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(Original Research)

Phenotypic Characterization of Philippine Native Pigs (*Sus scrofa domesticus* L.) in Selected Municipalities of Isabela Province

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Abstract

Background: Philippine native pigs (Sus scrofa domesticus L.) are valuable animal genetic resources because of their adaptability to low input farming systems, yet localized phenotypic data in Isabela Province remain limited. **Methods**: A survey of 209 native pigs (boars, sows, and gilts) was carried out in six municipalities in Isabela Province. Pigs were randomly selected from farms. Researchers recorded physical traits like coat color, ear type, and tail type, as well as body measurements such as body length and heart girth, following FAO guidelines. **Results**: Most native pigs showed uniform phenotypic traits, including absence of prominent tusks, straight snout and head profiles, erect forward-facing ears, black coat color, straight hair, smooth skin, and straight backlines. Curly tails were more frequent in males, while females more commonly had straight tails. Morphometric measurements were relatively consistent, with females generally exhibiting larger body dimensions than males. **Conclusion**: The phenotypic characteristics observed are consistent with other Philippine native pig populations and recognized strains reported by DOST-PCAARRD such as ISUbela®, Bohol and Romblon. These findings provide baseline data for conservation, breeding improvement, and sustainable utilization of native pig genetic resources in Isabela Province.

Keywords

Native Philippine native pigs, Phenotype, Morphometry, Province of Isabela

1. Introduction

The Philippine native pig (*Sus scrofa domesticus L.*) is an endemic animal genetic resource commonly grown under the small-hold farming system. The continued existence of native pig populations is anchored on their economic, social, religious, and/or cultural significance in specific segments of the Philippine society. Relative attributes favorable in the keeping of native pigs are commonly based on their adaptation to the local environment (which includes climate, feed resources, disease challenges, and management) that keeps the cost of maintaining them very low [1,2]. Similar observations were also documented in Mexico [3] and in Papua New Guinea [4].

The contributions and roles of the Philippine native pig are immensely valuable under the low-input production system. The international

community, through the Food and Agriculture Organization (FAO) of the United Nations, recognized the utility of the native, indigenous, or endemic animal genetic resources for food and other functions related to agriculture [5]. The Philippine native pig meat is viewed as a healthier alternative to commercial pig meat in the market and as a valuable source of income for producers, especially with the growing demand for organic products today. The decreasing ratio of indigenous breeds to exotic breeds in the country due to commercialization, its anticipated economic worth, and potential have led researchers to improve their production performance and product quality without reducing genetic diversity. Also, the production and marketing components of the local pig business are being improved to ensure its availability in the market. Recent advancements in native pig farming resulted in genetically modified native pigs that have higher levels of morphological homogeneity, larger litter sizes, and faster growth rates [1].

To date, there are currently seven genetic subgroups of native pigs being produced, each of which is called after the province in which it originated, such as Kalinga, Benguet, Isabela, Nueva Vizcaya, the *Bundok* Peninsula of Quezon province, Marinduque, and Eastern Samar. Certain distinctions seen in these native pig groups include the hues and patterns of the coat, the conformation of the body, the size of the adult body, behavior, and genetic distances. Moreover, the research and development efforts made on organized breeding and selection of native pigs have led to increased litter size, sped up growth, and pigs with highly uniform physical traits [6].

The African Swine Fever (ASF) outbreak reported in the Philippines in 2019 caused significant impact in both commercial and native pig populations. Thereby emphasizing the need to document and conserve indigenous pig resources.

Native animals depend on their unique phenotypic and genetic characteristics to adapt to their environment and the conditions of farming. Hence, documentation and establishment of accurate information on indigenous pig genetic resources, particularly on their phenotypic characteristics will help in the improvement, conservation, and sustainable use of the native pig population. In Isabela Province, provincial data on native pig populations remain limited.

This study aimed to characterize the qualitative phenotypic traits of native pigs in selected municipalities of Isabela, determine their quantitative morphometric measurements, and compare these traits with those of other native pig populations in the Philippines.

2. Materials and Methods

2.1 Study Area

The study was conducted from six municipalities from different districts of Isabela Province: Angadanan, Benito Soliven, City of Ilagan, Jones, Roxas, and San Guillermo.

2.2 Sampling Design and Animal Selection

Municipalities from six legislative districts were purposively selected based on the presence of native pig populations. Within each municipality, individual pigs (n=209) were randomly selected from participating farms. This sample size has a 5% margin of error at a 95% confidence level. The native status of pigs was determined based on farmer identification, observable phenotypic traits consistent with Philippine native pigs, and the absence of visible signs of crossbreeding with commercial breeds.

2.3 Data Collection

Qualitative traits such as sex, dominant color (black, dark red, light red, Qualitative traits such as sex, presence or absence of tusk in boars, shape of snout (curve or straight), shaped of the head (concave, convex or straight), ear type (droopy, semi-lop or erect), ear orientation (forward, backward or upward), dominant color (black, dark red, light red, spotted), color pattern (plain, patchy or spotted), hair type (curly or straight), hair distribution (dense or sparse), skin type (smooth or wrinkled), backline conformation (straight or swayed back), tail type (straight or curly), and temperament (docile: no avoidance or aggression), aggressive: charging, biting attempts or afraid: retreat, persistent avoidance) were visually observed and noted.

Quantitative traits were measured in cm using a tape measure following the guidelines of FAO [7] on phenotypic characterization of animal genetic resources including age at characterization. These traits include hair length

(measured by plucking three strands from different areas of the withers and computing the average value), snout length was measured at the level of the base of the medial canthus of the eye to the tip of the nose or rostrum, head length was measured from the base of the ear to the tip of the nose , width of the forehead was measured at the level between the base of the left and right ear, ear length was measured from the base to the tip, body length was measured on the mid lateral side of the animal from the cranial border of the scapula to the caudal tip of the rump, the heart girth was measured by taking the circumference of the thorax at the level immediately caudal to the elbow joint, the midbody girth was measured by taking the circumference of the abdomen at the level of the umbilicus, the rump girth was measured by taking the circumference of the abdomen at the area cranial to the stifle joint, height at wither was measured from the tip of the shoulder to distal end of the hoof, height at rump was measured from the tip of the hip joint to distal end of the hoof, circumference of the hock was measured, the tail length was measured from the base to the tip of the fully stretched tail, and teat number both from left and right was obtained.

2.4 Ethical Clearance

Before the conduct of the study, the data collection protocol was submitted for review to the Institutional Animal Care and Use Committee of Isabela State University for Approval. This was approved through the ISU-IACUC permit with protocol number: 07-2023.

2.5 Data Analysis

Data on the questionnaire were compiled in a spreadsheet program (Microsoft Excel©). Descriptive analysis was conducted for the phenotypic characteristics of native pigs using mean, frequency distributions and percentage. Standard Error of Means (SEM) was used to calculate and analyze the quantitative characteristics of the native pig.

3. Results

3.1 Profile of Native Pigs at Characterization

A total of 209 native pigs were surveyed across six municipalities of Isabela Province. Table 1 presents the general profile of native pigs at characterization in this study. Out of the 209 native pigs surveyed, 79.90% (167/209) were females (gilts and sows), while 20.10% (42/209) were males, including 13.88% intact boars and 6.22% castrated males. The computed boar-to-gilt/sow ratio in the population was 1:5.76.

In terms of age distribution, most of the native pigs surveyed were 6 to 8 months old (42.58%), followed by those aged 1 to 2 years (38.76%), 2.1–

Table 1. Profiles of native pigs of Isabela Province at characterization.

PROFILE AT CHARACTERIZATION	NATIVE PIGS FROM SIX MUNICIPALITIES OF ISABELA (n = 209)	
	f	%
Sex		
Male	**42**	**20.10**
Castrate	13	6.22
Intact	29	13.88
Female	**167**	**79.90**
Sow	85	40.67
Gilt	82	39.23
Age		
6-8 months	89	42.58
9-11 months	13	6.22
1 - 2 years	81	38.76
2.1-3.0 years	17	8.13
3.1 - 5.0 years	9	4.31
Weight		
10-20 kgs	40	19.14
21-30 kgs	43	20.57
31-40 kgs	40	19.14
41-50 kgs	23	11.00
51-60 kgs	63	30.14
Behavior		
Docile	145	69.38
Aggressive	23	11.00
Afraid	41	19.62

3.0 years (8.13%), 9–11 months (6.22%), and 3.1–5.0 years (4.31%) groups.

Regarding live weight, most pigs were within the 51–60 kg range (30.14%), followed by 21–30 kg (20.57%), while 19.14% were recorded in the 10–20 kg and 31–40 kg ranges, and 11.00% within 41–50 kg range.

Behavioral assessment showed that the majority of pigs were docile (69.38%), while 19.62% were classified as afraid and 11.00% as aggressive.

3.2 Qualitative Phenotypic Characteristics

The qualitative traits of native pigs in Isabela Province showed generally uniform characteristics across sex groups as shown in Table 2.

Table 2. Distribution of Isabela native pigs according to their qualitative phenotypic characteristics.

QUALITATIVE TRAITS	NATIVE PIGS FROM SIX MUNICIPALITIES OF ISABELA					
	Male n = 42		Sow n = 85		Gilts n = 82	
	f	%	f	%	f	%
Tusk						
Present	17	40.48	3	3.53	0	0
Absent	25	59.52	82	96.47	82	100.00
Snout						
Curve	7	16.67	20	23.53	6	7.32
Straight	35	83.33	65	76.47	76	92.68
Long and Thin	21	50.00	57	67.06	42	51.22
Short and Cylindrical	21	50.00	28	32.94	40	48.78
Head Profile						
Concave	9	21.43	17	20.00	8	9.76
Convex	1	2.38	1	1.18	2	2.44
Straight	32	76.19	67	78.82	72	87.80
Ear Type						
Droopy	7	16.67	24	28.24	9	10.98
Semi-loop	17	40.48	27	31.76	35	42.68
Erect	18	42.86	34	40.00	38	46.34
Ear Orientation						
Forward	26	61.90	59	69.41	46	56.10
Upward	10	23.81	14	16.47	25	30.49
Backward	6	14.29	12	14.12	11	13.41
Color (Dominant)						
Black	38	90.48	73	85.88	73	89.02
Dark red	0	0	1	1.18	3	3.66
Light red	2	4.76	1	1.18	1	1.22
White	2	4.76	10	11.76	5	6.10
Coat Color Pattern						
Plain	35	83.33	70	82.35	70	85.37
Patchy	4	9.52	11	12.94	8	9.76
Spotted	3	7.14	4	4.71	4	4.88
Hair Type						
Curly	6	14.29	7	8.24	6	7.32
Straight	36	85.71	76	89.41	76	92.68
Hair Distribution						
Dense	21	50.00	39	45.88	35	42.68
Sparse	21	50.00	45	52.94	47	57.32
Skin						
Smooth	39	92.86	78	91.76	82	100.00
Wrinkled	3	7.14	7	8.24		
Backline Conformation						
Straight	28	66.67	58	68.24	56	68.29
Swaybacked	14	33.33	27	31.76	26	31.71
Tail						
Straight	15	35.71	50	58.82	34	41.46
Curly	27	64.29	35	41.18	48	58.54

Tusks were absent in all gilts (100%) and were present only in a small proportion of boars (40.48%) and sows (3.53%), indicating that most pigs lacked tusks.

Snout profile was predominantly straight, observed in 83.33% of males, 76.47% of sows, and 92.68% of gilts, with fewer animals showing curved snouts. Snout shapes varied between long and thin and short and cylindrical, with males equally distributed (50% each), while sows more commonly exhibited long and thin snouts (67.06%).

Head profile was mainly straight across all groups (76.19% in males, 78.82% in sows, and 87.80% in gilts), with concave and convex types occurring less frequently. Snout and head profile is shown in Figure 1.

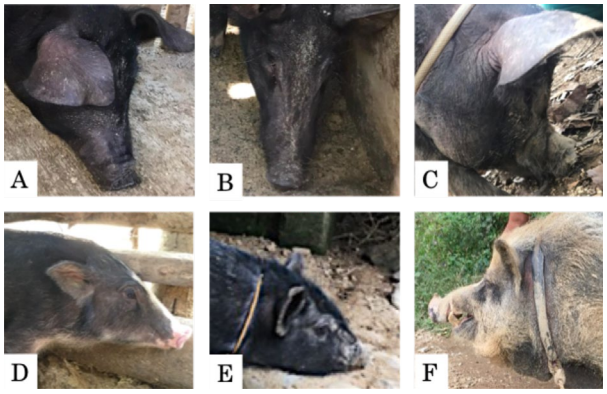

Figure 1. Snout and head profiles of Philippine native pigs from six municipalities of Isabela. A.) Straight, short and cylindrical snout; B.) Straight, long and thin snout; C.) Curve, short and cylindrical snout; D) Straight head profile; E) Concave head profile; and F) Convex head profile with tusks.

Erect ears were the most common ear type in males (42.86%) and gilts (46.34%), while sows showed a more varied distribution of erect (40.00%), semi-loop (31.76%), and droopy ears (28.24%). Semi-loop ears were also common in males (40.48%) and gilts (42.68%).

Ear orientation was primarily forward-facing across all groups (61.90% of males, 69.41% of sows, and 56.10% of gilts). Backward and upward orientations occurred in lower frequencies. Figure 2 shows the different types and orientation of the Isabela native pig ears.

Black was the dominant coat color (90.48% of males, 85.88% of sows, and 89.02% of gilts) with a

Figure 2. Type and orientation of ears of Philippine native pigs from six selected municipalities of Isabela. A) Droopy and forward orienting ears; B) Semi-lop and forward orienting ears, C) Erect with upward orienting ears, and D) Erect and backwards orienting ears.

predominantly plain coat pattern (83.33% of males, 82.35% of sows, and 85.37% of gilts), while other colors and patterns occur less frequently. Different color patterns of native pig in Isabela is shown in Figure 3.

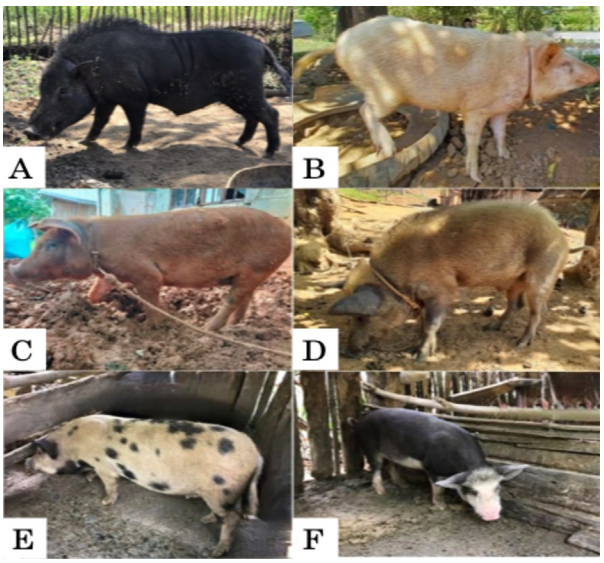

Figure 3. Different color patterns of the Philippine native pigs from different municipalities. A) Plain black color; B) Plain white color; C) Plain dark red; D) Plain light red color; E) Roan color with spots; and F) Black with patches of white in the abdomen, limbs, and face.

Hair type was mostly straight (5.71% in males, 89.41% in sows, and 92.68% in gilts), and hair density was variable, with males evenly distributed and females showing slightly higher proportions of sparse hair (52.94% of sows and 57.32% of gilts).

Skin type was predominantly smooth, observed in 92.86% of males, 91.76% of sows, and 100.00% of gilts with only a few cases of wrinkled skin. Different skin types are shown in Figure 4A,B.

Figure 4. Skin type, backline conformation, hair type, and tail type of Philippine native pigs from six selected municipalities of Isabela. A) Smooth type skin; B) Wrinkled type skin; C) Straight backline; D) Swaybacked; E) Curly, dense hair distribution and straight tail; and F) Straight, sparse hair distribution and curly tail.

Most pigs exhibited a straight backline, observed in 66.67% of males, 68.24% of sows, and 68.29% of gilts. Swayback conformation was present in a smaller proportion across all groups. Backline conformations are presented in Figure 4C,D.

Tail type varied between straight and curly forms. Curly tails were more common in males (64.29%) and gilts (58.54%), while sows showed a higher proportion of straight tails (58.82%). Tail types are presented in Figure 4E,F.

3.3 Quantitative Phenotypic Characteristics

The comparative mean (± SEM) measurements of selected quantitative phenotypic traits of Philippine native pigs in Isabela Province are presented in Table 3.

Male pigs exhibited a mean snout length of 14.53 ± 0.47 cm, which was lower than that of sows (15.48 ± 0.32 cm) but higher than gilts (12.37 ± 0.29 cm). A similar pattern was observed for head length, with males recording 28.29 ± 0.83 cm, sows 30.12 ± 0.51 cm, and gilts 24.83 ± 0.40 cm. Forehead width averaged 12.31 ± 0.29 cm in males, 12.81 ± 0.33 cm in sows, and 11.49 ± 0.32 cm in gilts.

Mean ear length (left) was recorded at 16.52 ± 1.25 cm in males, 17.63 ± 1.58 cm in sows, and 14.46 ± 1.27 cm in gilts. Body length was highest in sows (80.68 ± 2.24 cm), followed by males (73.18 ± 1.65 cm), and lowest in gilts (65.11 ± 2.50 cm).

Heart girth measurements followed the same trend, with sows having the highest mean (94.69 ±

Table 3. Comparative mean (± SEM) measurements of selected quantitative phenotypic traits (cm) in native pigs across male, sow, and gilt groups.

QUANTITATIVE TRAITS (CM)	NATIVE PIGS FROM SIX MUNICIPALITIES OF ISABELA		
	Male (n = 42)	Sow (n = 85)	Gilts (n = 82)
Snout Length	14.53 ±0.47	15.48 ±0.32	12.37 ±0.29
Head Length	28.29 ±0.83	30.12 ±0.51	24.83 ±0.40
Forehead Width	12.31 ±0.29	12.81 ±0.33	11.49 ±0.32
Ear Length (Lt)	16.52 ±1.25	17.63 ±1.58	14.46 ±1.27
Body Length	73.18 ±1.65	80.68 ±2.24	65.11 ±2.50
Heart Girth	87.10 ±2.20	94.69 ±1.49	74.86 ±2.89
Mid-Body Girth	96.45 ±2.41	109.00 ±1.98	87.92 ±2.64
Rump Girth	91.51 ±1.96	104.55 ±2.99	79.67 ±3.20
Pelvic Width	17.50 ±0.53	19.94 ±1.27	15.36 ±0.48
Tail Length	25.05 ±0.89	25.93 ±0.90	21.38 ±1.24
Height at Wither	54.31 ±1.54	56.15 ±1.49	48.12 ±1.98
Height at Rump	60.78 ±1.76	63.89 ±1.54	53.30 ±2.38
Hock Circumference	14.69 ±0.24	14.99 ±0.28	12.99 ±0.46
Hair Length	5.29 ±0.40	6.22 ±0.29	4.30 ±0.30
Teat Pairs	5.65 ±0.13	5.97 ±0.19	5.89 ±0.05

1.49 cm), followed by males (87.10 ± 2.20 cm), and gilts (74.86 ± 2.89 cm). Mid-body girth was also highest in sows (109.00 ± 1.98 cm), compared to males (96.45 ± 2.41 cm) and gilts (87.92 ± 2.64 cm). Similarly, rump girth measurements were greater in sows (104.55 ± 2.99 cm) than in males (91.51 ± 1.96 cm) and gilts (79.67 ± 3.20 cm).

Pelvic width measurements were 17.50 ± 0.53 cm in males, 19.94 ± 1.27 cm in sows, and 15.36 ± 0.48 cm in gilts. Tail length averaged 25.05 ± 0.89 cm in males, 25.93 ± 0.90 cm in sows, and 21.38 ± 1.24 cm in gilts.

Height at withers was recorded at 54.31 ± 1.54 cm in males, 56.15 ± 1.49 cm in sows, and 48.12 ± 1.98 cm in gilts. Height at rump measurements were 60.78 ± 1.76 cm in males, 63.89 ± 1.54 cm in sows, and 53.30 ± 2.38 cm in gilts.

Hock circumference averaged 14.69 ± 0.24 cm in males, 14.99 ± 0.28 cm in sows, and 12.99 ± 0.46 cm in gilts. Hair length was measured at 5.29 ± 0.40 cm in males, 6.22 ± 0.29 cm in sows, and 4.30 ± 0.30 cm in gilts.

