helmínticos, pelo manejo e pelas condições ambientais. No Sul do Brasil, por exemplo, as temperaturas baixas do inverno favorecem a ocorrência de *Teladorsagia circumcincta*, espécie que não tem sido registrada em ovinos criados em outras regiões do país (Amarante, 2014). Além dessa espécie, ainda é comum a ocorrência de *Nematodirus* spp. e *Oesophagostomum venulosum* (Ramos et al., 2004). Porém, no Rio Grande do Sul, a exemplo dos demais estados brasileiros, *H. contortus* é a espécie predominante, especialmente nos meses de verão, enquanto que *Trichostrongylus* spp. e *T. circumcincta* predominam nos períodos com baixas temperaturas, nos meses de inverno e primavera, quando causam problemas clínicos e redução na produtividade dos ovinos (Echevarria et al., 1996).

No estado de São Paulo, Vieira et al. (1989) realizaram estudos epidemiológicos nos quais 100% das infecções correspondeu a *H. contortus* seguido por 85% *Trichostrongylus colubriformis*, 69% *Oesophagostomum columbianum*, 50% *Cooperia punctata*, 12% *Cooperia pectinata* e 3,8% *Trichuris ovis*. Amarante e Barbosa (1995) constataram que em Botucatu, SP, os gêneros de helmintos de maior ocorrência foram *Haemonchus* spp., *Trichostrongylus* spp., *Cooperia* spp., *Oesophagostomum* spp. e *Strongyloides papillosus*, acometendo bovinos e ovinos adultos, e *Haemonchus* spp., *Oesophagostomum* spp. e *Trichostrongylus* spp. em cordeiros antes do desmama. Bassetto et al. (2009), durante o período de novembro de 2007 a julho de 2008, verificaram que na pastagem da região de Botucatu, predominavam larvas de *Haemonchus* spp., *Trichostrongylus* spp. e *Oesophagostomum* spp. Rocha et al. (2008) citam *H. contortus* e *T. colubriformis* como as espécies mais prevalentes na região de Tupi Paulista.

1.2.2 A resistência parasitária aos anti-helmínticos

As helmintoses em pequenos ruminantes continuam em aumento devido aos altos níveis de resistência anti-helmíntica (RA), atual cenário em quase todos os países do mundo com grande número de ovinos (Kaplan e Vidyashankar, 2012). A resistência desenvolve-se quando os parasitas sobrevivem ao tratamento e transmitem genes associados à resistência aos seus descendentes. Com a seleção e reprodução adicionais, estes genes aumentam em frequência na população. Para que a resistência se desenvolva em uma espécie, parasitas com genes de resistência devem sobreviver ao tratamento, continuar a se reproduzir e seus descendentes serem transmitidos para o hospedeiro seguinte (Sangster et al., 2018).

O primeiro relato de resistência ocorreu em 1957 (Drudge et al., 1957a, b). Detectou-se resistência de *H. contortus* aos benzimidazóis (BZs) em ovinos e foi o primeiro registro de uma droga moderna em animais de produção. A resistência ocorre em várias espécies de NGI, porém *H. contortus* demonstrou maior capacidade de desenvolver RA (Gilleard, 2013). Em muitos casos, a resistência apareceu menos de 10 anos após a introdução de uma nova classe de medicamentos (Waller, 1994). As populações de campo desta espécie agora mostram resistência a todas as classes de drogas anti-helmínticas (Gilleard, 2013). Essa situação, associada ao aparecimento precoce de resistência ao monepantel, ameaçam a sustentabilidade dos sistemas de produção de ovinos e caprinos em todo o mundo (Kotze e Prichard, 2016).

O desenvolvimento de RA ocorreu globalmente, mas os padrões diferem de região para região. É provável que essa variação esteja ligada a fatores conhecidos que aumentam a resistência, tais como padrões de uso de anti-helmínticos (por exemplo, maior número de tratamentos por intervalo de tempo), decisões de manejo (por exemplo, usando drogas de uma única classe), raças (por exemplo, raças com baixa imunidade podem exigir mais tratamentos), além da perda da proporção de vermes na população que não são expostos às drogas (Sangster et al., 2018).

1.2.2.1 Resistência aos anti-helmínticos na América do Sul

No final da década de 90, a América do Sul registrou indiscutivelmente os níveis mais altos e espacialmente mais difundidos de RA em criações de pequenos

ruminantes no mundo (Waller, 1997). Levantamentos realizados no norte da Argentina (Eddi et al., 1996), sul do Brasil (Echevarria et al., 1996), Paraguai (Maciel et al., 1996) e Uruguai (Nari et al., 1996) indicaram que os BZs e imidazotiazóis haviam quase alcançado o fim de sua vida terapêutica nesses países, o que continua até os tempos atuais (Kaplan e Vidyashanka, 2012; Salgado e Santos, 2016).

Após o primeiro relato de RA em ovinos no Brasil (Dos Santos e Gonçalves, 1967), multiplicaram-se os relatos de nematoides resistentes a todos os fármacos comercialmente utilizados. Na região Sul, a resistência foi detectada no Paraná (Thomaz-Soccol et al., 2004), Santa Catarina (Ramos et al., 2004) e novamente no Rio Grande do Sul (Echevarria e Trindade, 1989; Echevarria et al., 1996). Na região Sudeste foram observados relatos em São Paulo (Verissímo et al., 2002). No Nordeste, suspeitou-se de nematoides resistentes inicialmente em caprinos no Ceará (Vieira et al., 1989) e posteriormente outros relatos em caprinos e ovinos ocorreram (Vieira e Cavalcante, 1999; Melo, 2001). Ainda no Ceará, foi observada a presença de *H. contortus* resistente em ovinos provenientes do Paraná e Rio Grande do Sul (Vieira et al., 1992), o que facilitou a disseminação da resistência para todo o país. Estudos também indicaram RA em Pernambuco, Bahia e Alagoas (Charles et al., 1989; Barreto e Silva, 1999; Bispo et al., 2002).

No Brasil, muitos produtos contendo combinações de diferentes anti-helmínticos mostraram-se quase ineficazes como resultado da disseminação da RA (Da Cruz et al., 2010; Molento et al., 2011). Estudos conduzidos por Almeida et al. (2010) e Cezar et al. (2010) identificaram populações de *Haemonchus* spp. e *Trichostrongylus* spp. com RA a múltiplas drogas, como IVM e moxidectina por exemplo, resultando em eficácia aproximada de 1% para ambas drogas.

Baixa eficácia do MPT em *H. contortus* foi detectada no Uruguai por Mederos et al. (2014) e no Brasil (Martins, 2016; Albuquerque et al., 2017; Mallman Junior et al., 2018; Ramos et al., 2018).

1.2.2.2 Resistência aos anti-helmínticos na América do Norte

Na América do Norte, embora a ovinocaprinocultura não fosse muito representativa, pesquisas conduzidas na década de 1990 indicaram elevada RA para os BZs (Uhlinger et al., 1992). Nos estados do sul de Louisiana e Flórida detectaram

RA para todos os anti-helmínticos. A criação de raças importadas de ovinos se tornou difícil de manter por causa das mortalidades devidas a *H. contortus* (Waller, 1997).

No sudeste dos Estados Unidos, um estudo de prevalência de RA em fazendas de ovinos e caprinos mostrou populações de *H. contortus* resistentes a BZs, levamisol (LEV), IVM e moxidectina (Howell et al., 2008; Tsukahara et al., 2017). Esses achados corroboram relatos anteriores de RA múltipla em ovinos e caprinos por Zajac e Gipson (2000) e Terrill et al. (2001), o que levou à fundação do grupo multidisciplinar ACSRPC (Consórcio Americano para Pequenos Ruminantes para Controle de Parasitas), dedicado ao desenvolvimento de sistemas sustentáveis de manejo de NGI, recomendando o uso do método FAMACHA© e de pastagens com propriedades anti-helmínticas**.**

Relatos em Ontário, Canadá, mostraram inicialmente que a RA era baixa em rebanhos ovinos, como consequência de: (1) Baixa necessidade de tratamento devido às temperaturas extremas, que geralmente limitam o desenvolvimento de larvas; (2) Diversidade nas práticas de manejo; (3) Uso menos frequente de anti-helmínticos em comparação com outros países; e (4) Menor quantidade de fazendas e de animais nos rebanhos comparado a outros países (Guthrie et al., 2010). Entretanto, um estudo recente de Falzon et al. (2013), também em Ontário, registrou falha na eficácia de ivermectina em 88% das 39 fazendas estudadas. Além disso, uma investigação subsequente nas mesmas propriedades indicou 97% (28/29), 95% (19/20) e 6% (1/17) de resistência à IVM, fenbendazol e LEV, respectivamente, em *H. contortus* (espécie majoritária identificada nas coproculturas pós-tratamento).

1.2.2.3 Resistência aos anti-helmínticos na África

Os estudos de monitoramento de RA na África, embora poucos, sugerem motivo de preocupação (Vatta e Lindberg, 2006). No Quênia, 50% das 42 fazendas de pequenos ruminantes pesquisadas mostraram RA (Wanyangu et al., 1996). Em um estudo realizado em fazendas comerciais de ovelhas no Zâmbia, a RA ao albendazol foi encontrada em cinco das seis propriedades avaliadas (Gabriel et al., 2001). Um dos grandes problemas na África foi o rápido desenvolvimento de RA para o monepantel (MPT), que ocorreu praticamente tão rapidamente quanto para a ivermectina (IVM) (Scott et al., 2013; Love, 2014).

Na África do Sul, o primeiro registro de RA está entre os primeiros para o continente (Berger, 1975) e, desde então, tem se manifestado de forma variada e rápida (van Wyk et al., 1998). Assim como em outros países, a RA foi considerada uma situação de crise, pois envolveu praticamente todos os anti-helmínticos disponíveis na época (van Wyk et al., 1999; van Wyk, 2001). As espécies de NGI nas quais se verificou RA foram *H. contortus* (Berger, 1975; van Wyk e Malan, 1988), *T. colubriformis* (van Wyk et al., 1990), *T. circumcincta* (van Schalkwyk et al., 1983) e *Moniezia expansa* (Visser et al, 1987).

Ocorreram relatos RA em *H. contortus* na África do Sul para as lactonas macrocíclicas (IVM), salicilanilidas (ambos rafoxanida e closantel) e substitutos fenólicos (nitroxinil e dinitrofenol) (van Wyk e Geber, 1980; van Wyk et al., 1982; Carmichael et al., 1987; van Wyk e Malan, 1988; van Wyk et al., 1997). No caso das lactonas macrocíclicas (LMs), duas populações diferentes de *H. contortus* foram relatadas como resistentes à IVM três anos após o medicamento ter sido registrado para uso em ovelhas na África do Sul (Carmichael et al., 1987; van Wyk e Malan, 1988; van Wyk et al., 1989). Isto levou a uma hipótese de resistência cruzada entre grupos anti-helmínticos por van Wyk et al. (1989), sendo os BZs o candidato mais provável em relação às LMs (Mottier e Prichard, 2008). Por outro lado, Leathwick et al. (2009) e Bartram et al. (2012) consideraram que não há uma indicação clara da ocorrência de resistência cruzada.

1.2.2.4 Resistência aos anti-helmínticos na Ásia

Em todas as regiões do sudeste da Ásia e do Pacífico Sul, devido ao rápido desenvolvimento e intensificação das indústrias de pequenos ruminantes, o uso intensivo de anti-helmínticos para o controle de nematoides resultou em aumento na prevalência e no nível de RA (Waller, 1997). Levantamentos diagnosticaram RA para BZ e LEV em Fiji, RA emergente para IVM (Le Jambre, 1994), e situação semelhante na Malásia (Dorny et al., 1994; Chandrawathani et al., 2011). Na Índia RA múltipla em NGI foi detectada para BZs, IVM e LEV (Gill, 1993; Easwaran et al., 2009).

1.2.2.5 Resistência aos anti-helmínticos na Austrália

A RA também é um problema sério na Austrália. Duas décadas atrás pesquisas regionais mostravam que aproximadamente 80% das populações de NGI nas fazendas tinham RA tanto para BZs como para imidazotiazóis (Waller et al., 1995). A resistência amplamente disseminada de *H. contortus* à salicilanilida e ao closantel foi documentada por Rolfe (1993), possivelmente devido ao seu uso extensivo durante o programa “WormKill” (Dash et al., 1985) nas regiões chuvosas da Austrália (van Wyk, 2001, 2006).

Um estudo atual de resistência, após um período de quase 20 anos, revelou níveis inesperadamente altos e crescentes de resistência aos ativos mais amplamente utilizados: 54% para moxidectina e 96% para BZs e LEV (Playford et al., 2014). É preocupante que o primeiro caso de resistência ao MPT, lançado na Austrália na primavera de 2010, também já tenha sido confirmado (Love, 2014; Constantinoiu et al., 2015).

Na Nova Zelândia, RA para os BZs, imidazotiazóis e LMs está amplamente difundida em fazendas de ovinos (Hughes et al., 2005; 2007; Waghorn et al., 2014) e mais grave em fazendas de caprinos (West et al., 2004). O primeiro caso de RA para o MPT foi registrado em *T. colubriformis* e *T. circumcincta*, poucos anos após o seu lançamento (Scott et al., 2013).

1.2.2.6 Resistência aos anti-helmínticos na Europa

Numerosos países europeus diagnosticaram RA, principalmente aos BZs e aos imidazotiazóis, e subsequentemente, um número crescente de casos de resistência às LMs (Papadopoulos, 2008; Papadopoulos et al., 2012).

No Reino Unido, RA foi documentada para os BZs (Mitchell et al., 2011) e o mecanismo de seleção para a resistência foi recentemente caracterizado por Morrison et al. (2014), tanto genotipicamente como fenotipicamente. Também há relatos de resistência múltipla ao BZ, LEV e avermectina em *T. circumcincta* (Sargison et al., 2010a).

Na Escócia, a resistência a BZs, imidazotiazóis e LMs, separadamente e em combinação, foi diagnosticada (Sargison et al., 2010b), enquanto no País de Gales,

100 das 122 fazendas pesquisadas foram positivas para RA a BZs, LEV ou ambos (Mitchell et al., 2010). Em rebanho irlandês de ovelhas comerciais, Good et al. (2012) relataram RA em populações de NGI tanto para BZs (>88% do rebanho) quanto para LEV (>39% do rebanho), enquanto para LMs suspeitou-se de resistência em 11% das fazendas.

No nordeste da Espanha, de 107 fazendas de ovelhas pesquisadas, 11% foram resistentes aos BZs (Calavia et al., 2011). Em outro estudo, também na Espanha, a falha do tratamento foi documentada em 40,8%, 20,8% e 9,6% das fazendas para LEV, IVM e BZs, respectivamente (Martínez-Valladares et al., 2011).

Cernansta et al. (2006) encontraram RA em 4% e 23% das fazendas testadas na Eslováquia para BZs e IVM, respectivamente, enquanto na Holanda, Borgsteede et al. (1997; 2007; 2010) relataram RA para BZs, IVM e doramectina em fazendas de ovinos e posteriormente para moxidectina e MPT (van den Brom et al., 2013, 2015).

Na Itália, a resistência ao LEV e à IVM foi registrada (Traversa et al., 2007). No oeste da França, RA aos BZs foram detectadas em 83% das fazendas. Em 50% das fazendas ocorreu RA para o LEV, afetando principalmente *Teladorsagia*, *Trichostrongylus* e *Cooperia* spp (Chartier et al.,1998). Na Grécia, Papadopoulos et al. (2001), utilizando testes *in vitro*, encontraram populações de *Teladorsagia* sp. resistentes ao BZ, enquanto que, no mesmo país, Gallidis et al. (2011) relataram a presença de populações de *H. contortus* 100% homozigóticas, resistentes ao BZ em rebanhos ovinos de leite.

