The "leva" dilemma: Harnessing next-generation sequencing for the surveillance of levamisole resistance in *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* in sheep across New South Wales

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Abstract

The rapid escalation of anthelmintic resistance (AR) poses a major threat to the productivity and sustainability of the Australian sheep industry. Gastrointestinal nematodes (GINs) such as *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* are among the most pathogenic parasites of small ruminants, with widespread resistance now reported across multiple anthelmintic classes. Levamisole (LEV) remains one of the few effective compounds available and is used in combination drenches to manage mixed-species GIN infections. However, emerging reports of LEV resistance are cause for significant concern. The single nucleotide polymorphism (SNP) S168T within the *acr*-8 exon 4 region has been functionally validated in *Haemonchus contortus* as a marker of LEV resistance, providing a valuable molecular target for surveillance. This study aimed to establish a proof of concept for the molecular detection of the S168T SNP in *Tri. colubriformis* and *Tel. circumcincta* populations using metabarcoding and next-generation sequencing. Across 200 mixed-larval samples collected from New South Wales sheep flocks, the average frequency of the S168T mutation was 34.64% in *Tel. circumcincta* and 24.34% in *Tri. colubriformis*. These results provide the first molecular evidence of LEV resistance alleles in *Tel. circumcincta* and *Tri. colubriformis* in Australia. By integrating real-time parasite monitoring with SNP-based screening, resistance alleles can be detected at low frequencies before phenotypic drug failure occurs. This approach provides a foundation for improved anthelmintic stewardship and highlights the need for further research to define the threshold at which genotypic resistance translates to phenotypic drug failure. Overall, this work supports a proactive transition in AR management, shifting from reactive treatment to evidence-based prevention.

Keywords

Anthelmintic resistance, sheep, levamisole, next-generation sequencing, S168T

1. Introduction

Anthelmintic resistance (AR) in gastrointestinal nematodes (GINs) represents one of the most pressing threats to global small ruminant production, undermining animal health, productivity and the sustainability of livestock systems (Charlier et al., 2022; Hodgkinson et al., 2019; McLeod, 1995). In Australia, AR costs the livestock industry an estimated AUD $785 million annually through reduced growth, impaired wool quality, reproductive losses, and increased mortality (Shephard et al., 2022). This burden is particularly severe in temperate regions, where *Teladorsagia circumcincta* (small brown stomach worm) and *Trichostrongylus colubriformis* (black scour worm) are highly prevalent and pathogenic to sheep (Craig, 2009; Roeber et al., 2013a). The pathogenicity of *Tel. circumcincta* and *Tri. colubriformis* not only compromises animal health and welfare, but also amplifies the economic and sustainability challenges posed by AR in grazing systems (Barker & Titchen, 1982; Roeber et al., 2013a).

Levamisole (LEV), an imidazothiazole acetylcholine receptor agonist, has maintained efficacy longer than other anthelmintic compounds due to its unique mode of action and historically lower usage (Tyrrell & LeJambre, 2010). Its continued effectiveness against *Haemonchus contortus* has driven widespread adoption in mixed-species infections, where resistance to benzimidazoles and macrocyclic lactones is prevalent (Antonopoulos et al., 2022). However, intensified reliance on this compound within combination drenches has coincided with elevated levels of LEV resistance in *Tri. colubriformis* and *Tel. circumcincta*, as demonstrated in national and regional surveys (Playford et al., 2014). The "leva" dilemma encapsulates this dual challenge: LEV is indispensable for controlling *H. contortus*, especially in temperate regions, yet its extensive use in mixed species infections appears to accelerate resistance in other critical GIN species.

Large-scale surveillance studies underscore the scale of this issue. Over a decade ago, a national survey reported LEV resistance in 82% of *Trichostrongylus* isolates (n = 104 properties) and 86% of *Teladorsagia* isolates (n = 97 properties), with regional studies in Victoria and Queensland revealing comparable prevalence (Lyndal-Murphy et al., 2014; Playford et al., 2014; Preston et al., 2019). Internationally, declining LEV efficacy has been documented in Argentina, New Zealand and Europe, often despite the deployment of combination drenches (Hodgson & and Mulvaney, 2017; Luque et al., 2021; Waghorn et al., 2014). Collectively, these findings highlight the adaptability of GINs as a result of increased drug selection pressure and indicate that even combination drenches may fail to provide sustainable control without enhanced AR surveillance.

Contemporary diagnostic approaches for surveying AR largely rely on phenotypic assessments, using observable traits such as larval development and egg production. The faecal egg count reduction test (FECRT) lacks sensitivity and fails to identify resistance until approximately 25% of the nematode population harbours resistance alleles (Martin et al., 1989; Morgan et al., 2022). Compounding this challenge, species-level identification depends on larval differentiation, an approach that is time-consuming, prone to bias from unequal species survival, and incapable of resolving beyond the genus level (Dobson et al., 1992; Roeber & Kahn, 2014). Critically, their inability to detect preclinical resistance means intervention only occurs once clinical failure is evident, by which point the opportunity to prevent production losses and contain the proliferation of resistance has often passed (Martin et al., 1989). Consequently, resistance in *Tri. colubriformis* and *Tel. circumcincta* is likely underestimated relative to *H. contortus*, where genomic characterisation has enabled the development of molecular diagnostics (Doyle et al., 2022).

As resistance escalates, molecular diagnostics offer a transformative opportunity for detecting resistance earlier and with greater precision than conventional approaches, as they can reveal resistance at the genetic level before phenotypic failure becomes apparent (Roeber et al., 2013b). Among these, next-generation sequencing and single nucleotide polymorphism (SNP) screening permit the identification of resistance-associated alleles at low frequencies, enabling proactive management interventions (Avramenko et al., 2019; Kotze et al., 2020). A prominent advance has been the characterisation of the S168T mutation in the *acr-8* gene of exon 4 in *H. contortus*, which has been functionally-validated in field-applicable diagnostics to be associated with LEV resistance (Antonopoulos et al., 2024; Francis et al., 2024). Whether this mutation or analogous markers occurs in *Tri. colubriformis* or *Tel. circumcincta* remains unknown, representing a critical knowledge gap that constrains molecular surveillance and leaves producers vulnerable to emerging resistance until clinical treatment failure occurs.

To address this deficit, this study investigates the presence of the S168T SNP associated with LEV resistance in field populations of *Tri. colubriformis* and *Tel. circumcincta* collected from sheep across New South Wales. To date, no molecular screening of LEV resistance has been conducted for these species in Australia, representing a key knowledge gap in national resistance surveillance. The objectives of this study are to: (i) demonstrate the potential of next generation sequencing as a high-throughput surveillance tool for detecting the S168T mutation in *Tri. colubriformis* and *Tel. circumcincta*, and (ii) map the distribution and frequency of this resistance-associated SNP across 200 mixed-larval field samples. Collectively, this research establishes the groundwork for integrating next generation sequencing into resistance monitoring frameworks, contributing to broader efforts to safeguard LEV efficacy in Australian sheep flocks.

2. Materials and Methods

2.1 Parasitic larval samples

Aliquots of third stage mixed larvae (L3s) were provided by Elizabeth Macarthur Agricultural Institute (EMAI; NSW Department of Primary Industries, Menangle, NSW, Australia.) L3s were cultured from ovine faeces submitted to EMAI for routine gastrointestinal nematode diagnostic testing from 2022 to 2024 across properties in NSW. For each larval sample, the postcode of origin, EMAI identification code, host species and larval collection date was recorded. All samples were stored at 4 °C until required for further analysis.