The average number of teat pairs was 5.65 ± 0.13 in males, 5.97 ± 0.19 in sows, and 5.89 ± 0.05 in gilts.

4. Discussion

This study provides a phenotypic characterization of native pigs in Isabela Province, showing both uniformity and variation in traits.

The population structure observed was predominantly female, with a boar-to-gilt/sow ratio of 1:5.76, higher than the recommended 1:17 for natural mating. [8] Although this may reduce breeding efficiency, the large number of females indicates a strong breeding base that may support population continuity in the province.

The age distribution of native pigs was primarily within the 6–8 months and 1–2 years age groups, reflecting typical backyard farming management practices. Studies indicates that gilts are bred between 8 and 12 months of age. [9,10] But studies indicate that native pigs reach sexual maturity as early as 4 – 5 months [11] and 7.11 months [12]. These findings suggest that early maturity is characteristic of Philippine native pig populations.

The distribution of live weights recorded were consistent with reports describing smaller body size in native pigs, typically ranging from 40 to 60 kg at maturity or during fattening [11,13]. This indicates that the native pig population in Isabela follows similar growth patterns of Philippine native pigs.

Behavioral observations showed that most pigs in the study were docile, which is advantageous for backyard production systems. Few afraid and aggressive were also observed, likely associated with limited human interaction. Prior studies suggest that regular and positive handling can improve animal behavior [26], highlighting the importance of management practices in influencing temperament.

The observed variation in demographic, weight, and behavioral characteristics across municipalities may reflect differences in management practices and production conditions. It may also indicate the presence of localized subpopulations within the province. Such variation is consistent with findings from genetic studies of Philippine native pigs, which have shown measurable population structure and differentiation across geographic locations, including Isabela Province [21].

Qualitatively, the predominance of plain black coat color, along with traits such as straight head profile, erect or forward-oriented ears, smooth skin, and straight hair, is consistent with descriptions of Philippine native pigs from other regions, including Zamboanga Peninsula, Romblon, Batanes–Quezon–Marinduque, and Bohol [10,15,17,19]. These similarities are also consistent with documentation of provincially named native pig strains (including ISUbela®) presented in the DOST PCAARRD native pig breeds resource [6,18]. Molecular evidence further supports that Philippine native pigs are typically small, black-colored animals with considerable genetic diversity across provinces [20,21]. Black coat color is a common, genetically influenced phenotype in pigs (e.g., MC1R-related pigmentation), and native pigs are raised under tropical conditions where thermoregulation is a known physiological constraint in pigs [24,25]. The presence of minor coat color variations may reflect within - population variation and potential

introgression, which is consistent with evidence that Philippine native pigs represent multiple lineages and show population structure [20,21].

Ear morphology, particularly the dominance of erect ears, reflects a common trait among indigenous pig populations and is consistent with recognized strains such as ISUbela® [18]. This feature may indicate limited genetic dilution from commercial breeds, which typically exhibit drooping ears.

Snout and head profiles were largely straight, which is a typical morphological characteristic of majority of Philippine native pigs [15,17,19]. These features are functionally important for foraging behavior. In backyard farming system, pigs spend considerable time on foraging and retain behaviors similar to wild boars even under domestication [23]. Even in *ad libitum* feeding, rooting behavior persists, which implies that it is an innate and strongly motivated behavior rather than solely driven by hunger [22].

The predominance of smooth skin and straight hair reflects adaptation to the hot and humid climate of Isabela. Sparse to moderate hair density likely enhances thermoregulation by improving heat dissipation under tropical conditions [25].

Quantitative results indicate that native pigs in Isabela are smaller compared to commercial breeds but comparable to other native pigs in the Philippines [15,19]. The smaller body size is well-suited to low-input production systems and supports *in situ* conservation efforts [27], pigs are commonly fed locally available resources, including forage crops and agricultural by-products. [28,32] Native pigs are also recognized for adaptive traits such as tolerance to environmental stress and disease, which enhance their value for backyard farming system [27,31].

Differences in phenotypic characteristics across municipalities suggests the presence of localized variability. This may be influenced by geographic isolation, management practices, and environmental conditions, with limited animal movement contributing to phenotypic differentiation [21].

These findings emphasize the importance of conserving native pig genetic resources to maintain biodiversity and support resilient livestock systems [29]. Breeding programs should improve productivity while preserving adaptive traits [30]. Future studies should include molecular analyses to better understand genetic diversity and population structure, thereby enhancing conservation and improvement strategies.

5. Conclusion

In conclusion, majority of the male native pigs surveyed from the six selected municipalities of Isabela have predominant phenotypic characteristics of having no prominent tusks, straight snout and head profile, erect and forward orienting ears, plain black color, straight hair, smooth skin, straight backline and curly tail, while female native pigs have predominant phenotypic characteristics of having also no prominent tusk, straight, long and thin snout profiles, straight head profiles, erect and forward orienting ears, black plain color, with straight hair, smooth skin, straight backline and straight tail profile for sows and curly tail profile for mature male native pigs and native gilts. Predominant quantitative traits of native pigs from selected municipalities were almost similar to the native pig strains reported by DOST-PCAARRD, and also native pigs from Bohol and Romblon.

Author Contributions

Conceptualization, J.D.C., K.M.G.N.; Methodology, J.D.C.; Investigation, J.D.C.; Writing – Original Draft, J.D.C.; Writing – Review & Editing, J.D.C., K.M.G.N; Funding Acquisition, J.D.C.; Resources, J.D.C.; Supervision, J.D.C., K.M.G.N

Ethics Approval and Consent to Participate

The Institutional Animal Care and Use Committee permit was secured prior to the conduct of the study with Protocol Number: 07-2023 of the ISU-IACUC.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Histomorphometric Analysis of Cardiopulmonary and Renal Organs in Philippine Native Darag and Commercial Redbro Chickens

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Abstract

Background: Philippine native Darag chickens are widely distributed, locally adapted, and valued for preferred flavor and perceived hardiness despite slower growth, smaller body size, and leaner musculature than commercial broilers. However, baseline histomorphometric data on major organs of this breed remain limited. This study evaluated the developmental histomorphometry of the heart, lungs, and kidneys of Darag chickens compared with Redbro commercial hybrid broilers, selected because of their roles in oxygen delivery, gas exchange, and metabolic waste elimination, respectively, and the scarcity of baseline data in Philippine native chickens. **Methods:** Heart, lung, and kidney tissues were collected from 100 day-old male Darag and 100 Redbro chicks. Three birds per strain were sampled at weeks 1, 2, 4, 6, and 8 for histologic processing and quantitative analysis. Associations among organ parameters, body weight, and feed intake were assessed. **Results:** Age significantly influenced heart muscle fiber thickness, parabronchial density, and parabronchial lumen diameter, while strain affected heart muscle fiber thickness and air capillary area fraction. Significant age–strain interactions were observed across all kidney parameters. Body weight and feed intake showed positive correlations with selected cardiopulmonary morphometric parameters across strains. **Conclusions:** Findings provide preliminary structural baseline data; larger functional studies are recommended.

Keywords

Darag; Philippine native chicken; Redbro; Heart; Lungs; Kidneys; Histomorphology

1. Introduction

Chicken meat ranks second to pork in Filipino diets. In 2016, the Philippines produced 1,674.50 metric tons of chicken, following pork at 2,231.66 metric tons [1]. In 2018, per capita chicken

consumption averaged 6.89 kg nationally and 12.94 kg in the National Capital Region [2]. These figures underscore the sector's importance and sustained consumer demand. Demand remains elevated, particularly following the African swine fever surge. Generally, meat-type broilers are more production-efficient than local breeds, whereas indigenous chickens offer disease resistance, environmental adaptability, and distinctive meat quality [3].

The Darag chicken originates from Western Visayas. It is central to Visayan cuisine, supporting regional popularity and market potential. Compared with the 28–45 days to market typical of commercial broiler-type chickens, Darag chickens undergo a 21–45-day "hardening" period to acclimate juveniles to environmental conditions. After hardening, birds are released to range and typically harvested at 75–120 days—approximately three times longer than broilers. Rural consumers value its dual-purpose traits and palatable meat, characterized by lower fat, higher unsaturated fatty acids, high protein, and notable potassium content [4, 5].

Philippine native chickens combine resilience, favorable sensory attributes, and sustainability. Adaptation to local conditions reduces disease risk and reliance on chemicals. Free-range systems promote welfare, and slower growth may yield nutritionally favorable meat for consumers seeking natural products.

The heart, lungs, and kidneys were selected as target organs owing to their central roles in oxygen delivery, gas exchange, and metabolic waste elimination, respectively, and because histomorphometric baseline data for these organs in Philippine native chicken strains are lacking in the published literature. The present study evaluated histomorphometric differences in these organs between Darag chickens and Redbro commercial broiler-type chickens.

2. Materials and Methods

2.1. Birds and Management

One hundred day-old male Philippine Darag chicks and 100 Redbro commercial hybrid broiler-type chicks were obtained from the Genome-Wide Association Study Project of the Institute of Animal Science, College of Agriculture and Food Sciences, University of the Philippines Los Baños. The birds were housed at the Institute of Animal Science Experimental Poultry Farm for up to 16 weeks during November 2022–January 2023, or until Darag chickens reached 1 kg. Birds at weeks 1, 2, 4, 6, and 8 were sampled.

Each pen measured 3 ft (length) × 4 ft (width) × 2.5 ft (height) and housed 10 birds. Pens were maintained under 24-hour light at 31 °C for the first 21 days, then under 12-hour light at 28 °C thereafter. Animals had ad libitum access to clean water and received booster mash for brooding (0–21 days) and starter feed for early growth (22–56 days). For disease prevention, birds received two Newcastle disease vaccines: B1B1 on day 7 and La Sota P9 on day 14.

All procedures were approved by the University of the Philippines Los Baños Institutional Animal Care and Use Committee (approval number UPLB-2023-036).

2.2. Experimental Design

Two sets of 100 male day-old chicks were used (one set Darag, one set Redbro). Both sets were raised under the same husbandry conditions described above. Random sampling was conducted at weeks 1, 2, 4, 6, and 8. At each time point, three birds per group were sampled; at week 2, six birds per group were available and included. These sampling intervals were selected to capture early, rapid, and intermediate growth phases across the study period.

2.3. Sample Collection

Birds were randomly selected and euthanized by decapitation on prescheduled sampling days. Standard chicken necropsy procedures were followed: (1) the abdominal cavity was opened by midline incision and the abdominal wall reflected; (2) the entire gastrointestinal tract, liver, and spleen were removed; (3) the kidneys were bluntly dissected free from the vertebral column; (4) the lungs were separated from the ribs by careful blunt dissection and removed from the thorax; and (5) the heart was excised by severing its major vascular attachments. All organs were weighed and immediately immersed in neutral buffered formalin for a minimum of 72 hours.

2.4. Tissue Processing and Histological Examination

Histological processing followed a standard paraffin-embedding protocol in sequential order. After fixation, tissues were rinsed in running tap water. Dehydration was performed through a graded ethanol series (70%, 80%, 90%, 100%) with two changes at each concentration. Tissues were cleared in two changes of 100% xylene and infiltrated with paraffin wax prior to embedding. Paraffin blocks were sectioned at 5 µm on a rotary microtome; one section of every four was selected for staining.

For hematoxylin and eosin (H&E) staining, sections were deparaffinized in four changes of xylene (5 minutes each) and rehydrated through a descending ethanol series (100%, 90%, 80%, 70%). Slides were rinsed in running tap water for 5 minutes and stained with hematoxylin for 2 minutes. After bluing in tap water, slides were soaked five times in 80% ethanol, stained with eosin for approximately 5–10 minutes, and dehydrated through ascending ethanol concentrations. Before mounting, slides were cleared in two changes of xylene (5 minutes each), mounted with a drop of resinous mounting medium, and coverslipped.

Stained slides were examined under a compound light microscope (AmScope, China) to assess histomorphometric parameters of the heart, lungs, and kidneys across time points.

2.5. Histomorphometric Examination

All morphometric measurements were performed using Fiji (ImageJ, National Institutes of Health, Maryland, USA) by a single trained observer using standardized protocols.

Heart. Left and right ventricular wall thicknesses were measured at five locations on transverse sections at 4× magnification. For larger samples, Adobe Photoshop Photomerge was used to stitch separately captured images. Muscle fiber thickness was measured in the free wall of the left ventricle. Five fibers per animal were measured at their narrowest transverse diameter, perpendicular to the long axis of the fiber. Boundaries between adjacent fibers were demarcated where the sarcolemma of one fiber was visually distinct from that of the adjacent fiber. Cross-sections were selected to provide a consistent, reproducible fiber profile.

Lungs. Parabronchial density was quantified as the number of parabronchi per microscopic field (five fields per animal) at 4× magnification. Parabronchial lumen diameter was measured from cross-sectional profiles of five parabronchi per animal; the internal diameter was taken at the widest point of each cross-section. Air capillary area fraction was estimated by color-contrasting image analysis (blood capillaries assigned black; air capillaries assigned white) and expressed as the percentage of the tissue cross-sectional area occupied by air capillaries.

Kidneys. Renal corpuscle density was determined as the number of corpuscles in one intact kidney lobule at 10× magnification. Renal corpuscle diameter was measured as the internal lumen diameter of five corpuscles per animal. Proximal and distal convoluted tubules were differentiated based on standard H&E histological criteria: proximal convoluted tubules (PCT) were identified by their larger lumen, taller cuboidal-to-columnar epithelium with abundant eosinophilic cytoplasm and indistinct apical brush border; distal convoluted tubules (DCT) had a smaller, rounder lumen, lower cuboidal epithelium, and paler, less eosinophilic cytoplasm. Tubule densities were counted in five fields per animal at 10× magnification.

2.6. Statistical Analysis

Data were analyzed in Prism (GraphPad Software, San Diego, CA, USA). A mixed-effects model was applied to accommodate missing values and to test the effects of age, strain, and their interaction on cardiopulmonary and renal parameters. Significance thresholds were set at $P < 0.05$ (significant), $P < 0.01$ (highly significant), and $P < 0.001$ (very significant). Given that three animals were sampled per group at each time point, the present study is best interpreted as exploratory; statistical findings indicate trends that warrant confirmation in larger cohorts.

A two-tailed Pearson correlation coefficient was used to assess relationships between measured parameters, body weight (BW), and total feed consumption, applying the same significance thresholds.

3. Results

3.1. Left Ventricular Wall Thickness

Left ventricular wall thickness did not differ significantly across weekly measurements in either the Darag or Redbro strains. Temporal fluctuations were evident (Figure 1D); these irregular patterns likely reflect high intra-group biological variability given the small sample size. In the Darag group, values rose from week 1 to 2, declined at week 4, and increased again from week 6 to 8. In the Redbro group, values increased steadily from weeks 1–4, decreased at week 6, and rose slightly at week 8.

(B) Whole heart. (C) Left ventricular wall at 40× magnification; representative measurement of

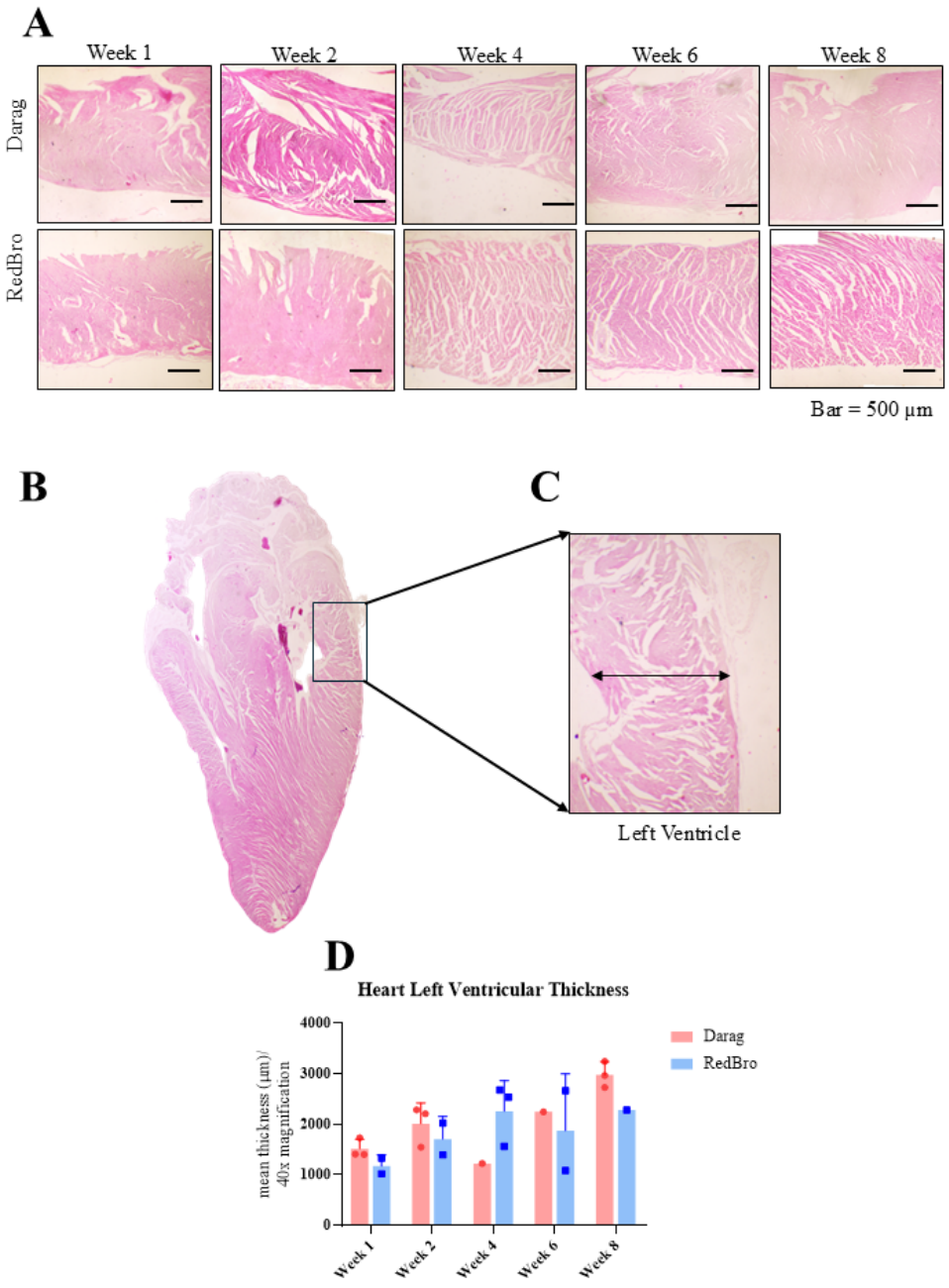

Figure 1. Left ventricular wall thickness did not differ significantly between Darag and Redbro. (A) Representative images of the left ventricular wall at weeks 1, 2, 4, 6, and 8. Scale bar = 500 µm.

thickness indicated by double-headed arrows. (D) Mean left ventricular wall thickness. Data are expressed as mean ± SD from five tissue sections per animal (n = 3 animals per group).

3.2. Right Ventricular Wall Thickness

Right ventricular wall thickness showed no significant weekly differences between strains. The pattern was irregular (Figure 2D), consistent with high intra-group variability. In the Darag group, a small increase occurred from week 1 to 2, followed by a decrease at week 4 consistent with the representative image (Figure 2A), and an increase at week 6. In the Redbro group, values increased at weeks 1, 2, 4, and 6, then decreased slightly at week 8.