Embora as investigações sobre RA na União Europeia tinham como foco principal os NGI de pequenos ruminantes, problemas também foram registrados em bovinos (Stafford e Coles, 1999; Demeler et al., 2009; Kleinschmidt et al., 2010; Taylor, 2010; Rose et al., 2015).

1.2.3 Consequências da resistência anti-helmíntica

Outra preocupação com o fenômeno da resistência aos anti-helmínticos está relacionada aos danos ambientais dos resíduos (Morgan et al., 2019). Seu impacto no meio ambiente pode ser detectado na fauna não-alvo (Cooke et al., 2017). Os efeitos no ambiente e nos sistemas de agricultura agroecológica, precisam ser bem compreendidos em relação aos benefícios do tratamento anti-helmíntico (Verdú et al.,

2018). Os prejuízos diretos da RA incluem o custo do medicamento, o uso intenso de mão-de-obra na administração do medicamento (usualmente ineficaz) e a redução da produção de carne e leite por hectare e por animal. No entanto, é provável que haja muitos outros prejuízos econômicos e ambientais indiretos, uma vez que serão necessários mais animais para produzir a mesma quantidade de alimentos (Herrero et al., 2015). Gerar essas percepções e integrá-las em estruturas econômicas tem grande potencial para apoiar programas sustentáveis de controle de helmintos nos níveis agrícola, regional e nacional. A valorização da sustentabilidade e os benefícios econômicos do controle dos helmintos nos sistemas agrícolas menos rentabilizados continuam a ser um desafio (Perry e Randolph, 1999).

A elevada frequência de tratamentos anti-helmínticos, muitas vezes desnecessária, leva a um risco crescente de resíduos nos alimentos, aumento da resistência e diminuição dos medicamentos úteis disponíveis (Kinsella et al., 2008).

Podem ocorrer resíduos de BZs nos tecidos dos animais devido a vários fatores: produtos inadequados para aquela espécie animal, uso de dose não recomendada, período de carência não observado ou consomo de ração contaminada pelos animais (Danaher et al., 2007). Os resíduos dependem da droga utilizada, via de administração, tecido alvo ou tempo decorrido desde o tratamento. Os limites máximos de resíduos (LMR) para medicamentos relacionados aos BZs foram estabelecidos em 100 µg/Kg para leite, músculo e gordura, 500 µg/Kg para rim, e 1000 µg/Kg para o fígado nas espécies bovina e ovina (EEC, 1990). O mebendazol e o triclabendazol são os BZs identificados como os mais persistentes em tecidos animais. Em estudos realizados em ovelhas tratadas com uma dose oral de febendazol (5 mg/Kg PV) detectou-se valores acima do LMR 7 dias após o tratamento (EMEA, 1997a, b).

O metabolismo das LMs em tecidos animais é bem descrito. Foi demonstrado que os medicamentos deste grupo químico utilizados por vias parentais são os principais resíduos encontrados nos tecidos dos animais (Danaher et al., 2006). Verificou-se que os resíduos de LMs ocorrem no fígado e nos tecidos gordurosos em níveis mais altos do que nos tecidos renais e musculares. Campbell et al. (1985) mostraram que a IVM não é extensivamente metabolizada em mamíferos e 90% da dose é excretada nas fezes. O medicamento é responsável por pelo menos 50% do

total de resíduos nos tecidos de bovinos, ovinos, suínos e ratos até 14, 5, 7 e 3 dias após o tratamento, respectivamente. Os principais metabólitos da IVM no fígado de bovinos, ovelhas e ratos foram identificados como 24-hidroximetil-$H_2B_{1\alpha}$ e o monossacarídeo 24-hidroximetil-$H_2B_{1\alpha}$ (Campbell et al., 1985; Danaher et al., 2006). Em ovelhas tratadas com doramectina via parental o medicamento representou 67-92% do total de resíduos na gordura, fígado, rim e músculo, 14 dias após o tratamento (EMEA, 1997c). Afzal et al. (1997) descobriram que a moxidectina representava 91, 51, 52 e 92% do total de resíduos nos tecidos adiposo, hepático, renal e muscular de ovelhas, respectivamente. A IVM não é licenciada para uso em espécies em lactação na união europeia, entretanto, Alvinerie et al. (1993) e Cerkvenik et al. (2002) constataram que os níveis de IVM (200 µg/Kg PV) atingiram uma concentração máxima de 23 µg/Kg no leite de ovelha 1,3 dias após o tratamento e os resíduos foram detectáveis 23 dias após o tratamento.

1.2.4 Estratégias para retardar a resistência aos anti-helmínticos

Na atualidade existem diferentes estratégias de manejo com o objetivo de prevenir a infecção e/ou reduzir a pressão de seleção dos anti-helmínticos. Isso inclui manejo de pastagens e abrigo e quarentena para animais recém-introduzidos em rebanho. O objetivo geral é reduzir a necessidade de anti-helmínticos e, consequentemente, retardar o desenvolvimento de resistência.

Uso correto dos anti-helmínticos: as recomendações de uso racional atualmente estabelecidas são geralmente baseadas em uma compreensão profunda da epidemiologia parasitária (Sargison, 2011). Têm como objetivo geral direcionar o tratamento de modo a reduzir a exposição desnecessária (tratamento seletivo) e, assim, limitar o risco de resistência. A sub-dosagem e/ou o uso frequente de anti-helmínticos pertencentes à mesma classe aumentarão o risco de seleção de resistência. Recomenda-se que os anti-helmínticos de ação prolongada sejam aplicados apenas em situações em que a estação de pastejo é mais longa do que a duração do efeito (Rathbone e McDowell, 2012). A manutenção da *refugia* através da implementação de rotinas adequadas de tratamento anti-helmíntico e gestão de pastagens é importante para diminuir a pressão de seleção e reduzir o risco de desenvolvimento de resistência (Muchiut et al., 2018).

Refugia: visa manter proporção baixa de NGI resistentes dentro da população e, portanto, é defendida como uma ferramenta para retardar o avanço da resistência anti-helmíntica (Van Wyk, 2001; Sargison, 2011; Muchiut et al., 2018; Forbes, 2019). Parasitas em *refugia* são aqueles que não foram expostos a um anti-helmíntico, incluindo aqueles presentes como estágios de vida livre no ambiente, indivíduos não tratados, e aqueles em qualquer fase do ciclo de vida no hospedeiro que não são afetados pelo tratamento anti-helmíntico. O conceito de *refugia* é amplamente aceito, mas ainda está cercado por várias suposições, como a dependência do nível de *refugia* às circunstâncias prevalecentes (por exemplo, climáticas) (Cornelius et al., 2016). A *refugia* como conceito tem sido aplicado principalmente aos NGI, mas seu papel no manejo da resistência em outros helmintos requer mais pesquisas (Hodgkinson et al., 2019; Morgan et al., 2019). Além disso, não está claro seu papel na inversão da RA (Leathwick et al., 2015), assim como seu uso nas estratégias de substituição de populações parasitárias para recuperar a suscetibilidade anti-helmíntica nas propriedades (Kenyon et al., 2013; Muchiut et al., 2018).

Uso de produtos anti-helmínticos de ação múltipla: ainda está em discussão se os produtos que contêm duas ou mais substâncias ativas direcionadas para o mesmo helminto, mas com diferentes modos de ação, podem ser vantajosos no que diz respeito a desacelerar o surgimento de resistência. Estudos de modelagem e alguns dados de campo indicaram que tais produtos podem retardar o desenvolvimento de resistência a novas substâncias ativas (Learmount et al., 2012; Leathwick, 2012; Leathwick et al., 2012) ou retardar o desenvolvimento de resistência anti-helmíntica a classes de anti-helmínticos existentes (Leathwick e Hosking, 2009; Leathwick et al., 2015). No entanto, o uso de anti-helmínticos de ação múltipla também pode selecionar RA múltipla (Leathwick e Besier, 2014). Se esses produtos oferecem um benefício em relação ao desenvolvimento de RA, que superaria o risco de promover RA múltipla, isso deve ser mais fundamentado.

1.2.5 Diagnóstico da resistência anti-helmíntica por testes *in vitro*

Há um consenso na comunidade científica de que os programas de controle de nematoides e o retardo da RA dependem da disponibilidade de testes sensíveis e eficazes para a detecção da resistência (Taylor et al., 2002). Neste contexto, o

conhecimento prematuro do status de resistência nas propriedades é importante para a manutenção da atividade dos grupos químicos que ainda são eficazes. Já que o diagnóstico da resistência é possível de ser estimado *in vitro*, a caracterização de isolados é imperativa para o estabelecimento de valores de referência e para que esse diagnóstico se torne menos dependente dos experimentos *in vivo* (Chagas et al., 2013).

Como os criadores necessitam de uma medida confiável do grau de resistência presente em seus rebanhos, o desenho experimental apropriado do teste para os gêneros de NGI que infectam os animais será importante. O monitoramento do status de resistência anti-helmíntica e a adoção de recomendações racionais para prevenir o estabelecimento da resistência a determinado grupo químico, podem ser consideradas ferramentas extremamente úteis. Assim, o monitoramento da resistência anti-helmíntica é uma chave para atrasar o desenvolvimento de resistência, preservar classes anti-helmínticas e talvez promover a reversão em direção à susceptibilidade, o que pode ser considerado de grande interesse para a indústria de drogas veterinárias.

Há uma grande necessidade de ampliar nosso conhecimento sobre as forças motrizes do desenvolvimento da RA, para estabelecer ferramentas de detecção de resistência aplicáveis e significativas no campo e, assim, fornecer informações mais atualizadas e confiáveis sobre a ocorrência da RA (Morgan et al., 2019). Em uma era de revolução de tecnologia nas indústrias de diagnóstico, melhorias dos testes ou, eventualmente, automação, têm grande potencial para permitir diagnóstico mais rápido, eficiente e precoce.

O uso de bioensaios *in vitro* com os estágios de vida livre dos nematoides representa uma alternativa para medir a sensibilidade a anti-helmínticos e, portanto, detectar que a resistência está surgindo. Esses bioensaios são muito mais baratos que os ensaios *in vivo*, relativamente rápidos, evitam quaisquer efeitos de animais hospedeiros e algumas das imprecisões associadas ao Teste de redução da contagem de ovos nas fezes (TRCOF). Além disso, tais testes podem usar uma gama de concentrações do fármaco a fim de verificar seu efeito na população de NGI, por exemplo, a concentração que mata 50% da população exposta (valores CL_{50}).

1.2.5.1 Teste de eclosão de ovos – TEO

Esse método foi inicialmente descrito por Le Jambre (1976) para detectar RA aos BZs em *H. contortus* e *T. circumcincta*. Posteriormente, um protocolo padronizado foi adotado pela WAAVP (Coles et al., 1992). O TEO mede o efeito dos BZs na inibição da embriogênese e na eclosão das larvas. O tiabendazol foi elencado para o ensaio devido à sua maior solubilidade em meio aquoso em comparação com alguns outros compostos do grupo dos BZs. Posteriormente, Dobson et al. (1986) adaptaram o ensaio para detectar resistência ao LEV.

A utilização do TEO para detecção de resistência a BZ em isolados a campo foi descrita por Coles et al. (1992). Mais tarde estabeleceu-se a dose discriminante como a dose da droga que inibe a eclosão de 99% dos ovos susceptíveis. As doses discriminantes foram estabelecidas usando isolados susceptíveis de *H. contortus*, *T. circumcincta* e *Trichostrongylus colubriformis* (Coles et al., 2006). Os dados atuais sugerem que uma dose de 0,1 µg/mL de tiabendazol pode inibir a eclosão dos ovos em 99% dessas espécies (Coles et al., 2006).

Em estudo de padronização entre laboratórios da Europa foi proposto um protocolo padrão para a detecção de resistência aos BZs (von Samson-Himmelstjerna et al., 2009a). A maioria dos estudos que avaliaram o uso do TEO para a detecção de nematoides resistentes aos BZs mostrou uma boa concordância com os resultados obtidos com o TRCOF em ovinos (Díez-Baños et al., 2008) e bovinos (Demeler et al., 2012).

1.2.5.2 Testes de desenvolvimento larvar– TDL

O teste de desenvolvimento larvar (TDL) mede os efeitos de diferentes drogas anti-helmínticas no desenvolvimento de ovos a larvas infectantes de terceiro estágio (L_3) (Hubert e Kerboeuf, 1992). Várias adaptações já foram descritas, mas geralmente o cultivo ocorre em meio líquido (Taylor, 1990; Hubert e Kerboeuf, 1992) ou em ágar 1 % (Gill et al., 1995; Coles et al., 2006; Dolinská et al., 2012).

Vários estudos mostraram que o ensaio é capaz de discriminar entre isolados de *H. contortus* que são susceptíveis ou resistentes às principais classes de anti-helmínticos (Gill et al., 1995; Dolinska et al., 2013; Kotze et al., 2014b, 2018). O TDL

também é capaz de detectar resistência das larvas ao organofosforado naftalofós (Kotze et al., 1999). Recentemente, Raza et al. (2016) demonstraram que o TDL também permite a diferenciação entre isolados susceptíveis e resistentes ao MPT.

O TDL é considerado sensível e prático, permitindo a avaliação da eficácia de mais de um grupo químico ao mesmo tempo, e não depende de ovos embrionados (Kaplan et al., 2007). Os resultados podem ser comparados com isolados de referência (susceptíveis e resistentes), de modo que é possível determinar a CL_{50} e anular a interferência inter-ensaios (Craven et al., 1999). Muitos estudos demonstraram boa correlação entre o TRCOF e a CL_{50} discriminatória para a resistência obtida *in vitro* (Várady et al., 2006; Kaplan et al., 2007; Díez-Baños et al., 2008; Taylor et al., 2009).

Gill et al. (1995) adaptaram o TDL para detectar RA às avermectinas e milbemicinas, o que levou ao lançamento de um teste hoje disponível no mercado, DrenchRite®. O teste é eficaz para BZs, levamisole e lactonas macrocíclicas em *H. contortus* e as outras espécies importantes na Austrália, no entanto, teve um desempenho muito ruim para a última classe de medicamentos em *T. circumcincta*. Assim, ele foi retirado do mercado na Austrália porque não era aplicável a outras espécies (Kotze e Prichard, 2016).

1.2.5.3 Teste de motilidade e migração larvar – TML

Os testes de motilidade e migração larvar podem ser usados para avaliar o efeito dos anti-helmínticos que causam paralisia na musculatura somática dos parasitas. A motilidade de larvas pode ser determinada por meio de observação visual, detectores eletrônicos (instrumentos que medem o grau de refração da luz e fornecem um índice de motilidade) ou migração através de peneiras. Estes ensaios são frequentemente usados como ferramentas de triagem em programas de descoberta de novas drogas, além de serem também aplicados para a detecção de RA (Gill e Lacey, 1998; d'Assonville et al., 1996; Molento e Prichard, 2001; Kotze et al., 2006; Fortes et al., 2013; Kotze e Prichard, 2016), visto que os testes a campo têm uso limitado. Por isso, muitas variações de protocolo foram descritas.

Em comparação ao TDL, o TML é um teste fácil e simples, possível de ser realizado na maioria dos laboratórios. Além disso, requer larvas de terceiro estágio,

que podem ser facilmente obtidas a partir de coproculturas, e mantidas em geladeira (Demeler et al., 2010a). O uso deste teste para detecção de resistência às LMs foi padronizado para diferentes espécies de nematoides, com amostras coletadas em ovinos e bovinos (Demeler et al., 2010b). Entretanto, sua utilidade para detecção de níveis baixos de resistência permanece desconhecida.

