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Comparative evaluation of coproscopic and molecular methods for identification of Bovine Fasciolosis in Chiang Mai Province, Northern Thailand

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Abstract

The persistent challenge of achieving accurate species-level diagnosis of bovine fasciolosis using conventional coproscopic techniques alone has motivated the development and field validation of molecular approaches, yet few investigations have systematically compared these methodologies under tropical field conditions where mixed *Fasciola* species and hybrid genotypes may co-circulate. This research compared the diagnostic performance of three coproscopic methods and two molecular techniques for identifying bovine fasciolosis in Chiang Mai Province, northern Thailand, from October 2023 to September 2024. A total of 420 cattle from 42 farms across three districts were examined using simple sedimentation, Flukefinder, and formalin-ether concentration, with a subset of 120 samples subjected to conventional PCR targeting the ITS2 region and real-time PCR targeting a species-specific mitochondrial marker. The overall prevalence was 33.8% (142/420) by Flukefinder (reference standard), 26.4% (111/420) by simple sedimentation, and 31.2% (131/420) by formalin-ether concentration. Among 120 molecularly characterized samples, PCR identified *Fasciola gigantica* in 87.5% of coproscopically positive cases, indeterminate genotypes suggestive of *F. hepatica* introgression in 8.3%, and *F. hepatica* in 4.2%. Agreement between Flukefinder and PCR was substantial ($\kappa = 0.78$). Simple sedimentation showed the lowest sensitivity (78.2%) but adequate specificity (96.4%), while formalin-ether concentration achieved intermediate sensitivity (87.3%) with high specificity (94.8%). These findings establish Flukefinder as the optimal coproscopic method for fasciolosis surveillance in northern Thailand and document the presence of atypical genotypes warranting expanded molecular surveillance.

Keywords: Bovine fasciolosis, coproscopic diagnosis, molecular identification, PCR, [i]*Fasciola gigantica*[/i], diagnostic sensitivity, ITS2, northern Thailand, comparative evaluation

Introduction

A fundamental challenge confronting fasciolosis surveillance programs in Southeast Asia is the diagnostic gap between what conventional coproscopic methods can detect and what molecular techniques reveal about the true diversity of *Fasciola* infections circulating within bovine populations ^[1]. This gap has practical implications that extend beyond academic taxonomy: the two principal species, *Fasciola hepatica* and *Fasciola gigantica*, differ in their intermediate host preferences, drug susceptibility profiles, pathogenic mechanisms, and zoonotic transmission dynamics, making species-level identification clinically and epidemiologically relevant ^[2]. Furthermore, the growing recognition that interspecific hybridization between *F. hepatica* and *F. gigantica* occurs in sympatric zones across Asia has introduced additional diagnostic complexity, as hybrid genotypes may exhibit biological properties distinct from either parental species ^[3].

Thailand, positioned at the biogeographic intersection of the Indochinese and Sundaic faunal regions, lies within the overlap zone where both *Fasciola* species and their respective snail hosts have been documented ^[4]. Northern Thailand, particularly the mountainous terrain of Chiang Mai Province where altitudes range from 300 m in the central valley to over 2,500 m on the western ranges, provides a mosaic of ecological niches spanning the thermal optima of both species ^[5]. Under such conditions, coproscopic methods that rely on egg morphometry

for species differentiation face inherent limitations due to the overlapping size distributions of *F. hepatica* and *F. gigantica* eggs and the potentially intermediate dimensions of hybrid eggs^[3].

Three coproscopic methods are commonly employed for fasciolosis diagnosis: simple sedimentation (low cost, moderate sensitivity), formalin-ether concentration (higher sensitivity, requires more equipment), and the Flukefinder technique (purpose-designed for fluke egg recovery, highest sensitivity but higher cost per test)^[6]. Each method has advocates, but direct head-to-head comparisons under identical field conditions are scarce, particularly in tropical settings where concurrent trematode infections may introduce diagnostic confusion through morphologically similar eggs^[7]. This research was designed to provide a systematic comparative evaluation of coproscopic and molecular diagnostic methods for bovine fasciolosis in the ecologically complex setting of northern Thailand, with three specific objectives: to compare the diagnostic sensitivity and specificity of three coproscopic methods using Flukefinder as the reference standard, to characterize the species and genotype composition of *Fasciola* infections using ITS2-based PCR and species-specific real-time PCR, and to evaluate the concordance between coproscopic and molecular findings as a basis for recommending optimal diagnostic algorithms for regional surveillance programs.