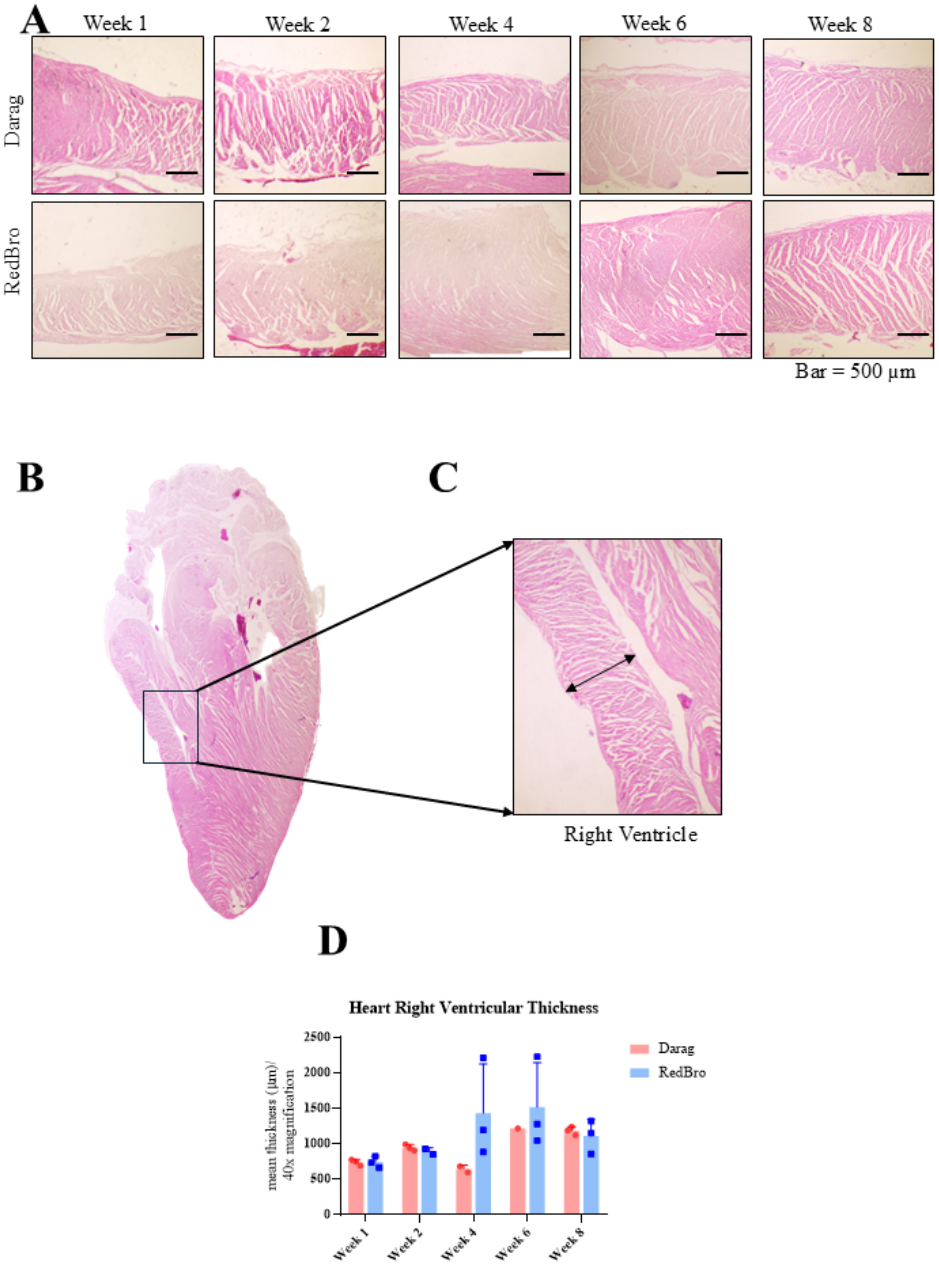

Figure 2. Right ventricular wall thickness did not differ significantly between Darag and Redbro. (A) Representative images of the right ventricular wall at weeks 1, 2, 4, 6, and 8. Scale bar = 500 µm.

(B) Whole heart. (C) Right ventricular wall at 40× magnification; representative measurement of thickness indicated by double-headed arrows. (D) Mean right ventricular wall thickness. Data are expressed as mean ± SD from five tissue sections per animal (n = 3 animals per group).

3.3. Muscle Fiber Thickness

Muscle fiber thickness in the left ventricular free wall differed significantly between strains at weeks 4 and 8, whereas no significant differences were observed at other time points. For both strains, values increased steadily from week 1 to week 8 (Figure 3B), with Redbro consistently demonstrating greater fiber thickness at the weeks where significant differences were observed.

3.4. Parabronchial Density

Parabronchial density did not differ significantly between strains. Both exhibited a

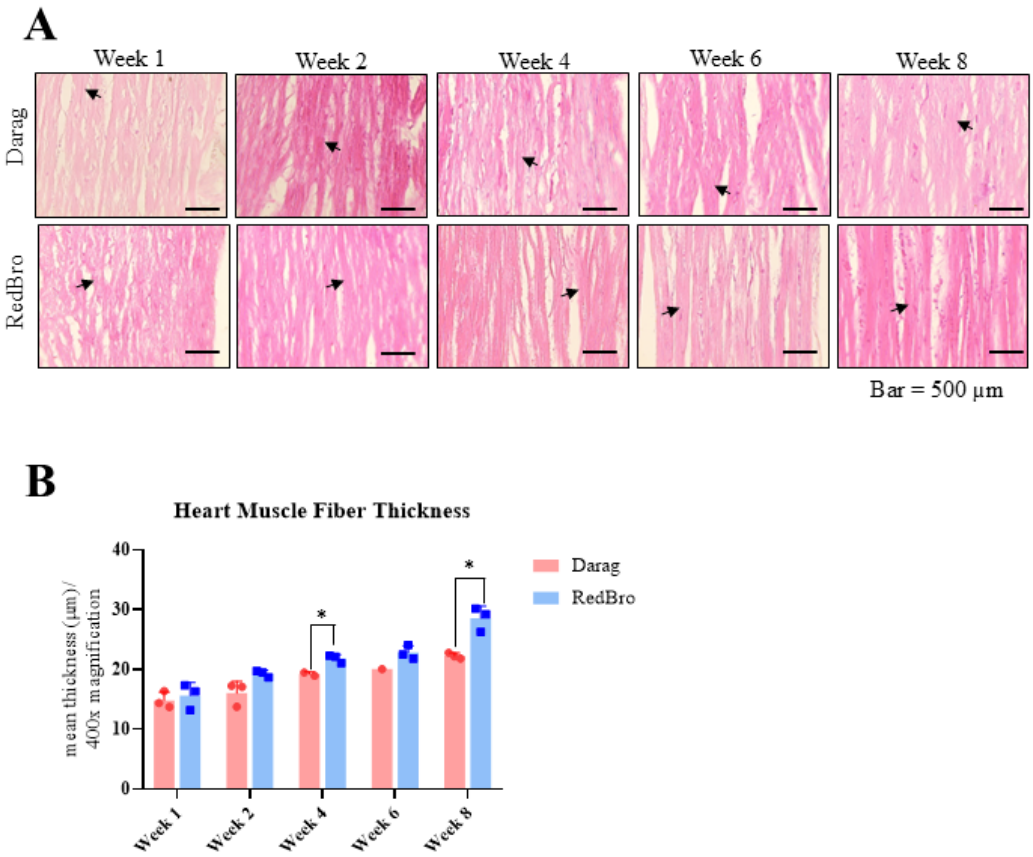

Figure 3. Heart muscle fiber thickness differed significantly between strains at weeks 4 and 8. (A) Representative images of left ventricular cardiomyocytes at weeks 1, 2, 4, 6, and 8; cardiac muscle fibers are labeled with arrows. Scale bar = 500 µm. (B) Mean muscle fiber thickness. Data are expressed as mean ± SD from five fibers per animal (n = 3 animals per group).

clear downward trend from week 1 to week 8 (Figure 4B). Representative images likewise illustrate a progressive decline in the number of parabronchi per field (Figure 4A).

(Figure 4C). In the Darag group, values increased from week 1 to week 6 and decreased slightly at week 8.

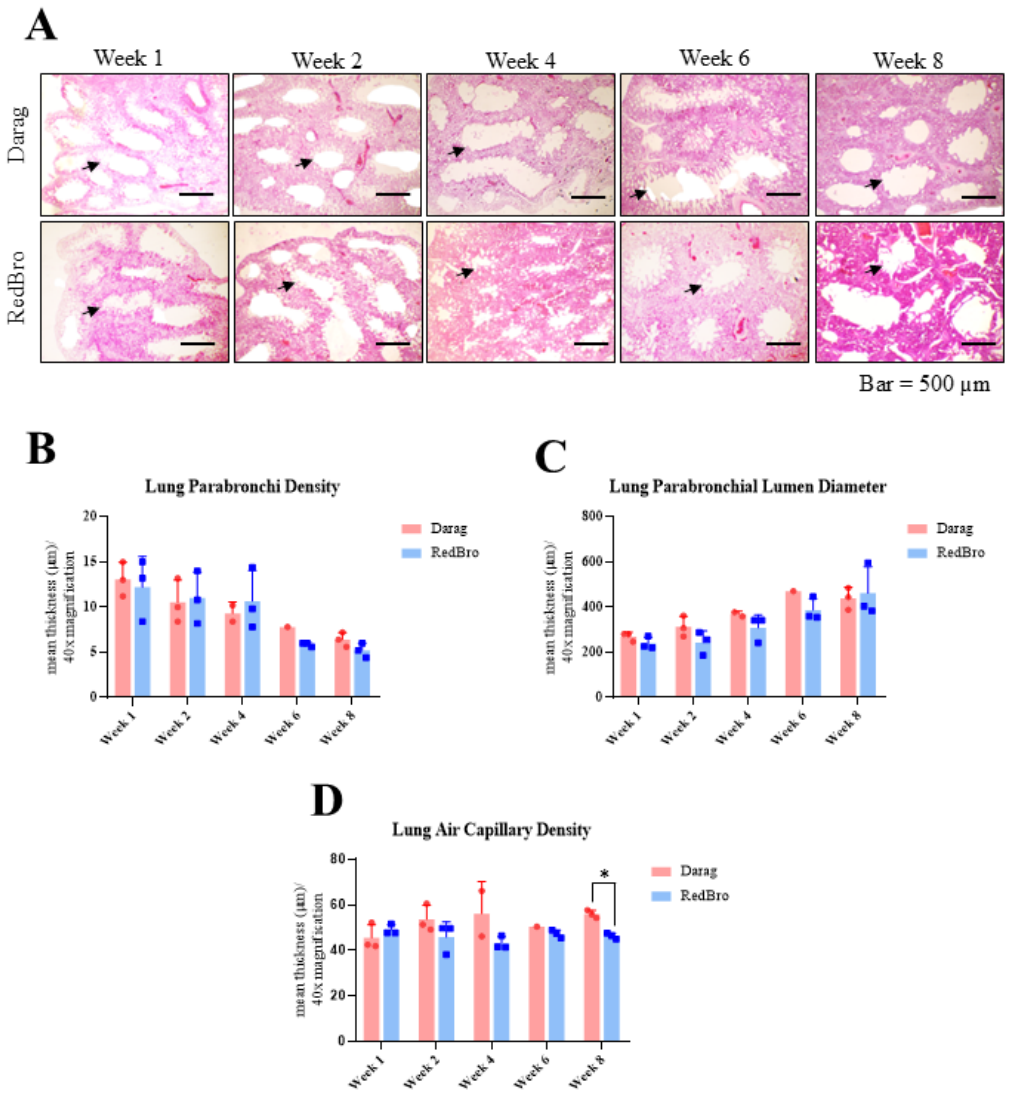

Figure 4. Lung air capillary area fraction differed significantly between strains at week 8, whereas parabronchial density and parabronchial lumen diameter did not. (A) Representative lung images at weeks 1, 2, 4, 6, and 8; parabronchi are labeled with arrows. Scale bar = 50 µm. (B) Mean lung parabronchial density. (C) Mean lung parabronchial lumen diameter. (D) Mean lung air capillary area fraction. Data are expressed as mean ± SD from five fields or structures per animal (n = 3 animals per group).

3.5. Parabronchial Lumen Diameter

Parabronchial lumen diameter did not differ significantly between strains. In the Redbro group, values increased steadily from week 1 to week 8

3.6. Air Capillary Area Fraction

Air capillary area fraction differed significantly between strains at week 8. The bar graph showed irregular patterns for both strains

(Figure 4D). In the Darag group, values increased slightly at weeks 1, 2, and 4, decreased at week 6, and increased again at week 8. In the Redbro group, values decreased from weeks 1–4, increased at week 6, and decreased slightly at week 8. One Redbro slide at week 8 showed processing artifacts and was excluded from analysis; this is acknowledged as a limitation.

(Figure 5). In the Darag group, counts were high at week 1, decreased sharply at week 2, and then fluctuated thereafter. In the Redbro group, counts varied but remained near the average of 10–20 corpuscles per week. Irregular trends in both strains likely reflect biological variability inherent to the small sample size.

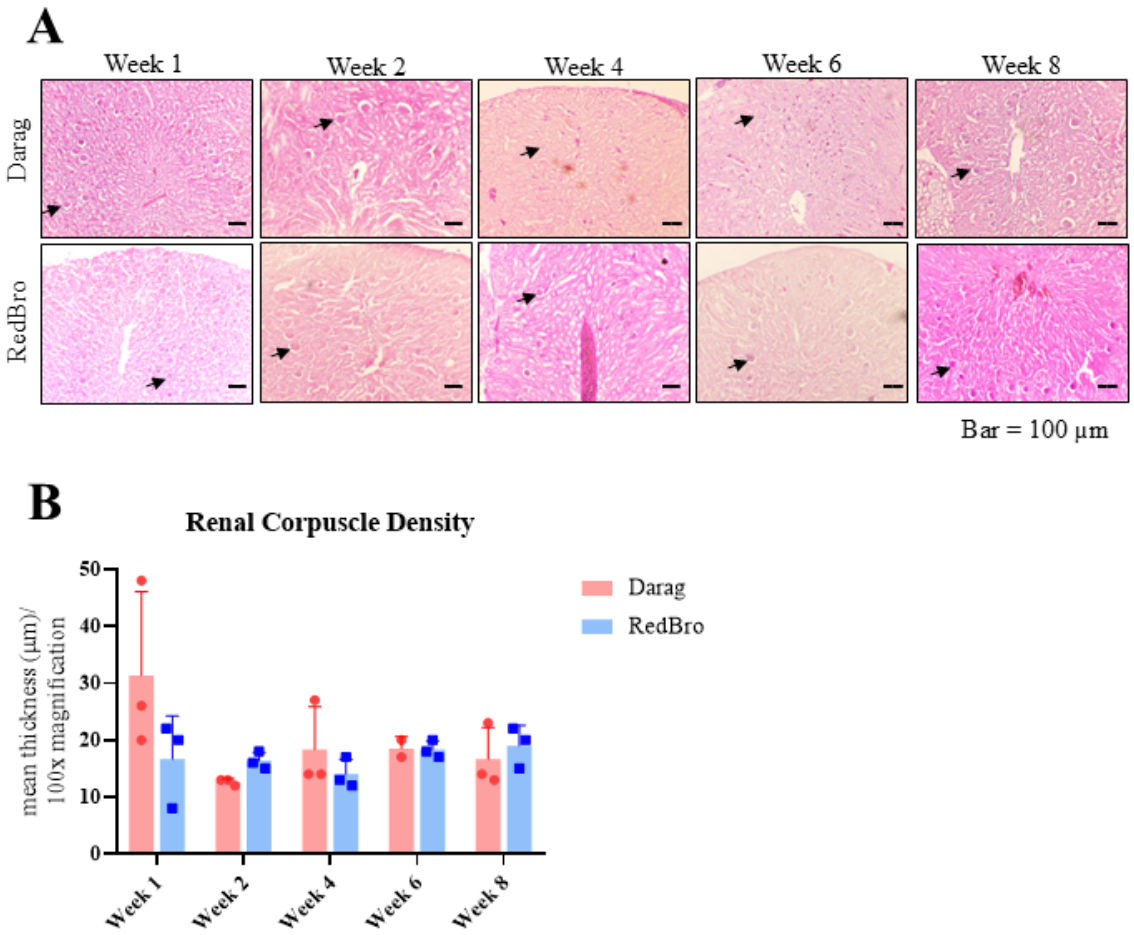

Figure 5. Histomorphology of renal corpuscle density in Darag and Redbro strains. No significant differences were observed between strains. (A) Representative kidney images at weeks 1, 2, 4, 6, and 8; mammalian-type renal corpuscles are labeled with arrows. Scale bar = 100 µm. (B) Bar graph showing mean renal corpuscle density. Data are mean ± SD from one intact kidney lobule per animal (n = 3 animals per group).

3.7. Renal Corpuscle Density

Renal corpuscle density did not differ significantly between strains. No consistent temporal trend was evident from week 1 to week 8

3.8. Renal Corpuscle Diameter

Renal corpuscle diameter did not differ significantly between strains. The graph showed an inconsistent pattern with comparable values in

both strains (Figure 6B). In the Darag group, values exceeded 40 µm at weeks 2 and 6; in the Redbro group, values exceeded 40 µm at weeks 4 and 6. These observations indicate minimal between-strain differences in corpuscle lumen diameter.

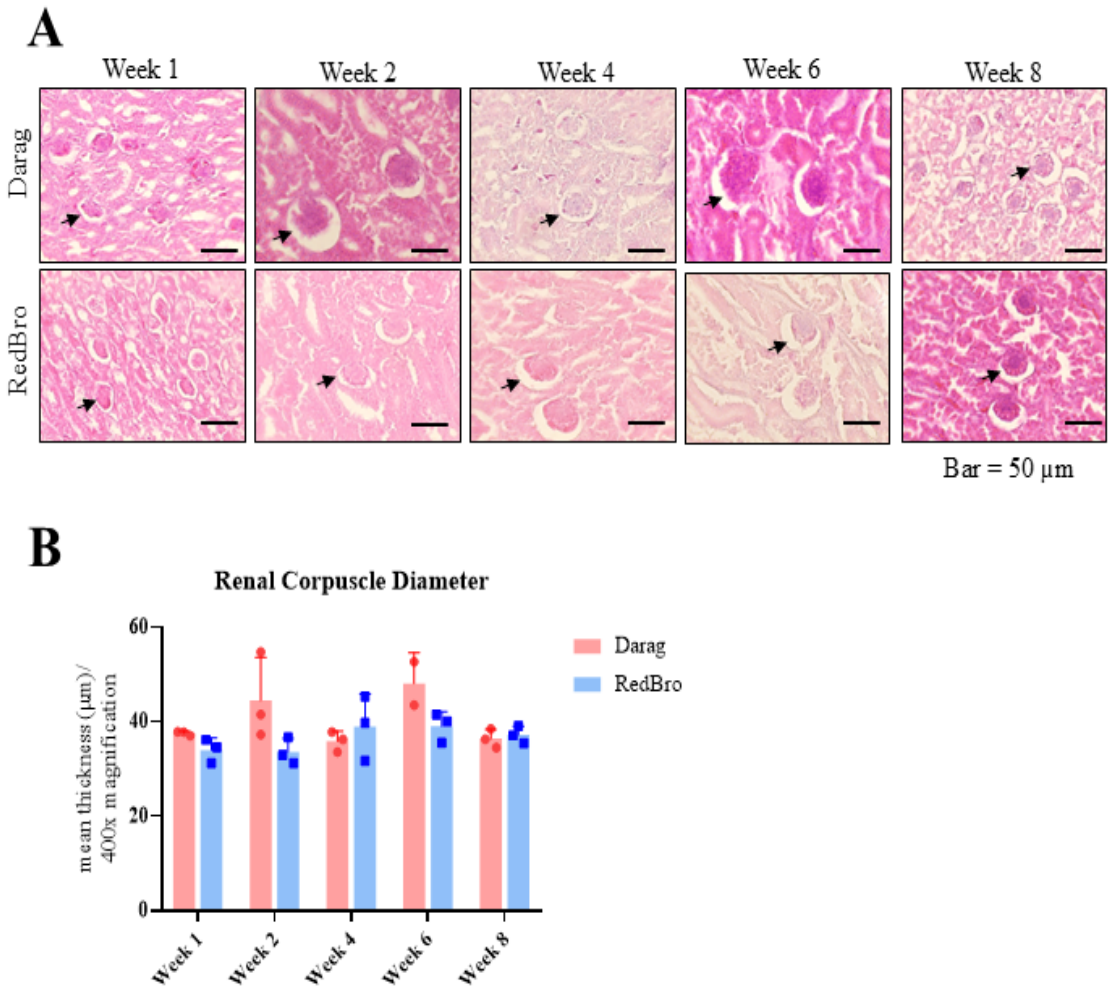

Figure 6. Histomorphological assessment of renal corpuscle diameter in Darag and Redbro strains, showing no significant differences between strains. (A) Representative images of renal corpuscles at weeks 1, 2, 4, 6, and 8; corpuscles are labeled with arrows. Scale bar = 50 µm. (B) Bar graph showing mean renal corpuscle diameter. Data are presented as mean ± SD from five corpuscles per animal (n = 3 animals per group).

3.9. Convoluted Tubule Diameters

Proximal convoluted tubule (PCT) diameter did not differ significantly between the Darag and Redbro groups (Figure 7B). No consistent pattern was observed from week 1 to week 8. The same applied to DCT diameter; Figure 7C shows an approximately stable distribution across weeks for both groups. For both PCT and DCT, the strains were comparable.