1.2.5.4 Testes moleculares

Os avanços na compreensão da base molecular da resistência de *H. contortus* a determinados anti-helmínticos levaram ao desenvolvimento de testes para o diagnóstico molecular de RA. Esta área de pesquisa tem sido foco do grupo de pesquisadores do Consórcio de Resistência e Susceptibilidade Anti-Helmíntica (CARS), e o progresso em direção ao desenvolvimento de marcadores moleculares tem sido descrito em vários artigos do grupo (Gilleard e Beech, 2007; Prichard et al., 2007; von Samson-Himmelstjerna et al., 2007; Beech et al., 2011; Kotze et al., 2014a).

As possibilidades para o desenvolvimento de tais testes em curto prazo são, no entanto, bastante diferentes para as classes químicas, devido ao nível de compreensão da natureza molecular dos vários tipos de resistência. Para os BZs, o desenvolvimento de testes moleculares foi bem-sucedido, já que é a base química mais bem compreendida em nível molecular. Para os outros grupos de fármacos, no entanto, a incerteza sobre a natureza específica dos mecanismos de resistência em isolados de campo e o fato de que as mudanças moleculares relatadas para alguns isolados não ocorreram em outros isolados, atrasaram o desenvolvimento de diagnósticos moleculares (Kotze e Prichard, 2016).

O primeiro fenótipo de resistência descrito vem da evidência de que frações enriquecidas com tubulina de NGI resistentes aos BZ ligam menos compostos radioativos BZ em comparação com NGI susceptíveis (Sangster et al., 1985), o que definiu a tubulina como o local de ação. O segundo foi a demonstração de que NGI resistentes a LEV requeria concentrações *in vitro* mais elevadas para alcançar o mesmo efeito na contração muscular (Sangster et al., 1991). Esses NGI também eram resistentes ao neurotransmissor acetilcolina, o que indica que a resistência provavelmente se deve a mudanças na farmacologia do receptor. Apesar das descrições da sequência da subunidade do receptor e das diferenças de transcrição,

falta evidência convincente de um genótipo para a resistência ao LEV (Kotze et al., 2014a)

Coles et al. (2006) descreveram uma PCR alelo-específica para a detecção de polimorfismos únicos de nucleotídeos (SNPs- single nucleotide polymorphism) F200Y associado à resistência a BZ em *H. contortus* usando DNA extraído de larvas L_3. Outros métodos moleculares também foram descritos para a detecção e quantificação deste SNP, assim como os outros dois associados à resistência a BZ (F167Y, E198A), incluindo PCR-RFLP (Tiwari et al., 2006; Ghisi et al., 2007), PCR em tempo real (Alvarez-Sanchez et al., 2005; Walsh et al., 2007; von Samson-Himmelstjerna et al., 2009b) e pirosequenciamento (von Samson-Himmelstjerna et al., 2009b).

Este último estudo descreveu ensaios de pirosequenciamento para os códons de *H. contortus* 167, 198 e 200 dos isotipos 1 e 2 de β-tubulina (Arafa et al., 2016). O método mostrou-se capaz de avaliar o status de resistência a BZ de vários isolados de *H. contortus*, indicando que pode ser adequado para o diagnóstico de rotina de resistência nesta espécie. Uma comparação de dados moleculares com dados de eclosão de ovos mostrou que o teste molecular nem sempre foi correlacionado com o grau de resistência; no entanto, foi capaz de discriminar isolados resistentes e susceptíveis. Curiosamente, os SNPs observados nas regiões 200 e 198 (para os BZs) também estão associados à resistência à lactonas macrocíclicas (Kotze et al., 2014a).

1.2.6 Diagnóstico da resistência anti-helmíntica por testes *in vivo*

A eficácia dos anti-helmínticos pode ser determinada por testes envolvendo contagem de NGI pós-morte, após o tratamento anti-helmíntico, comparando-se animais tratados e não tratados (Wood et al., 1995). No entanto, tal procedimento requer abate de animais e seu custo é alto, por isso não é amplamente utilizado (Fortes e Molento, 2013). O método padrão e mais frequentemente usado para estimar a eficácia dos anti-helmínticos contra NGI, incluindo *H. contortus,* é o TRCOF.

No TRCOF, populações de parasitas são consideradas susceptíveis quando a eficácia do medicamento excede 95%. Por outro lado, a resistência está presente quando a eficácia é < 95% (Coles et al., 1992). O ponto de corte de 95% é mais complexo do que parece, porque algumas drogas têm eficácia muito alta (99,9%)

contra algumas espécies de parasitas, mas menores (95%) para outros no mesmo hospedeiro. Portanto, reduções na eficácia requerem interpretação à luz de diferentes situações.

O TRCOF, no entanto, apresenta diversas restrições em termos de sensibilidade entre espécies animais, variações individuais e presença de infecções múltiplas (Levecke et al., 2012; Traversa e von Samson-Himmelstjerna, 2016; George et al., 2017; Wang et al., 2017). Isso ocorre especialmente quando espécies com baixas taxas de fecundidade, como por exemplo *Trichostrongylus* spp., estão envolvidas em infecções naturais de ovinos descritas como resistentes aos fármacos (Palcy et al., 2010).

Algumas drogas podem causar uma supressão temporária na postura dos ovos, levando a uma superestimativa da eficácia anti-helmíntica, se avaliada nesse período. Uma redução superior a 95% no TRCOF indica eficácia do anti-helmíntico, mas uma pequena porcentagem de vermes sobreviventes pode indicar resistência, que pode aumentar com tratamentos subsequentes (Coles et al., 2006).

Outras considerações são que as contagens de ovos nas fezes: (1) se relacionam com infecções patentes, mas não pré-patentes (Thienpont et al., 1986), (2) não fornecem nenhuma informação sobre NGI machos ou imaturos que possam estar presentes (McKenna, 1981), e (3) pode ser influenciada pela variação na excreção de ovos por NGI adultos (Villanua et al., 2006), idade da população de NGI e imunidade, sexo e idade do hospedeiro (Thienpont et al., 1986).

O TRCOF é considerado um teste laborioso e demorado e, portanto, ensaios *in vitro* têm sido desenvolvidos para a avaliação da RA a diferentes classes de drogas (Babják et al., 2018).

Os próximos capítulos se referem à descrição da otimização do Teste de Desenvolvimento Larvar com isolados de *H. contortus* (Capítulo 2) e à sua validação para a detecção da resistência em *H. contortus* oriundos de rebanhos do estado de São Paulo (Capítulo 3).

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CAPÍTULO 2 – Improvement of an *in vitro* test (RESISTA-*Test*) for *Haemonchus contortus* resistance diagnosis in small ruminant[2]

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2.1 Abstract

Parasitic resistance monitoring by the larval development test (LDT) can be a tool to delay its occurrence in flocks and preserve the effectiveness of anthelmintics. The aim of this study was to optimize an *in vitro* diagnostic test (RESISTA-*Test*) of *Haemonchus contortus* resistance to the main commercially anthelmintics available in Brazil. The efficacy of thiabendazole (TBZ), levamisole (LEV), ivermectin-monosaccharide (IVM-M), monepantel (MPT) and Zolvix® (ZLV) in the larval development test (LDT) was evaluated in susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) *H. contortus* isolates, in 24- and 96-well culture plates. Complementary tests were subsequently performed with ivermectin aglycone (IVM-A). After standardization of concentration ranges in HcEc91 e HcBot, *H. contortus* isolated from fecal samples of five sheep flocks (HcFlocks) were also evaluated. LDT data were analyzed using logit dose-response by the Probit model and the degree of parasitic resistance was expressed by the resistance factor (RF). Resistance factors above 3 indicated that the test was able to differentiate susceptible and resistant isolates to TBZ, LEV, MPT, IVM-A, and in a less consistent way for IVM-M. The high similarity profile of dose-response

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curves between plates and interaction plate*concentration (R^2 from 98.4 to 99.0% for HcEc91/HcBot and from 99.4 to 99.0% for HcFlocks), as well as low differences in mean efficacy (0.02 to 2.26% for HcEc91/HcBot and 0.02 to 4.90% for HcFlocks) for all anthelmintic, except MPT, indicated reliable LDT agreement in both plates, for the evaluation of HcEc91/HcBot and for HcFlocks. In the latter case, resistance detection by ZLV was clearer and more stable than by MPT. Results obtained with IVM-A were much more reliable than with IVM-M. Adapting the test to 96-well plates resulted in cost savings of at least 51.9%. The LDT validation by comparing the results with those of the Fecal Egg Count Reduction Test (FECRT) in flocks may make it available for routine laboratory use, supporting decisions in parasite control programs.

Index terms: diagnostic test, RESISTA-*Test*, gastrointestinal nematodes, resistance, sheep.

Otimização de um teste *in vitro* para diagnóstico da resistência de *Haemonchus contortus* em pequenos ruminantes (RESISTA-*Test*)

Resumo

O monitoramento da resistência anti-helmíntica pelo teste de desenvolvimento larvar (TDL) pode ser uma ferramenta para atrasar sua ocorrência nos rebanhos e para preservar a eficácia dos anti-helmínticos. O objetivo deste estudo foi otimizar um teste diagnóstico (RESISTA-*Test*) *in vitro* da resistência de *Haemonchus contortus* aos principais anti-helmínticos comercialmente disponíveis no Brasil. A eficácia do tiabendazol (TBZ), levamisol (LEV), ivermectina-monossacárida (IVM-M), monepantel (MPT) e Zolvix® (ZLV) no teste de desenvolvimento larvar (TDL) foi avaliada em isolados de *H. contortus* susceptível (Echevarria1991 - HcEc91) e resistente (Botucatu - HcBot), em placas de cultura de 24 e 96 poços. Testes complementares foram posteriormente realizados com a ivermectina aglicona (IVM-A). Após padronização dos intervalos de concentração em HcEc91 e HcBot, *H. contortus* isolados de amostras de fezes coletadas de cinco rebanhos ovinos (HcRebanhos) também foram avaliados. Os dados do TDL foram analisados usando logit dose-resposta pelo modelo Probit e o grau de resistência anti-helmíntica foi expresso pelo fator de resistência

(FR). FRs acima de 3 indicaram que o teste foi capaz de diferenciar os isolados susceptível e resistente a TBZ, LEV, MPT, ZLV e IVM-A, e de maneira menos consistente para IVM-M. O alto perfil de similaridade das curvas dose-resposta entre as placas e interação placa*concentração (R^2 de 98,4 a 99,0% para HcEc91/HcBot e de 99,4 a 99,0% para HcRebanhos), bem como baixas diferenças na eficácia média (0,02 a 2,26% para HcEc91/HcBot e 0,02 a 4,90% para HcRebanhos) para todos os anti-helmínticos, exceto MPT, indicaram concordância confiável do TDL em ambas as placas, tanto para a avaliação de HcEc91/HcBot quanto para HcRebanhos. No último caso, a detecção de resistência pelo ZLV foi mais clara e mais estável do que pelo MPT. Os resultados obtidos com a IVM-A foram muito mais confiáveis do que com a IVM-M. A adaptação do teste para placas de 96 poços resultou em economia de pelo menos 51,9%. A validação do TDL por meio da comparação de seus resultados com os do teste de redução da contagem de ovos nas fezes (TRCOF) em rebanhos podem torná-lo disponível para uso laboratorial de rotina, apoiando a tomada de decisão em programas de controle parasitário.

Termos para indexação: teste diagnóstico, RESISTA-*Test*, nematoides gastrintestinais, resistência, ovinos.

2.2 Introduction

The breeding of small ruminants is a global activity for meat, milk, and leather production (SARGISON, 2016). Brazil has approximately 18 million of sheep (ANUALPEC, 2018), a number that is growing due to factors such as better management and genetic improvement (AQUINO et al., 2016). However, sheep meat consumption in Brazil is still low (ANUALPEC, 2018). Human population growth has prompted the conversion of many pasture areas into farmland for increased food production. Breeding large ruminants is becoming more difficult due to the lack of grazing land. Properties in densely populated areas may be smaller than 0.5 ha. In these places, the importance of sheep and goats in relation to meat and milk supply has been recognized. In this sense, in the subsistence sector, farmers and breeders depend more on small ruminants. This generates income and allows the sale of surpluses, improving the quality of life. Thus, any intervention that increases sheep

and goat productivity is important (HIRPA; ABEB, 2008).

The main health problem of small ruminants is gastrointestinal nematode infections (GIN). Animals suffer reduced weight gain, decreased fertility rate and increased mortality, causing significant economic losses (NOVA et al., 2014). Parasites such as *Haemonchus* and *Trichostrongylus* cause huge economic losses each year to the sheep production chain. In a survey conducted in the southern region of the state of Rio Grande do Sul, Brazil, the economic losses were estimated at US$ 500,000/year (OLIVEIRA et al., 2017). Excessive reliance on anthelmintic for parasite control has led to the development of multiple parasite resistance, so this approach is not considered sustainable for GIN control (SUTHERLAND; LEATHWICK, 2011; VAN WYK; REYNECKE, 2011). Several studies report GIN resistance to multiple drugs in sheep flocks from all regions of Brazil. The first report of resistance to benzimidazole was in Rio Grande do Sul (DOS SANTOS; GONÇALVES, 1967), where the first record of ivermectin-resistant nematodes also occurred (ECHEVARRIA; TRINDADE, 1989). In the northeastern of Brazil, resistance in goat nematodes was reported in Pernambuco to levamisole, albendazole and parbendazole (CHARLES et al., 1989) and in the state of Bahia to albendazole and ivermectin (BARRETO; SILVA, 1999). In Ceará, resistance occurred in goats using oxfendazole and levamisole (VIEIRA; CAVALCANTE, 1999), in sheep and goats using closantel, oxfendazole and ivermectin (MELO et al., 1998), and so on. Monepantel belongs to the group of aminoacetonitrile derivatives (AAD) (KAMINSKY et al., 2008a; KAMINSKY et al., 2008b) and its commercial product Zolvix® is the most recently released anthelmintic in Brazil. However, resistance to this group has already been reported in Brazil (CINTRA et al., 2016; ALBUQUERQUE et al., 2017; CIUFFA et al., 2017) as well as in other countries (SCOTT et al., 2013; MEDEROS et al., 2014; VAN DEN BROM et al., 2015).

Therefore, when a new anthelmintic is launched and widely used, nothing prevents the rapid development of resistance (CHAGAS, 2015). This makes the validation of practices for monitoring and controlling anthelmintic resistance in flocks extremely important. The main method to detect resistance *in vivo* is the fecal egg count reduction test (FECRT), which can be performed with all chemical groups. However, this method is expensive, laborious and time consuming (VÁRADY et al., 2007). When the resistance diagnosis is not routinely adopted in flocks, the main consequence is a lack

of information about the anthelmintic efficacy and of the proper management of the chemical groups.

Compared to the FECRT, *in vitro* tests are less expensive, faster, and more accurate, and in many cases, are a reproducible alternative (LACEY et al., 1991; HAZELBY et al., 1994; VÁRADY et al., 1996). Depending on the method adopted, the test can generate linear dose-response curves for the chemical groups (HUBERT; KERBOUEF, 1992). The larval development test (LDT) is considered sensitive and practical, allowing the evaluation of different chemical groups at the same time (KAPLAN et al., 2007). The results can be compared with reference isolates (susceptible and resistant) so that the lethal concentration (LC_{50}) can be determined and the inter-assay interferences eliminated (CRAVEN et al., 1999). Many studies have shown good correlation between the FECRT and the discriminatory LC_{50} for resistance obtained *in vitro* (TAYLOR, 1990; LACEY et al., 1991; KÖNIGOVÁ et al., 2003; VÁRADY et al., 2006; KAPLAN et al., 2007; DÍEZ-BAÑOS et al., 2008; TAYLOR et al., 2009). Gill et al. (1995) improved the LDT to detect resistance to avermectins and milbemycins, which led to the launch of a commercial test kit, DrenchRite® (KAPLAN et al., 2007). However, this kit is expensive, difficult to import to Brazil and has been used mainly by parasitological diagnostic laboratories in the US.