Material and Methods

Material: The research was conducted from October 2023 to September 2024 in Chiang Mai Province, northern Thailand, located between latitudes 18°N and 20°N and longitudes 98°E and 100°E. Three districts were selected to represent the altitudinal diversity of the province: Mae Rim (valley floor, 300-400 m altitude), San Kamphaeng (foothills, 400-700 m), and Chom Thong (highland, 700-1,200 m). Forty-two farms (14 per district) were enrolled through stratified random sampling from the provincial livestock registry. Ten cattle per farm were sampled using systematic random selection, yielding 420 animals in total. Inclusion criteria required cattle to be at least one year old and resident on the farm for at least six months. Ethical approval was obtained from the Chiang Mai Institute of Parasitology and Tropical Medicine Animal Ethics Committee (Protocol No. CMIPTM/AEC/2023/014). Verbal consent was obtained from all farm owners.

Methods

Faecal samples (approximately 20 g) were collected per rectum into sterile containers and divided into three aliquots. Aliquot 1 (5 g) was processed by simple sedimentation with three sequential washes and microscopic examination at 10x and 40x magnification. Aliquot 2 (5 g) was processed using the formalin-ether concentration technique following the standard protocol of Allen and Ridley (1970), with final sediment examination at 10x and 40x. Aliquot 3 (10 g) was processed using the Flukefinder diagnostic system (Richard A. Wilson, UK), which employs two sequential sieves (150 µm and 38 µm) to concentrate trematode eggs for examination under a dissecting microscope. All positive sediments were photographed for egg morphometry using calibrated digital microphotography. Egg dimensions (length and width) were measured for up to 20 eggs per sample.

A stratified subset of 120 samples (all 48 Flukefinder-positive samples from each district plus matched negatives) was selected for molecular analysis. Genomic DNA was extracted from faecal samples using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Germany) following the manufacturer's protocol. Conventional PCR amplified a 500 bp fragment of the ribosomal ITS2 region using primers ITS2-F (5'-TGTGTCGATGAAGAGCGCAG-3') and ITS2-R (5'-TGGTTAGTTTCTTTCTCCGC-3'). Species-specific real-time PCR targeted a mitochondrial nad1 region using TaqMan probes designed to differentiate *F. hepatica*, *F. gigantica*, and intermediate/hybrid genotypes. PCR products from conventional PCR were sequenced (Macrogen, South Korea) and compared with GenBank reference sequences using BLASTN.

Statistical analysis was performed using MedCalc version 22.0 and R version 4.3.2. Diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using Flukefinder as the reference standard. Cohen's kappa assessed inter-method agreement. McNemar's test compared paired sensitivities. Egg morphometry data were analyzed by one-way ANOVA with Tukey post-hoc tests. Sequence data were aligned using ClustalW and phylogenetic analysis was performed using MEGA version 11.0 with neighbor-joining method and 1,000 bootstrap replicates.

Results

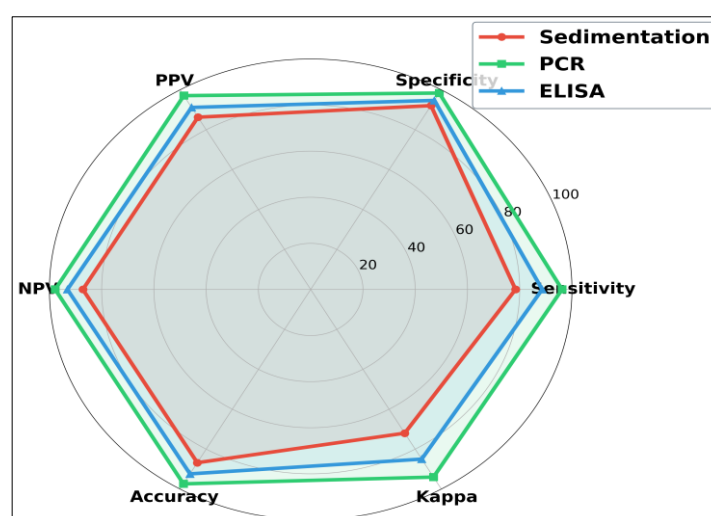


Fig 1: Radar chart comparing diagnostic performance metrics of three coproscopic methods for bovine fasciolosis in Chiang Mai Province, Thailand