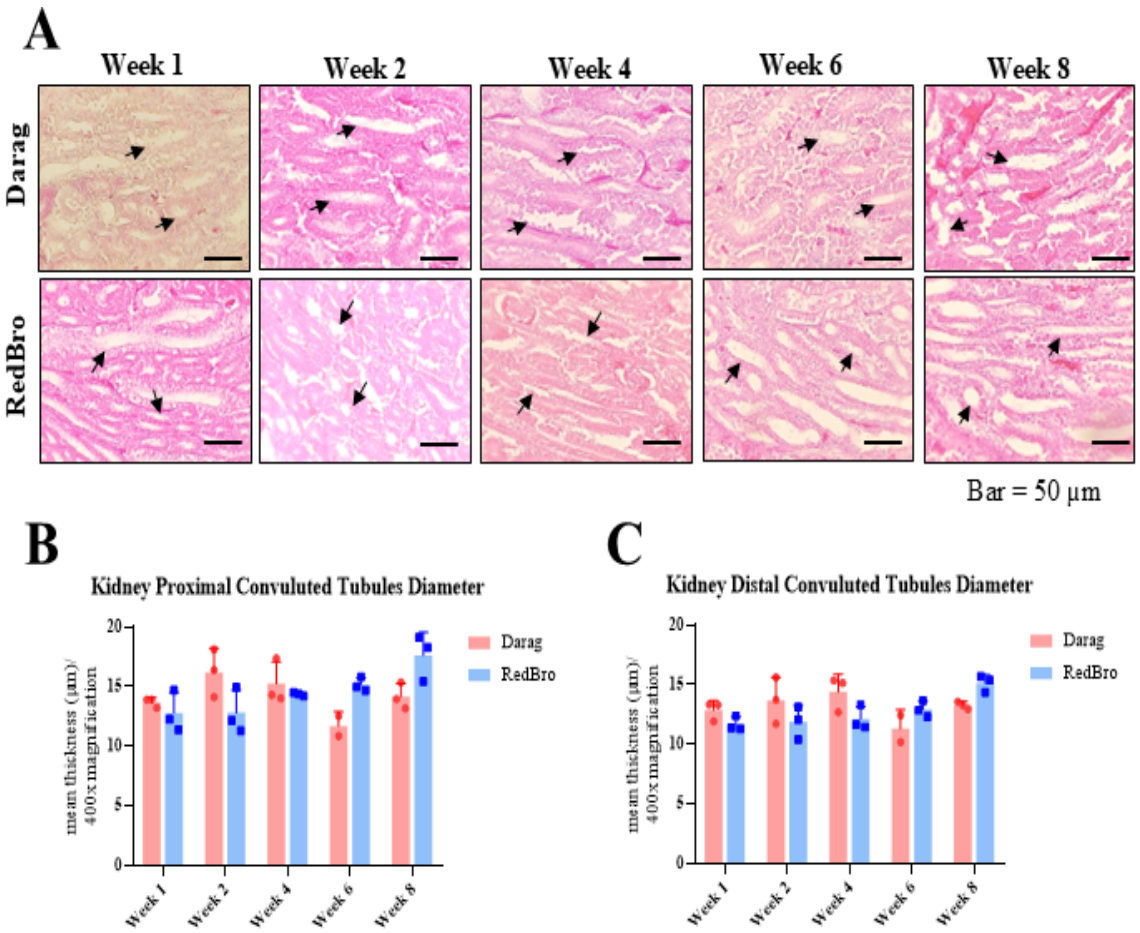

Figure 7. Histomorphology of proximal and distal convoluted tubule diameter in Darag and Redbro strains. No significant differences were observed between strains. (A) Representative kidney images at weeks 1, 2, 4, 6, and 8; proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) are labeled with arrows. Scale bar = 50 µm. (B) Bar graph showing mean PCT diameter. (C) Bar graph showing mean DCT diameter. Data are mean ± SD from five tubules per field across five fields per animal (n = 3 animals per group).

3.10. Effect of Age, Strain, and Age–Strain Interaction

Table 1 indicates significant effects of age on muscle fiber thickness, parabronchial density, and parabronchial lumen diameter. Strain significantly affected muscle fiber thickness and air capillary area fraction. The age–strain interaction significantly influenced all kidney parameters assessed (renal corpuscle density, renal corpuscle diameter, and PCT and DCT diameter).

3.11. Correlation between Heart Parameters and Body Weight

Muscle fiber thickness was the only heart parameter that correlated significantly with body BW. In both strains, BW was strongly and positively correlated with muscle fiber thickness (Darag: r = 0.97, P = 0.008; Redbro: r = 0.97, P = 0.007). Left and right ventricular wall thickness showed no significant correlation with BW in either strain (Table 2).

3.12. Correlation between Lung Parameters and Body Weight

Two lung parameters correlated with BW in both strains. In the Darag group, BW showed a strong negative correlation with parabronchial density (r = −0.94, P = 0.02) and a positive correlation with parabronchial lumen diameter (r = 0.88, P = 0.048). In the Redbro group, BW similarly showed a strong negative correlation with parabronchial density (r = −0.95, P = 0.02) and a very strong positive correlation with parabronchial lumen diameter (r = 0.996, P = 0.0004). Air capillary area fraction did not correlate with BW in either strain (Table 2).

3.13. Correlation between Kidney Parameters and Body Weight

For kidney parameters, only Redbro chickens showed significant correlations with BW. A strong positive correlation was observed between BW and PCT diameter (r = 0.996, P = 0.0003) and between BW and DCT diameter (r = 0.94, P = 0.02). Renal corpuscle density and diameter were not

Table 1. Mixed-effects model results for age and strain effects on each dependent variable.

Dependent Variable	Age	Strain	Interaction
Left ventricular wall thickness	F(1.699, 2.974) = 3.889 *P = 0.1475*	F(1, 4) = 0.2622 *P = 0.6356*	F(4, 7) = 1.505 *P = 0.2984*
Right ventricular wall thickness	F(1.256, 5.024) = 2.062 *P = 0.2155*	F(1, 16) = 1.827 *P = 0.1953*	F(4, 16) = 1.412 *P = 0.2749*
Muscle fiber thickness	F(1.968, 6.395) = 44.38 *P = 0.0002***	F(1, 4) = 28.89 *P = 0.0058***	F(4, 13) = 2.992 *P = 0.0592*
Parabronchial density	F(1.933, 6.283) = 12.70 *P = 0.0064***	F(1, 4) = 0.3086 *P = 0.6081*	F(4, 13) = 0.6921 *P = 0.6105*
Parabronchial lumen diameter	F(1.624, 5.279) = 21.78 *P = 0.0033***	F(1, 4) = 1.160 *P = 0.3421*	F(4, 13) = 1.052 *P = 0.4183*
Air capillary area fraction	F(1.168, 3.796) = 0.4199 *P = 0.5846*	F(1, 4) = 7.884 *P = 0.0484**	F(4, 13) = 2.014 *P = 0.1518*
Renal corpuscle density	F(1.167, 4.378) = 3.853 *P = 0.114*	F(1, 4) = 0.6042 *P = 0.4804*	F(4, 15) = 3.761 *P = 0.026**
Renal corpuscle diameter	F(2.298, 8.618) = 2.971 *P = 0.1003*	F(1, 4) = 2.481 *P = 0.1904*	F(4, 15) = 3.515 *P = 0.0325**
PCT diameter	F(1.649, 6.185) = 3.179 *P = 0.1161*	F(1, 4) = 0.4001 *P = 0.5614*	F(4, 15) = 6.063 *P = 0.0041***
DCT diameter	F(1.792, 6.720) = 3.178 *P = 0.1093*	F(1, 4) = 0.6806 *P = 0.4557*	F(4, 15) = 4.243 *P = 0.0171**

** P < 0.05 = significant; ** P < 0.01 = highly significant; *** P < 0.001 = very significant (two-tailed)*

Table 2. Pearson correlation coefficients between histomorphometric parameters and body weight.

Dependent Variable		Darag	Redbro
Left ventricular wall thickness	r	0.7949	0.764
	P (two-tailed)	0.108	0.1327
	N	5	5
Right ventricular wall thickness	r	0.727	0.5536
	P (two-tailed)	0.1641	0.333
	N	5	5
Muscle fiber thickness	r	0.9656	0.9676
	P (two-tailed)	0.0076**	0.007**
	N	5	5
Parabronchial density	r	−0.9448	−0.9452
	P (two-tailed)	0.0154*	0.0153*
	N	5	5
Parabronchial lumen diameter	r	0.8821	0.9955
	P (two-tailed)	0.0477*	0.0004***
	N	5	5
Air capillary area fraction	r	0.5192	−0.1892
	P (two-tailed)	0.37	0.7606
	N	5	5
Renal corpuscle density	r	−0.3887	0.6083
	P (two-tailed)	0.5178	0.2763
	N	5	5
Renal corpuscle diameter	r	−0.06011	0.6669
	P (two-tailed)	0.9235	0.2189
	N	5	5
PCT diameter	r	−0.3741	0.9964
	P (two-tailed)	0.5351	0.0003***
	N	5	5
DCT diameter	r	−0.2439	0.9408
	P (two-tailed)	0.6926	0.0172*
	N	5	5

** P < 0.05; ** P < 0.01; *** P < 0.001 (two-tailed)*

significantly correlated with BW in either strain, and PCT and DCT diameters were not significantly correlated in Darag chickens (Table 2).

3.14. Correlation between Heart Parameters and Total Feed Intake

Both strains exhibited significant correlations between heart parameters and total feed intake. In Darag chickens, total feed intake was strongly and positively correlated with muscle fiber thickness ($r = 0.98$, $P = 0.002$). In the Redbro group, left ventricular wall thickness showed a significant positive correlation ($r = 0.90$, $P = 0.04$), and muscle fiber thickness showed a highly significant positive correlation ($r = 0.98$, $P = 0.004$). Right ventricular wall thickness showed no significant correlation in either strain (Table 3).

Table 3. Pearson correlation coefficients between histomorphometric parameters and total feed intake.

Dependent Variable		Darag	Redbro
Left ventricular wall thickness	r	0.7423	0.9027
	P (two-tailed)	0.1508	0.0359*
	N	5	5
Right ventricular wall thickness	r	0.719	0.675
	P (two-tailed)	0.1711	0.2112
	N	5	5
Muscle fiber thickness	r	0.9844	0.9769
	P (two-tailed)	0.0023**	0.0042**
	N	5	5
Parabronchial density	r	−0.9713	−0.871
	P (two-tailed)	0.0058**	0.0545
	N	5	5
Parabronchial lumen diameter	r	0.932	0.94
	P (two-tailed)	0.0211*	0.0175*
	N	5	5
Air capillary area fraction	r	0.5519	−0.4354
	P (two-tailed)	0.3348	0.4637
	N	5	5
Renal corpuscle density	r	−0.44	0.3997
	P (two-tailed)	0.4584	0.505
	N	5	5
Renal corpuscle diameter	r	0.007675	0.7588
	P (two-tailed)	0.9902	0.1369
	N	5	5
PCT diameter	r	−0.3946	0.9455
	P (two-tailed)	0.5109	0.0152*
	N	5	5
DCT diameter	r	−0.261	0.8575
	P (two-tailed)	0.6715	0.0632
	N	5	5

** P < 0.05; ** P < 0.01; *** P < 0.001 (two-tailed)*

3.15. Correlation between Lung Parameters and Total Feed Intake

In the Darag group, total feed intake showed a highly significant negative correlation with parabronchial density (r = −0.97, P = 0.006) and a positive correlation with parabronchial lumen diameter (r = 0.93, P = 0.02). In the Redbro group, total feed intake showed a positive correlation with parabronchial lumen diameter (r = 0.94, P = 0.02).

3.16. Correlation between Kidney Parameters and Total Feed Intake

Redbro chickens showed a significant positive correlation between total feed intake and PCT diameter (r = 0.95, P = 0.02). In the Darag group, none of the kidney parameters were significantly correlated with total feed intake.

4. Discussion

Philippine native chicken strains such as Darag grow more slowly, typically requiring 75–120 days to reach slaughter weight [5]; consumers, particularly in rural areas, often prefer native chickens for their meat quality. Consequently, native chicken breeds are important for maintaining poultry genetic diversity and provide considerable economic value [6]. In contrast, Redbro chickens reflect years of genetic selection to improve growth rate and feed conversion efficiency [7].

Histomorphometric analysis of the heart showed that Darag chickens had thinner cardiac muscle fibers at weeks 4 and 8 than Redbro chickens (Figure 3B). Although Darag chickens are typically managed under free-range systems and Redbro chickens under intensive commercial production in field settings, both strains were maintained under identical controlled conditions in the present study. The observed between-strain differences in cardiac muscle fiber thickness may reflect inherent strain-related differences rather than management divergence. The irregular fluctuations observed in ventricular wall thickness for both strains are likely attributable to high intra-group biological variability within the small samples. Redbro demonstrated a more consistent increase in cardiac muscle fiber thickness from week 1 to week 8, whereas Darag appeared to exhibit a more gradual pattern of cardiac structural development within the sampling period. Further work at the cardiomyocyte level is needed to clarify the structural basis of these differences. It should be noted that no physiological measurements (e.g., cardiac output) were performed; the findings herein are strictly structural in nature [8,9].

Parabronchial density and lumen diameter did not differ significantly between strains. Both strains exhibited a decline in parabronchial density and an increase in lumen diameter over time (Figures 4B and 4C). These trends are attributed to apparent changes in density as the lung parenchyma expands with growth; however, organ size measurements were not incorporated into the current analysis, and this interpretation remains preliminary. Although organ weights were recorded as part of the necropsy protocol, comparative organ-to-body-weight analysis was not performed in the present study; future work should incorporate these measures. Air capillary area fraction differed significantly between strains at week 8 (Figure 4D); the structural basis for this difference remains unclear and warrants further investigation. It is acknowledged that air capillary area fraction alone does not permit inferences about gas exchange capacity without functional measurements [10]. Across more than 9,000 avian species, respiratory architecture is broadly conserved, and strain-level variation in parabronchial density or structure has been little described [11–13].

Kidney parameters showed no statistically significant between-strain differences across all measures (Figures 5–7), suggesting that Darag and Redbro chickens possess comparable renal filtration architecture at the structural level, particularly in mammalian-type renal corpuscles and proximal and distal convoluted tubules. However, the significant age × strain interaction effects observed in the mixed-effects model suggest that renal maturation trajectories may differ temporally, with each strain potentially following distinct developmental patterns before converging toward similar adult renal morphology. It is acknowledged that the avian kidney contains two structural types of renal corpuscles: the larger mammalian-type (cortical) corpuscle and the smaller reptilian-type (medullary) corpuscle. In the present study, measurements were limited to mammalian-type corpuscles, which are more readily identifiable in H&E-stained sections. Future studies should stratify measurements by corpuscle type to more comprehensively characterize renal filtration architecture in these strains. Comparable findings extended to PCT and DCT diameters, with both strains following similar trends (Figures 7B and 7C). Few studies have directly compared chicken strains, although notable interspecies differences in renal structure have been reported [14].

The mixed-effects model showed that age had a very significant effect on heart muscle fiber thickness, reflecting cardiac structural growth. Age also had highly significant effects on parabronchial density and lumen diameter; parabronchi decreased in apparent number as lungs grew, whereas lumen diameter increased. Strain effects were observed for muscle fiber thickness and air capillary area fraction; Redbro chickens had thicker muscle fibers, and Darag

chickens had a higher air capillary area fraction at week 8. Significant age–strain interactions were detected for all kidney parameters, indicating that renal maturation trajectories, while ultimately converging, may differ temporally between strains.

Body weight correlated strongly and positively with muscle fiber thickness in both strains, consistent with somatic growth. Parabronchial density correlated negatively with body weight in both strains, likely because fewer parabronchi are captured within a fixed microscopic field as lungs enlarge. In the Redbro group, parabronchial lumen diameter showed a very strong positive correlation with body weight; in the Darag group, the correlation was positive but more modest, consistent with a proportional growth pattern [15]. For kidney parameters, PCT and DCT diameters showed significant positive correlations with body weight only in Redbro chickens. Because this pattern was absent in the Darag group, a strain-specific developmental difference may exist, although this interpretation remains speculative given the small sample size. Reported effects of glucocorticoid growth promoters on broiler renal tubule morphology may provide context for these observations, but their contribution here remains to be investigated [16].

Analysis of correlations between total feed intake and histomorphometric parameters identified several significant associations consistent with those for body weight. Feed intake is a primary driver of poultry growth, development, and production [17,18]. The Redbro group consumed more than twice as much feed as the Darag group over the study period. Pelleted diets used in commercial broiler production are designed to improve feed efficiency and intake [19]; genetic and management differences between commercial broilers and native strains are also relevant [20].

Overall, the results provide preliminary evidence of between-strain differences in selected parameters, whereas most parameters were comparable between Darag and Redbro under the controlled experimental conditions of this study.

5. Conclusions

This study compared the histomorphology of the heart, lungs, and kidneys of the Philippine native Darag chicken strain with those of commercial hybrid Redbro chickens to characterize between-strain differences. The findings provide preliminary structural baseline data on Darag cardiopulmonary and renal histomorphometry and identify parameters where the strain is comparable to its slow-growing broiler counterpart under controlled conditions.

The present study is exploratory given the small number of animals per sampling period (three per group per time point), and all findings should be regarded as indicative trends pending confirmation in larger cohorts. No physiological measurements were performed; conclusions are limited to structural observations. The irregular trends observed in several parameters likely reflect high intra-group biological variability inherent to the sample size used. A formal inter-observer reliability assessment was not conducted and is acknowledged as a limitation.

Specific areas for future investigation include: incorporating organ-to-body-weight ratios and organ size measurements; distinguishing mammalian-type from reptilian-type renal corpuscles in morphometric analyses; increasing sample sizes to improve statistical power; and examining additional Philippine native chicken strains such as ZamPen, Caraga Black [21,22], Paroakan, Bolinao, Banaba, and Joloano [23]. Sample processing precision should be ensured to minimize slide artifacts. These collective efforts will clarify the histomorphometric traits of native Philippine chicken strains and expand baseline anatomical data for future comparative and functional studies.

Ethics Approval and Consent to Participate

All procedures were approved by the University of the Philippines Los Baños Institutional Animal Care and Use Committee (approval number UPLB-2023-036).

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Molecular Detection of Virulence Genes Associated with Salmonella Pathogenicity Islands (SPIs) 1 and 2 in *Salmonella enterica* Isolates from Poultry in Selected Areas of the Philippines

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Abstract

Background: Salmonellosis, particularly infections caused by *Salmonella enterica*, remains a major concern in poultry production and food safety. The disease poses significant economic losses due to mortality, reduced growth rates, and treatment costs, highlighting the need to characterize virulence gene patterns of Philippine isolates. This study aimed to molecularly confirm *S. enterica* isolates recovered from poultry-derived samples, particularly eggs, giblets, and cloacal swabs, collected from selected regions of the Philippines using 16S rRNA-targeted PCR, and to determine the presence and distribution of the *invA* (SPI-1) and *sseC* (SPI-2) virulence genes among these isolates. **Methods:** A total of 865 samples, including eggs, giblets, and cloacal swabs, were collected from Philippine poultry farms and wet markets. Standard microbiological procedures were employed: pre-enrichment in buffered peptone water, enrichment in Rappaport Vassiliadis broth, plating on xylose lysine deoxycholate agar, and purification on nutrient agar. Putative isolates were further analyzed using morphological and selected biochemical tests, followed by PCR targeting of *16S rRNA*, *invA*, and *sseC* genes. Amplicons were visualized by gel electrophoresis. **Results:** All 56 suspected isolates were confirmed as *S. enterica*. Among these, 69.6% carried both *invA* and *sseC*, 14.3% carried *invA* alone, 3.6% lacked both genes, and none harbored *sseC* alone. These genes are associated with pathogenicity islands SPI-1 (*invA*) and SPI-2 (*sseC*). **Conclusions:** The presence of virulence genes linked to SPI-1 and SPI-2 among Philippine *S. enterica* isolates highlights their role in pathogenesis. This provides baseline data on virulence gene prevalence in poultry-derived Salmonella and emphasizes the importance of continued surveillance to mitigate risks of salmonellosis from poultry-derived isolates.