2.2 DNA preparation

All parasitic material was pelleted by centrifugation at 2000 rpm for 2 minutes, and approximately 20 µL of the pellet was extracted for DNA isolation: equating to ~2000 larvae. DNA was extracted from the pellets using the Monarch Genomic DNA Purification kit (New England Biolabs, Australia), following the manufacturer's instructions for tissue lysis. Blank extraction controls (ddH_2O) were included in each batch to detect potential contamination during the DNA extraction process. Resultant DNA isolates were eluted into 100 µL of elution buffer (10 mM Tris-HCl, pH 9.0; 0.1 mM EDTA) and stored at -20 °C until further use. Internal Transcribed Spacer 2 (ITS2) nemabiome metabarcoding was performed to determine the species composition of mixed-larval samples. Samples which acquired less than 1000 ITS2 total reads were discarded from further analysis, with samples that had over 10% DNA for both *Tri. colubriformis* and *Tel. circumcincta*, selected for PCR assays.

2.3 Real-Time PCR assays

Species-specific SYBR chemistry real-time PCRs were conducted to confirm the presence of *Tri. colubriformis* and *Tel. circumcincta* in each mixed-larval sample. Reactions were prepared using a Myra robotic liquid handling system (Bio Molecular Systems, Australia) at a final volume of 20 µL, containing SensiFAST™ SYBR® No-ROX mix (Meridian Bioscience, Australia), 2 µL of template DNA (1:1000 dilution), 400 nM each of forward and reverse primers, and nuclease-free water added to volume. Primer sequences utilised were: *Tri. colubriformis* (forward primer S1188, reverse primer S1191) and *Tel. circumcincta* (forward primer S1183, reverse primer S1184).

PCR cycling was performed on Bio-Rad CFX96 real-time detection system (Bio-Rad, Australia), with an initial denaturation at 95 °C for 3 minutes, followed by 28-32 amplification cycles of 95 °C for 5 seconds, 60 °C for 15 seconds and 72°C for 15 seconds. A melt-curve analysis was performed at the end of each run to confirm specificity. No-template controls (NTCs) were included in each assay to verify that amplification was specific to the target sequence and that no contamination occurred within the reaction setup or PCR reagents. 200 samples producing adequate amplification (Relative Fluorescence Units above 2000) in both *Tri. colubriformis* and *Tel. circumcincta* primer sets were selected to send for Illumina sequencing.

2.4 Illumina next generation sequencing

PCR products for *Tri. colubriformis* and *Tel. circumcincta* from each sample were pooled at a 1:1 ratio and submitted for paired-end sequencing at the Ramaciotti Centre for Genomics at the University of New South Wales. Sequencing libraries were prepared according to standard Illumina protocols, and raw sequencing reads were provided as FASTQ files.

2.5 Bioinformatic analysis

FASTQ files were processed in RStudio (R version 2024.12.1+563) using the DADA2 pipeline (https://benjjneb.github.io/dada2/tutorial.html). Forward and reverse primer sequences for both *Tri. colubriformis* and *Tel. circumcincta* were imported (Table 1). Quality filtering, primer removal, error estimation, dereplication, and chimera removal were performed to infer amplicon sequence variants (ASVs). Additional cleaning steps were applied by transforming raw read counts to relative abundances and removing low-abundance ASVs (<0.5%) to reduce potential sequencing noise (Francis et al., 2024). Separate ASV tables were generated for *Tri. colubriformis* and *Tel. circumcincta* and imported into CLC Genomics Workbench (Version 25.0.1).

Table 1. Primer sequences imported into RStudio.

Primer ID	Sequence
LEV *Tri. colubriformis* forward primer	GGTAAYTGTMGYACAKCCAGCAG
LEV *Tri. colubriformis* reverse primer	GCGGATGGKGAGCCTCAYATCAC
LEV *Tel. circumcincta* forward primer	GGYAACTGCCGYRCATSTAACAG
LEV *Tel. circumcincta* reverse primer	GCTGATGGTGAACCGCATATCAC

ASVs were aligned against NCBI reference genomes for *Tri. colubriformis* TcoSO (HQ215518) and *Tel. circumcincta* TciSO (HQ215517), with a minimum threshold of 97% greatest identity for species assignment for *Tel. circumcincta* (Mejías-Alpízar et al., 2024). ASVs below this threshold were discarded from further analysis. Due to there being multiple species within the *Trichostrongylus* genus which infect sheep such as *Tri. vitrinus*, *Tri. axei* and *Tri. rugatus*, and there being only a reference genome available for *Tri. colubriformis*, there is inherent uncertainty in sequence assignment within that genus. Consequently, a lower identity threshold of 90% was applied for *Tri. colubriformis* ASVs. Species identity was further confirmed through plotting ASVs against a phylogenetic tree with confirmed *Tri. colubriformis*, *Tel. circumcincta* and *H. contortus* samples to visualise species clustering. An annotated *H. contortus* chromosome 5 genome containing the LEV-resistant S168T SNP was simultaneously imported and utilised as a reference to guide alignment and identify the corresponding codon in *Tri. colubriformis* and *Tel. circumcincta* (Figure 1). Resultant ASVs with confirmed species identity were used to screen the acetylcholine receptor subunit gene (*acr*-8) located on exon-4 to determine the presence of susceptible or resistant alleles at codon 168 (S168T SNP).

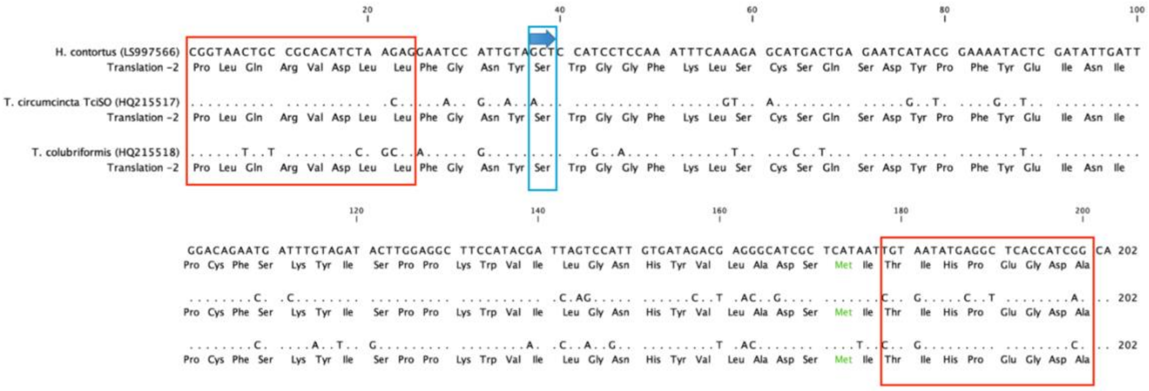


Figure 1: Alignment of the *acr*-8 exon 4 region from *H. contortus* (LS997566), *Tel. circumcincta* (HQ215517) and *Tri. colubriformis* (HQ215518) generated in CLC Workbench. The alignment illustrates conservation across species and the position of the LEV-resistant S168T SNP within the *acr*-8 gene. Primer binding sites are indicated by red boxes, and the blue box marks the codon corresponding to the serine-to-threonine substitution at residue 168.