Due to this situation, we sought to develop an *in vitro* diagnostic test (RESISTA-*Test*), for *H. contortus* resistance to the main commercially available anthelmintic groups in Brazil. The diagnostic test was designed to perform a liquid-based test using drug standards representing different chemical groups. Some procedures were refined by adapting the diagnostic test to 96-well plates and checking which drugs could be used to obtain a reliable and affordable laboratory diagnosis. The validation of the test in the future may allow more rational and guided parasite control in sheep and goats, preserving chemical classes.

2.3. Material and methods

2.3.1 *Anthelmintics*

In order to establish lethal concentrations (LC) of anthelmintics in susceptible and resistant *H. contortus* isolates and in *H. contortus* collected from flocks, *in vitro* tests were standardized for thiabendazole - TBZ (Sigma-Aldrich T8904), levamisole - LEV

(Sigma-Aldrich 31742), ivermectin monosaccharide - IVM-M (Sigma-Aldrich I8898), monepantel - MPT (PGS P11144) and Zolvix® - ZLV (Novartis). Additional tests were latter performed with ivermectin aglycone – IVM-A (Bioaustralis I1151-1) as well.

2.3.2 *GIN evaluation*

All procedures involving parasite donor animals were approved by the Committee on Ethical Use of Animals (CEUA) of Embrapa Pecuária Sudeste (Protocol No. 04/2017). Four Santa Inês lambs, aged between three and four months with average weight of 27 kg, were treated with Zolvix® (monepantel, 2.5 mg/kg LW) to eliminate natural infection by GIN parasites still susceptible to that drug (CHAGAS et al., 2013). On the 7th, 10th and 15th days after treatment, fecal egg counts (FEC) were performed using the McMaster X 50 technique (UENO; GONÇALVES, 1998) to confirm that the animals were worm-free. On the 15th day after treatment, two animals were artificially infected with 4000 third stage larvae (L_3) of the *H. contortus* Echevarria1991 isolate (HcEc91: isolate from Rio Grande do Sul State, Brazil, susceptible to all anthelmintic, ECHEVARRIA et al. (1991) and two other animals with the *H. contortus* Botucatu isolate (HcBot: resistant to monepantel, ALBUQUERQUE et al. (2017)). The animals were kept in pairs in separate pens, supplemented daily with 400 g of corn silage and with free access to water and mineral salt. With the establishment of infection, feces were collected for egg recovery to perform *in vitro* tests.

Samples were also collected from five sheep-producing properties in the state of São Paulo, Brazil, where the animals were not subjected to deworming for at least 40 days and presented FEC>200 in a previous analysis (day before the experiment). In each flock, samples were collected from 49 animals individually, placed in vacuum-sealed plastic bags, identified with the animal's number and immediately taken to the laboratory (D0). Fecal cultures (pool) were performed for GIN identification (VAN WYK et al., 2004) and FEC for egg recovery for LDT. The flocks were visited 14 days later (D14) to collect feces to perform a new LDT (independent repetitions).

2.3.3 *Egg recovery*

Feces collected directly from the animals' rectal ampoule were dissolved in water and filtered using sequential sieves. A saturated NaCl solution was added to the eggs

retained in the last sieve and centrifuged at 3,000 rpm for five minutes. The supernatant was washed with distilled. The eggs were then divided into five aliquots, and their suspensions were added to the 24- and 96-well culture plates (Kasvi®) (COLES et al., 1992).

2.3.4 *Larval development test (LDT)*

The LDT, adapted from HUBERT; KERBOEUF (1992), was performed in 24- and 96-well plates. Solutions containing 100 and 70 *H. contortus* eggs were added to each well in 24- and 96-well plates, respectively, which also received culture medium (*Escherichia coli* [EC11303] and amphotericin B [A9528] - Sigma-Aldrich). This culture medium allows the development from first-stage larvae (L_1) to L_3. The plates were identified, sealed with PVC film and kept in an incubator for 24 h (27 °C, RH ≥ 80%) for larval development (L_1). After this period, each well received serial anthelmintic dilutions and the plates were incubated again for six days, when larvae L_1, L_2 and L_3 from each well were quantified with an inverted microscope. For the tests with HcEc91/HcBot, all anthelmintic concentrations and the negative control with water were tested in six replicates (six wells) and in three independent experiments (on different dates). For the tests with HcFlocks, all anthelmintic concentrations and the negative control were tested in two replicates (two wells following the commercial kit model, Drenchrite®) and in two independent experiments (D0 and D14).

Regarding the HcFlocks analyses, plates were examined to estimate the critical well for each anthelmintic (well in which 50% of parasites did not develop to L_3; interpolation between 2 wells was performed when necessary) (KAPLAN et al., 2007). All larvae in the control wells, and above and below the critical well, were counted and identified to determine the species composition of the sample. *H. contortus* resistance (estimated by the resistance factor - RF) was determined when the predominant parasite (L_3) in the control and in the critical wells consisted of this species (HcFlocks) (ANONYMOUS, 1996), which occurred in all flocks.

2.3.5 *Statistical analysis*

The anthelmintic efficacy against each isolate was determined based on the arithmetic mean of larval development according to the following equation (COLES et al., 1992), where: $Inhibition(\%) = 100(\bar{X}_{test} / \bar{X}_{total})$, $\bar{X}_{test}$ refers to the number of larvae that did not reach the L_3 stage, and $\bar{X}_{total}$ corresponds to the number of $L_1 + L_2 + L_3$.

LDT results were analyzed by Probit logistic regression model to determine LC_{50} and LC_{99} values, which were defined as the anthelmintic concentrations at which 50% and 99% of L_1 to L_3 development was inhibited. The degree of anthelmintic resistance was expressed as the resistance factor (RF), calculated as the LC_{50} and LC_{99} values of the resistant isolate divided by the respective susceptible isolate values (DOLINSKÁ et al., 2013). Analyses were performed with XLSTAT-Premium 2019.2.2 (Addinsoft 2019 – XLSTAT, Boston, USA) and a significance level of $p \leq 0.05$ was considered.

Two-way ANOVA was applied by the SAS GLM procedure (SAS 2010) considering the effects of plate (24 and 96 wells), concentrations (1 to 12) and the interaction plate*concentration. LSMEANS was adopted for multiple comparison between average effects of the plates and of the interaction, using the Tukey test and the F-test, respectively, with a significance level of 5% ($p \leq 0.05$).

2.4 Results

2.4.1 *Echevarria1991 and Botucatu isolates*

Table 1 summarizes the mean efficacy ($\bar{X}$ ± SE) for each of the isolates compared between the 24- and 96-well plates by two-way ANOVA. For TBZ, LEV, IVM-M and ZLV there were subtle differences (DIF) between the plates. Although low, these differences were significant ($p \leq 0.05$) according to the plate p-value, as well as for some concentrations, expressed by the plate x concentration interaction. However, biologically these differences (DIF) could be neglected (did not exceed 2.26%). In the case of MPT, the differences between the plates were higher (42.06 and 22.49% for HcEc91 and HcBot, respectively), with a significant ($p \leq 0.001$) plate effect and plate x concentration interaction. The values of R^2 (98.4 to 99.9%) indicated the accuracy of the analysis, i.e., the higher R^2, the more explanatory the model is and the better it fits the sample. In Figure 1 the dose-response curves reinforce the confidence of the

results obtained for TBZ, LEV, IVM-M and ZLV in both plates, since the results were very similar for these drugs. The inhibition percentage of larval development was thus very evident

Table 1. Results of the larval development inhibition of *H. contortus* isolates Echevarria1991 (HcEc91) and Botucatu (HcBot), in 24 and 96-well plates, for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Isolates	Anthelmintic	Plates (well)	$\bar{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcEc91	TBZ	24	93.91±0.09	69.07	100	0.44	0.02	0.05	0.984
		96	93.47±0.16	66.66	100				
	LEV	24	86.13±0.16	46.54	100	0.02	0.94	0.0003	0.984
		96	86.11±0.28	48.40	100				
	IVM- M	24	47.60±0.18	0.00	100	1.85	0.006	0.006	0.997
		96	49.45±0.30	0.00	100				
	MPT	24	83.64±0.07	41.30	100	42.06	10^{-9}	10^{-11}	0.999
		96	41.58±0.12	0.00	100				
	ZLV	24	70.82±0.11	5.73	99.95	0.48	0.03	0.05	0.997
		96	70.34±0.19	5.63	100				
HcBot	TBZ	24	36.88±0.20	0.00	95.64	2.26	0.004	0.004	0.994
		96	34.62±0.34	0.00	89.62				
	LEV	24	63.73±0.16	1.93	100	0.19	0.54	0.88	0.996
		96	63.54±0.27	2.27	100				
	IVM- M	24	52.06±0.13	1.70	99.95	0.33	0.20	0.003	0.997
		96	51.72±0.23	1.57	97.64				
	MPT	24	22.18±0.71	1.09	100	22.49	10^{-11}	10^{-11}	0.927
		96	12.81±1.23	0.00	66.67				
	ZLV	24	39.21±0.10	0.00	91.39	0.85	0.003	0.55	0.998
		96	40.06±0.17	0.00	91.74				

* Significant at 5% ($p \leq 0.05$) by ANOVA and the Tukey test. $\bar{X}$ ± SE: Mean efficacy ± standard error; Min: minimum efficacy; Max: maximum efficacy; DIF: Difference in mean efficacies between 24-well and 96-well plates; R^2 (coefficient of determination, 0 to 1): measure of adjustment of the statistical model to the observed values.

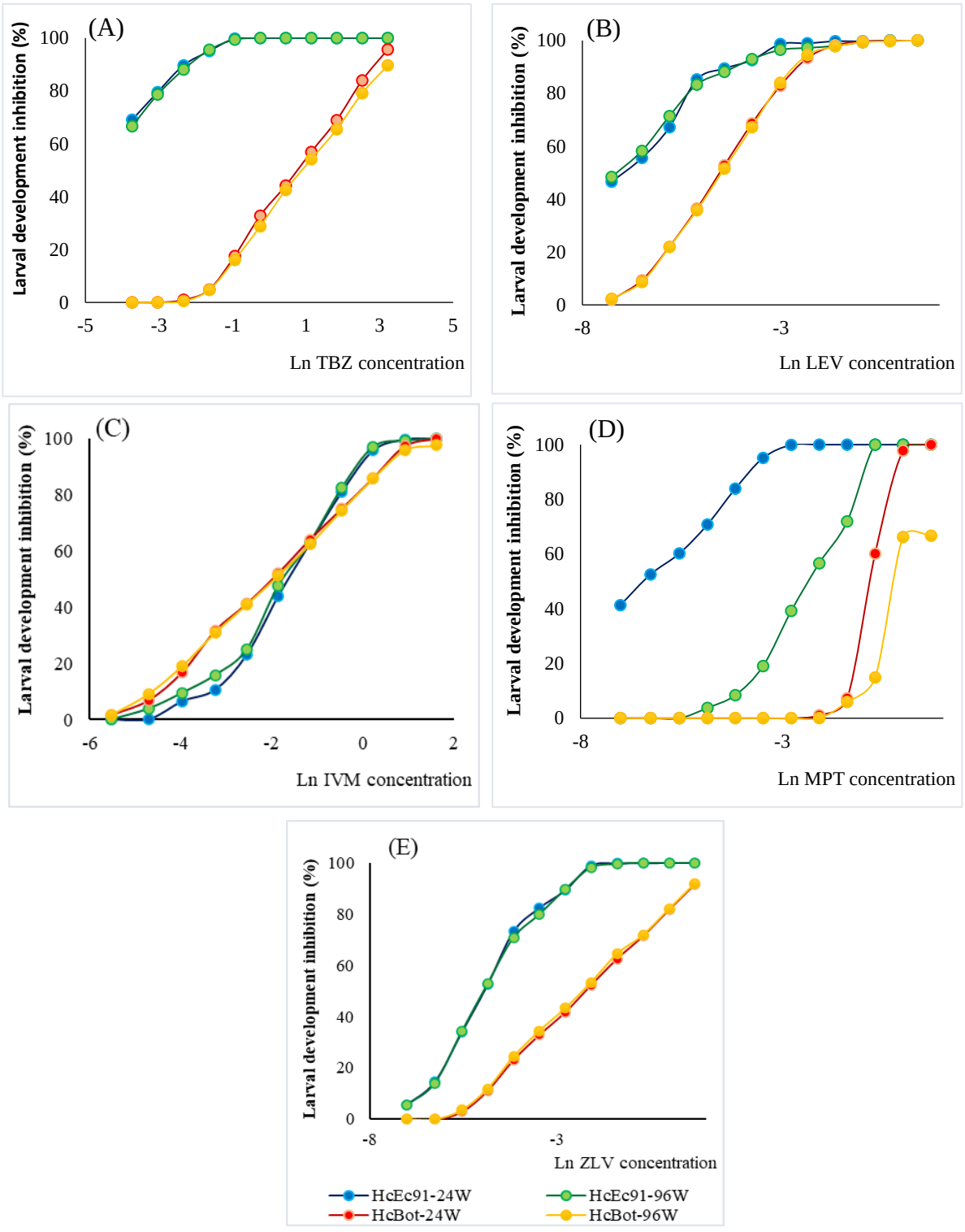

Figure 1. Log-dose and logit-response curves for *H. contortus*: Echevarria1991 susceptible isolate in 24-well (HcEc91-24W) and 96-well (HcEc91-96W) plates, and Botucatu resistant isolate in 24-well (HcBot-24W)) and 96-well plates (HcBot-96W) to Thiabendazole-TBZ (A), Levamisole-LEV (B), Ivermectin-IVM-M (C), Monepantel-MPT (D) and Zolvix-ZLV (E).

Another criterion that reinforces these results was the LC_{50} (Table 2), the usual cutoff that separates resistant from susceptible isolates. The results obtained for each drug and isolate in the 24-well plates were within the confidence interval (95%) of the 96-well plate results. This only did not occur for MPT, since 0.086 µg/mL was not within the confidence range of 0.0009 to 0.0011 µg/mL and 1.041 µg/mL did not fit within the confidence interval of 0.437 to 0.453 µg/mL. In the comparison between isolates for IVM-M, LC_{50} obtained for the susceptible isolate did not differ much from the resistant isolate. This can also be seen in Figure 1, where the curves of both isolates intersect close to the development inhibition of 50% of the parasites. Such results indicate the reliability of the results obtained in both plates, except for MPT and IVM-M.

The LC results were used to determine the RFs of the isolates in both plates (Table 2). RF_{99} and RF_{50} values were above 3 for all drugs in both plates and only the RF_{99} values for LEV (1.88 and 1.32 in the 24- and 96-well plates, respectively) and RF_{50} for IVM-M (0.83 to 0.99 in 24- and 96-well plates, respectively) were below this level. MPT allowed better differentiation of isolates in 24-well plates, although the values obtained in 96-well plates were also reliable. RFs above 3 demonstrate that the test was able to distinguish isolates, which therefore did not occur only for IVM-M considering the RF_{50}.