Keywords

Salmonella enterica, Pathogenicity islands, Poultry, invA, sseC, virulence genes

1. Introduction

The Philippine Statistics Authority (PSA) reported that as of September 2023, the total chicken inventory reached 202.82 million birds. This figure represents a 1.1 percent increase compared to the year's inventory of 200.64 million birds. Of this total, native/improved chickens accounted for 43.0 percent, followed by broiler chickens at 34.7 percent, and layer chickens at 22.3 percent. The highest chicken inventory was noted in Central Luzon, with 35.80 million birds, followed by CALABARZON with 27.30 million birds, and Northern Mindanao with 26.74 million birds. Together, these regions represented 44.3 percent of the country's total chicken inventory during the review period. Per capita poultry consumption in the Philippines for 2024 is estimated at 14.91 kilograms [1].

Worldwide, non-typhoidal Salmonella is one of the leading bacterial causes of diarrhea, causing around 150 million illnesses and approximately 60,000 deaths each year [2]. Of the food poisoning cases with a known bacterial cause, it is the predominant pathogen, as evidenced by recorded cases from 2005 to June 2018 [3]. In this context, contaminated chicken meat can likely serve as a source of the pathogen for consumers of poultry products such as eggs, giblets, and meat.

The traditional classification system for Salmonella, based on Kauffmann-White (1934), utilizes O and H antigens to divide the organism into serovars. As of 2018, a total of 2,659 serovars of *Salmonella* spp. have been identified, with 1,586 of these belonging specifically to *S. enterica* subsp. *enterica* [4]. However, current taxonomy recognizes two species: *S. enterica* and *S. bongori*. Within *S. enterica*, there are six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae*, and *indica*. Serovars and serogroups are contained within those taxonomic levels. These subspecies are further categorized into serogroups based on their antigenic characteristics [5,6]. A serovar (serotype) is a specific strain defined by its unique combination of surface antigens, while a serogroup is a broader category that clusters serovars sharing common O (somatic) antigen(s) (5). Almost all Salmonella organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica* [7]. Thus, reporting a strain's serovar (or serogroup) conveys antigenic identity used for surveillance and epidemiology, while species/subspecies indicate its broader genetic and evolutionary placement [4,5,6,7].

Primers targeting the *16S rRNA* gene allow for more robust, reproducible, and accurate bacterial identification than that obtained by phenotypic testing [8]. However, if the goal is to differentiate species within a genus, other genes must be utilized; therefore, the study employed primers targeting the virulence genes.

Numerous virulence genes have been identified in Salmonella, contributing to its complex life cycle within infected birds and humans [9]. One of these is a bacterial secretion system known as the type III secretion system (T3SS), which is the most crucial virulence factor for *Salmonella* spp. [10]. This T3SS translocates effector proteins across bacterial and host membranes into the host cytosol, where they modulate signaling and cytoskeletal pathways to facilitate colonization and virulence [11]. The effector proteins disrupt various aspects of the host cell's physiology and immune response, thereby enhancing bacterial virulence [12].

Salmonella spp. has a chromosomal region where most T3SS genes are concentrated, known as Salmonella pathogenicity islands (SPIs), with 17 SPIs documented to date [13]. Among these SPIs, SPI-1 and SPI-2 are the most extensively studied and represent two key determinants of pathogenesis [14-16]. The SPI-1 secretion system enables bacterial invasion of epithelial cells [12], which then leads to inflammation of the intestinal epithelium and symptoms of diarrhea [9]. On the other hand, the principal role of SPI-2 is to promote the replication of intracellular bacteria found in the Salmonella-containing vacuoles (SCVs) [12]. The SPI-2 promotes intracellular survival and replication by modifying SCV thereby, interfering with host endosomal trafficking and vesicle fusion, and enabling systemic infection [15]. The SPI-1 and SPI-2 secretion systems facilitate Salmonella pathogenesis.

Comparative sequence analysis of 12 conserved chromosomal housekeeping genes and invasion-associated genes demonstrated that Group V (*S. bongori*) is strongly distinct from other Salmonella groups, supporting its status as a separate species [17]. Many of these genes, which are clustered in the SPIs, are responsible for virulence. However, *S. bongori* has reduced virulence as it carries a limited, ancestral set of virulence functions and subsequently elaborated this in a different direction than *S. enterica* [18].

The *invA* gene (also known as invasion A protein) and the *sseC* gene (Salmonella secreted protein C) are located in SPI-1 and SPI-2, respectively. Consequently, the presence of SPI-1 and SPI-2, along with their associated genes, serves as an indication of a virulent Salmonella [10]. The invasion-associated SPI-1, specifically *invA*, mediates epithelial cell invasion and is induced during the intestinal/epithelial contact phase, while the survival-associated SPI-2, namely *sseC*, contributes to intracellular survival and replication, induced following internalization in host cells [11]. Indeed, the presence of the virulence genes *invA* and *sseC* is necessary for Salmonella organisms to have an effect on both human and animal hosts, among other factors that depend on gene expression, regulatory networks, additional effectors, and host factors.

This study was henceforth aimed to molecularly confirm *Salmonella enterica* isolates recovered from poultry-derived samples (eggs, giblets, and cloacal swabs) collected from selected regions of the Philippines using 16S rRNA-targeted PCR, and to determine the presence and distribution of the *invA* (SPI-1) and *sseC* (SPI-2) virulence genes among these isolates.

2. Materials and Methods

2.1 Ethical Consideration

The research protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of the three participating institutions: Cavite State University with protocol number 2019-001, the University of Eastern Philippines with protocol number 2020-0001, and the University of the Philippines Los Baños with protocol number 2019-0027.

2.2 Sample Collection

The sample size calculation was conducted according to Thompson's procedure [19]. A total of 865 samples were collected from poultry farms and wet markets across Regions 1, 2, 3, 4, 5, 6, 7, 8, 10, and 11, as well as from the National Capital Region and the Cordillera Administrative Region in the Philippines.

Stratified random sampling was used, in which the overall population was divided into strata (regions 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, NCR, and CAR), followed by province, then cities/municipalities, and finally farms. Using 50% prevalence estimate which is the default prevalence to calculate for the largest possible sample size, accuracy at 5% and 95% level confidence.

2.3 Microbiological Methods

Sterile cotton-tipped applicator sticks were used to collect cloacal and organ/tissue samples from sick and necropsied poultry. The cloacal and organ/tissue samples were collected from individual birds. The protocols established by Kebede et al. [20] and Shirota *et al.* [21] for isolating *S. enterica* from swabs, organ/tissue, and eggshells were followed with slight modifications. The eggs were pooled with 10 eggs per pool. The swab samples were then aseptically placed in test tubes containing buffered peptone water (BPW) (Himedia®, HiMedia Laboratories, India). These tubes were accurately labeled and placed in an ice box for transportation to the laboratory for further isolation.

The samples submerged in BPW were incubated at 37 °C for 18 hours. Next, 0.1 mL of the broth culture was inoculated into 9.9 mL of Rappaport Vassiliadis (RV) broth (HiMedia®, HiMedia Laboratories, India) as an enrichment medium and incubated at 42 °C for 24 hours. Then, a loopful of the sample from the RV broth was streaked onto xylose lysine deoxycholate (XLD) agar (HiMedia®, HiMedia Laboratories, India) and incubated at 37 °C for 24 hours. Red colonies, with or without black centers, that developed on the XLD agar were presumed to be *S. enterica* serovars and were subsequently purified on nutrient agar (NA) plates using the quadrant streak plate method. The purified isolates were then transferred onto NA slants, incubated at 37 °C for 24 hours, and stored at 4 °C until use. All

procedures for isolation and handling of live bacteria were conducted in a Class II biosafety cabinet (Telstar Bio II Advance Plus®).

2.4 Morphological Observation and Biochemical Characterization

The presumptive *S. enterica* isolates were subjected to the Gram staining technique described by Cheesbrough [22]. As previously reported, all Gram-negative, rod-shaped organisms underwent standard biochemical methods [21,22] primarily oxidase test, catalase test, indole, methyl red, Voges-Proskauer, citrate utilization (IMViC) tests, and urease test. A reference strain of *S. enterica* was used as a positive control, and uninoculated media served as the negative control for all the tests mentioned above, allowing for comparison.

2.5 Genomic DNA Extraction

Isolates exhibiting typical biochemical characteristics of *Salmonella* spp. underwent genomic DNA extraction. Fifty-six bacterial isolates were inoculated onto XLD agar (HiMedia®, HiMedia Laboratories, India) for 24 hours. After incubation, three to five isolated colonies were selected and suspended in 1 mL of phosphate-buffered saline (PBS) (Thermo Fisher Scientific®, North Carolina, USA), and DNA extraction was performed using a commercial DNA extraction kit (DNeasy®, Qiagen, California, USA), adhering to the manufacturer's instructions.

2.6 Molecular Confirmation of *S. enterica* Isolates by Amplification of 16S rRNA and Virulence Genes Associated with SPI-1 and SPI-2

Polymerase chain reaction (PCR) targeting the bacterial *16S rRNA* gene was performed to confirm that the isolates were bacteria. Subsequently, the isolates that were confirmed to have the *16S rRNA* gene were subjected to PCR to detect the presence of the virulence genes of *S. enterica,* the *invA* and *sseC* genes.

Amplification of the target gene was performed using a commercial PCR kit (SapphireAmp® Fast PCR Master Mix, Takara Bio Inc., Shiga, Japan), along with forward and reverse primers and the extracted DNA template. Table 1 presents the sequences of the primers used, including those for the target gene and the reference. The samples were analyzed in a gradient thermocycler (SimpliAmp™ Thermal Cycler, Thermo Fisher Scientific®, North Carolina, USA). The PCR cycling conditions employed are outlined in Table 2.

Table 1. Primer sequences used in the study.

PRIMER	SEQUENCE (5' TO 3')	TARGET GENE	AMPLICON SIZE	REFERENCE
F- *S. enterica* 16S rRNA	TGTTGTGGTTAATAACCGCA	*S. enterica 16S rRNA*	572	*Nyabundi et al.* (2017) [23]
R- *S. enterica* 16S rRNA	CACAAATCCATCTCTGGA			
F-invA	GTGAAATTATCGCCACGTTCGGGCAA	*invA*	284	*Salehi et al.* (2005) [24]
R-invA	TCATCGCACCGTCAAAGGAACC			
F-sseC	TATGGTAGGTGCAGGGGAAG	*sseC*	121	*Fazl et al.* (2013) [25]
R-sseC	CTCATTCGCCATAGCCATTT			

Table 2. Thermal profiles of the PCR protocols used in the study.

Target Gene	Initial Denaturation	Denaturation	Annealing	Extension	Cycles	Final Extension
16S rRNA	95˚C, 3 min	95˚C, 2 min	55˚C, 30 s	72˚C, 1 min	35	72˚C, 10 min
invA	94˚C, 1 min	94˚C, 1 min	64˚C, 30 s	72˚C, 30 s	35	72˚C, 5 min
sseC	95˚C, 5 min	95˚C, 1 min	50˚C, 1 min	72˚C, 1 min	35	72˚C, 5 min

PCR amplicons were analyzed through electrophoresis using a 1.2% (w/v) agarose gel (Vivantis®, Malaysia) in 1X Tris Acetate EDTA buffer (Vivantis®, Malaysia), which included GelRed® (Biotium, CA, USA) for staining. A molecular weight standard (Vivantis®, Malaysia) was used to estimate the size of the amplified fragments. Genomic DNA from a known *S. enterica* strain and sterile distilled water served as positive and negative controls in each assay, respectively. Electrophoresis was performed at 120 volts for 35 minutes using a Mupid® system (USA), and the results were visualized with a gel documentation system (GelOne®, Cleaver Scientific Ltd., United Kingdom).

3. Results

From the 865 poultry and market samples, 56 or 6.5%, were identified as putative *S. enterica* isolates which appeared as red colonies with or without black centers, or as completely black colonies, on XLD agar (Himedia®), and were gram-negative rod-shaped bacteria. The results in the biochemical tests were as follows: oxidase negative, catalase positive, indole negative, methyl red positive, Voges-Proskauer negative, citrate utilization positive (IMViC) or - + - +, and urease negative. These isolates were subsequently confirmed using 16S rRNA primers. These samples originated from various regions in the Philippines, with 62.5% coming from Cavite (Table 3).

Table 4 shows the percentage of positive results for the putative Salmonella isolates for the 16S rRNA gene and the SPI-1 and SPI-2 virulence genes of interest. Among the 56 *S. enterica* isolates, 69.6% (39/56) possessed *invA* and *sseC*, 14.3% (8/56) harbored *invA* alone (Fig. 1), 3.6% (2/56) lacked both virulence genes, and none of the isolates harbored *sseC* alone.

Table 3. Geographical location of the putative Salmonella isolates (n=56) and type of samples collected.

Geographical location	%	Type of sample
National Capital Region	25	Eggs
Pampanga (Region III)	7.1	Broiler chicks
Cavite (Region IV)	62.5	Broiler and poultry giblets
Laguna (Region IV)	3.6	Breeder
Iloilo (Region VI)	1.8	Broiler chicks

Table 4. Percent positive of the putative Salmonella isolates for the *16S rRNA*, and the SPI-1 and SPI-2 virulence genes of interest.

Genes	Number Tested	Number Positive	% Positive (95% CI)
16S rRNA	56	56	100.0 (93.6, 100.0)
invA	56	48	85.7 (74.6, 93.0)
sseC	56	39	69.6 (57.0, 81.6)

4. Discussion

Salmonellae are widespread bacteria present in animals, humans, and the environment, promoting transmission and cross-contamination [20]. Janda and Abbott emphasized the routine use of *16S rRNA* gene sequences as a key tool in bacterial taxonomy and phylogeny [26]. It serves as a valuable complement to routine clinical microbiology [27]. The *16S rRNA* gene is the region of DNA that is most frequently used to classify bacteria because it is conserved [28]. The degree of conservation arises from its critical role in cell function [29]. No gene has demonstrated as broad applicability across all taxonomic groups as the 16S rRNA gene, which can be problematic. Thus, Clarridge noted that if the goal is to differentiate species within a particular genus, a better gene than the 16S rRNA gene may be found for identifying species [8], which is why in this study, both the *invA* and *sseC* genes were also targeted.

All putative isolates were confirmed as *S. enterica* by PCR targeting the 16S rRNA gene, and their virulence potential was further characterized by PCR detection of the invA and sseC genes. However, it has been reported that validated primers targeting the *invA* and *16S rRNA* genes may yield false-positive signals with isolates of *Citrobacter* spp., *E. coli*, and *Serratia* spp. [30], prompting the development of alternative assays targeting the *ttrA/C* genes to distinguish *S. enterica* from non-Salmonella isolates. Building on these observations, future studies on local poultry strains could integrate more advanced synthetic DNA tools [31,32], alongside whole-genome sequencing and BLAST-based comparative analysis, to further refine the detection and characterization of *S. enterica* populations in the Philippines [33].

The *invA* gene promotes and controls the initial phase of Salmonella pathogenesis. It is the most common and clinically significant genetic marker for Salmonella serovars [34]. The use of *invA* in PCR for *Salmonella* spp. was first proposed by Rahn *et al.* in 1992 and is employed to differentiate Salmonella from other bacteria. It serves as a common molecular target for Salmonella-specific detection methods [35]. The most prevalent serovars associated with human salmonellosis worldwide are *S.* Typhimurium and *S.* Enteritidis, and both commonly carry the *invA* virulence gene [36].

Numerous studies from abroad have demonstrated the utility of *invA* as a target gene for Salmonella detection in various animal and environmental samples. The *invA* gene plays a crucial role not only in Salmonella detection but also in the processes of colonization and invasion within the intestinal epithelium of free-range chickens [37]. It serves as a dependable PCR target for Salmonella detection in different samples worldwide, as evidenced by Sunar *et al.* (2010) where the *invA* gene exhibited a highly specific alignment with sequences from Salmonella species found in compost samples [38]. The *invA* virulence gene was identified in milkfish samples in Indonesia [39]. In one study, the *invA* gene was found in 100% of Salmonella isolates from poultry farms [40].

Eight isolates (14.3%) that tested positive for the *invA* gene alone were identified in eggs (50%), cloacal swabs (37.5%), and giblets (12.5%). Additionally, 14 (25%) of the *S. enterica* isolates were sourced from eggs, and among these, 71.4% exhibited both virulence genes, while 28.6% had *invA* only. A report has indicated that phylogenetic analysis of the *invA* gene sequences from their Salmonella isolates in human and egg samples clustered together, suggesting a common source of infection [36]. Their results emphasized that chickens are a significant source of Salmonella and that humans primarily acquire the infection from these sources [41]. A local study also evaluated the presence of the *invA* gene and other genes related to SPI-1 in *S. enterica* isolates from swine and poultry in the Philippines, highlighting the potential risk these animal isolates pose to humans [33].

Additionally, giblets can harbor Salmonella when macrophages ingest the bacteria but fail to eliminate it, allowing systemic infection [42]. A study found an overall detection rate of 29% of *S.* Typhimurium in giblets from retail chickens sold in Egypt [43].

The overall detection rate of *invA* in this study is 85.7% (48/56), while the detection rate for *sseC* is 69.6% (39/56). Theoretically, isolates that contain *invA* but lack *sseC* can still be virulent because *invA* enables the organism to invade eukaryotic cells [44]. None of the isolates found to possess the 16S rRNA gene were only positive for the *sseC* gene. The *sseC* gene allows the organism

to affect biological processes and transport proteins directly into the host cell cytoplasm [45].

The SPI-1 encodes the first type III secretion system (SPI-1 T3SS) that Salmonella uses to facilitate the infiltration of non-phagocytic cells [46]. In contrast, SPI-2 encodes a second type III secretion system (SPI-2 T3SS) that enables evasion of NADPH oxidase–mediated oxidative defenses by preventing oxidase localization to Salmonella-containing vacuoles [47]. The *sseC* gene is crucial for establishing systemic infection [48], and SseC, together with SseD, facilitates bacterial translocation [49].

Dual targeting of the *invA* and *sseC* genes was conducted because Salmonella virulence is regulated by a complex network [44]. Esquivel-Hernandez *et al.* (2018) confirmed that dual detection yields robust and artifact-free amplicons [50].

The presence of virulence genes in Salmonella is associated with salmonellosis in humans [51]. Mutations in these genes lead to decreased virulence [7]. Serovars pathogenic to poultry include *S.* Pullorum and *S.* Gallinarum, while zoonotic ones include *S.* Typhimurium and *S.* Enteritidis [52]. Garcia *et al.* reported *S.* Enteritidis in eggs and broilers [53].

Two isolates (3.6%) that lacked both virulence genes may represent reduced virulence strains. Other virulence factors include plasmids, flagella, capsules, and alternative SPIs [48]. The *spv* gene, plasmid-encoded, is one example [54].

Molecular analyses of Salmonella virulence factors *invA* and *sseC* shed light on its diversity as well as the regulation of pathogenicity. These findings, together with ecological and epidemiological information, are crucial for developing control measures at the farm and community level. Therefore, effective biosecurity measures, including vaccination, must consider the complexity of gene expression, host-pathogen interaction, and environmental factors.

5. Conclusions

Determining the presence of *invA* and *sseC* genes, which are key virulence factors associated with SPI-1 and SPI-2 in *S. enterica*, is essential for evaluating the organism's virulence potential. Additionally, it has been found that poultry, including eggs and giblets from various regions in the Philippines, harbors potentially virulent strains of *S. enterica.* As the *invA* and *sseC* genes contribute to regulatory networks underlying Salmonella pathogenicity in poultry, their detection could help in the development of diagnostic approaches to improve prevention, treatment and control strategies of Salmonella infections in these animals, ultimately benefiting the poultry-consuming public in the long term.

Availability of Data and Materials

All data are presented in the manuscript.

Author Contributions:

Conceptualization – M.C.R.D.C., G.A.C., and D.V.U.; Methodology – M.C.R.D.C., G.A.C., Y.R.M.T., R.D.D., M.L.G.A., G.M.R.G. and D.V.U.; Investigation – M.C.R.D.C., G.A.C., Y.R.M.T., R.D.D., M.L.G.A., G.M.R.G., and D.V.U.; Writing – Original Draft, M.C.R.D.C., G.A.C., Y.R.M.T., M.L.G.A., and D.V.U.; Writing – Review & Editing, M.C.R.D.C.; Funding Acquisition – M.C.R.D.C., G.A.C., and D.V.U.; Resources – M.C.R.D.C., G.A.C., R.D.D., and D.V.U.; Supervision – M.C.R.D.C., G.A.C., and D.V.U.; Project Administration – M.C.R.D.C., G.A.C., and D.V.U.