2.6 Data analysis and quality control

For each sample, the total counts of LEV-resistant and susceptible ASVs were determined in Microsoft Excel, alongside the total number of Illumina sequencing reads. Allele frequencies for the S168T SNP in *Tri. colubriformis* and *Tel. circumcincta* were calculated in Microsoft

Excel by dividing the number of resistant reads by the total reads for each sample. For visualisation of allele frequency distributions, figures were generated in GraphPad Prism and Microsoft Excel.

2.7 Mapping

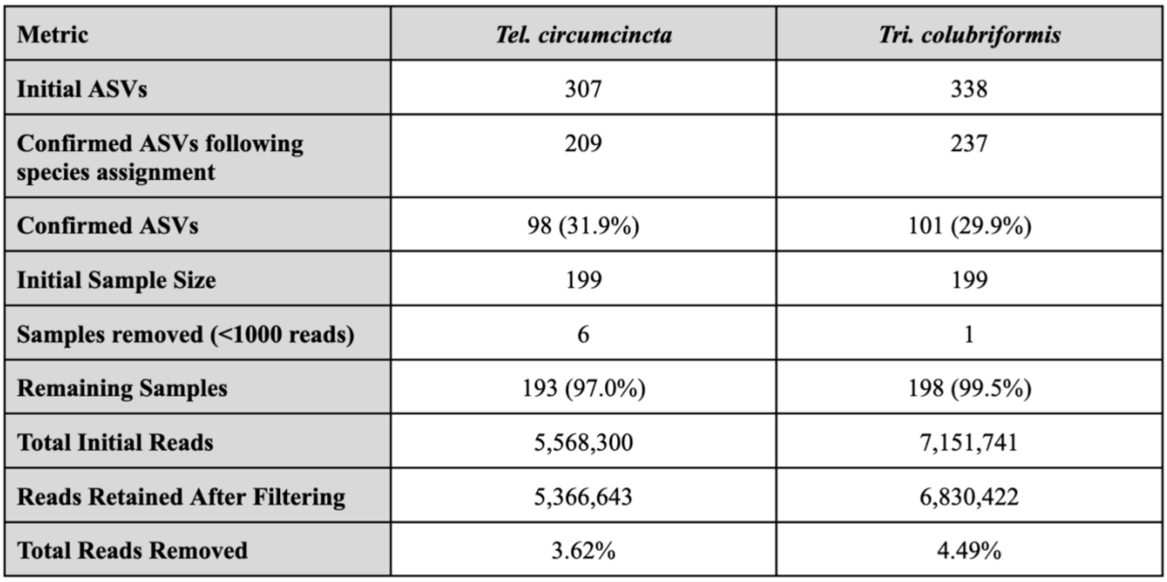
Shapefiles for Australia and Local Land Service (LLS) regions were sourced from the Australian Bureau of Statistics (1270.0.55.001 – Australian Statistical Geography Standard (ASGS): Volume 1 – Main Structure and Greater Capital City Statistical Areas, July 2016, Australian Government). These shapefiles were processed and resistance allele frequency data were integrated using R packages 'sf', 'ggplot2' and 'dplyr' (current versions as of 2025), enabling the spatial visualisation of samples across NSW, showcasing the average, maximum and minimum SNP frequency for each GIN species.

3. Results

3.1 Sequencing and species confirmation of *Tri. colubriformis* and *Tel. circumcincta*

A total of 200 mixed-larval samples underwent ITS-2 metabarcoding to detect and differentiate *Tri. colubriformis* and *Tel. circumcincta*. Across all samples, *Tri. colubriformis* and *Tel. circumcincta* represented an average of 22.93% (S.D. ±0.25) and 24.96% (S.D. ±0.21) of total ITS-2 reads respectively. Quantitative PCR assays employing SYBR-chemistry yielded mean cycle threshold (Ct) values of 27.69 (S.D. ±2.92) for *Tri. colubriformis* primers, and 28.30 (S.D. ±2.12) for *Tel. circumcincta* primers. Initial amplicon sequence variants (ASVs) were rigorously filtered through NCBI BLAST analysis and phylogenetic tree placement to confirm species identity. ASVs failing to meet species-specific sequence identity thresholds, as well as samples with fewer than 1000 reads, were excluded from subsequent analyses to maintain data integrity and analytical reliability. For *Tel. circumcincta*, 307 ASVs were initially detected, of which 98 (31.9%) satisfied the 97% BLAST identity criterion. Six samples (EF0288, EF0369, EF0436, EF0565, EF0685, EF0734) were excluded due to insufficient sequencing depth. Following these quality control measures, 5,366,643 of 5,568,300 initial reads were retained, corresponding to a 3.62% reduction.

Table 2. Summary of data filtering and sequencing read retention for *Tri. colubriformis* and *Tel. circumcincta*.



Metric	*Tel. circumcincta*	*Tri. colubriformis*
Initial ASVs	307	338
Confirmed ASVs following species assignment	209	237
Confirmed ASVs	98 (31.9%)	101 (29.9%)
Initial Sample Size	199	199
Samples removed (<1000 reads)	6	1
Remaining Samples	193 (97.0%)	198 (99.5%)
Total Initial Reads	5,568,300	7,151,741
Reads Retained After Filtering	5,366,643	6,830,422
Total Reads Removed	3.62%	4.49%

For *Tri. colubriformis*, 338 ASVs were initially detected. Given the species' elevated sequence diversity and the availability of only a single reference genome for the entire *Trichostrongylus* genus, a 90% BLAST identity threshold was applied, yielding 101 confirmed ASVs (29.9%). Three ASVs that clustered phylogenetically with *Tel. circumcincta* were excluded to prevent misassignment. One sample (EF0369) was removed for low read count. Post-filtering, 6,830,422 of 7,151,741 reads were retained, representing a 4.49% loss. The combined read-retention and filtering outcomes for both species are summarised in Table 2. Across *Tri. colubriformis*, the average Illumina sequencing depth was 34152.2 (S.D. ±29009.9), whilst for *Tel. circumcincta* it was 26833.2 (S.D. ±25,898.3); reflecting high variability in sequencing depth within species. Phylogenetic reconstruction demonstrated clear species-specific clustering for both taxa (Figure 2), validating the accuracy of ASV assignment and establishing a robust foundation for downstream SNP analyses.