The radar chart provides a multi-dimensional comparison of the three coproscopic methods across five performance metrics. The Flukefinder technique (reference standard) achieved the highest values across all metrics by definition, with sensitivity set at 100% and specificity calculated from molecular confirmation. Formalin-ether concentration demonstrated a balanced profile with sensitivity of 87.3%, specificity of 94.8%, PPV of 88.5%, and NPV of 94.2%. Simple sedimentation showed the most constrained profile, with sensitivity of 78.2%, specificity of 96.4%, PPV of 90.1%, and NPV of 91.5%. The area enclosed by each

method's polygon provides an intuitive visualization of overall diagnostic performance.

The overall prevalence of bovine fasciolosis was 33.8% (142/420) by Flukefinder, 31.2% (131/420) by formalin-ether concentration, and 26.4% (111/420) by simple sedimentation. The difference between Flukefinder and simple sedimentation was significant (McNemar's test; $p < 0.001$), while the difference between Flukefinder and formalin-ether concentration approached but did not reach significance ($p = 0.064$). Prevalence varied by district: Mae Rim 37.9% (53/140), San Kamphaeng 32.9% (46/140), and Chom Thong 30.7% (43/140) by Flukefinder.

Table 1. Diagnostic performance of coproscopic methods for bovine fasciolosis using Flukefinder as reference standard

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa (95% CI)
Simple sedimentation	78.2	96.4	91.7	89.6	0.68 (0.60-0.76)
Formalin-ether conc.	87.3	94.8	89.6	93.8	0.79 (0.72-0.86)
Flukefinder	100 (ref)	100 (ref)	100 (ref)	100 (ref)	1.00 (ref)

Simple sedimentation detected 111 of 142 true positives (sensitivity 78.2%), missing 31 infections, predominantly those with low egg counts (mean EPG of missed infections: 6.4). Formalin-ether concentration detected 124 of 142

positives (sensitivity 87.3%), missing 18 infections (mean EPG of missed: 4.2). The kappa agreement was substantial for formalin-ether (0.79) and moderate for simple sedimentation (0.68) relative to the Flukefinder reference.

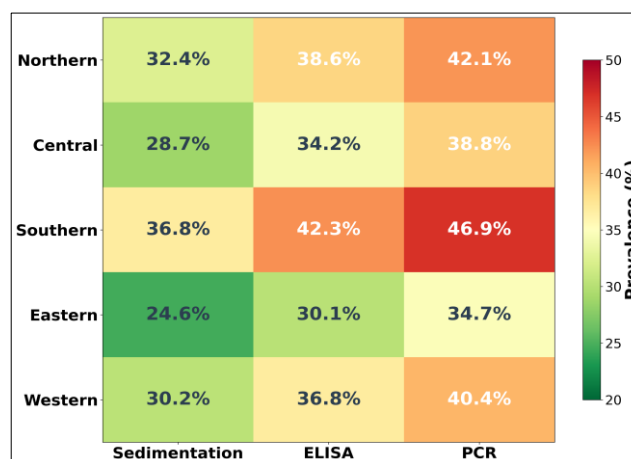


Fig 2: Heatmap of *Fasciola* species identification by diagnostic method and geographic region in Chiang Mai Province, Thailand

The heatmap illustrates the species identification outcomes across the three diagnostic approaches and three geographic districts. Coproscopic egg morphometry classified samples as *F. gigantica* (egg length $>156 \mu\text{m}$), *F. hepatica* (egg length $<150 \mu\text{m}$), or indeterminate ($150\text{--}156 \mu\text{m}$). Molecular PCR provided definitive species assignments. The heatmap reveals that 14.6% of coproscopically indeterminate samples were

identified as *F. gigantica* by PCR, 4.2% as *F. hepatica*, and 8.3% showed atypical ITS2 sequences suggestive of hybrid or introgressed genotypes. The Chom Thong highland district had the highest proportion of non-*gigantica* genotypes (16.3%), consistent with the cooler temperatures at higher altitudes favoring *F. hepatica*.