Ethics Approval and Consent to Participate

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Cavite State University, the University of Eastern Philippines, and the University of the Philippines Los Baños, and permissions were obtained from farm owners/market authorities prior to sample collection; no human participants were involved.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Gastrointestinal Parasites in Dairy Cattle in Batu City, Indonesia: Prevalence, Management-Related Risk Factors, and Farmer Practices

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Abstract

Background: Gastrointestinal (GI) parasitism remains an important endemic problem in tropical dairy systems, yet epidemiological evidence integrating infection patterns with management-related risk factors is limited in Indonesia. Identifying determinants of infection at the population level is essential for targeted parasite control strategies. **Methods:** A cross-sectional epidemiological study was conducted on 400 dairy cattle in Batu City, East Java. Gastrointestinal parasites were identified through microscopic fecal examination using flotation, sedimentation, and the McMaster technique to determine their prevalence in dairy cattle. Data on animal and farm-level variables were collected, and a structured questionnaire was administered to 50 farmers. Associations between risk factors and infection status were analysed using Chi-square tests, odds ratios (OR), and relative risks (RR). **Results:** The overall prevalence of GI parasites was 11.75%. Infections were dominated by *Eimeria* spp. (5.50%), followed by strongyle-type nematodes (2.75%), *Moniezia* spp. (2.50%), *Fasciola* spp. (1.25%), and Amphistome eggs (0.25%). Calves had a significantly higher infection risk than adults (OR = 6.04; RR = 4.12). Closed housing (OR = 2.13; RR = 1.95) and small pen size (<10 cattle; OR = 5.47; RR = 4.77) were identified as key epidemiological risk factors. **Conclusions:** GI parasitism in Batu City is primarily driven by *Eimeria* spp. infections in calves and the use of closed housing systems. These findings indicate that control programs must shift from broad-spectrum deworming to targeted coccidiosis management for young stock and environmental modifications to reduce humidity and fecal accumulation in closed stalls.

Keywords

Cattle; Epidemiology; Gastrointestinal Parasites; Helminth; Prevalence

1. Introduction

Gastrointestinal (GI) helminth infections remain a major constraint on cattle health, welfare, and productivity in tropical livestock systems. These infections arise from interactions among environmental conditions, husbandry practices, and host factors, leading to impaired nutrient absorption, mucosal damage, and metabolic disturbances [1]. Cattle are frequently infected by strongyle-type nematodes (e.g.,

Ostertagia, Haemonchus, Trichostrongylus, and *Cooperia*) and other GI parasites like *Trichuris*, *Capillaria*, *Ascaris*, and *Strongyloides* spp.; trematodes including *Fasciola hepatica* and *Paramphistomum* spp.; cestodes such as *Moniezia* spp.; and protozoa including *Eimeria* spp. and *Cryptosporidium* [2,3]. These infections may elevate serum pepsinogen and gastrin levels, indicating gastrointestinal damage that compromises growth and milk production [4].

In Indonesia, GI helminths are endemic in both dairy and beef cattle systems, facilitated by a warm and humid tropical climate that supports continuous parasite transmission. Meta-analyses report an average prevalence of approximately 46%, while regional studies frequently exceed 50%, reflecting considerable ecological and management-related variability [5–8]. Inter-provincial heterogeneity has been associated with grazing exposure, sanitation, and farming systems [3], with local studies reporting prevalence ranging from 16.5% to 66.7% influenced by manure management, farmer education, age, sex, and production system [5,8,9].

Environmental and management-related risk factors strongly influence the distribution and intensity of infection. Extensive grazing systems, poor sanitation, inadequate manure disposal, insufficient drainage, and earthen flooring enhance larval survival and transmission [10]. Climatic factors such as humidity and rainfall further promote parasite development, while younger animals generally exhibit higher infection rates due to immature immunity [5,8]. Control strategies rely primarily on anthelmintic use, with albendazole being the most commonly administered drug among Indonesian smallholders due to its affordability and broad efficacy [11,12]. However, inappropriate dosing, irregular treatment schedules, and limited diagnostic guidance may reduce treatment effectiveness and accelerate the development of anthelmintic resistance [2,13–15].

Batu City in East Java is an important dairy-producing area and provides a relevant setting for examining GI helminth infections in smallholder systems. The dairy cattle population declined markedly from 13,053 animals in 2022 to 8,500 in 2023 following the first major foot-and-mouth disease (FMD) outbreak in Indonesia [16], intensifying pressure on herd health and productivity [17]. Addressing endemic diseases such as GI helminthiasis is therefore critical for supporting herd recovery. This study aims to assess the prevalence and infection intensity of GI parasites in dairy cattle in Batu City and to examine associated environmental risk factors and farmer behavioral practices to inform practical and sustainable parasite control strategies in tropical dairy systems.

2. Materials and Methods

Ethical approval

All procedures were observational and non-invasive, with informed consent from farm owners. Ethical approval was granted by the Universitas Brawijaya IACUC (No. 092-KEP-UB-2023).

Study period and sites

The study was conducted in three sub-districts of Batu City, East Java, Indonesia: Batu, Bumiaji, and Junrejo, which are located in the upland dairy-farming region of Malang Raya at elevations ranging from 739 to 1,000 m above sea level. The study area is positioned around 7°50′01″S and 112° 32′ 03″ E (Fig. 1), representing a cool highland agro-ecological zone characterized by smallholder dairy farming systems. Sampling was conducted from July to October 2023 to capture dry season conditions. To validate the environmental context, climatological data were sourced from the Indonesian Agency for Meteorology, Climatology, and Geophysics (www.bmkg.go.id). During this period, the region recorded average temperatures of 25°C–29°C and exceptionally low rainfall (0–20 mm/month). Precipitation was categorized as 'below normal' (0–30%), indicating an intensified dry season. Study sites were selected using a purposive sampling method based on the density of smallholder dairy farms and the prevalence of household-level cattle management.

Study population and sample collection

Sample size estimation was performed using EpiTools (https://epitools.ausvet.com.au/) at 95% confidence and 10% precision, resulting in a target of 400 dairy cattle. Stratified sampling was applied at the farm level, and animals were selected through simple random sampling from official

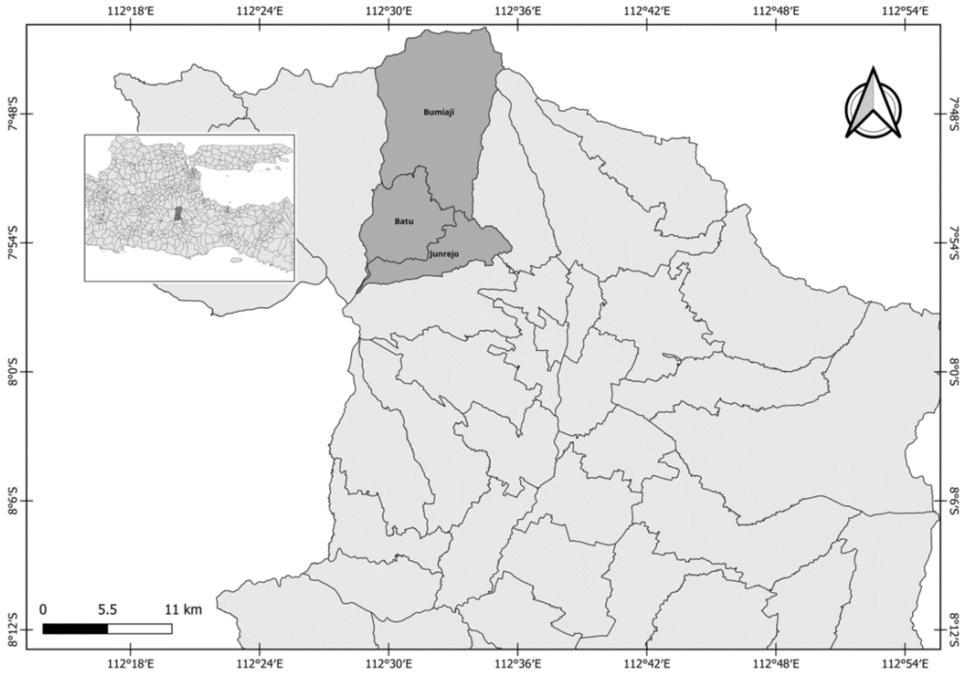

Figure 1. Study area encompassing three sub-districts and the dairy cattle population of Batu City, Indonesia. [Source: The base map was obtained from Statistics Indonesia (BPS) East Java, and the figure was generated using QGIS version 3.4.5].

farm rosters. Individual fecal samples were collected per rectum using sterile disposable gloves, placed in labeled containers, and accompanied by basic animal data (sex, age, number ear tags, health status, housing type, and deworming history). Samples were kept in a cool box during transport and processed within 14 hours at the Veterinary Parasitology Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya.

Survey instrument

A structured questionnaire was developed to assess farmers' Knowledge, Attitudes, and Practices (KAP) regarding helminthiasis and anthelmintic use. To ensure clarity and validity, the instrument was pre-tested on a small group of dairy farmers (n = 10) with characteristics similar to those of the target population but who were not included in the final analysis. Based on the pre-test results, minor adjustments were made to the phrasing of questions to ensure they were easily understood by the respondents.

The finalized questionnaire collected demographic variables, including age, educational attainment, years of farming experience, and herd size. The survey consisted of eight items: four attitude-related questions and four practice-related questions. Attitude items were measured using a three-point response scale (agree, disagree, and unknown), while practice items employed a four-point frequency scale (always, sometimes, seldom, and never). Following the scoring framework described by Sazmand *et al.* [13], farmers with an attitude score ≥1 were classified as having a positive attitude toward parasite control, while those with a practice score ≥7 were considered to have appropriate anthelmintic practices.

Parasitological analysis

Fecal samples were processed and examined using native smears, sedimentation, and flotation with saturated sodium chloride (NaCl; SG 1.200). Trematode eggs were identified using the Parfitt and Banks sedimentation method [2,18]. The procedure involved homogenizing 1 g of feces in 20 mL of filtered water and allowing it to sediment. The resulting sediment was stained with methylene blue to provide a contrasting background for microscopic examination at 100-400x magnification. *Fasciola* spp. eggs were identified by their golden-yellow coloration and

distinct operculum, whereas amphistome eggs appeared transparent to bluish-grey after methylene blue staining. Because amphistome eggs are morphologically similar to those of several taxa within the superfamily Paramphistomoidea, identification in this study was conservatively limited to amphistome eggs based on fecal egg morphology alone [2,26]. Methylene blue facilitated differentiation by darkening the fecal debris while preserving the natural appearance of the eggs.

Eimeria spp. were differentiated via sporulation in 2.5% potassium dichromate ($K_2Cr_2O_7$). Infection intensity was quantified using the McMaster chamber technique by homogenizing 4 g feces in flotation solution and converting chamber counts to EPG/OPG [19,20]. To specifically screen for *Cryptosporidium* spp., a subset of fecal samples was further processed using Lugol's iodine and modified acid-fast (Ziehl-Neelsen) staining. Stained slides were examined under oil immersion (1000× magnification) to identify small, spherical oocysts (4–6 µm) [39]. All preparations were examined using an Olympus CX-21 microscope (Olympus Corp., Tokyo, Japan) (100×–400×), with morphometrics captured using an OptiLab Advanced Plus camera (Miconos Corp., Yogyakarta, Indonesia) and ImageJ®. Infection intensity was categorized (low, medium, high) based on species-specific EPG thresholds: strongyle-type eggs <200, 200–1000, >1000 EPG; *Moniezia* spp. <100, 100–800, >800 EPG; *Toxocara vitulorum* <100, 100–800, >800 EPG; and *Trichuris* spp. and *Capillaria* spp. <50, 50–200, >200 EPG [40,41].

Statistical Analysis

Data were analyzed using the Chi-square test to evaluate differences in proportions. Risk factor analysis was based on overall prevalence because the low prevalence of individual species resulted in expected frequencies below five, which would compromise statistical validity. Associations were reported as Odds Ratios (OR) and Relative Risks (RR) with 95% confidence intervals (CI). Survey validity and internal reliability were assessed using the Pearson Correlation and Cronbach's alpha ($\alpha > 0.6$), respectively. All analyses were performed using SPSS® version 28 (IBM Corp., Armonk, NY, USA).

3. Results

Prevalence of Gastrointestinal Parasites in Dairy Cattle in Batu City, East Java, Indonesia

A total of 400 dairy cattle were examined across 50 farms located in Batu, Junrejo, and Bumiaji subdistricts of Batu City, East Java. The cattle population consisted predominantly of Friesian Holstein crossbreeds. Overall, 11.75% (47/400) of the animals were infected with at least one type of gastrointestinal (GI) parasite (Table 1). Junrejo showed the highest prevalence (5.25%), followed by Batu (3.75%) and Bumiaji (3.25%). The environmental conditions were significantly arid, with rainfall remaining below 0 – 20 mm.

Across the sampled population, four major GI parasite groups were identified: protozoa (*Eimeria* spp.), nematodes (strongyle-type eggs), cestodes (*Moniezia* spp.), and trematodes (*Fasciola* spp. and Amphistome eggs) (Fig. 2). *Eimeria* spp. exhibited the highest overall prevalence (5.50%), confirming that coccidiosis was the most frequently detected infection. Strongyle-type nematodes accounted for 2.75%, followed by *Moniezia* spp. (2.50%). Trematode infections were comparatively rare, with *Fasciola* spp. detected in 1.25% and Amphistome eggs in only 0.25% of animals. In addition to single infections, the study identified specific instances of mixed parasitic burdens. A co-infection involving strongyle-type nematodes and *Moniezia* spp. was observed in 0.75% of the examined cattle. Infection intensity also varied among parasite taxa. Strongyle-type nematodes showed EPG values ranging from 250 to 450, while *Eimeria* spp. showed OPG levels between <50 and 200. Trematode consistently exhibited low shedding (<50 OPG). Furthermore, no *Cryptosporidium* spp. oocysts were confirmed via acid-fast staining in the selected subset of samples.

Risk Factors Analysis

The prevalence of GI parasite infection among Friesian Holstein crossbred cattle varied significantly with age, housing type, and pen density (Table 2). Age was strongly associated with infection status ($\chi^2 = 29.87$, $p < 0.001$). Calves (<1 year) exhibited a markedly higher prevalence of GI

Table 1. Prevalence of gastrointestinal (GI) parasites and egg/oocyst shedding (EPG/OPG) in dairy cattle from Batu District, East Java, Indonesia.

Location	N examined	N positive (%)	Parasite Class	Species	Predilection Site	% Positive (species level)	EPG/OPG (Mean ± SD, Range)
Batu	238	15 (3.75)	Cestode	*Moniezia spp.*	Small intestine	0.25	400 ± 77.46 (300–500)
			Nematode	Strongyle-type egg.	Intestine	1.50	318.18 ± 52.53 (250–450)
			Trematode	*Fasciola* spp.	Liver	0.25	<50 ± 0
			Coccidia	*Eimeria* spp.	Small and large intestine	1.75	82.50 ± 55.81 (<50–200)
Junrejo	121	21 (5.25)	Cestode	*Moniezia* spp.	Small intestine	1.75	400 ± 77.46 (300–500)
			Nematode	Strongyle-type egg.	Intestine	1.25	318.18 ± 52.53 (250–450)
			Trematode	*Fasciola* spp.	Liver	0.25	<50 ± 0
			Coccidia	*Eimeria* spp.	Small and large intestine	2.00	82.50 ± 55.81 (<50–200)
Bumiaji	41	11 (3.25)	Cestode	*Moniezia* spp.	Small intestine	0.50	400 ± 77.46 (300–500)
			Trematode	*Fasciola spp.*	Liver	0.75	<50 ± 0
			Trematode	Amphistome eggs	Rumen	0.25	<50 ± 0
			Coccidia	*Eimeria* spp.	Small and large intestine	1.75	82.50 ± 55.81 (<50–200)
Overall	400	47 (11.75)	-	-	-	-	-

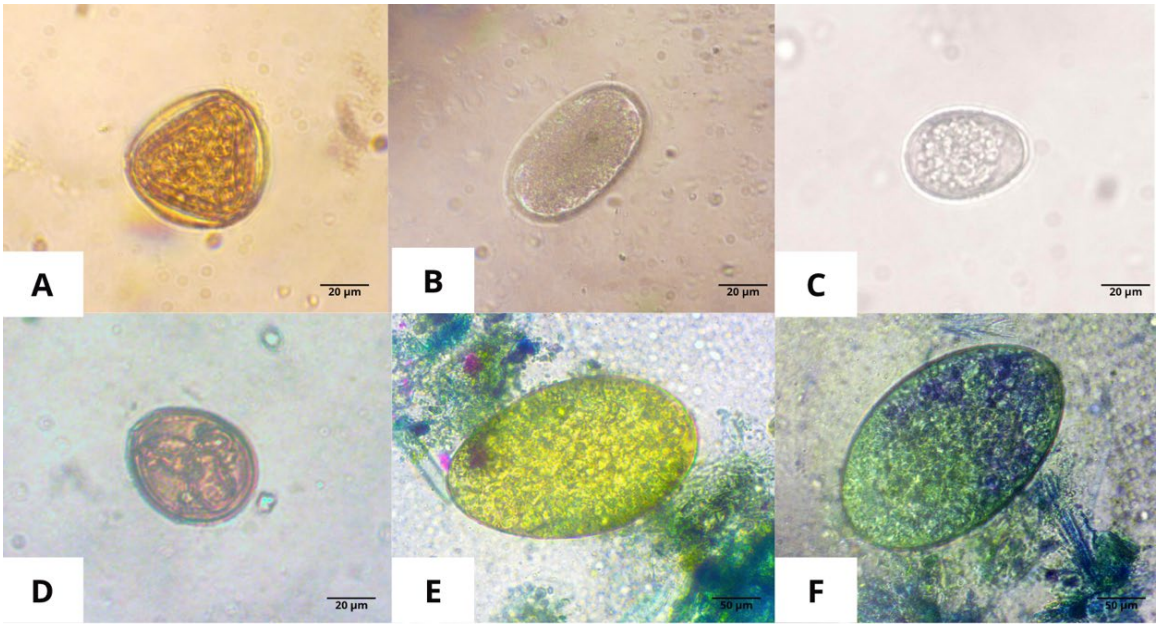

Figure 2. Gastrointestinal (GI) parasite eggs and oocysts identified in fecal samples. (A) *Moniezia* spp.; (B) *Strongyle*-type egg.; (C) unsporulated oocyst of *Eimeria* spp.; (D) sporulated oocyst of *Eimeria* spp.; (E) *Fasciola* spp.; and (F) Amphistome eggs. Photos were taken under 400x magnification.

parasite infection than adult cattle (>1 year), with 38.2% (13/34) of calves testing positive compared with 9.3% (34/366) of adults. Calves had 6.04-fold higher odds of infection (OR = 6.04) and a 4.12-fold higher risk of infection (RR = 4.12) than adult cattle.

Housing type was significantly associated with GI parasite infection. Cattle kept in closed housing systems had a higher prevalence of infection than those kept in open housing systems, with 15.8% (30/190) and 8.1% (17/210) testing positive, respectively. Closed housing was

Table 2. Prevalence of gastrointestinal (GI) parasites in dairy cattle based on age, housing type, and pen density in Batu City, East Java, Indonesia.

Variable	No. Examined n (%)	Positive n (%)	Negative n (%)	X^2	p-value
Age					
Calf (<1 year)	34 (8.5%)	13 (38.2%)	21 (61.8%)		
Adult (>1 year)	366 (91.5%)	34 (9.3%)	332 (90.7%)	29.87	<0.001
Housing Type					
Open	210 (52.5%)	17 (8.1%)	193 (91.9%)		
Closed	190 (47.5%)	30 (15.8%)	160 (84.2%)	5.71	0.017
Number of Cattle per Pen					
<10	277 (69.3%)	43 (15.5%)	234 (84.5%)		
>10	123 (30.7%)	4 (3.3%)	119 (96.7%)	15.46	<0.001
Total	400 (100%)	47 (11.8%)	353 (88.2%)	-	-

associated with 2.13-fold higher odds of infection (OR = 2.13) and a 1.95-fold higher risk of infection (RR = 1.95) compared with open housing. Figure 3 illustrates the observed housing types, consisting of closed barns located inside or adjacent to households with limited light and ventilation (A–B), and open barns built separately with adequate ventilation (C–D).

Figure 3. Housing types observed in dairy cattle farms in Batu City, East Java, Indonesia. (A-B) Closed housing, located inside the farmer's house, with limited light and restricted ventilation, and (C-D) open housing, constructed as a separate building near the house, providing good light and open ventilation.

Pen size was significantly associated with GI parasite infection. Cattle housed in pens containing fewer than 10 animals had a higher prevalence of infection than those housed in pens containing more than 10 animals, with 15.5% (43/277) and 3.3% (4/123) testing positive, respectively. Smaller pens were associated with 5.47-fold higher odds of infection (OR = 5.47) and a 4.77-fold higher risk of infection (RR = 4.77) compared with larger pens.