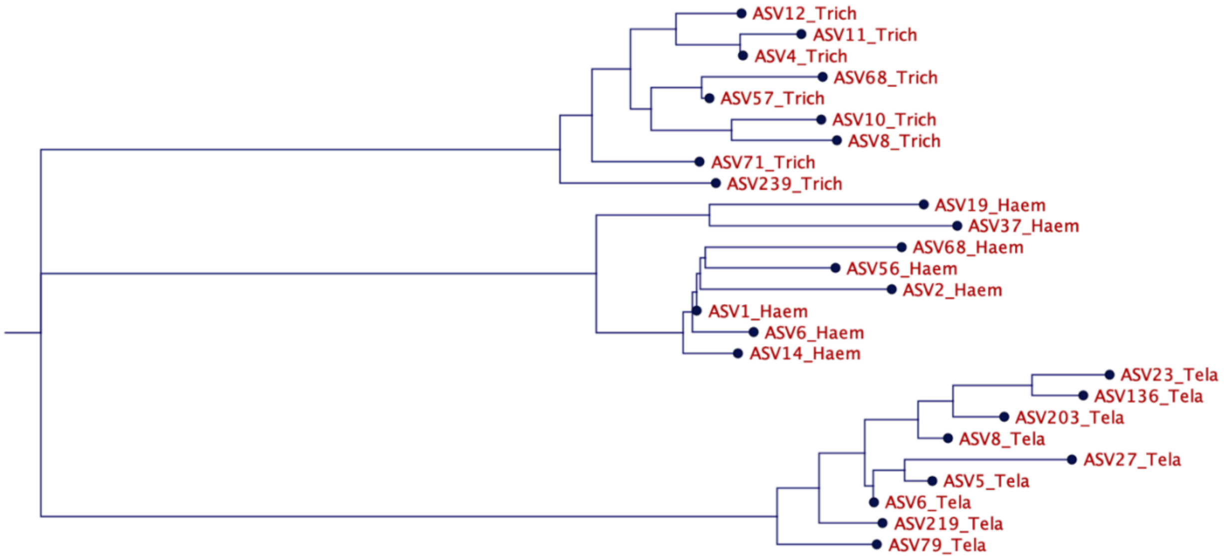


Figure 2: Reference phylogenetic tree of known GIN sequences (*T. circumcincta*, *H. contortus* and *Tri. colubriformis*). Branch lengths indicate genetic distances, and the tree illustrates the distinct clustering of species. This reference framework was used to assign unknown ASVs to their respective species, ensuring accurate species assignment for subsequent S168T SNP analysis.

3.2 Identification of the LEV-resistant S168T SNP

To examine the distribution of S168T SNP frequencies within populations, a cumulative histogram was constructed (Figure 3). In *Tri. colubriformis*, allele frequencies were heavily skewed toward the lower bins (0-20%), indicating that LEV resistance largely persists at low prevalence within most populations. In contrast, *Tel. circumcincta*, exhibited a broader and more uniform distribution across frequency bins, suggesting that resistance-associated alleles are more widely established and entrenched within these GIN populations. While all low-abundance ASVs were removed prior to analysis to minimise sequencing noise, a small subset of samples still exhibited allele frequencies below 5%. These may reflect genuine low-prevalence alleles, although the potential contribution of background noise cannot be entirely excluded.

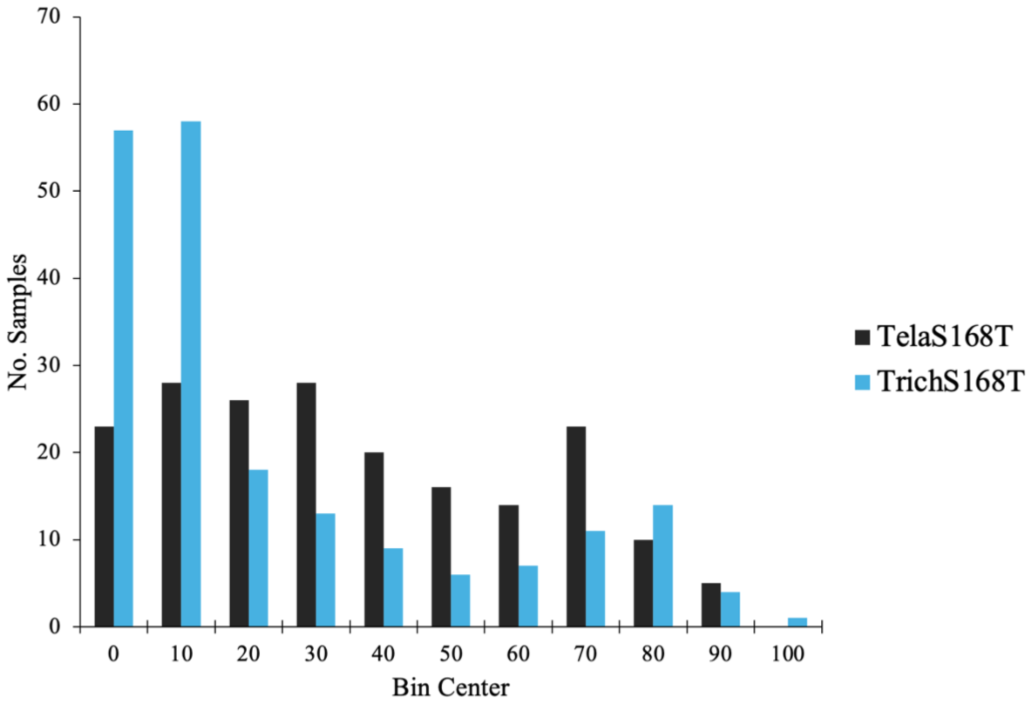
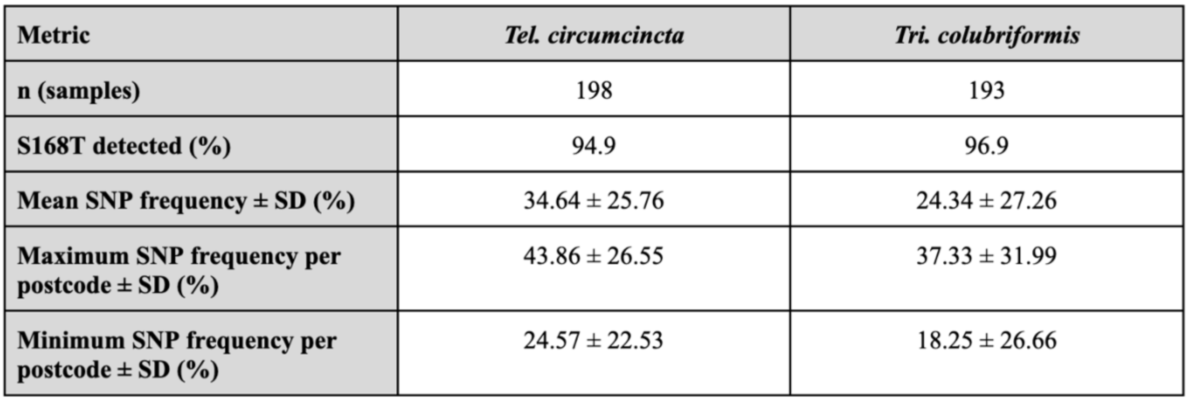


Figure 3: Cumulative histogram of the S168T SNP allele frequencies in *Tel. circumcincta* and *Tri. colubriformis*. Data were binned in 10% intervals, with the y-axis representing the cumulative portion of samples at or below each frequency bin.

Analysis of the S168T SNP revealed species-specific differences in prevalence (Table 3). The S168T SNP was detected in 188/198 (94.9%) *Tel. circumcincta* samples, and 187/193 (96.9%) *Tri. colubriformis* samples. Across all samples which fulfilled the eligibility criteria, the average frequency of the S168T mutation for *Tel. circumcincta* was 34.64% (S.D. ±25.76%), whereas in *Tri. colubriformis*, the mutation was detected an at an average frequency of 24.34%

(S.D. ±27.26%) across all confirmed samples (Figure 4; Figure 5). Frequencies were calculated following quality filtering and confirmation thresholds for each species. For *Tel. circumcincta*, the average maximum SNP frequency per postcode was 42.86% (S.D. ±26.55%), whilst the average minimum SNP frequency was 24.57% (S.D. ±22.53%). Conversely, the average maximum SNP frequency per postcode for *Tri. colubriformis* was 37.33% (S.D. ±31.99%), whereas the average minimum SNP frequency was 18.25% (S.D. ±26.66%).