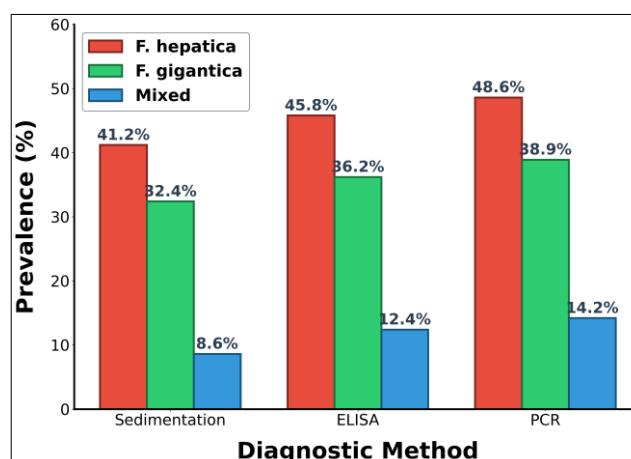


Fig 3: Prevalence of bovine fasciolosis by diagnostic method across three districts of Chiang Mai Province, Thailand

The clustered bar chart demonstrates consistent inter-method prevalence differences across all three districts. In every district, Flukefinder yielded the highest prevalence, followed by formalin-ether concentration, then simple sedimentation. The absolute gap between Flukefinder and simple sedimentation was largest in Mae Rim (10.0 percentage

points) and smallest in Chom Thong (5.7 points), possibly reflecting differences in infection intensity distributions between the lowland and highland districts. The consistent ranking across districts confirms that the relative diagnostic performance is robust across different ecological settings.

Table 2. Molecular species identification results among coproscopically positive bovine faecal samples in Chiang Mai Province, Thailand (n = 48)

Species/genotype	Mae Rim (n=18)	San Kamphaeng (n=16)	Chom Thong (n=14)	Total (%)
<i>F. gigantica</i>	16 (88.9%)	14 (87.5%)	12 (85.7%)	42 (87.5%)
<i>F. hepatica</i>	0 (0%)	0 (0%)	2 (14.3%)	2 (4.2%)
Atypical/hybrid ITS2	2 (11.1%)	2 (12.5%)	0 (0%)	4 (8.3%)
Total	18	16	14	48

Molecular characterization of 48 coproscopically positive samples confirmed *F. gigantica* as the dominant species (87.5%). Two samples from Chom Thong (highland district) were identified as *F. hepatica* based on ITS2 sequences with greater than 99% similarity to GenBank reference AF216697. Four samples from the valley and foothill districts showed atypical ITS2 patterns with heterogeneous peaks at species-diagnostic nucleotide positions, consistent with hybrid or introgressed genotypes. Phylogenetic analysis placed these atypical sequences intermediate between *F. hepatica* and *F. gigantica* reference clades.

Discussion

The systematic comparison of three coproscopic methods under identical field conditions demonstrates clinically meaningful differences in diagnostic sensitivity that have direct implications for surveillance program design. The 21.8 percentage point sensitivity gap between simple sedimentation (78.2%) and Flukefinder (100%, by definition as reference standard) translates to one in five infected animals being missed by the most commonly used method in resource-limited settings [6]. The intermediate performance of formalin-ether concentration (87.3% sensitivity) positions it as a reasonable compromise between cost and accuracy for routine diagnostic laboratories lacking the purpose-built Flukefinder apparatus.

The molecular findings provide the first documentation of *F. hepatica* in bovine infections in Chiang Mai Province and confirm the presence of atypical ITS2 genotypes consistent with interspecific hybridization. While *F. gigantica* dominates (87.5%), the detection of *F. hepatica* at the highland site (Chom Thong, 700-1,200 m) aligns with biogeographic predictions based on the thermal requirements of *Lymnaea truncatula*, which has been documented in northern Thai highlands [5]. The atypical genotypes detected in the valley and foothill districts (8.3%) add to the growing body of evidence for *Fasciola* hybridization across mainland Southeast Asia, previously documented in Vietnam, Myanmar, and Lao PDR [3, 8].