Table 2. Lethal concentrations LCs, confidence limits (95%, µg/mL) and resistance factors (*RFs) in LDT to *Haemonchus contortus* susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) isolates, in 24- and 96-well plates, for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Anthelmintic	LCs	HcEc91		HcBot		RF_{50}		RF_{99}	
		24-well	96-well	24-well	96-well	24-well	96-well	24-well	96-well
TBZ	LC_{50}	0.011 (0.0110-0.0120)	0.010 (0.0060-0.0140)	2.166 (2.0856-2.2501)	2.650 (2.4440-2.8851)				
	LC_{99}	2.325 (2.0583-2.6402)	0.621 (0.3678-1.3548)	95.871 (86.2741-107.0918)	157.244 (123.8728-204.9480)	191.52	253.15	41.23	253.32
LEV	LC_{50}	0.002 (0.0017-0.0019)	0.001 (0.0004-0.0011)	0.011 (0.0103-0.0111)	0.011 (0.0101-0.0117)				
	LC_{99}	0.175 (0.1491-0.2071)	0.251 (0.1325-0.6083)	0.328 (0.3021-0.3576)	0.331 (0.2797-0.3967)	5.85	14.58	1.88	1.32
IVM - M	LC_{50}	0.131 (0.1244-0.1380)	0.158 (0.1346-0.1869)	0.129 (0.1243-0.1345)	0.132 (0.1211-0.1443)				
	LC_{99}	3.115 (2.6900-3.6539)	3.978 (2.7511-6.2582)	9.603 (8.6688-10.6836)	13.260 (10.5670-16.9982)	0.99	0.83	3.08	3.33
MPT	LC_{50}	0.001 (0.0009-0.0011)	0.086 (0.0743-0.1008)	0.445 (0.4365-0.4528)	1.041 (0.9749-1.1145)				
	LC_{99}	0.058 (0.0513-0.0669)	1.306 (0.9414-1.9630)	1.216 (1.1672-1.2709)	9.668 (7.7975-12.5113)	447.76	12.03	20.86	7.40
ZLV	LC_{50}	0.008 (0.0074-0.0079)	0.008 (0.0066-0.0095)	0.112 (0.1067-0.1171)	0.103 (0.0935-0.1141)				
	LC_{99}	0.206 (0.1903-0.2244)	0.244 (0.1669-0.3939)	21.715 (18.7480-25.3428)	21.433 (15.7926-30.1104)	14.65	12.95	105.27	87.68

*LC_{50} and LC_{99} of the resistant isolate (Botucatu) divided by the respective value of the susceptible isolate (Echevarria1991). RF values greater than 3 show that the test was able to distinguish susceptible and resistant isolates.

2.4.2 *GINs from flocks*

The fecal cultures indicated *Haemonchus* as the predominant genre in the flocks on D0 and D14 (Figure 2).

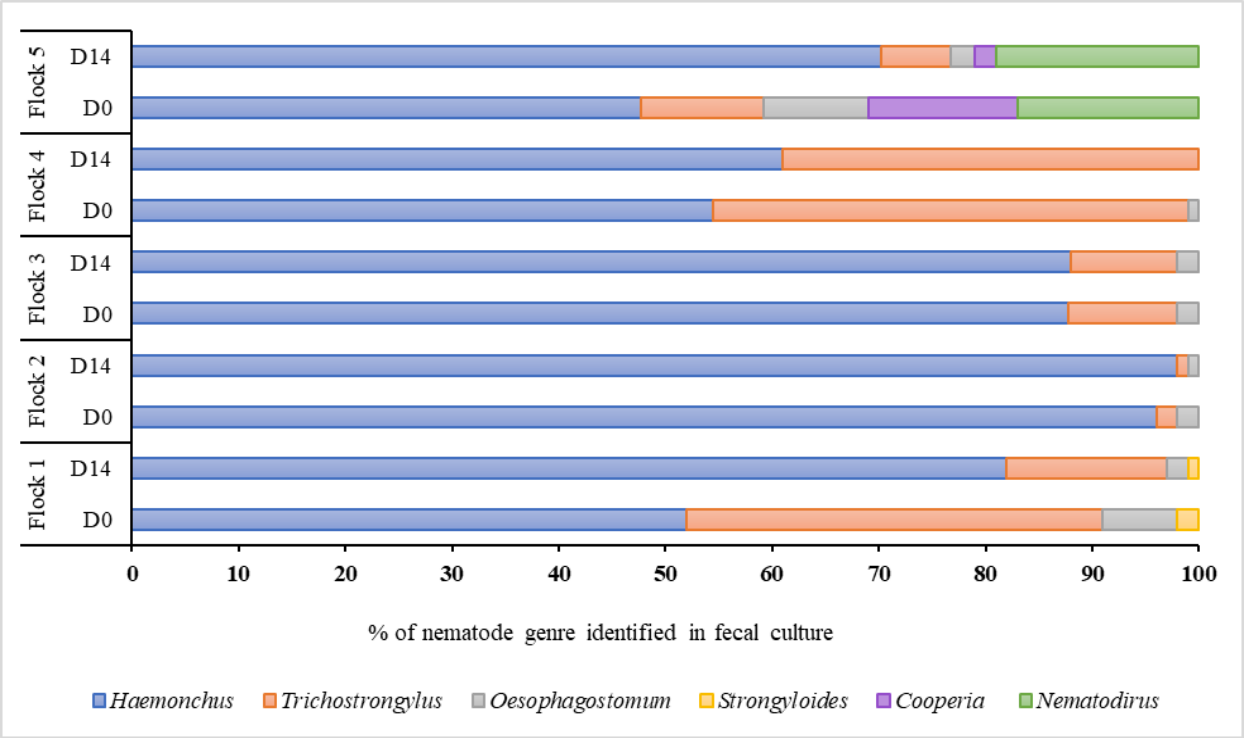

Figure 2. Percentage of nematode genre in fecal culture from flocks (Flock 1 to Flock 5) on days 0 and 14.

Table 3 shows again that although there were significant differences ($p \leq 0.05$), the percentage of these differences (DIF) between plates and between days were subtle for all drugs (the lowest being 0.02% to ZLV HcFlock-3 and the highest 4.90% to LEV HcFlock-5), except for MPT, which was higher on HcFlock-3 (10.56% and 8.41% for D0 and D14, respectively) and on HcFlock-5 (8.03% and 6.35% for D0 and D14, respectively). Differences detected for the others anthelmintic can be neglected as they did not exceed 5.25% (HcFlock-1). Again, high R^2 values (99.4 to 99.9%) indicate the accuracy of the analysis. The results show the agreement of LDT when performed in different plates and days of field collection, being sensitive and functional, except for MPT.

Table 3. Results of the inhibition of *H. contortus* larval development by flock (HcFlocks 1 to 5), in 24- and 96-well plates, on days 0 and 14, for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Flock	Anthelmintic	Day	Plates (well)	$\overline{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-1	TBZ	D0	24	34.48±0.20	0.00	100	0.98	0.00003	0.00003	0.999
			96	33.50±0.21	0.00	100				
		D14	24	35.07±0.18	0.00	100	4.77	0.00004	0.00004	0.999
			96	39.84±0.18	0.00	100				
	LEV	D0	24	85.81±0.16	43.50	100	1.55	0.00004	0.00004	0.999
			96	84.26±0.16	34.91	100				
		D14	24	90.52±0.16	52.53	100	1.11	0.00004	0.0005	0.998
			96	89.41±0.17	53.42	100				
	IVM-M	D0	24	47.82±0.27	0.47	100	3.64	0.00004	0.00003	0.999
			96	44.18±0.27	1.31	100				
		D14	24	47.98±0.21	0.00	100	4.13	0.00003	0.00003	0.999
			96	43.85±0.21	0.00	100				
	MPT	D0	24	33.76±0.14	0.00	100	5.25	0.00003	0.00003	0.999
			96	28.51±0.15	0.00	100				
		D14	24	37.02±0.17	0.00	100	4.14	0.00004	0.00003	0.999
			96	32.88±0.16	0.00	100				
	ZLV	D0	24	70.06±0.26	7.24	100	0.20	0.59	0.00003	0.999
			96	70.26±0.26	6.95	100				
		D14	24	71.35±0.19	11.27	100	1.74	0.00003	0.02	0.999
			96	73.09±0.21	14.96	100				

Flock	Anthelmintic	Day	Plates (well)	$\bar{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-2	TBZ	D0	24	50.77±0.27	0.45	100	0.16	0.69	0.007	0.999
			96	50.93±0.26	0.00	100				
		D14	24	48.79±0.18	0.00	94.77	0.60	0.03	0.006	0.999
			96	48.19±0.17	0.00	95.03				
	LEV	D0	24	96.13±0.17	76.13	100	0.08	0.74	0.003	0.994
			96	96.05±0.17	79.17	100				
		D14	24	87.73±0.15	57.01	100	2.63	0.00003	0.00004	0.998
			96	90.36±0.15	62.03	100				
	IVM-M	D0	24	83.70±0.18	43.00	100	0.62	0.03	0.00004	0.999
			96	83.08±0.19	37.79	100				
		D14	24	79.72±0.20	36.79	100	0.85	0.007	0.609	0.999
			96	80.57±0.20	37.58	100				
	MPT	D0	24	17.48±0.13	0.00	100	1.39	0.00004	0.00003	0.999
			96	18.87±0.14	0.00	100				
		D14	24	18.26±0.14	0.00	76.36	0.96	0.0001	0.0001	0.999
			96	17.30±0.15	0.00	74.33				
	ZLV	D0	24	30.64±0.17	0.00	89.81	0.36	0.17	0.00003	0.999
			96	31.00±0.18	0.00	91.17				
		D14	24	29.80±0.13	0.00	89.96	0.06	0.74	0.02	0.999
			96	29.74±0.13	0.00	88.96				

Flock	Anthelmintic	Day	Plates (well)	$\bar{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-3	TBZ	D0	24	35.39±0.21	0.00	88.43	1.18	0.03	0.0002	0.999
			96	34.72±0.21	0.00	90.64				
		D14	24	34.58±0.15	0.00	79.99	0.46	0.05	0.0005	0.999
			96	35.04±0.16	0.00	81.04				
	LEV	D0	24	54.77±0.19	2.37	99.06	1.99	0.00004	0.00004	0.999
			96	56.76±0.19	6.54	100				
		D14	24	41.64±0.20	0.00	89.76	0.99	0.002	0.00003	0.999
			96	42.63±0.20	0.65	96.10				
	IVM-M	D0	24	72.84±0.21	20.49	100	0.08	0.79	0.001	0.999
			96	72.76±0.20	21.13	100				
		D14	24	64.64±0.22	30.62	93.91	4.17	0.00003	0.03	0.999
			96	68.81±0.21	32.64	99.34				
	MPT	D0	24	50.03±0.16	0.00	100	10.56	0.00003	0.00003	0.999
			96	60.59±0.16	0.00	100				
		D14	24	51.43±0.14	0.00	100	8.41	0.00004	0.00003	0.999
			96	59.84±0.14	0.00	100				
	ZLV	D0	24	96.39±0.07	83.25	100	0.02	0.87	0.00003	0.998
			96	96.41±0.06	83.56	100				
		D14	24	94.60±0.08	76.92	100	0.37	0.005	0.0002	0.998
			96	94.97±0.08	75.89	100				

Flock	Anthelmintic	Day	Plates (well)	$\bar{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-4	TBZ	D0	24	36.57±0.13	0.00	89.91	2.22	0.00004	0.00003	0.999
			96	34.35±0.13	0.00	91.11				
		D14	24	32.56±0.15	0.00	81.14	0.04	0.86	0.00002	0.999
			96	32.52±0.16	0.00	80.54				
	LEV	D0	24	39.68±0.14	0.00	95.35	0.34	0.10	0.00003	0.999
			96	39.34±0.14	0.00	96.73				
		D14	24	31.92±0.15	0.00	86.48	1.04	0.00003	0.001	0.999
			96	32.96±0.15	0.00	86.00				
	IVM-M	D0	24	50.51±0.17	2.84	94.39	0.36	0.15	0.07	0.999
			96	50.87±0.17	2.60	95.89				
		D14	24	39.40±0.11	0.00	87.56	2.83	0.00003	0.00002	0.999
			96	42.23±0.11	0.00	89.73				
	MPT	D0	24	17.27±0.10	0.00	100	2.32	0.00003	0.00003	0.999
			96	14.95±0.10	0.00	100				
		D14	24	32.31±0.11	0.00	100	1.62	0.00002	0.00002	0.999
			96	30.69±0.11	0.00	100				
	ZLV	D0	24	86.65±0.10	48.38	100	1.15	0.00004	0.00003	0.999
			96	87.80±0.10	51.00	100				
		D14	24	86.69±0.08	50.23	100	0.36	0.007	0.002	0.999
			96	87.05±0.09	50.70	100				

Flock	Anthelmintic	Day	Plates (well)	$\bar{X}$ ± SE (%)	Min. (%)	Max. (%)	DIF (%)	Plate (p-value)	Plate x Conc. (p-value)	R^2
HcFlock-5	TBZ	D0	24	43.30±0.30	0.00	95.84	0.99	0.03	0.48	0.999
			96	42.31±0.30	0.00	97.22				
		D14	24	44.04±0.17	0.95	93.50	1.32	0.00003	0.00003	0.999
			96	42.72±0.18	0.00	95.18				
	LEV	D0	24	63.47±0.17	17.21	100	4.90	0.00004	0.00003	0.999
			96	68.37±0.18	20.81	100				
		D14	24	45.11±0.13	2.39	91.18	4.77	0.00003	0.00004	0.999
			96	49.88±0.14	8.06	92.83				
	IVM-M	D0	24	88.74±0.12	55.92	100	1.18	0.00003	0.00003	0.999
			96	89.92±0.11	50.68	100				
		D14	24	70.38±0.15	31.25	100	4.53	0.00004	0.00003	0.999
			96	74.91±0.15	34.94	100				
	MPT	D0	24	39.17±0.14	0.00	100	8.03	0.00003	0.00003	0.999
			96	47.20±0.14	0.00	100				
		D14	24	33.77±0.12	0.00	100	6.35	0.00002	0.00002	0.999
			96	40.12±0.12	0.00	100				
	ZLV	D0	24	85.36±0.10	42.05	100	0.41	0.001	0.00001	0.999
			96	84.95±0.10	47.94	100				
		D14	24	84.91±0.10	41.55	100	1.20	0.00003	0.00003	0.999
			96	83.71±0.10	40.29	100				

* Significant at 5% ($p \leq 0.05$) level of probability by ANOVA and the Tukey test. $\bar{X}$ ± SE: Average efficacy ± standard error; Min: minimum efficacy; Max: maximum efficacy; DIF: Difference in mean efficacies between 24-well and 96-well plates; R^2 or coefficient of determination (0 to 1): measure of adjustment of the statistical model to the observed values.