Table 3. Summary of LEV-resistance associated S168T SNP detection and allele frequencies in *Tel. circumcincta* and *Tri. colubriformis*.



Metric	***Tel. circumcincta***	***Tri. colubriformis***
n (samples)	198	193
S168T detected (%)	94.9	96.9
Mean SNP frequency ± SD (%)	34.64 ± 25.76	24.34 ± 27.26
Maximum SNP frequency per postcode ± SD (%)	43.86 ± 26.55	37.33 ± 31.99
Minimum SNP frequency per postcode ± SD (%)	24.57 ± 22.53	18.25 ± 26.66

Two samples were excluded from New South Wales analysis due to Victorian postcodes. The remaining samples were distributed across New South Wales as follows: Central Tablelands (n = 55), Central West (n = 31), Greater Sydney (n = 1), Hunter (n = 1), Murray (n = 12), North West (n = 5), Riverina (n = 34), South East (n = 54), Western (n = 3) and Australian Capital Territory (n = 3). Geographic visualisation of sample distribution and frequency is shown in Figure 6. The prevalence of the S168T mutation was mapped to these regions (Figure 4; Figure 5), with frequencies displayed for each Local Land Service region (Figure 7).

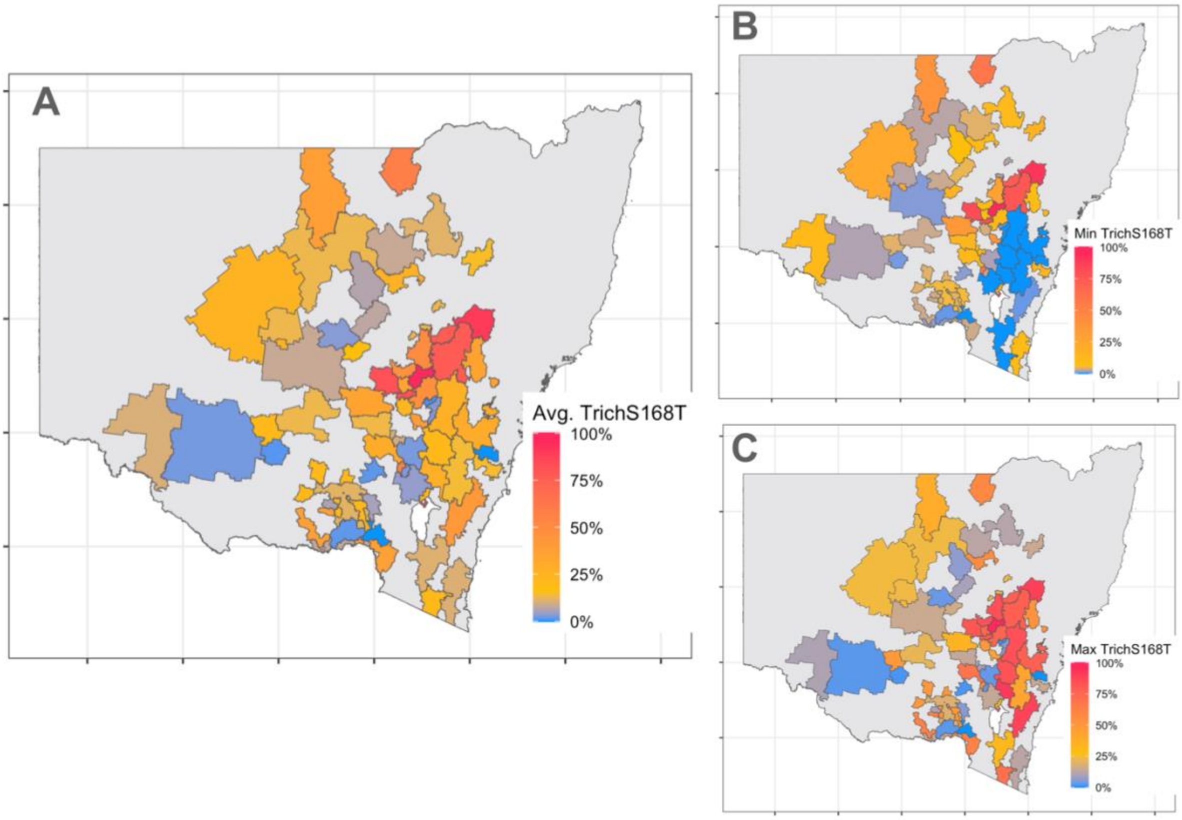


Figure 4: Spatial distribution of the S168T SNP in *Tri. colubriformis* across New South Wales. Panel (A) shows the average frequency of the mutation per postcode, providing an overview of resistance prevalence. Panels (B) and (C) depict the maximum and minimum frequencies observed per postcode, respectively, highlighting the range of variation within each sampled location. Colour intensity corresponds to mutation frequency.