Socio-demographic Characteristics and Attitudes / Practices of Dairy Farmers in Batu City, East Java, Indonesia

A total of 50 dairy farmers participated in the study (Table 3). Most were over 50 years old (40%, n = 20), followed by those aged 41–50 years (32%, n = 16) and under 40 years (28%, n = 14). Nearly all farmers had more than 10 years of farming

Table 3. Socio-demographic characteristics of dairy farmers participating in the study (n = 50).

Characteristic	Category	n (%)
Age (years)	< 40	14 (28)
	41–50	16 (32)
	> 50	20 (40)
Experience in Dairy Farming	0–10 years	1 (2)
	> 10 years	49 (98)
Number of Cattle Owned	1–10	39 (78)
	11–20	9 (18)
	> 20	2 (4)
Sex	Male	37 (74)
	Female	13 (26)
Breeding activity status	As the main job	38 (76)
	As a side job	12 (24)

experience (98%, n = 49). Most owned 1–10 cattle (78%, n = 39), while 18% (n = 9) owned 11–20 animals and 4% (n = 2) owned more than 20. Male farmers predominated (74%, n = 37), and dairy farming served as the primary occupation for most participants (76%, n = 38).

Farmer attitudes and practices regarding helminthiasis management are summarized in Table 4. Overall, respondents demonstrated high awareness of anthelmintic guidelines, with 88% (n = 44) agreeing that treatments should strictly follow label recommendations and 92% (n = 46) recognizing that forage should be harvested at least 10 cm above the ground to minimize parasite risk. Regarding veterinary intervention, 68% (n = 34) acknowledged the importance of professional consultation, while only 10% (n = 5) believed that drug price correlated with efficacy.

In practice, this proactive approach was reflected by 80% (n = 40) of farmers consulting veterinarians and 88% (n = 44) reading drug instructions before administration. Despite these positive attitudes toward forage height, the study

Table 4. Attitudes and practices of dairy farmers toward helminthiasis and anthelmintic administration.

Question	Agree/Often n (%)	Disagree/Sometimes n (%)	Not Sure/Never n (%)
Attitudes*			
- More expensive anthelmintics work better.	5 (10)	18 (36)	27 (54)
- Consultation with a veterinarian is necessary before administering anthelmintics.	34 (68)	7 (14)	9 (18)
- Anthelmintic administration should follow the dosage indicated on the label.	44 (88)	3 (6)	3 (6)
- Forage should be cut 10 cm above the ground.	46 (92)	0 (0)	4 (8)
Practices**			
- I consult a veterinarian regarding anthelmintic treatment.	40 (80)	4 (8)	6 (12)
- I read the instructions before administering the drug.	44 (88)	2 (4)	3 (8)
- I use more than one type of anthelmintic (bolus, injection, pour-on).	3 (6)	2 (4)	45 (90)
- I administer deworming drugs by placing them directly into the animal's mouth.	28 (56)	3 (6)	19 (38)

area relies entirely on a cut-and-carry system from communal areas, which may act as a persistent transmission route. The most common administration route was oral drenching (56%, n = 28), with very few farmers (6%, n = 3) utilizing multiple types of anthelmintics.

Discussion

The prevalence of GI helminths in Batu City is notably lower than in other regions of Indonesia. For comparison, beef cattle in Sumedang, West Java, exhibit a 47.6% overall prevalence, with 38.1% attributed to strongyle types, while Bangkalan, East Java, recorded a 20% helminthiasis rate during the dry season. Similarly, Malang District and Boyolali have reported fasciolosis rates of 30% and 16.50%, respectively [33–36,42]. This comparatively low prevalence in the study area is likely influenced by a combination of environmental constraints and proactive management.

The low prevalence of *Moniezia* spp. in this study may be attributed to specific forage management practices. Moniezia infections are transmitted through the ingestion of oribatid mites containing infective cysticercoids. Because these mites primarily reside in the lower layers of the pasture, farmers' adherence to appropriate forage cutting heights likely limited the ingestion of these intermediate hosts [23,24]. This suggests that current feeding management serves as a mechanical barrier against tapeworm transmission.

In contrast, infections with Fasciola spp. and amphistomes occur through the ingestion of metacercariae encysted on vegetation near water sources. The arid conditions and below-normal rainfall documented during the study period likely restricted the development of the snail intermediate hosts (*Lymnaea* and *Indoplanorbis*) and the subsequent encystment of metacercariae. Consequently, the combination of environmental stressors and controlled forage harvesting appears to significantly reduce the infection pressure for both cestodes and trematodes in this highland dairy system [37,38].

Eimeria spp. infections were detected in dairy cattle but remained subclinical, with no cases of clinical coccidiosis observed. This study identified Eimeria only at the genus level; therefore, species-level attribution was not possible. Pathogenic bovine Eimeria species, such as *E. bovis* and *E. zuernii*, are known to be associated with clinical coccidiosis in cattle, but the absence of clinical signs in this study may reflect low infection levels and management conditions that limited disease expression. Although this study was conducted during the dry season, previous studies have shown that Eimeria transmission may increase under humid conditions. The detection of *Eimeria* spp. during the dry season suggests that environmental contamination may persist even under less favorable conditions. Transmission risk is enhanced by inadequate hygiene, high pen density, shared feeding and watering facilities, poor ventilation, and infrequent cleaning, particularly in intensive and semi-intensive housing systems [30,31].

Age and housing type emerged as important epidemiological determinants in this study. Calves showed a higher prevalence of GI parasite infection than adults (38.2% vs. 9.3%), with 6.04-fold higher odds and 4.12-fold higher risk of infection. This finding likely reflects immature immune responses and limited prior exposure to parasitic antigens in young animals. Cattle kept in closed housing systems also showed a higher prevalence than those in open housing systems (15.8% vs. 8.1%), with 2.13-fold higher odds and 1.95-fold higher risk of infection, probably due to reduced sunlight exposure, limited ventilation, and increased moisture retention that may favor the survival of oocysts and infective stages. In contrast, open housing allows for greater sunlight exposure and ventilation, which suppresses larval development [29].

Notably, while the third-stage larvae (L3) of strongyles possess a double cuticle that enhances environmental resilience, the hot and dry conditions documented during the study period likely accelerated metabolic depletion, thereby reducing larval longevity on the pasture and within open-stall environments. The strategic timing of this study indicates that detectable parasite infections persisted during unfavorable dry-season conditions, suggesting a baseline level of infection pressure that may increase during the rainy season [25,26]. The dominance of strongyle-type nematodes in terms of infection intensity aligns with reports from smallholder dairy systems in Ethiopia and India [21–23].

Farmer behavioral practices significantly influence the therapeutic landscape of the region. While the frequent consultation with veterinarians is a positive indicator for reducing anthelmintic resistance risk, the observed price-quality bias—where farmers equate higher cost with higher efficacy—suggests a need for better evidence-based education [2,13]. Furthermore, the occasional practice of mixing anthelmintics with feed or water risks underdosing, which can accelerate the development of resistant parasite strains [14,27,32]. The increased veterinary supervision following the FMD outbreak likely contributed to the current low infection intensity, as farm-level biosecurity and health awareness were heightened during this period [17].

Despite providing a critical baseline, this study has limitations regarding diagnostic depth. The reliance on morphological identification by microscopy precluded species-level differentiation for several parasite taxa, particularly strongyle-type nematodes, *Moniezia spp.*, and amphistome eggs. In addition, dry-season sampling does not capture the full scale of seasonal fluctuations. Future research should incorporate longitudinal sampling and molecular diagnostics, alongside Fecal Egg Count Reduction Tests (FECRT), to monitor for anthelmintic resistance and provide a comprehensive year-round epidemiological profile.

5. Conclusions

This study demonstrates that gastrointestinal parasitism in Batu City's dairy sector is a multifactorial challenge primarily driven by host age and intensive management, with *Eimeria* spp. emerging as the dominant pathogen (5.50%). The significant association between closed housing systems (OR = 2.13; RR = 1.95) and infection status—likely due to increased humidity and fecal accumulation—highlights a critical environmental risk factor that sustains parasite transmission cycles. Furthermore, the high vulnerability of calves (OR = 6.04; RR = 4.12) suggests that current control measures are insufficient for young stock. Therefore, effective parasite management in East Java must transition from generic deworming to an integrated paradigm that prioritizes coccidiosis control in calves, improves hygiene within closed stall designs to disrupt oocyst maturation, and supports veterinarian-guided anthelmintic use with periodic efficacy monitoring to mitigate the future risk of anthelmintic resistance.

Abbreviations

EPG, Egg Per Gram; FECRT, Fecal Egg Count Reduction Test; FMD, Foot-and-Mouth Disease; GI, Gastrointestinal; L3, Third-stage larvae; OPG, Oocyst Per Gram; OR, Odds Ratio; RR. Relative Risk (or Risk Ratio).

Availability of Data and Materials

All data related to this study is available in the manuscript.

Author Contributions

Conceptualization: S.K., and N.N.; Methodology: S.K., R.N.R., and N.N.; Investigation: A.S., G.A.D., and R.N.R.; Writing - original draft: S.K., A.S., and R.N.R., G.A.D.; Writing - review & editing: S.K., and N.N.; Finding Acquisition: S.K.; Resources, S.K.; Supervision, S.K., and N.N.

Ethics Approval and Consent to Participate

This study was approved by the University Brawijaya Ethics Committee (approval no. 092-KEP-UB-2023).

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Histomorphometric and Immunohistochemical Characterization of Hepatic Cirrhosis in Algerian Dromedary Camels (*Camelus dromedarius*)

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Abstract

Background: The dromedary camel is a socio-economically important species in arid regions, and hepatic cirrhosis represents a major threat to its health and productivity. Despite its clinical relevance, a comprehensive pathomorphological description of cirrhosis in this species has been lacking. **Methods**: Liver samples from 24 adult dromedary camels (both sexes, aged approximately 6–12 years), with gross lesions suggestive of cirrhosis and 24 age-matched healthy controls were collected from an abattoir in El Oued, Algeria. Histochemical stains (H&E, Azan trichrome) and immunohistochemical markers for macrophages (CD68) and activated myofibroblasts (α-SMA) were applied. Quantitative histomorphometric analysis was conducted to determine tissue composition. **Results**: Macroscopically, cirrhotic livers showed a significant reduction in mass (8.12 ± 1.86 kg to 6.30 ± 1.25 kg, $p < 0.01$), accompanied by marked nodularity and a firm consistency. Histologically, normal hepatic architecture was replaced by fibrous septa and regenerative nodules. Immunohistochemistry confirmed increased $CD68^+$ macrophages and $\alpha\text{-SMA}^+$ stellate cells in fibrotic areas. Morphometric analysis demonstrated a marked increase in the volume density (Vv) of connective tissue (33.25 ± 2.31% vs. 7.25 ± 2.65%, $p < 0.001$) accompanied by a significant reduction in the Vv of hepatocyte (54.34 ± 4.24% vs. 82.64 ± 4.87%, $p < 0.001$) in cirrhotic livers compared with controls. **Conclusions**: This study provides the first quantitative immunomorphometric characterization of hepatic cirrhosis in Algerian dromedary camels and establishes pathological benchmarks for diagnosis and future research.

Keywords

Dromedary, Cirrhosis, Hepatic pathology, Immunohistochemistry, Morphometry, Liver

1. Introduction

The dromedary camel (*Camelus dromedarius)* is an economically and culturally important livestock species well adapted to the arid and semi-arid environments of North Africa, including Algeria [1,2]. It serves as a major source of meat, milk, and transport for rural communities. The liver, being central to metabolism, detoxification, and synthesis, plays a crucial role in maintaining animal health and productivity.

Hepatic pathology in dromedary camels has significant economic consequences as liver lesions often cause partial or total organ condemnation at slaughterhouses and chronic liver disease can impair growth, productivity, and carcass quality,

ultimately affecting local and international camel trade [3].

Hepatic cirrhosis represents the terminal stage of chronic liver injury, characterized by irreversible replacement of hepatic parenchyma by fibrous tissue and the formation of regenerative nodules [4,5]. While this condition has been extensively studied in humans and common domestic animals, its occurrence and pathological characteristics in camels remain poorly documented [6,7]. In camels, several factors have been suggested as possible causes of chronic hepatic damage, including parasitic infections such as hydatidosis and fascioliasis [8,9], exposure to hepatotoxic plants and mycotoxins, as well as nutritional imbalances [10,11].

These insults trigger the activation of hepatic stellate cells, which transform into α-smooth muscle actin (α-SMA)–positive myofibroblasts, leading to excessive extracellular matrix deposition and fibrosis [12].

From a pathogenic perspective, hepatic fibrosis is driven by the interaction between inflammatory cells and fibrogenic effector cells. CD68-positive macrophages play a central role by releasing pro-fibrotic cytokines, while activation of hepatic stellate cells into α-SMA–positive myofibroblasts represents the main source of extracellular matrix deposition [13].

Despite the suspected importance of chronic liver diseases in camel health, comprehensive pathological descriptions and quantitative data are lacking. The present study was therefore designed to characterize hepatic cirrhosis in Algerian dromedary camels through a combination of gross pathology, morphometric analysis, histochemical evaluation, and Immunohistochemical assessment. This approach aimed to establish objective diagnostic criteria and provide reference data for future veterinary investigations.

2. Materials and Methods

2.1 Study Design and Sampling Site

A cross-sectional study was conducted between January 2022 and January 2024 at the municipal abattoir of El Oued Province, southeastern Algeria, a region with an arid Saharan climate. Liver specimens were collected post-slaughter from 48 adult dromedary camels *(Camelus dromedarius)* aged between five (5) and 12 years of both sexes. The control group (n = 24) consisted of livers without visible gross lesions, while the cirrhotic group (n = 24) included livers with macroscopic alterations consistent with cirrhosis, such as nodularity, firmness, and discoloration.

Livers were classified as cirrhotic based on the presence of diffuse fibrous septa forming regenerative nodules, disruption of normal lobular architecture, and associated macroscopic alterations such as nodularity and firmness. Samples showing limited fibrous expansion without nodule formation or architectural distortion were not included in the cirrhotic group. All samples were obtained during routine meat inspection procedures, and therefore no specific ethical approval was required. Each liver was photographed and weighed using a precision electronic balance.

2.2 Gross Examination

Gross examination included assessment of liver color, surface features (nodularity, capsular thickening, adhesions), consistency, associated lesions (e.g., parasitic cysts), and finally liver mass. Liver mass was measured using a precision electronic balance (±1 g accuracy). Representative tissue samples (≈1 cm^3) were taken from each liver, fixed in 10% neutral buffered formalin for at least 72 h, processed using the routine paraffin embedding technique, and sectioned at 4–5 µm.

Gross examination was performed according to standard veterinary pathological criteria as previously described by [14].

2.3 Histological and Immunohistochemical Analysis

Histochemical staining with hematoxylin and eosin (H&E) and Azan trichrome was performed following the manufacturers' standard protocols (Merck, Darmstadt, Germany). Briefly, deparaffinized sections were rehydrated through graded alcohols, stained with hematoxylin for 5 min, differentiated, counterstained with eosin for 2 min, dehydrated, and mounted. Azan trichrome staining was performed according to the

manufacturer's instructions for collagen visualization.

Immunohistochemistry was performed using a mouse monoclonal anti-CD68 antibody (clone KP1, Dako, Denmark; dilution 1:100) and a mouse monoclonal anti-α-smooth muscle actin antibody (clone 1A4, Dako, Denmark; dilution 1:200). Sections were incubated with primary antibodies overnight at 4°C. Detection was carried out using a streptavidin–biotin peroxidase system with diaminobenzidine (DAB) as chromogen.

2.4 Histomorphometric analysis

Quantitative histomorphometric analysis was performed to assess the relative composition of liver tissue. Digital photomicrographs of H&E-stained sections were captured at 200× magnification using a light microscope equipped with a digital camera (Olympus BX51, Olympus Corporation, Tokyo, Japan) and analyzed using ImageJ software (National Institutes of Health, USA), and the underlying structures were classified as hepatocytes, connective tissue, blood vessels/sinusoids, or bile ducts/ductules.

The volume density (Vv) was calculated as:
Vv = (Number of points hitting the structure / Total number of points) × 100.
The methodology followed the approach described by Avtondilov [15].

Digital photomicrographs were analyzed using calibrated morphometric software. All slides were independently evaluated by two observers blinded to group allocation.

2.5 Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics v26.0. Data for liver mass and morphometric parameters are expressed as mean ± standard deviation (SD). The normality of distribution was assessed using the Shapiro–Wilk test. Differences between the control and cirrhotic groups were analyzed using an independent samples Student's t-test. If normality assumptions were not met, the Mann–Whitney U test was applied as a non-parametric alternative. All statistical tests were two-tailed.

Differences were considered statistically significant at $p < 0.05$, with highly significant differences indicated at $p < 0.01$ and $p < 0.001$.

3. Results

3.1 Macroscopic Findings

Gross examination revealed distinct differences between healthy and cirrhotic livers. Healthy livers exhibited a characteristic dark brown color, a smooth capsular surface, and a soft, uniform consistency, with no associated lesions (Fig. 1A). In contrast, cirrhotic livers showed discoloration, a markedly nodular surface architecture ranging from micronodular to macronodular patterns, firm to hard consistency upon palpation, and occasional associated lesions such as parasitic cysts (Fig. 1B).

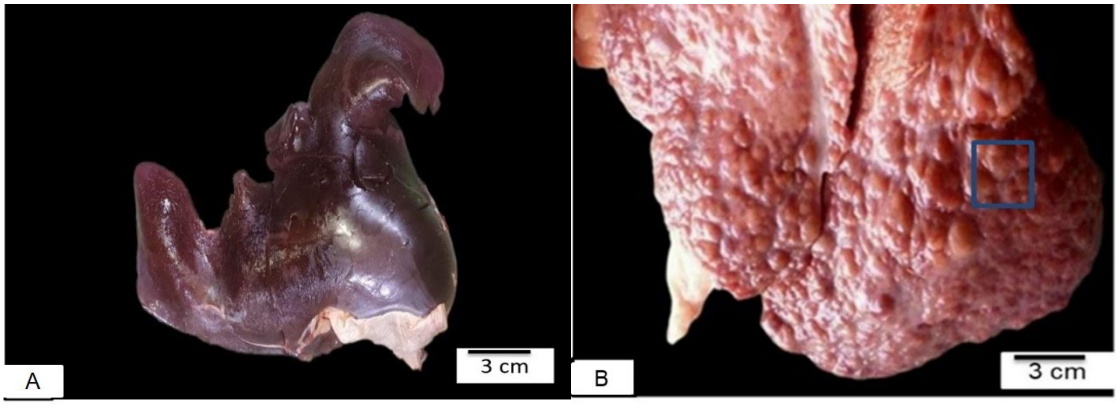

Figure 1. Macroscopic comparison: Healthy liver (A) and macronodular cirrhotic liver (B) of a dromedary camel *(Camelus dromedarius)* from the El Oued Region.

Macroscopic lesions in cirrhotic livers were predominantly concentrated in the peripheral regions of the hepatic parenchyma, with nodules extensively involving the subcapsular and peripheral areas of the liver lobes. Fibrotic changes were pronounced in these peripheral zones and extended inward, resulting in severe and generalized architectural distortion.

No significant differences in age range or sex distribution were observed between the control and cirrhotic groups.

Quantitative analysis confirmed a significant reduction ($p < 0.01$) in the mean mass of cirrhotic livers (6.30 ± 1.25 kg) compared to the healthy control group (8.12 ± 1.86 kg), representing a mean mass loss of approximately 22.41% (Table 1).

3.2 Histopathological Findings

Histological examination of healthy livers revealed a well-preserved lobular architecture. Hepatocytes were organized in regular radial plates extending from the central vein, with intact hepatic sinusoids interposed between the hepatocellular cords. The hepatocytes exhibited a polygonal morphology with homogeneous cytoplasm and centrally located nuclei, without evidence of cellular degeneration, necrosis, fibrosis, or inflammatory infiltration. Portal tracts appeared unremarkable, confirming the structural integrity of the hepatic parenchyma. At low magnification, the liver tissue displayed a homogeneous appearance with preserved lobular organization and an absence of fibrous septa or regenerative nodules, consistent with normal histological architecture (Fig. 2A and 2B).

Table 1**.** Morphometric comparison of liver mass (mean ± SD) between healthy and cirrhotic dromedary camels.