The estimated LC_{50} for HcFlocks are shown in Table 4. The confidence intervals (95%) obtained in the 24-well plates on the same day of collection were within the confidence interval of the 96-well plates, except for TBZ D14 and MPT D0 and D14 for HcFlock-1, ZLV D14 HcFlock-2, LEV D0 and MPT D0 and D14 HcFlock-3, and again MPT D0 and D14 HcFlock-5. Most LC_{50} results were consistent and in agreement with their respective RF_{50} values (Table 5). RF above 3 demonstrates that the test was able to detect resistance in HcFlocks. For TBZ this occurred for all HcFlocks in both plates and collection days. For LEV, HcFlock-1 and 2 were susceptible (RF_{50} from 0.05 on HcFlock-2 to 2.10 on HcFlock-1), while the others were resistant (RF_{50} from 5.19 to 278.41 on HcFlock-5). For IVM-M, RF_{50} values rated all HcFlocks as susceptible, while RF_{99} presented opposite results for HcFlock 3, 4 and 5. Detection of resistance to MPT proved to be inconsistent. HcFlock-2 was clearly resistant. The others presented contradictory values, since results obtained in 24-well plates showed resistant status while in the 96-well plates the results indicated susceptible status for HcFlock 1, 3, 4 and 5. On the other hand, RF_{50} and RF_{99} values for ZLV rated HcFlock-2 as resistant (15.61 to 48.70) and the others as susceptible (0.06 on HcFlock-3 to 2.11 on HcFlock-1). These results indicate that with the use of ZLV the difference in status was clearer and more stable between collection days and plates than with MPT.

Table 4. Lethal concentrations LC_{50} and confidence limits (95%, µg/mL) in LDT for HcFlocks in 24 and 96-well plates, on collection days zero (D0) and fourteen (D14), for thiabendazole -TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Anthelmintic	Day	Plates	HcFlock-1	HcFlock-2	HcFlock-3	HcFlock-4	HcFlock-5
TBZ	D0	24	2.690 (2.325-3.125)	0.737 (0.631-0.861)	2.540 (2.161-3.002)	2.332 (1.964-2.787)	1.420 (1.184-1.711)
		96	2.783 (2.386-3.257)	0.715 (0.597-0.854)	2.583 (2.146-3.132)	2.747 (2.251-3.386)	1.451 (1.177-1.800)
	D14	24	2.605 (2.228-3.060)	0.879 (0.748-1.033)	2.942 (2.411-3.642)	3.325 (2.778-4.023)	1.275 (1.061-1.538)
		96	1.750 (1.452-2.118)	0.926 (0.762-1.124)	2.764 (2.214-3.500)	3.443 (2.748-4.397)	1.434 (1.158-1.787)
LEV	D0	24	0.001 (0.001-0.001)	0.001 (0.001-0.001)	0.043 (0.036-0.051)	0.084 (0.071-0.100)	0.009 (0.008-0.012)
		96	0.002 (0.001-0.002)	0.001 (0.001-0.001)	0.018 (0.014-0.023)	0.086 (0.076-0.106)	0.006 (0.004-0.008)
	D14	24	0.001 (0.00-0.001)	0.001 (0.001-0.001)	0.073 (0.061-0.088)	0.172 (0.143-0.208)	0.055 (0.044-0.070)
		96	0.001 (0.00-0.001)	0.001 (0.001-0.001)	0.064 (0.051-0.080)	0.157 (0.127-0.197)	0.034 (0.026-0.046)
IVM - M	D0	24	0.179 (0.156-0.205)	0.007 (0.006-0.009)	0.021 (0.017-0.026)	0.152 (0.127-0.184)	0.003 (0.002-0.004)
		96	0.221 (0.189-0.258)	0.008 (0.006-0.010)	0.021 (0.016-0.027)	0.146 (0.117-0.182)	0.004 (0.003-0.005)
	D14	24	0.182 (0.158-0.209)	0.011 (0.009-0.013)	0.033 (0.024-0.043)	0.384 (0.319-0.467)	0.022 (0.017-0.027)
		96	0.242 (0.206-0.286)	0.010 (0.008-0.013)	0.022 (0.015-0.030)	0.311 (0.249-0.394)	0.014 (0.010-0.018)
MPT	D0	24	0.164 (0.148-0.183)	0.722 (0.623-0.851)	0.044 (0.040-0.049)	0.666 (0.619-0.718)	0.108 (0.097-0.120)
		96	0.257 (0.235-0.283)	0.631 (0.535-0.755)	0.018 (0.015-0.020)	0.798 (0.704-0.913)	0.055 (0.049-0.062)
	D14	24	0.127 (0.112-0.143)	0.683 (0.581-0.814)	0.067 (0.061-0.074)	0.186 (0.168-0.206)	0.166 (0.148-0.187)
		96	0.177 (0.156-0.201)	0.733 (0.611-0.900)	0.019 (0.016-0.022)	0.218 (0.194-0.245)	0.098 (0.086-0.111)
ZLV	D0	24	0.008 (0.007-0.009)	0.233(0.199-0.274)	0.001 (0.001-0.001)	0.001 (0.001-0.002)	0.002 (0.001-0.002)
		96	0.008 (0.007-0.010)	0.228 (0.189-0.278)	0.001 (0.001-0.001)	0.001 (0.001-0.001)	0.002 (0.001-0.002)
	D14	24	0.007 (0.006-0.008)	0.119 (0.103-0.138)	0.001 (0.001-0.001)	0.001 (0.001-0.002)	0.002 (0.001-0.002)
		96	0.006 (0.005-0.007)	0.251 (0.208-0.306)	0.001 (0.001-0.001)	0.001 (0.001-0.002)	0.002 (0.001-0.003)

Table 5. Resistance factors (*RFs) in LDT in 24 and 96-well plates, on different collection days (D0 and D14), for thiabendazole - TBZ, levamisole - LEV, ivermectin monosaccharide - IVM-M, monepantel - MPT and Zolvix® - ZLV.

Anthelmintic	Day	Plates	HcFlock-1		HcFlock-2		HcFlock-3		HcFlock-4		HcFlock-5	
			RF_{50}	RF_{99}	RF_{50}	RF_{99}	RF_{50}	RF_{99}	RF_{50}	RF_{99}	RF_{50}	RF_{99}
TBZ	D0	24	237.86	34.79	65.18	16.53	224.62	59.81	206.16	86.68	125.59	82.46
		96	265.46	59.90	68.16	42.96	246.40	189.31	262.03	278.38	138.41	235.89
	D14	24	230.37	49.43	77.70	23.83	260.17	237.45	293.99	173.19	112.75	80.39
		96	166.92	128.41	88.29	97.37	263.66	577.91	328.39	764.05	136.82	282.80
LEV	D0	24	0.61	0.35	0.36	0.05	23.48	25.01	45.86	44.27	5.19	16.65
		96	2.10	0.23	1.17	0.05	24.34	17.31	116.01	24.93	7.85	8.29
	D14	24	0.38	0.16	0.36	0.73	39.99	68.50	93.73	105.17	30.14	278.41
		96	0.99	0.18	0.88	0.29	85.78	38.68	211.53	67.43	46.27	206.49
IVM - M	D0	24	1.36	1.22	0.06	0.22	0.16	1.23	1.16	8.97	0.02	0.16
		96	1.40	0.89	0.05	0.12	0.13	1.07	0.92	5.93	0.03	0.05
	D14	24	1.38	1.41	0.08	0.36	0.25	41.53	2.93	19.45	0.16	3.44
		96	1.53	1.41	0.06	0.24	0.14	10.93	1.97	14.27	0.09	1.35
MPT	D0	24	165.18	18.98	727.42	238.28	44.43	4.21	671.18	28.05	108.64	10.91
		96	2.98	0.51	7.30	8.13	0.21	0.12	9.23	3.42	0.64	0.18
	D14	24	127.56	25.19	688.10	263.74	67.58	6.14	186.95	18.05	167.63	26.21
		96	2.04	0.89	8.48	11.69	0.22	0.21	2.52	0.80	1.13	0.47
ZLV	D0	24	1.04	2.11	30.50	48.70	0.07	0.08	0.16	0.56	0.22	0.40
		96	1.00	1.38	28.60	43.79	0.06	0.10	0.14	0.34	0.19	0.58
	D14	24	0.91	1.79	15.61	20.01	0.07	0.16	0.15	0.63	0.23	0.44
		96	0.75	1.26	31.51	46.78	0.06	0.11	0.14	0.49	0.25	0.48

* LC_{50} and LC_{99} of the HcFlock on the collection days divided by the respective value of the susceptible isolate (Echevarria1991). RF values greater than 3 show that the test was able to distinguish resistance to the Anthelmintic.

RF_{50} below 3 were obtained for IVM-M demonstrating that this molecule was not able to distinguish isolates. Thus, complementary tests were performed with IVM-A in 96-well plates to get LCs and RFs results. Comparative values between IVM-M and IVM-A are shown in Table 6 indicating that there are major differences between these drugs. RF_{50} and RF_{99} much higher than 3 obtained for IVM-A were unquestionably more reliable in the distinction of *H. contortus* resistant and susceptible isolates (640 and 6480.33, respectively).

Table 6. Lethal concentrations LC_{50}, confidence limits (95%, µg/mL) and resistance factors (RFs) for ivermectin monosaccharide - IVM-M and aglycone - IVM-A in LDT to *Haemonchus contortus* susceptible (Echevarria1991-HcEc91) and resistant (Botucatu-HcBot) isolates, in 96-well plates.

Anthelmintic	HcEc91		HcBot		RFs*	
	LC_{50}	**LC_{99}**	**LC_{50}**	**LC_{99}**	**RF_{50}**	**RF_{99}**
IVM-M	0.158 (0.1346-0.1869)	3.978 (2.7511-6.2582)	0.132 (0.1211-0.1443)	13.260 (10.5670-16.9982)	0.81	3.33
IVM-A	0.001 (0.0001-0.0004)	0.026 (0.0088-0.0446)	0.637 (0.5482-0.7394)	194.410 (135.1915-292.9334)	640	6480.33

*LC_{50} and LC_{99} of the resistant isolate (Botucatu) divided by the respective value of the susceptible isolate (Echevarria1991). RF values greater than 3 show that the test was able to distinguish susceptible and resistant isolates.

2.5 Discussion

The validation of laboratory methods for parasite resistance diagnosis to anthelmintic in small ruminants is extremely important, since they may support adjustments of the drug management and preserve the effectiveness of active principles in the flocks (VON SAMSON-HIMMELSTJERNA et al., 2009). However, the dilution ranges of the chemical groups and the behavior of the reference isolates are not described, so not *in vitro* tests have been standardized and validated for routine use in Brazil. Thus, from national isolates of *H. contortus*, the present study was able to determine the dose-response curves to the main anthelmintic groups in 24 and 96-well plates. In addition, the responses of parasite samples from flocks were compared to HcEc91/HcBot, so that the correlation between both could be verified.

The observed DIFs were exceptionally low when comparing isolates between plates. Results indicated the reliability of the LDT when performed in both plates for all drugs, except for MPT. With respect to the dose-response curves, LC_{50} and RF values obtained from HcEc91/HcBot, we observed that they could be differentiated regarding MPT, especially in 24-well plates, which did not occur for IVM-M. Regarding the results of the HcFlocks, again the percentage of difference between plates was low, being more pronounced for MPT. ZLV was more effective in the detection of resistance than MPT (being able to replace the latter), while the results obtained for IVM-M were mostly inconsistent.

KELLY, HALL (1979) stated that RF values greater than 3 demonstrate that the test was able to distinguish susceptible and resistant isolates. The RF values obtained in the present study allowed this distinction, meaning that the results obtained for the HcFlocks are valid. However, this distinction was not possible for IVM-M considering the $RF_{50,}$ which may be due to the use of monosaccharide IVM as standard substance. DOLINSKÁ et al. (2013, 2014) reported that the use of avermectin analogs and especially IVM-A significantly increased the ability of the LDT to differentiate isolates. This fact was confirmed by the RF values obtained for IVM-A in the present study. However, despite the strong potential of IVM aglycone in detecting resistance in the LDT, it is believed to have low sensitivity in cases of mixed parasitic populations (DOLINSKÁ et al., 2012). The lowest LC_{50} values in different IVM analogs were demonstrated in *H. contortus* (LACEY et al., 1991; DEMELER, 2005). These values were 2 to 4 times lower than those for *Ostertagia circumcincta* and *T. colubriformis* (DOLINSKÁ et al., 2012).

In the resistance diagnosis, we expected the larval development of both isolates and both plates for MPT would be similar to that observed for ZLV, since MPT is the chemical base of ZLV. However, this was not observed. The commercial product showed a more coherent and statistically similar dose-dependent curve for both isolates and in both plates. In the case of MPT, higher concentrations were required to obtain better efficacy. Figure 1 clearly shows the distinction between isolates for ZLV in both plates. Although for MPT, the behavior of the isolates was different between plates, their differentiation was possible, according to the RFs obtained: RF_{50} values of 447.76 and 12.03 and RF_{99} values of 20.86 and 7.40, in 24 and 96-well plates,

respectively. For HcFlocks, however, 24-well plates gave opposite results regarding the resistance status from those obtained in the 96-well plates. Other researchers have reported difficulty in detecting *H. contortus* resistance status to MPT. LECOVÁ et al. (2013) attempted to determine the *in vitro* efficacy of MPT by micro-agar LDT (MALDT) against resistant and susceptible isolates of *H. contortus*. Remarkably similar LC_{50} values were obtained for both isolates (0.0034 and 0.0037 µg/mL, respectively), making the resistance diagnosis unfeasible. Recently, KOTZE et al. (2018) evaluated the resistance of isolates to this chemical group through LDT using ZLV, which allowed the isolates' differentiation (RF=6). In the present study, the RFs obtained were much higher than in the previous study. In other studies, in which MPT resistance was detected in *H. contortus*, this substance was subjected to previous chemical reactions in order to transform it into MPT-sulfone (KAMINSKY et al., 2008a; LECOVÁ et al., 2013; STUCHLÍKOVÁ et al., 2013, 2016). Perhaps the use of MPT for diagnostic tests requires structural modification, so use of ZLV may be a more efficient and less costly option. In addition, ZLV it is more stable than monepantel (ultra-pure reference substance) given its chemical composition. The pharmacologically active substance is monepantel with monepantel-sulfone as marker residue and excipients (RRR-α-tocopherol, beta-carotene, maize oil, propylene glycol, macrogolglycerol hydroxystearate, polysorbate 80, propylene glycol monocaprylate and propylene glycol dicaprylocaprate) that stabilize this commercial formulation (EPAR, 2009).

The results clearly detected strong resistance to TBZ in all HcFlocks, to LEV in HcFlock 3, 4 and 5 and to MPT and ZLV in HcFlock-2. Our RF_{50} results for TBZ are higher than those reported by VÁRADY, CORBA (1999) (RF_{50} =14.3). This leads us to believe that the resistant isolate of the present study, originally from a SP flock, was strongly selected for resistance (ALMEIDA et al., 2010). The same situation was detected in all flocks. This can be explained by the fact that in Brazil (especially São Paulo), benzimidazoles have been the anthelmintic most used in recent decades, with a high percentage of resistance in the flocks of that state (VERÍSSIMO et al., 2012). The LEV RF_{50} values obtained for the isolates (5.85 and 14.58 in 24 and 96-well plates, respectively) were lower than those reported by VÁRADY, CORBA (1999) (32.52) and those obtained for *H. contortus* LevR and Lawes resistant isolates reported by SARAI et al. (2013) (668 and 903, respectively). For HcFlock 3, 4 and 5, the highest RF_{50}

values were 85.78, 211.53 and 46.27, indicating resistance in more than 50% of the flocks evaluated. The low resistance reported for MPT was expected since it is the drug with the highest cost in the Brazilian market.