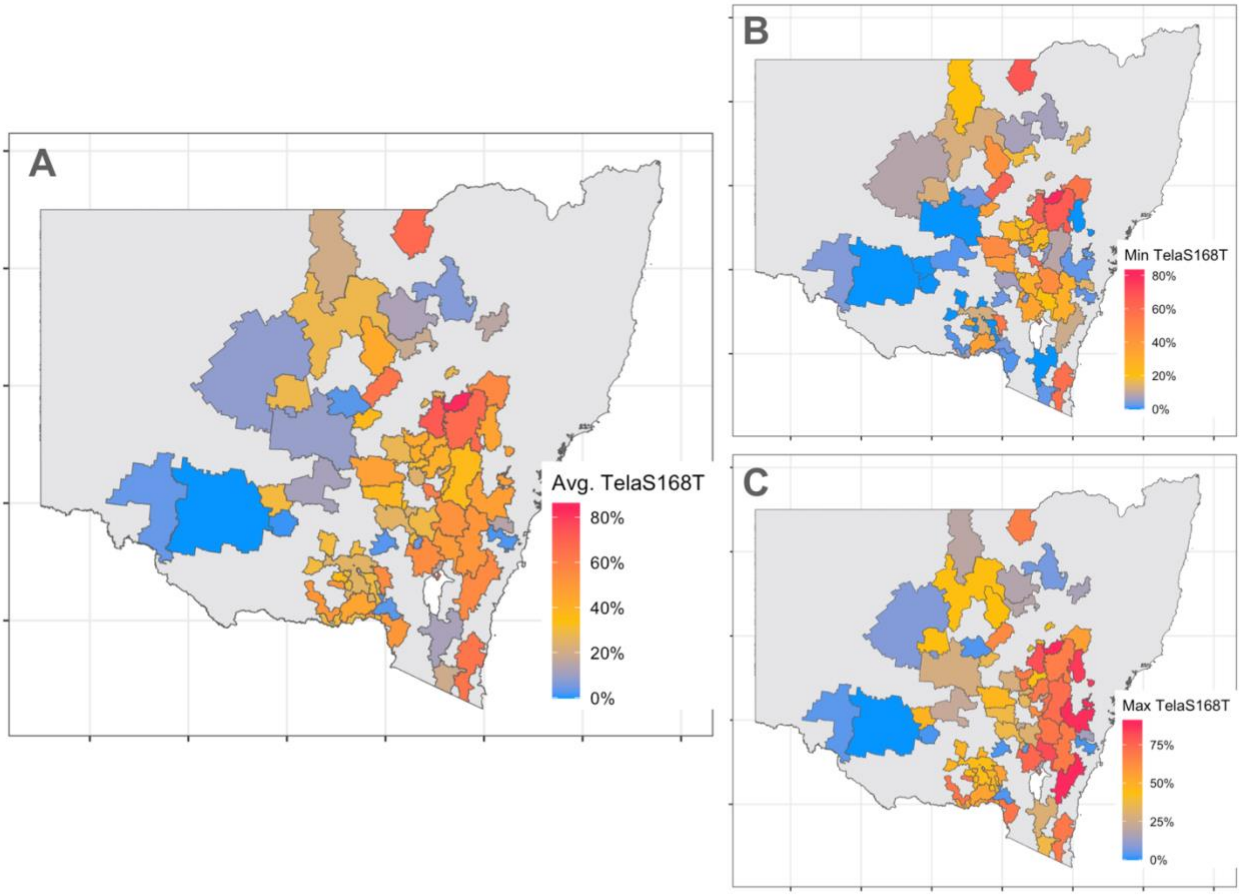


Figure 5: Spatial distribution of the S168T SNP in *Tel. circumcincta* across New South Wales. Panel (A) shows the average frequency of the mutation per postcode, providing an overview of resistance prevalence. Panels (B) and (C) depict the maximum and minimum frequencies observed per postcode, respectively, highlighting the range of variation within each sampled location. Colour intensity corresponds to mutation frequency.

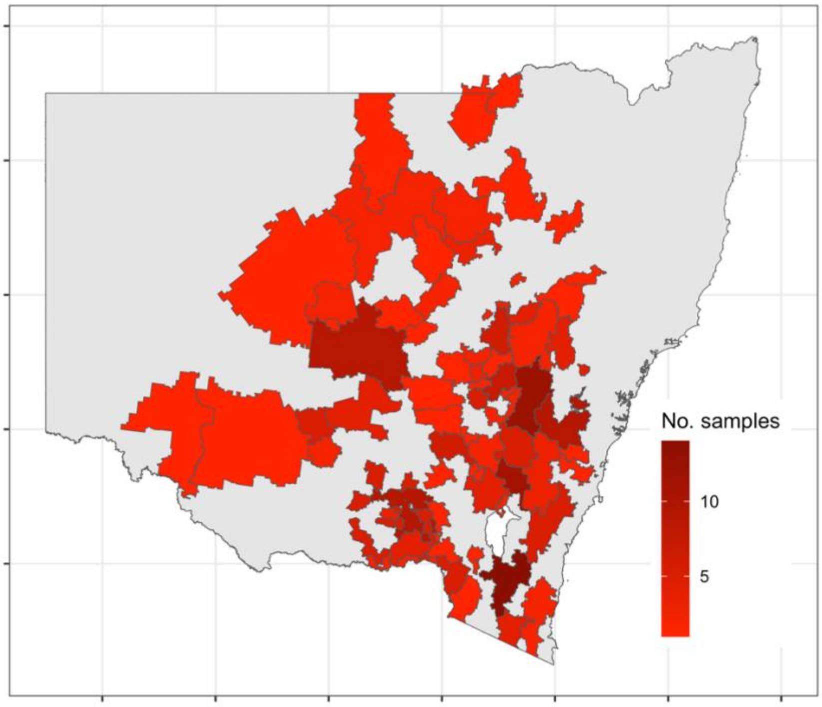


Figure 6: Frequency of collected samples per postcode in New South Wales for *Tri. colubriformis* and *Tel. circumcincta*. Darker red indicates higher sampling density, providing a spatial overview of the dataset used for S168T SNP analyses.

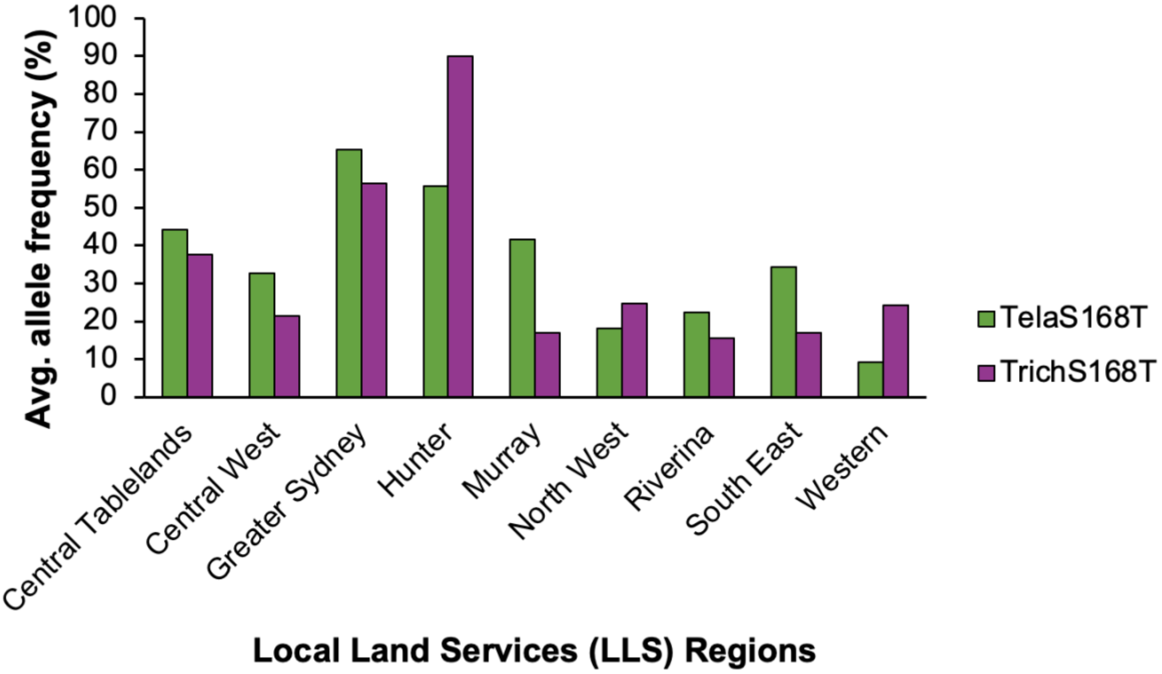


Figure 7: Average frequency of the S168T SNP in *Tri. colubriformis* and *Tel. circumcincta*

across Local Land Service (LLS) Regions of New South Wales. Each bar represents the mean mutation frequency per species in each LLS region, allowing direct comparison of resistance prevalence between the two GIN species.

4. Discussion

This study represents the first molecular screening of the LEV resistance-associated S168T SNP in *Tel. circumcincta* and *Tri. colubriformis* populations across New South Wales, Australia. Using ITS-2 metabarcoding and next-generation sequencing, this proof-of-concept investigation successfully demonstrated the feasibility of identifying species-specific resistance alleles directly from mixed-field larval populations. Across 200 samples, the S168T mutation was detected at an average frequency of 34.64% in *Tel. circumcincta* and 24.34% in *Tri. colubriformis*, providing foundational evidence that the LEV-resistant SNP is not only present but may already be established within NSW nematode populations.