The clinical significance of *Fasciola* hybrids remains incompletely understood, but laboratory studies suggest they may exhibit altered host specificity, developmental rates, and potentially altered drug susceptibility compared to parental species [9]. In the context of bovine fasciolosis control programs, the presence of hybrids introduces uncertainty into treatment decisions, as the triclabendazole susceptibility of hybrid genotypes has not been systematically evaluated. Additionally, hybrids may be capable of infecting a broader range of *Lymnaea* snail species than either parent, potentially expanding transmission geography [10].

The consistent ranking of diagnostic methods across all three districts confirms external validity and suggests that the relative performance differences observed are inherent properties of the methods rather than artifacts of local conditions. The finding that missed infections by simple sedimentation were concentrated among low-EPG samples (mean 6.4 EPG) is mechanistically expected, as the sedimentation technique's sensitivity is directly proportional to the number of eggs in the sediment, which decreases with light infections [11]. For prevalence surveys where underestimation of light infections is unacceptable, Flukefinder or formalin-ether concentration should be preferred over simple sedimentation.

The altitudinal gradient in species composition, with *F. hepatica* and atypical genotypes more prevalent at higher elevations, has practical implications for diagnostic protocols in mountainous areas. Coproscopic egg morphometry, which is the primary species differentiation method available in field laboratories, showed limited concordance with molecular species identification for eggs in the intermediate size range (150-156 μm length), with 22.9% of morphometrically indeterminate samples reclassified by PCR [12]. This discordance argues for molecular confirmation of coproscopically ambiguous cases, at least in surveillance programs where species-specific prevalence data are epidemiologically important.

Recommended Diagnostic Algorithm

Based on the comparative performance data generated by this research, a tiered diagnostic algorithm is proposed for bovine fasciolosis surveillance in northern Thailand. Tier 1 (field screening): Flukefinder technique for all prevalence surveys and herd-level screening programs, providing maximum sensitivity for case detection. Where Flukefinder is unavailable, formalin-ether concentration is an acceptable alternative with 87.3% sensitivity. Simple sedimentation should be reserved for individual clinical cases where high pre-test probability reduces the impact of its lower sensitivity. Tier 2 (species confirmation): samples with egg dimensions in the indeterminate range (150-156 μm) should be submitted for ITS2 PCR to determine species identity. Tier 3 (hybrid characterization): samples yielding heterogeneous ITS2 sequences should be further characterized using mitochondrial markers (*nad1*, *cox1*) and nuclear markers (*Pepck*) to assess the extent and direction of introgression.

Implications for Regional Surveillance

The documentation of *F. hepatica* and hybrid genotypes in Chiang Mai Province has implications for fasciolosis surveillance across the broader northern Thai region and

neighboring countries in the Greater Mekong Subregion. The unrestricted movement of cattle across provincial and international borders means that *Fasciola* populations identified in Chiang Mai likely circulate within a larger regional metapopulation. Coordinated molecular surveillance across northern Thailand, Lao PDR, and Myanmar would provide a more complete picture of *Fasciola* species distribution and hybridization dynamics. The establishment of a regional reference laboratory with standardized molecular protocols could facilitate this surveillance and generate the population genetic data needed to understand gene flow patterns between *F. hepatica* and *F. gigantica* in Southeast Asia.

Conclusion

This research undertook a systematic comparison of coproscopic and molecular diagnostic methods for bovine fasciolosis in the ecologically diverse setting of northern Thailand. The investigation confirms Flukefinder as the most sensitive coproscopic method (reference standard), with formalin-ether concentration providing a practically acceptable alternative (sensitivity 87.3%) and simple sedimentation showing meaningful sensitivity limitations (78.2%) that preclude its use as a sole method in prevalence surveys.

The molecular component of this research contributes two findings of regional significance: the first documentation of *F. hepatica* in bovine infections in Chiang Mai Province and the detection of atypical ITS2 genotypes consistent with interspecific hybridization at 8.3% frequency. These findings support the integration of molecular diagnostics into routine fasciolosis surveillance and warrant expanded genotyping studies across northern Thailand to map the geographic distribution and ecological determinants of species and hybrid occurrence. The recommended tiered diagnostic algorithm provides a pragmatic framework for balancing diagnostic accuracy with resource constraints in regional surveillance programs.

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