FECRT and LDT costs were investigated to check if the latter would be economically advantageous. FECRT costs US$ 220.45 for a trial of three chemical groups and a control group, with an additional cost of US$ 57.50 per extra group (LOVE; HUTCHINSON, 2003). In a seven-group experiment, the cost of FECRT would be US$ 392.95. The price for performing FEC on stool samples from seven experimental groups is US$ 538.60 (DPI, 2012). In the present study, considering FECRT costs related to the visits (travel expenses and anthelmintics costs) were in average US$ 187.03 for a farm located in the São Paulo state, with the cheapest being US$ 169.37 and the highest being US$ 195.20. We also estimated the cost of resistance diagnosis for a flock by LDT in both plates (culture plates, chemical molecules and reagents of the culture medium), with two repetitions per concentration (according to the commercial test). For the 24-well plate, the costs would be US$ 166.06 and US$ 58.46 if the test was done with MPT or ZLV, respectively. In the 96-well plate, the costs would be US$ 46.06 and US$ 28.13, respectively. Therefore, by performing the test in the 96-well plate, cost reductions of 72.3% and 51.9% would occur, respectively, compared to the 24-well plate. In all those cases, the costs were calculated using the IVM-A since it presented higher LDT performance. It should be considered that the synthesis/production of MPT needs to be ordered from suppliers outside Brazil, which adds to the costs. In addition, the lowest cost to perform FECRT was greater than the highest cost to perform the LDT.

2.6 Conclusions

RF values allowed the differentiation of isolates, especially for TBZ, LEV, MPT, ZLV and IVM-A, and less significantly for IVM-M. The small differences in the average efficacy of anthelmintics indicated reliable agreement between plates, both in the evaluation of isolates and for *H. contortus* from flocks. Adapting the test to 96-well plates resulted in cost savings of at least 51.9% compared to the 24-well plates. RESISTA-*Test* could be a diagnostic tool in the future, supporting the adjustments of the drug management and preserving chemical classes. The LDT results confirmed the reliability of this diagnostic test.

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CAPÍTULO 3 – Anthelmintic resistance of *Haemonchus contortus* from sheep flocks in Brazil: concordance of *in vivo* and *in vitro* (RESISTA-*Test*) methods[3]

Resistência anti-helmíntica de *Haemonchus contortus* em rebanhos ovinos no Brasil: concordância de métodos *in vivo* e *in vitro* (RESISTA-Test).

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3.1 Abstract

This study evaluated the resistance status of *Haemonchus contortus* from sheep flocks in São Paulo state, Brazil, through a comparative study between the fecal egg count reduction test (FECRT) and the larval development test (LDT). For the FECRT, 49 sheep were selected in each one of five flocks and divided in seven groups: control (untreated) and treated with benzimidazole, levamisole, closantel, ivermectin, moxidectin and monepantel. Feces were collected for fecal egg counts and fecal cultures. The LDT was performed with thiabendazole-TBZ, levamisole-LEV, ivermectin aglycone-IVM-A and Zolvix-ZLV. *H. contortus* was the predominant parasite, presenting a multi-resistant profile. Resistance to all drugs was detected by FECRT to 100% of the flocks, except to ZLV (40% resistant and 20% suspected of resistance). LDT indicated resistance to TBZ and IVM-A in all flocks, in 80% of flocks to LEV and 20% to ZLV. Total agreement was obtained between both tests for TBZ and IVM

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[3] Este capítulo corresponde ao artigo científico submetido na Revista Brasileira de Parasitologia Veterinária

(k = 1.0), while for LEV and ZLV was obtained substantial agreement (k = 0.8 and 0.6, respectively). Concordance between tests was good, showing that it is possible to use the outcome of one test to predict the other, validating LDT as a fast diagnosis test for sheep farmers in Brazil.
Keywords: Anthelmintic resistance, FECRT, laboratory diagnosis, RESISTA-*Test*, small ruminants.

Resumo

Este estudo avaliou o status de resistência de *Haemonchus contortus* em rebanhos ovinos do estado de São Paulo, Brasil, através de um estudo comparativo entre o teste de redução da contagem de ovos nas fezes (TRCOF) e o teste de desenvolvimento larvar (TDL). Para o TRCOF, 49 ovinos foram selecionados em cada um dos cinco rebanhos e divididos em sete grupos: controle (não tratado) e tratados com benzimidazol, levamisol, closantel, ivermectina, moxidectina e monepantel. Fezes foram coletadas para a contagem de ovos nas fezes e coproculturas. O TDL foi realizado com tiabendazol-TBZ, levamisol-LEV, ivermectina aglicona-IVM-A e Zolvix-ZLV. *H. contortus* foi o parasita predominante, apresentando um perfil de multirresistência. Resistência a todos os fármacos foi detectada por meio do TRCOF em 100% dos rebanhos, exceto para ZLV (40% resistentes e 20% suspeitos de resistência). O LDT indicou resistência ao TBZ e IVM-A em todos os rebanhos, em 80% dos rebanhos ao LEV e 20% ao ZLV. Foi obtida concordância total entre os dois testes para TBZ e IVM (k = 1,0), enquanto para LEV e ZLV foi obtida concordância substancial (k = 0,8 e 0,6, respectivamente). A concordância entre os testes foi boa, sendo possível usar o resultado de um teste para prever o outro, validando o LDT como um teste diagnóstico rápido para criadores de ovinos no Brasil.
Palavras-chave: Resistência anti-helmíntica, TRCOF, diagnóstico laboratorial, RESISTA-*Test*, pequenos ruminantes.

3.2 Introduction

Helminthiasis in small ruminants is aggravated by high levels of parasitic resistance, a current scenario in almost all countries in the world in which sheep farming is present (Kaplan and Vidyashankar, 2012). The increasing occurrence of

gastrointestinal nematodes (GIN) resistant to anthelmintic and the need for reliable information about their establishment and dissemination indicate the need for standardized laboratory tests (Coles et al., 1992). Many advances in the resistance research have been occurred in the past 50 years, including diagnostics, important physiological and genetic discoveries, and the development of predictive mathematical models and tools to help farmers to manage resistance (Kotze et al., 2014). However, the challenge was only partially solved. A key issue that requires resolution for farmers is the use of faster and easier diagnostic techniques to estimate the presence of anthelmintic resistance.

Among *in vivo* techniques for the detection of resistance, the fecal egg count reduction test (FECRT) stands out. This test is based on the comparison of the infection estimated by the mean pre-treatment and post-treatment fecal egg counts (FEC) and must meet the recommendations made by Coles et al. (1992). However, FECRT presents some limitations ignoring the variability introduced by the counting process and by different infection levels across animals (Traversa and von Samson-Himmelstjerna, 2016; George et al., 2017; Wang et al., 2017). In addition, there are flaws in the detection of resistance in FECRT, especially in species with low egg production, such as *Teladorsagia circumcincta* or *Trichostrongylus* spp. (Palcy et al., 2010). There are reports of false positive results in *H. contortus, T. colubriformis* and *T. circumcincta* for levamisole (Cawthorne and Cheong, 1984; Grimshaw et al., 1994) and false negative results for ivermectin (Jackson, 1993). Moreover, FECRT is considered a laborious, time-consuming and expensive test (US$ 392.95 for a test with six chemical groups and a control group) (Love and Hutchinson, 2003; DPI, 2012; Chagas et al., 2013; Babják et al., 2018).

Therefore, *in vitro* assays have been developed to assess parasitic resistance to different classes of anthelmintic. The larval development test (LDT) (Hubert and Kerboeuf, 1992) is considered sensitive and practical, and allows *in vitro* evaluation of the efficacy of more than one chemical group at the same time and it does not depend on embryonated eggs (Kaplan et al., 2007). The LDT results provide reliable dose-response curves for benzimidazoles (BZs), levamisole (LEV) and ivermectin (IVM) (Taylor et al., 1990) and reference parasite strains may be included to give a measure of inter-assay variation (Craven et al., 1999).

Seeking to deal with the advancement of resistance, the use of sensitive tests to determine the degree of efficacy of a given drug, in a specific population of parasites, can help in the planning of control strategies (Taylor et al., 2002). However, even though it is of fundamental importance, the diagnosis of parasitic resistance is not yet a practical reality in Brazil. Thus, the validation of practical methods for diagnostic laboratory is extremely important. *In vitro* analyzes are less costly, relatively easy and able to provide reproducible parameters in the measurement of drug resistance, enabling a diagnosis less dependent on animal experiments (Chagas et al., 2013). Therefore, the objective of this study was to evaluate the resistance status of *H. contortus* from sheep flocks for the chemical groups of benzimidazoles, imidothiazoles, avermectins and amino-acetonitrile derivatives (AADs), performing a comparative study between FECRT and LDT.

3.3 Material and Methods

3.3.1 Sampling procedures for FECRT

The trial was carried out in the state of São Paulo, Brazil, in five flocks of sheep kept on pasture. To be included in the study, the flocks needed to: (1) have a minimum of 49 animals with FEC ≥ 200; and (2) keeping the animals with no anthelmintic treatment for at last 12 weeks before the study (Coles et al., 1992). In total, 245 animals of different breeds participated in the experiment following the methodology of Coles et al. (2006). The breed composition of the animals was represented as follows: crosses of ½ Texel and ½ Santa Inês (0.82%), ½ Île-de-France and ½ Santa Inês (1.22%), ½ Dorper and ½ Santa Inês (24.08%), ¾ Dorper (24.08%) and other breeds such as Dorper (0.41%), Texel (2.86%), White Dorper (3.27%), Île-de-France (6.12%), Santa Inês (17.14%), Morada Nova (20.00%).

In each of the five flocks, 49 sheep were divided into seven groups (n = 7): untreated-control (C), Benzimidazole - BZ (Valbazen 10 oral Cobalt, Pfizer, 0.5 mL/10 kg BW), Levamisole - LEV (Ripercol L 150 F injectable, Fort Dodge, 0.33 mL/10 kg BW), Closantel - CLO (Diantel 10% oral, Hipra, 1 mL/10 kg BW), Ivermectin - IVM (Ivomec 1% injectable, Merial, 0.2 mL/10 kg BW), Moxidectin - MOX (Cydectin 1% injectable, Zoetis, 0.2 mL/10 kg BW) and Zolvix- ZLV (Monepantel 2.5% oral, Novartis, 1 mL/10 kg BW). For the constitution of the groups, the flocks were visited the day

before the treatment (D-1) to collect feces for individual FEC and for fecal culture (pool) to determine the occurrence of the parasite's genera (van Wyk et al., 2004). Each group received seven animals in decreasing order of FEC (≥ 200), to obtain a similar average per group. The animals were then weighed and dewormed (D0) according to the specifications of the anthelmintic manufacture. After 14 days (D14), a third visit was carried out in the flocks for a new collection of feces for individual FEC and fecal culture (group) analysis.

3.3.2 Sampling procedures for LDT

Feces were collected individually for FECRT in D0 and D14, placed in vacuum-sealable plastic bags (Vac Freezer-Sanremo-Sr375), identified and immediately taken to the Veterinary Parasitology Laboratory of Embrapa Pecuária Sudeste for the LDT. The recovery of eggs was performed with the sequential use of sieves following the methodology of Coles et al. (1992). About 70 nematode eggs were added to each well of 96-well plates, along with a nutrient medium according to Hubert and Kerboeuf (1992). The plates were identified, sealed with PVC film and kept in an incubator for 24 hours (27 °C, RH≥ 80%) for the development of larvae (L_1). After that period, each well received the serial dilutions of thiabendazole-TBZ (Sigma-Aldrich T8904), levamisole-LEV (Sigma-Aldrich 31742), ivermectin aglycone-IVM-A (Bioaustralis BIA-I1151), and Zolvix®. Comparative study with monepantel-MPT (chemical base, GS P11144) and Zolvix® (commercial product), indicated that the use of Zolvix® is recommended (Raza et al., 2016; Kotze et al., 2018; Gainza et al., 2020). The negative control consisted of distilled water and nutrient medium. The plates were again incubated at the same conditions for more 6 days. All concentrations of anthelmintic and the negative control were tested in two replicates.

After incubation, eggs and larvae (L_1, L_2 and L_3) from each well were quantified using an inverted microscope and identified at the genus level (van Wyk et al., 2004). The numbers of L_3 vs. eggs + L_1 + L_2 in each well containing the treatments were compared with the control wells to determine the critical well, defined as the well where development to the L_3 stage is inhibited by 50% compared with controls. Critical wells correspond closely to the calculated values of LC_{50} (Kaplan et al., 2007) and can be used in a similar way to make inferences about the resistance status of the parasite population for a given flock.

3.3.3 FECRT and LDT analysis and resistance determination

The efficacy of the anthelmintic in the FECRT was estimated by the RESO 4.0 program to define the status of resistance or susceptibility of the treated groups in relation to the control group. Resistance is present if (i) the reduction percentage in egg count is less than 95% and (ii) the 95% confidence level is less than 90%. If only one of the two criteria is met resistance is suspected (Coles et al., 1992; 2006).

In LDT, all larvae in the control wells, and in the wells above and below the critical well, were counted and identified to determine the predominant genus. The resistance of *H. contortus* to anthelmintic was determined when the parasite (L_3) predominant in the control and critical wells of the plates was composed by this genus (ANONYMOUS, 1996).

Results of the LCs obtained for susceptible and resistant *H. contortus* isolates can be seem in Chapter 2 (Table 2). Comparisons of these values were used to determine the status of anthelmintic resistance in each flock. It was determined that, for *H. contortus*: 1) the critical wells associated with the values of LC_{50} correspond to FECRT ≥ 95% and were classified as susceptible, 2) wells associated with the values of the delineating doses (LC_{90}- LC_{95}) correspond to FECRT between 80% to 94% and were classified as suspected of resistance, 3) the wells associated with the discriminatory dose values (LC_{99}) correspond to FECRT <79% and were classified as resistant. We also added a value equivalent to half-well (0.5 critical well) to the value of the critical wells used as a cut-off value. Therefore, resistance status estimates were conservative so as not to overestimate resistance status. Thus, the results at the resistance limit would be classified as suspected of resistance.

3.3.4 Statistical analysis

A concordance was made between the efficacy (susceptible, suspected of resistance and resistance) obtained for each chemical group (TBZ, LEV, IVM-A and ZLV) in the animals of the different flocks with the critical well obtained in the LDT (where there was a 50% inhibition of larval development) + 95% CIs) occurring therefore an association with the concentration of the drug that was in that critical well (Crook et al., 2016). Paired t-tests were used to compare the values of the critical wells associated with the LC_{50} and LC_{99}, respectively, for the four chemical groups. The Chi-

square and Fisher's exact tests were used to compare the resistance status of *H. contortus* for the chemical groups in each flock, and to analyze whether there is a good agreement between the *in vivo* field test (FECRT) and the *in vitro* laboratory test (LDT), for validation of the last one. Good agreement thresholds change from one field or question to another. However, Landis and Koch (1977) have established the scale below to describe agreement quality according to Kappa values: <0: no agreement; 0-0.2: small; 0.2-0.4: fair agreement; 0.4-0.6: moderate; 0.6-0.8: substantial; 0.8–1: almost perfect. All analyses were performed using the XLSTAT-Premium 2020.1.1 software (Addinsoft 2020 -XLSTAT statistical and data analysis solution. Boston, USA). All effects were evaluated at a 5% significance level.

3.4 Results

The anthelmintic resistance status of *H. contortus* was determined when this was the predominant genus, counted in the control and critical wells of the plates, and this criterion was met in all flocks evaluated. The fecal cultures carried out, on days -1 and 14 post-treatment, indicated the presence of the following genera: Flock 1: 52.0% and 82.0% *Haemonchus*, 39.0% and 15.0% *Trichostrongylus*, 7.0% and 2.0% *Oesophagostomum* and 2.0% and 1.0% *Strongyloides*; Flock 2: 96.0% and 98.0% *Haemonchus*, 2.0% and 1% *Trichostrongylus* and 2.0% and 1% *Oesophagostomum*; Flock 3: 87.8% and 88.0% *Haemonchus*, 10.2% and 10.0% *Trichostrongylus* and 2.0% and 2.0% *Oesophagostomum*; Flock 4: 54.5% and 61.0% *Haemonchus*, 44.5% and 39.0% *Trichostrongylus* and 1.0% and 0% *Oesophagostomum*; and Flock 5: 47.7% and 70.2% *Haemonchus*, 11.5% and 6.5% *Trichostrongylus*, 9.8% and 2.3% *Oesophagostomum*, 14.0% and 2.0% *Cooperia* and 17.0% and 19.0% *Nematodirus*.