These findings align with the broader trajectory of rising AR in Australian livestock industries (Fissiha & Kinde, 2021; Kaplan, 2004; Playford et al., 2014), highlighting the value of molecular surveillance approaches in complementing phenotypic assays such as FECRTs, allowing detection of resistance at a genetic level before phenotypic failure translates into production losses (Avramenko et al., 2019; Francis & Šlapeta, 2024). While this study was not designed to assess LEV efficacy, the molecular evidence presented through this investigation provides a valuable proxy for emerging resistance. By validating the S168T marker in these two key GIN species, this research contributes a critical step toward integrating SNP-based diagnostics into national resistance monitoring frameworks. These approaches will be instrumental in facilitating earlier detection of resistance alleles, shifting AR management from reactive responses following drug failure toward proactive, evidence-based prevention (Francis et al., 2024; Kaplan, 2004).

4.1 Species-specific variation in S168T prevalence

Sheep in temperate grazing systems are routinely exposed to multiple GIN species, including *Tel. circumcincta*, *Tri. colubriformis* and *H. contortus* (Roeber et al., 2013a). While LEV remains effective against *H. contortus*, its broad and extensive application to control mixed-species nematode burdens may inadvertently impose selection pressure on *Tel. circumcincta* and *Tri. colubriformis*, both of which have exhibited elevated levels of LEV resistance in field studies (Playford et al., 2014). The marked difference in S168T frequency between *Tel. circumcincta* and *Tri. colubriformis* highlights substantial interspecific variation in resistance dynamics. The higher prevalence in *Tel. circumcincta* (34.64%) compared to *Tri. colubriformis* (24.34%) may arise from differences in drug exposure history, genetic diversity and species-specific selection pressures. This disparity likely reflects a combination of biological and pharmacokinetic factors, whereby the abomasal niche of *Tel. circumcincta* may confer greater and more sustained LEV exposure than that experienced by intestinal *Trichostrongylus* spp., compounded by different treatment histories and host immune pressures that collectively favour the persistence of resistant *Tel. circumcincta* genotypes. These findings are consistent with previous reports demonstrating species-specific variation amongst trichostrongylid nematodes in the development and persistence of LEV resistance (Edwards et al., 1986; Playford et al., 2014; Preston et al., 2019).

The detection of S168T in both species further supports the hypothesis of a conserved molecular mechanism underlying LEV resistance across trichostrongylid nematodes. However, as Antonopoulos et al. (2024) argue, resistance in *Tel. circumcincta* and *Tri. colubriformis* is unlikely to be explained by a single genetic determinant. Alternative or additive mutations may influence the acetylcholine receptor subunit targeted by LEV, modifying receptor sensitivity or drug binding affinity (Antonopoulos et al., 2024). Consequently, while the S168T marker provides a valuable diagnostic entry point, it should be viewed within a broader, multifactorial resistance framework that integrates multiple molecular and ecological drivers.

4.2 Comparison with historical phenotypic data

Although phenotypic assays were not performed within this study, comparison with historical FECRT data offer meaningful interpretive context. The last national resistance survey conducted between 2009 and 2012 confirmed phenotypic LEV resistance in 82% of *Teladorsagia* and 89% of *Trichostrongylus* isolates from Australian sheep flocks, with NSW trends mirroring national averages (Playford et al., 2014). The S168T frequencies reported in this study parallel these high resistance rates, suggesting that the molecular signature of LEV resistance has persisted over the past decade.

In *H. contortus*, phenotypic resistance has been associated with a population-level S168T frequency of approximately 16% (Francis et al., 2024). Alternatively, based on previous FECRT data, a study exploring benzimidazole resistance in *H. contortus* applied a 10% threshold utilising SNPs at codons 167, 198 and 200 of the β-tubulin gene (Barrere et al., 2013). While these thresholds cannot be directly extrapolated across drug classes or nematode species, the substantially higher S168T frequencies reported likely exceed any plausible diagnostic threshold for LEV resistance, implying phenotypic resistance is already widespread.

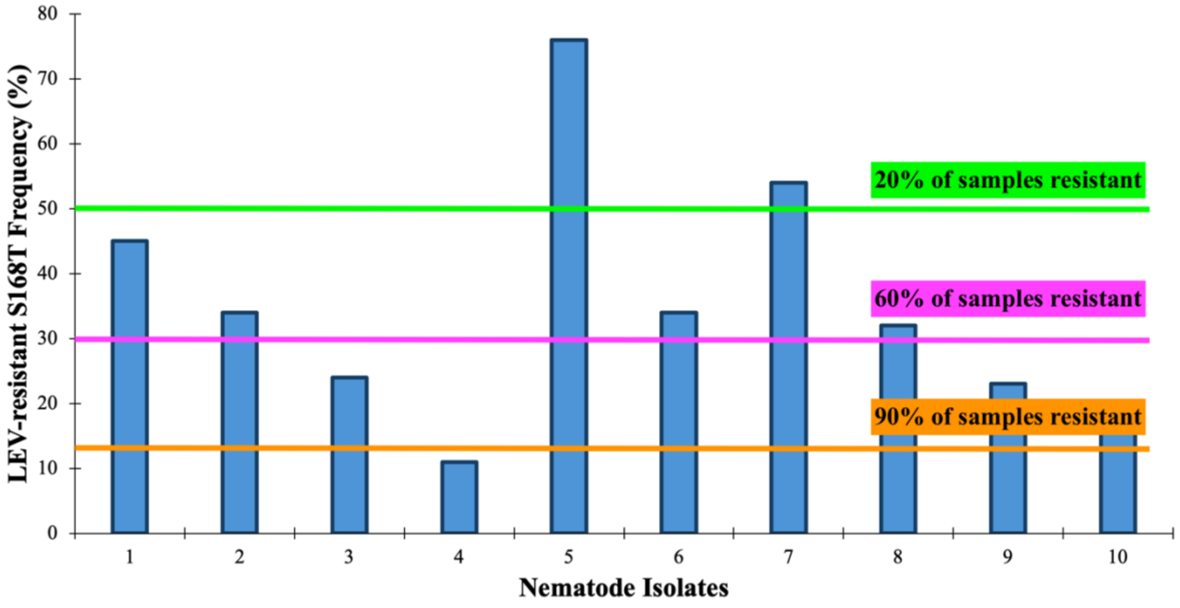


Figure 8: Bar graph showcasing the frequency (%) of the LEV-resistant S168T SNP across 10 hypothetical *Tri. colubriformis* and *Tel. circumcincta* larval isolates, assessed against three theoretical diagnostic thresholds for levamisole resistance. The green threshold indicates the LEV-resistance is conferred when 50% of the isolate has the SNP, pink is indicative of a 30% threshold and orange represents a 15% threshold.

To illustrate this relationship where different thresholds would result in different levels of phenotypic resistance, Figure 8 compares hypothetical S168T allele frequencies against three theoretical diagnostic thresholds. However, cross-species extrapolation must be approached critically. The functional implications of S168T may differ depending on gene expression profiles and receptor subunit composition unique to each species. Without concurrent efficacy testing, it remains uncertain whether similar allele frequencies translate to equivalent clinical resistance. Nonetheless, the alignment between molecular and historical phenotypic data reinforces that LEV resistance in *Tel. circumcincta* and *Tri. colubriformis* is not an emerging trend but an entrenched phenomenon. The persistence of this signal suggests sustained selection pressure, likely driven by intensive drench use and the continued inclusion of LEV within combination drenching products (Bartram et al., 2012; Fissiha & Kinde, 2021; Gilleard et al., 2021). The absence of coordinated molecular surveillance since the last national survey conducted over a decade ago has likely obscured the true progression of resistance evolution, underscoring the urgency of adopting genomic monitoring tools capable of detecting early shifts in allele frequency before treatment failure becomes irreversible.

4.3 Methodological considerations

While this study successfully establishes a proof of concept for SNP-based detection of LEV resistance, several methodological factors warrant rigorous consideration. The modest sample size (n = 200) and uneven distribution across NSW Local Land Service regions restrict generalisation as some regions contributed only a single sample, precluding robust spatial comparisons. The absence of information on drenching practices across sampled flocks limits interpretation, as potential associations between treatment history and the uneven geographic distribution of the S168T SNP could not be assessed. Moreover, larval samples were pre-selected for ITS-2 positivity for *Tri. colubriformis* and *Tel. circumcincta* to ensure dual-species representation and mitigate confounding from *H. contortus* dominance, although this approach may have favoured populations with higher infection intensities of the target species. Consequently, the reported allele frequencies should be interpreted as indicative rather than representative of statewide prevalence.

Furthermore, unlike *Tel. circumcincta* which is the only *Teladorsagia* species within the genus to infect sheep (Cortés et al., 2020), the *Trichostrongylus* genus encompasses multiple species of importance for ovine species; inclusive of *T. vitrinus*, *T. rugatus* and *T. axei* (Craig, 2009). As the only complete genome currently available within this genus is that of *Tri. colubriformis*, this taxonomic resolution gap introduces additional uncertainty in ASV assignment. Although the conservative 90% identity threshold minimised false positives, it concurrently may have inadvertently excluded true ASVs or misclassified others. The implementation of phylogenetic placement assisted in the mitigation of these risks, by serving as a tool to individually visualise where ASVs were clustering in accordance with confirmed *Tel. circumcincta, Tri. colubriformis* and *H. contortus* ASVs. Nonetheless, this limitation underscores the urgent need for high-quality reference sequences across non-*Haemonchus* nematodes to enhance assignment accuracy and reliability for future molecular surveillance of drug resistance.

Additionally, the study's molecular-only design, while advantageous for throughput and sensitivity, precludes phenotypic validation. Without paired drench efficacy data, the results of this experiment cannot define the precise frequency at which the S168T mutation confers measurable drug inefficacy. This knowledge gap reflects a broader challenge in resistance genetics as linking genotype to phenotype requires integrated datasets that capture both molecular and field efficacy measures. Without such integration, genotypic markers remain predictive rather than confirmatory. Despite these constraints, the methodological framework employed here exemplifies a scalable, cost-efficient approach for routine resistance surveillance. The ability to derive molecular resistance data from existing diagnostic samples demonstrates strong potential for integration into ongoing parasite monitoring programs, enhancing the efficiency and reach of surveillance efforts without imposing additional sampling demands.

4.4 Implications for LEV-resistance surveillance and anthelmintic stewardship

The broader significance of these findings lies in their implications for sustainable parasite control. Traditional resistance detection in Australia relies heavily on FECRT, which although informative, is time-consuming, costly and often insensitive to low-frequency resistance alleles with 25% of a population needing to be resistant for detection (Martin et al., 1989). Molecular diagnostics, particularly SNP-based screening, offer a transformative alternative by enabling early detection of resistance before phenotypic failure occurs. When combined with nemabiome metabarcoding, such tools allow simultaneous identification of parasite species and resistance status from mixed infections, representing a critical advancement for realistic field surveillance. However, no coordinated resistance monitoring has occurred since 2012 (Playford et al., 2014), underscoring the need for molecular-screening integration into national diagnostic frameworks.

The identification of substantial S168T frequencies in both *Tel. circumcincta* and *Tri. colubriformis* suggests that LEV resistance has become entrenched within NSW nematode populations; catalysing significant implications for anthelmintic stewardship. Continued use of LEV in combination drenches may temporarily mask treatment failure but risks accelerating selection pressure across co-infecting species. Given the co-occurrence of *Tel. circumcincta* and *Tri. colubriformis* in Australian sheep, differential selection could drive asynchronous resistance trajectories (Evans et al., 2023), complicating control outcomes and hastening multi-drug resistance.

These findings support the urgent implementation of proactive management strategies that incorporate drench preservation, rotational drenching, and evidence-based decision-making. Integrating molecular surveillance into these frameworks would enable producers to align drench decisions with the resistance profiles of their flocks, representing an essential step toward implementing targeted selective treatment that maximises efficacy while minimising selection pressure (Busin et al., 2014; Charlier et al., 2014; Laurenson et al., 2013). By detecting resistance alleles at sub-clinical levels, producers and veterinarians can adjust treatment regimes before efficacy declines, reducing unnecessary drenching and preserving the longevity of remaining effective compounds (Francis & Šlapeta, 2024; Kotze et al., 2020; Roeber et al., 2013b).

4.5 Further directions

This study highlights both the potential and contemporary limitations of molecular resistance surveillance in Australian parasitic GIN populations. Future research should fundamentally address the following three key areas. (1) The development of comprehensive reference genomes for *Trichostrongylus* and other non-*Haemonchus* species to improve ASV assignment and potentially discover additional resistance SNPs. (2) The integration of genotypic and phenotypic data to define allele frequency thresholds predictive of functional resistance, allowing molecular markers for *Tel. circumcincta* and *Tri. colubriformis* to transition from exploratory to robust diagnostic tools. (3) The implementation of longitudinal surveillance across diverse geographic regions to track LEV-resistant allele dynamics and resistance evolution over time, supporting predictive modelling and evidence-based drench strategies. Beyond technical advancements, these findings harbour broader implications for national parasite control policy, as the persistently high S168T frequencies reported in this investigation indicate that existing anthelmintic stewardship frameworks are insufficiently adaptive. Alternatively, coordinated, data-driven approaches incorporating molecular screening are indispensable to maintain the efficacy of critical anthelmintics such as LEV and support sustainable livestock production (Francis & Šlapeta, 2024).

5. Conclusion

This study establishes a proof of concept for the molecular detection of the LEV-resistant S168T SNP in *Tel. circumcincta* and *Tri. colubriformis* using next-generation sequencing;

addressing the growing diagnostic bottleneck for these species relative to *H. contortus*. The high frequencies of this mutation across NSW field populations indicate that LEV resistance is likely entrenched within Australian sheep flocks. While limited by sample representation and the absence of phenotypic validation, the results nonetheless emphasise the feasibility and value of SNP-based diagnostics for large-scale resistance surveillance. By bridging molecular insight with practical management, this research contributes to a growing body of evidence supporting integrated frameworks for anthelmintic stewardship in Australian livestock industries, promoting the transition from reactive drenching to preventative strategies that preserve drug efficacy and sustain long-term parasite control. Overall, these findings underscore the need for national adoption of genomic-driven surveillance to preserve LEV efficacy and sustain long-term parasite control.

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