Table 1 summarizes the results of the FECRT for each experimental group in each of the sheep flocks evaluated. Of the five flocks, 3 (60%) showed resistance to all anthelmintic (flocks 1, 2 and 4), while 2 flocks (40%) showed susceptibility to ZLV (flocks 3 and 5). Flock 4 demonstrated the worst resistance scenario for BZ, LEV, CLO and IVM. Although ZLV presented 97% efficacy, the 95% confidence level was from 87 to 100%, classifying it as suspected of resistance. In flock 1 in its turn efficacy higher than 80% were detected for LEV, CLO, MOX and ZLV.

In the LDT, the resistance of *H. contortus* from the flocks for each chemical

group was confirmed based on the concentration of the critical well (LC_{50}) and the discriminant concentration (LC_{99}). *H. contortus* from all flocks were classified as resistant to TBZ and, comparing the average concentrations in the critical wells (P = 0.028). For LEV, *H. contortus* from all flocks, excepted Flock 2, were considered resistant, with no significant difference among the mean concentrations of critical wells (P = 0.077) among farms. *H. contortus* from all flocks were resistant to IVM-A with significant difference between the averages of the critical wells (P = 0.009). Finally, or ZLV resistance was detected only in Flock-2 (P = 0.514), while susceptibility occurred in Flocks 1, 3, 4, 5, with no difference between the averages (P = 0.119).

Table 1. The arithmetic means of fecal egg counts on day 14 after treatment (FEC D14), fecal egg count reduction percentages (R%) and the lower and upper 95% confidence intervals (CI), in sheep flocks (N=49, 7 animals per group) tested for the anthelmintics (AH) benzimidazole (BZ), levamisole (LEV), closantel (CLO), ivermectin (IVM), moxidectin (MOX), and zolvix (ZLV), including an untreated control group (C).

AH	Flock 1		Flock 2		Flock 3		Flock 4		Flock 5	
	FEC D14	R% (CI)	FEC D14	R% (CI)	FEC D14	R% (CI)	FEC D14	R% (CI)	FEC D14	R% (CI)
BZ	8579	0 (0-74)	871	54 (10-76)	2450	32 (0-79)	836	0 (0-63)	1667	2 (0-72)
LEV	900	89 (79-94)	350	81 (44-94)	2471	31 (0-70)	529	37 (0-74)	825	51 (0-85)
CLO	1207	85 (71-92)	400	79 (27-94)	1164	67 (0-90)	650	22 (0-70)	350	79 (40-93)
IVM	11264	0 (0-66)	1050	44 (0-81)	3171	11 (0-69)	2893	0 (0-0)	1400	18 (0-71)
MOX	1486	82 (24-96)	1229	35 (0-72)	579	84 (61-93)	1979	0 (0-9)	2600	0 (0-42)
ZLV	1086	87 (50-96)	721	62 (0-90)	71	**98 (93-99)**	2143	97 (87-100)	43	**97 (94-99)**
C	8157		1886		3579		836		1700	

* Resistance is present if (i) the percentage reduction in egg count is less than 95% and (ii) the 95% confidence level is less than 90%. If only one of the two criteria is met resistance is suspected (Coles et al., 1992). Bold indicates that the anthelmintic was effective.

When comparing the results of LDT and FECRT, there was total agreement between the tests (k = 1.00) regarding the resistance of *H. contortus* in all flocks to benzimidazoles/TBZ, with no significant difference (P = 0.655) (Table 2). LDT showed susceptibility to LEV in Flock 2, while FECRT demonstrated resistance. The critical cut-off point for LEV resistance was 0.001 µg/mL. Flock 2 showed critical values below this indication, while Flocks 1, 3, 4 and 5 showed values above, indicating resistance to LEV (well 7.5, 9.5 and 6.5 respectively). However, the chi-square analysis revealed that there was no significant difference between the results obtained in FECRT and LDT for LEV (P = 0.151) and a substantial agreement between the tests was detected (k = 0.8). In the case of IVM-A, there was total agreement (k = 1.0) between the tests regarding the detection of *H. contortus* resistance in all flocks, with no significant difference (P = 0.666). Finally, for ZLV, the critical cut-off point for resistance in the LDT was 0.008 µg/mL. Flock 2 presented values above this indication (well 9.5) and Flock 3 and 5 with values below, indicating susceptibility, showing total agreement with the results of the FECRT (k = 1.0). However, the results reflected total disagreement (k = -1.0) in Flock 1 and 4. The chi-square analysis revealed that there was no significant difference (P = 0.197) between the results obtained in FECRT and LDT for ZLV, which presented substantial agreement (k = 0.6), according to the classification of Landis and Koch (1977).

Table 2. Comparison of the efficacy obtained in the fecal egg count reduction test (FECRT) and the critical cut-off points obtained in the larval development test (LDT) for benzimidazole (BZs), levamisole (LEV), ivermectin (IVM), and Zolvix (ZLV). Results were reported as resistant (R), suspected of resistance (SR) or susceptible (S).

Flocks	Tests	BZs				LEV				IVM				ZLV			
		$\bar{X}$	Status	K-Value*		$\bar{X}$	Status	K-Value		$\bar{X}$	Status	K-Value		$\bar{X}$	Status	K-Value	
1	**FECRT**	0	R	1.0		89	R	1.0		0	R	1.0		87	R	-1.0	
	LDT	2.783	R			0.002	R			0.783	R			0.008	S		
2	**FECRT**	54	R	1.0		81	R	-1.0		44	R	1.0		62	R	1.0	
	LDT	0.715	R			0.001	S			0.512	R			0.228	R		
3	**FECRT**	32	R	1.0	1.0	31	R	1.0	0.8	11	R	1.0	1.0	98	S	1.0	0.6
	LDT	2.583	R			0.018	R			0.492	R			0.001	S		
4	**FECRT**	0	R	1.0		37	R	1.0		0	R	1.0		97	SR	0.8	
	LDT	2.747	R			0.086	R			1.037	R			0.001	S		
5	**FECRT**	2	R	1.0		51	R	1.0		18	R	1.0		97	S	1.0	
	LDT	1.451	R			0.006	R			0.261	R			0.002	S		

*Cohen's Kappa (k) varies between -1 and +1 with: -1 reflecting total disagreement; +1 reflecting total agreement; 0 reflecting total randomness. Good agreement thresholds change from one field or question to another. However, Landis and Koch (1977) have established the scale below to describe agreement quality according to Kappa values: < 0: no agreement; 0 - 0.2: small; 0.2 - 0.4: fair agreement; 0.4 - 0.6: moderate; 0.6 - 0.8: substantial; 0.8 – 1: almost perfect.

3.5 Discussion

This is the first study carried out in Brazil comparing FECRT and LDT to detect the resistance of GIN in small ruminants. The chemical groups used in LDT were selected based on previous research that demonstrates a dose-response relationship to detect resistance to anthelmintic *in vitro* (Coles et al., 1992; 2006). In the present study, LDT revealed resistance of *H. contortus* to TBZ (100%), IVM-A (100%), LEV (60%) and ZLV (20%) of flocks. In FECRT, resistance to anthelmintic evaluated was detected in 60% for all drugs, being 40% susceptible to ZLV. These results were expected, since Salgado et al. (2019) related that multiple anthelmintic resistance is a seriously problem to sheep production in Brazil.

In order to set up a comparison between *in vitro* and *in vivo* tests the discriminatory doses for resistance detection in LDT was established for all anthelmintic. The critical cut-off points (LC_{50}) for resistance were: 0.01 µg/mL for TBZ, 0.001 µg/mL for LEV, 0.001 µg/mL for IVM-A and 0.008 µg/mL for ZLV. The LC_{50} values used as cut-off values or discriminatory doses for the detection of resistance have been determined. Discriminant doses were also determined in other studies for use in LDT: 0.1 µg/mL TBZ and 1 µg/mL LEV to *H. contortus* (Hong, 1992; Mitchell et al., 2010), 0.02 µg/mL TBZ and 0.5 µg/mL LEV to *H. contortus*, *T. circumcincta* and *T. colubriformis* (Coles et al., 2006), 0.01 µg/mL TBZ, 2.5 µg/mL LEV and 0.0008 µg/mL IVM to *H. contortus* (Taylor, 1990). It should be noted that our results differ in the cut-off values for LEV to those reported by Taylor (1990), Hong (1992) and Mitchell et al. (2010) (0.001 vs 1.0 µg/mL). However, these authors had used commercial anthelmintic in their assays to perform the LDT and to determine the LC_{50}. Likewise, our results differ from those reported by Taylor (1990) for IVM (0.001 vs 0.0008 µg/mL). Due to this, it is necessary to highlight the importance of using standard substances to establish cut-off criteria and to reduce the variability of criteria to a minimum. Although in the present study there is an approach to determine the cut-off values (LC_{50}) for MPT through the commercial anthelmintic (Zolvix®), it is justified by the fact that in previous studies Zolvix® presented a better performance than MPT (chemical base) to detect resistance against resistant *H. contortus* isolates in LDT (Lecová et al., 2013; Raza et al., 2016; Kotze et al., 2018; Gainza et al., 2019).

The present study indicated that we have got a good association between FECRT and LDT in the detection of *H. contortus* resistant to benzimidazoles/TBZ. Similar findings were reported by Grimshaw et al. (1994) using FECRT, Egg hatch test and LDT in sheep farms in southern England, and by Crook et al. (2016) in sheep and goat farms in the Central Atlantic region of the United States. The high frequency of resistance to benzimidazoles (BZs) in the São Paulo state was previously described by Verissimo et al. (2012). Anthelmintic from this chemical group have been the basis for GIN control in many countries for decades (Chaudhry et al., 2015; Ali et al., 2019). Resistance to BZs is an established problem worldwide, in regions where the production of small ruminants is essential (Lalljee et al., 2019). Despite this, that chemical group is still widely used, a fact that probably causes the increase and/or maintenance of the high frequency of resistance alleles in sheep flocks.

Despite the different results between FECRT and LDT regarding resistance to LEV and ZLV in some flocks, there were no significant differences. In these flocks, LDT showed susceptibility to LEV and ZLV, while FECRT showed resistance and suspect of resistance. Although LDT assesses resistance to chemical compounds, it's important to highlight that the anthelmintic can present some efficacy when the level of resistance is not very high (period of resistance establishment). This provides an explanation of why LEV and ZLV were more effective by LDT in Flock 2, and Flocks 1 and 4 respectively, than in the others that had a higher critical resistance value. Previous studies have reported conflicting results only for LEV resistance between FECRT and LDT (Maingi et al., 1998). However, the data from the present study indicate that the results of the two tests are comparable. Therefore, small ruminant producers can use any of these methods to determine the efficacy of the anthelmintic, depending on the status of their flocks and the resources available.

The cut-off values (LC_{50}) for IVM recorded in the present study for isolates classified as resistant or susceptible to IVM were higher than those reported by Taylor (1990). It may be occurred probably due to the use of a commercial anthelmintic by the aforementioned authors vs the use of the standard substance in the present study. Here, the anthelmintic resistance detected by LDT for *H. contortus* isolates from flocks was also detected in FECRT. Thus, the agreement of the results is due to the adoption of IVM-A in LDT. The use of this molecule significantly increases the ability of LDT to

differentiate susceptible and resistant isolates (Dolinská et al., 2013; 2014).

Anthelmintic resistance is an evolutionary process that is impossible to prevent if anthelmintic drugs are used in a flock (Kaplan, 2020), but it is possible to reduce the rate with which resistance develops. For this reason, an early diagnostic is primordial. In the current work we were conservative in assigning cut-off points for resistance and included a borderline category (suspected of resistance) to avoid classifying a resistant flock when it is indeed susceptible. The cut-off points for assigning a low resistance status presented values of critical well from 1.5 to 2.0 higher than the cut-off point for assigning a susceptible status, which corresponded to an increase in the drug concentration. Thus, we believe that suspected flocks represented an important shift towards resistance. Probably the anthelmintic resistance is in process of establishment, but the resistance allele frequency did not reach 25% in the GIN studied population. The cut-off points for assigning a resistance status presented values of critical well ≥ 2.5 greater than the cut-off point for assigning a susceptible status, which corresponded to a 5-fold increase in drug concentration.

FECRT is an important tool for resistance diagnosis, but it can be extremely laborious and expensive for the producer. In addition, sometimes this method may not be an option to small farmers, due to the small number of animals and the minimum of animals required to test multiple anthelmintic agents. On the other hand, TDL has also limitations as contamination of the culture medium, the labor of parasite counting, dependence on the purchase of molecules. Even so, it clearly has advantages over FECRT. The LDT validated in the present study offers a faster and reliable laboratory alternative for the resistance monitoring and would be used in the sustainable integrated parasite management by Brazilian sheep farmers and technicians.

3.6 Conclusion

The data from the present study indicate that *H. contortus* has a multiple-resistance profile in the evaluated flocks. Total agreement was obtained regarding the results from both tests for TBZ and IVM-A, and substantial agreement for LEV and ZLV. Usually, correlations between the tests were good and it was possible to use the outcome of one test to predict the outcome of another, validating the LDT for the diagnosis of *H. contortus* resistance for farmers, the main parasite of small ruminants

in tropical countries.

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CAPÍTULO 4 – CONSIDERAÇÕES FINAIS

Com base nos dados experimentais obtidos, pode-se estabelecer as seguintes conclusões:

O uso de IVM-A em comparação com IVM-M aumenta significativamente a capacidade do TDL em diferenciar isolados susceptíveis e resistentes. A IVM-A é específica para a inibição do desenvolvimento larvar, portanto, aumenta a sensibilidade para detectar resistência em isolados oriundos do campo e as CLs permitem melhor diferenciação entre isolados susceptíveis e resistentes.

O uso do MPT para testes de diagnóstico exige modificação estrutural. Portanto, o uso do Zolvix® resulta em uma opção mais eficiente e menos onerosa. Além disso, o Zolvix® é mais estável que o monepantel dada a sua composição química.

A adaptação do teste em placas de 96 poços permitiu avaliar a eficácia *in vitro* de todas as drogas em um único teste e resultou em economia de pelo menos 51.9% em comparação às placas de 24 poços.

A concordância entre os testes *in vitro* (TDL) e *in vivo* (TRCOF) foi boa, permitindo o uso do resultado de um teste para prever o resultado do outro, validando o TDL para o diagnóstico da resistência aos anti-helmínticos em *H. contortus,* o principal parasita de pequenos ruminantes nos países tropicais.

Contribuição técnico-científico: Constitui uma opção a mais, a ser utilizada na rotina laboratorial para monitoramento da resistência anti-helmíntica em rebanhos de pequenos ruminantes.

Contribuição econômica e política: Esta ferramenta de diagnóstico contribui para a redução dos custos provenientes da situação atual de multirresistência (compra frequente de antiparasitários, mão-de-obra, queda da produtividade, perda de animais, etc.).

Contribuição social: O teste aqui validado, permite a identificação da situação da RA visando ajustes de manejo, desacelerando o estabelecimento da resistência e, consequentemente, reduzindo a chance de existência de resíduos de antiparasitários nos produtos de origem animal (especialmente leite e carne) e no ambiente.