Characteristic	Minimum (kg)	Maximum (kg)	Mean ± SD (kg)	p-value
Healthy liver mass	5.50	10.27	8.12±1.86	
Cirrhotic liver mass	4.20	8.75	6.30±1.25**	0.008

Values are expressed as mean ± SD. Statistical significance: *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

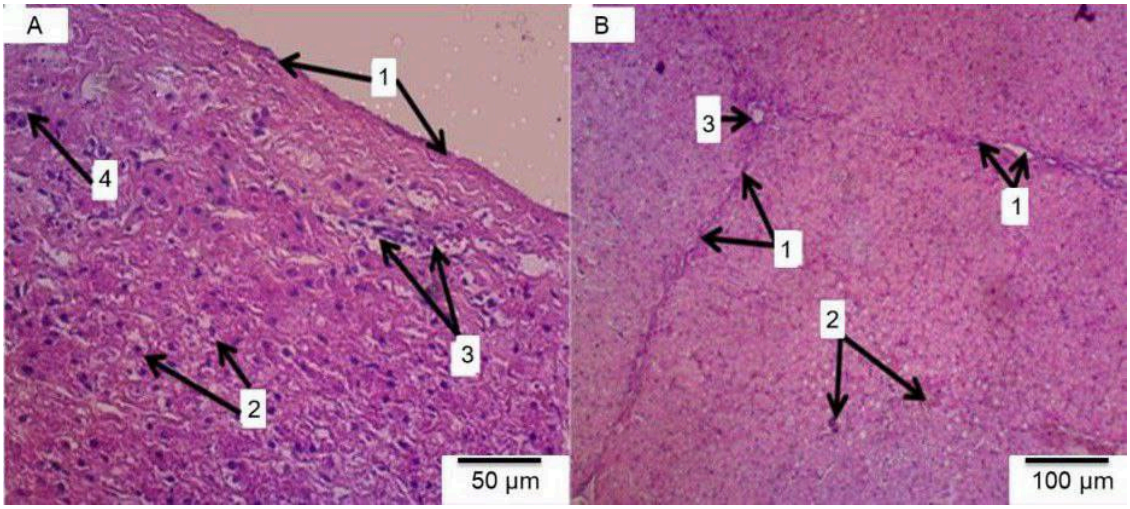

Figure 2. Histological section of a healthy dromedary liver, H&E staining, A: 1- Glisson's capsule; 2- hepatocytes, 3- hepatic sinusoids; 4- Kupffer cells, X400. B: 1- interlobular connective tissue, 2- hepatocytes, 3- central vein, X100.

In cirrhotic livers, the normal hepatic architecture was completely disrupted. Figure 3 illustrates cirrhotic dromedary liver tissue stained with Azan trichrome. At higher magnification (Fig. 3A), thick collagen-rich fibrous septa intensely stained blue are observed extensively subdividing the hepatic parenchyma. These septa contain proliferating bile ducts and small vascular structures and exert compressive effects on the adjacent hepatic tissue, consistent with advanced fibrotic remodeling. At lower magnification (Fig. 3B), a well-defined regenerative nodule is evident, surrounded by dense fibrotic bands. The fibrous septa are associated with inflammatory cell infiltrates and vascular elements, reflecting marked architectural distortion characteristic of established hepatic cirrhosis.

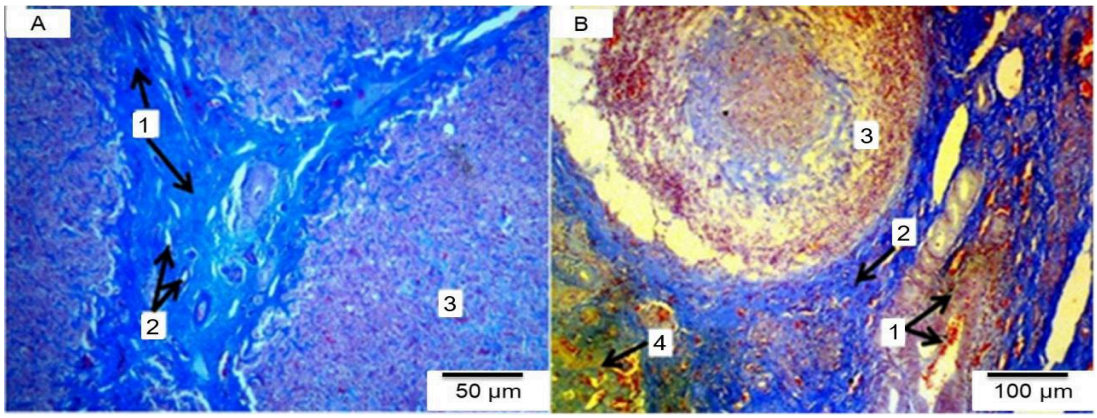

Figure 3. Histological section of dromedary liver tissue, affected by hepatic cirrhosis, Azan trichrome staining, A: 1- Fibrous septa; 2- Blood vessels; 3- Hepatic parenchyma, X400. B: 1- Blood vessels; 2- Fibrous tissue; 3- regeneration nodules; 4- Inflammatory infiltrate, X100.

3.3 Immunohistochemical Findings

In cirrhotic livers, α-SMA immunoreactivity was intensely localized within numerous elongated, spindle-shaped cells distributed along thick fibrous septa. Strong brown cytoplasmic DAB staining was observed in cells embedded within densely collagenized connective tissue and arranged parallel to fibrotic bands, whereas surrounding hepatocytes exhibited minimal to no immunoreactivity (Fig. 4A). CD68 immunostaining revealed numerous positively stained macrophages predominantly localized within septal inflammatory infiltrates and peri-nodular regions. These CD68-positive cells appeared either individually dispersed or clustered within fibrotic areas (Fig. 4B).

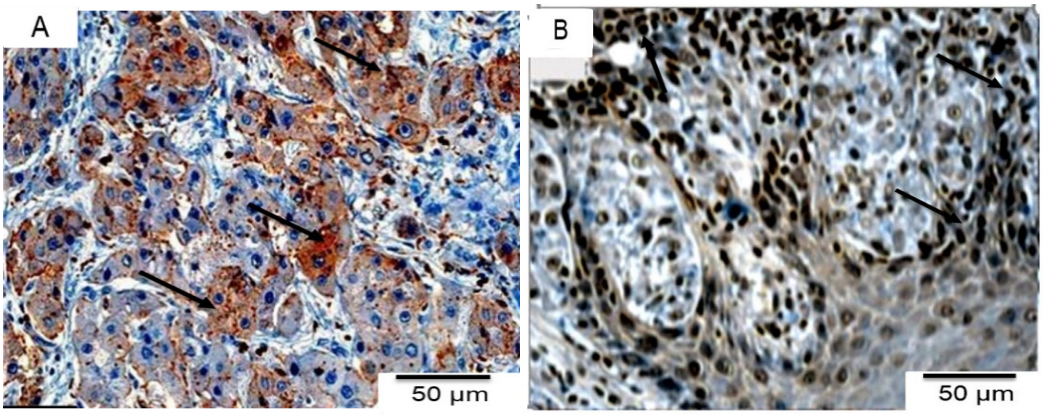

Figure 4. Immunohistochemical staining of hepatic parenchyma from dromedary camels with hepatic cirrhosis (×400). A: positive staining with α-SMA antibody . B: positive staining with CD68 antibody.

The spatial colocalization of activated stellate cells and macrophages within fibrotic septa underscores their coordinated contribution to sustained extracellular matrix deposition and progressive architectural remodeling. Collectively, these immunohistochemical findings confirm the pivotal and synergistic involvement of hepatic stellate cells and macrophages in the pathogenesis and progression of camel hepatic cirrhosis.

3.4 Histomorphometric analysis

The point-counting morphometric analysis provided quantitative data on the extensive tissue remodeling observed in cirrhotic livers (Table 2). The most pronounced change was a greater than 4.5-fold expansion of connective tissue, which increased from 7.25 ± 2.65% in healthy livers to 33.25 ± 2.31% in cirrhotic livers ($p < 0.001$). Concurrently, the proportion of functional hepatocytes was markedly reduced from 82.64 ± 4.87% to 54.34 ± 4.24% in cirrhotic livers ($p < 0.001$). Smaller but statistically significant increases were also observed in the vascular (blood vessels and sinusoids) and biliary compartments ($p < 0.05$).

Table 2. Histomorphometric composition (%) of hepatic tissue in healthy and cirrhotic dromedary camels.

Tissue Component	Healthy Liver (% ± SD)	Cirrhotic Liver (% ± SD)	p-value
Hepatocytes	82.64 ± 4.87	54.34 ± 4.24***	$p < 0.001$
Blood vessels + Sinusoids	9.57 ± 1.44	11.68 ± 2.05*	0.041
Bile ducts + Canaliculi	0.18 ± 0.12	0.41 ± 0.21*	0.032
Connective tissue	7.25 ± 2.65	33.25 ± 2.31***	$p < 0.001$

Values are expressed as mean ± SD. Statistical significance: *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

This study provides the first comprehensive histopathological and histomorphometric characterization of hepatic cirrhosis in the dromedary camel, a disease of considerable economic and animal welfare relevance in arid regions. The observed pathological features are broadly consistent with the established mechanisms of cirrhosis described in other species, while also reflecting aspects that may be particularly relevant to camel physiology [16].

The most prominent gross alteration was the significant reduction in liver mass (approximately 22%) in cirrhotic animals, as demonstrated in the present study. Such hepatic atrophy is a characteristic feature of advanced cirrhosis and results from progressive loss of functional hepatocytes and their replacement by dense fibrous tissue [17]. The nodular surface and increased firmness observed macroscopically directly correspond to the microscopic processes of regenerative nodule formation and fibrotic septation.

Histologically, the complete disruption of normal lobular architecture and its subdivision into regenerative nodules by broad fibrous septa represent the defining hallmarks of cirrhosis [15]. The associated ductular proliferation and mononuclear inflammatory infiltrates observed within fibrous septa reflect a chronic reparative response to sustained hepatic injury, a pattern commonly reported in chronic liver diseases across species [18,19].

The severity of these structural alterations was objectively confirmed by quantitative histomorphometric analysis. Connective tissue content increased more than 4.5-fold in cirrhotic livers, while hepatocyte volume density decreased markedly from approximately 82% to 54%. This quantitative shift provides a robust and objective measure of parenchymal loss and functional compromise, reinforcing the advanced stage of disease in the examined animals [20].

Immunohistochemical findings, interpreted qualitatively rather than quantitatively, further elucidated the cellular mechanisms underlying fibrogenesis. The marked expansion of α-SMA-positive myofibroblasts within fibrous septa confirms the activation of hepatic stellate cells,

which are universally recognized as the principal source of pathological collagen deposition in liver fibrosis [21]. Concurrently, the increased presence of CD68-positive macrophages indicates persistent inflammatory activity. These cells secrete profibrotic mediators such as transforming growth factor-ß and platelet-derived growth factor, which sustain stellate cell activation and perpetuate extracellular matrix accumulation [22].

The interaction between activated myofibroblasts and inflammatory macrophages likely establishes a self-perpetuating cycle of injury, inflammation, and fibrosis, accounting for the progressive and irreversible nature of cirrhosis [23]. Although similar pathogenic pathways have been described in other species, their demonstration in camels is of particular importance given the scarcity of detailed pathological data in this species.

From a species-specific perspective, the functional implications of these architectural changes may be especially pronounced in camels dromedary. The extensive replacement of hepatocellular parenchyma by fibrous tissue is expected to impair essential hepatic functions, including metabolism, detoxification, and protein synthesis. Considering the camel's physiological adaptation to prolonged nutritional and water stress in arid environments, such severe structural damage may remain clinically unapparent until advanced stages, potentially explaining delayed detection despite extensive histopathological lesions [24].

Conclusions

In conclusion, this study establishes a definitive histopathological and immunomorphometric profile of hepatic cirrhosis in dromedary camels. We have quantitatively demonstrated the replacement of functional parenchyma by fibrous tissue and identified the key cellular players such as activated myofibroblasts and macrophages that are involved in this process. These findings provide robust diagnostic criteria and quantitative benchmarks that will serve as a valuable tool for veterinary pathologists and clinicians in diagnosing and understanding the pathogenesis of chronic liver disease in this economically vital species.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

Conceptualization, A.C.B.; Methodology, A.C.B.; Investigation, A.C.B.; Data Analysis, A.C.B.; Writing – Original Draft, A.C.B.; Review & Editing, D.E.R.; Supervision, D.E.R.

Ethics Approval and Consent to Participate

All samples were collected post-slaughter during routine meat inspection; hence no ethical approval was required.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Determinants of Pet Owners' Knowledge, Attitudes, and Practices on Animal Bites and Rabies Prevention and Control in the Municipality of Alaminos, Laguna: A Health Belief Model Approach

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Abstract

Background: Despite ongoing national efforts to eliminate rabies, the Philippines continues to experience high animal bite incidence and human rabies deaths. Local governments face challenges compounded by limited understanding of pet owners' beliefs and behaviors. This study aimed to determine how pet owners' knowledge, attitudes and practices (KAP) toward animal bites and rabies prevention and control in Alaminos, Laguna are influenced by the Health Belief Model. **Methodology:** A cross-sectional survey was conducted among 402 randomly selected pet-owning households using an HBM-based questionnaire. KAP scores were analyzed using descriptive statistics and non-parametric tests. **Results:** There were significant positive correlations between knowledge versus attitudes (ρ=0.385, $p<0.001$), and between attitudes versus practices (ρ=0.372, $p<0.001$), but the correlation between knowledge versus practices was positive but weak despite being statistically significant (ρ=0.157, p=0.002). Dog owners who acquired pets from breeders had higher rates of vaccination and registration. Age, employment status, information source, and exposure history significantly influenced KAP, while education and sex did not show consistent patterns. **Conclusion:** Pet owners' knowledge of animal bites and rabies does not consistently lead to safe practices. Strategies that will focus on improving attitudes, addressing behavioral barriers, and engaging communities through practical, trust-building approaches will be more effective than knowledge alone in strengthening rabies prevention and control.

Keywords

Animal bites, Rabies, KAP survey, Health Belief Model

1. Introduction

Rabies is a neglected tropical disease (NTD) that poses a significant public health concern, primarily affecting impoverished and vulnerable populations. It exists in over 150 countries, resulting in approximately 59,000 human deaths worldwide annually, with the majority occurring in Asia and Africa. Alarmingly, 40% of these fatalities affect children under 15 years old. Rabies is almost 100% fatal once clinical signs appear, making swift action crucial [1,2].

Rabies is a zoonotic disease caused by the rabies virus (RABV) of the Lyssavirus genus [3,4]. Dogs are the primary reservoir, causing 90-99% of human rabies deaths worldwide [4,5,6,7]. Accurately estimating its burden remains difficult

due to scarce dog bite data. Dog bites cause millions of injuries yearly across low- and middle-income countries (LMICs). These countries have higher dog bite fatality rates due to rabies prevalence and healthcare access barriers, causing an estimated economic cost of US$8.6 billion [6]. Challenges in LMICs include maintaining dog vaccination coverage, affordability of post-exposure prophylaxis (PEP), and weak surveillance systems [1,8].

In the Philippines, animal bites persist as a significant cause of morbidity despite declines in reported cases. Animal bite cases are still alarmingly high [9] with 503,167 documented cases and a case rate of 450.98 cases per 100,000 people in 2022 [10]. Dog bites are the primary source of human rabies, with 285 reported cases of rabies in 2007 and 276 cases in 2018 [9]. DOH aims to declare the Philippines rabies-free by 2030 [10,11,12] by implementing strategies such as providing PEP to all Animal Bite Treatment Centers (ABTCs), allocation of pre-exposure prophylaxis (PrEP) to high-risk individuals and school children in high incidence zones; enhanced public awareness through information, education and communication (IEC) promotion, observance of World Rabies Day every September 28 and Rabies Awareness Month every March; training of medical providers on treating rabies victims, and establishment of ABTCs in every interlocal health zones [9,13].

In 2018, Region 4-A ranked as having the second-highest cases of human rabies in the Philippines, with 30 reported cases [9]. All human rabies cases reported resulted in death, which makes case rate and mortality rate synonymous. As reported by DOH Region 4-A, there has been a 42% reduction in rabies cases during the first two months of 2023. However, this reduction in rabies cases does not reflect the incidence of animal bites, which continue to pose a risk factor for rabies transmission [14].

Republic Act No. 9482, or The Anti-Rabies Act of 2007, aims to control, prevent, and eventually eradicate human and animal rabies in the Philippines. It established the National Rabies Prevention and Control Program (NRPCP), which promotes responsible pet ownership and requires local government units (LGUs) to conduct dog registration and vaccination, manage stray dogs, and establish dog pounds [15].

Although pre- and post-exposure prophylaxis help prevent human rabies, evidence shows that mass dog vaccination (MDV) is more cost-effective in reducing human rabies exposure [7]. Dog population management and environmental control measures are also instrumental in deterring free-roaming animals from going through human garbage. It demonstrates the One Health approach by controlling canine rabies and reducing human transmission [16].

NRPCP reported in 2015 that vaccine coverage among dogs in Region 4-A was about 38.9%, which is way below the target of 70% coverage. Furthermore, this estimate is based on a calculation of 1 dog to 10 human ratios, which is refuted by other studies showing a higher dog population. Hence, the reported dog vaccination coverage may be an overestimation [17].

The Mandanas-Garcia Ruling mandated the devolution of governmental functions, including the NRPCP, to LGUs in the Philippines [11]. This ruling increased LGUs' share of national taxes by expanding the internal revenue allotment (IRA) base to include Bureau of Customs (BOC) collections and other revenues [18]. This enables LGUs to better address the specific needs of their constituents, considering disparities in local capabilities [19], emphasizing decentralization and local empowerment. However, LGUs show varying technical readiness for devolution [20], and studies report misconceptions and non-compliance to regulations among stakeholders [21,22,23].

The Health Belief Model (HBM) is a key framework in health behavior research [24]. It guides health behavior interventions by considering personal beliefs about a disease and strategies available to decrease its occurrence [25,26]. These beliefs are shaped by unconscious associations formed over time and influenced by the political context that defines the relationship between the individual and the State [24]. The link between these factors and dog owners' intent to vaccinate remains unclear, highlighting the need for further insight into psychological determinants of protective behavior. Rabies elimination in dogs depends on the commitment and collaboration of the stakeholders involved. HBM helps define factors influencing health behaviors, such as perceived benefits, risks, barriers, technological needs, and attitudes toward rabies control. Studies

in Ethiopia [25]; Australia [27]; Indonesia [26,28,29]; Nepal [30]; and the Philippines (Camarines Norte and Sur) [22] identified that socio-demographic, cultural beliefs, bite history, access to healthcare, ownership status of the biting animal, bite severity, monthly spending, information sources, level of knowledge, and political initiative influence health seeking behavior and willingness to participate in One Health collaboration.

This study aimed to determine how pet owners' KAP towards animal bites and rabies prevention and control in Alaminos, Laguna are influenced by their socio-demographic characteristics and pet ownership history within the HBM.

The findings provide evidence-based insights for local government units, public health professionals and veterinary stakeholders to improve information and education campaigns, enforce policies, and enhance community engagement. This also contributes to the knowledge pool on health behavior and zoonotic disease prevention at the grassroots level.

2. Methodology

2.1. Research Design and Study Setting

This study used a quantitative cross-sectional design with an interview-guided questionnaire administered to pet owners in Alaminos, Laguna.

Alaminos is a third-class municipality in Laguna's third congressional district, spanning 5,363 hectares across 15 barangays. According to the 2020 census, it had 51,619 residents and 13,249 households. The local rabies control program is managed by the Municipal Agriculture Office (MAO) and the Rural Health Unit (RHU). The MAO handles animal control and vaccination, while the RHU administers PEP to humans.

The municipality recorded four rabies cases in 2020. Despite this, animal bite cases increased from 406 in 2020 to 624 in 2022 [31].

2.2. Study Population and Sampling Design

The survey was conducted among households of pet owners across the 15 barangays of Alaminos, Laguna. The recruitment of households for interviews used cluster random sampling based on their barangay residents' listings.

The estimated proportion of pet-owning households in Alaminos, Laguna was derived from Dizon *et al*. (2022), which reported that 54.1% of households in Central Luzon owned dogs [21]. This yielded a sample size of 408 households at a 95% confidence level and 5% margin of error, including a 10% allowance for non-response. The sample per barangay was proportionally weighted based on the number of registered households.

2.3. Inclusion and Exclusion Criteria

Only households currently owning pets (dogs or cats) and having an adult (18 years old and older) available for interview were included in the survey. Persons who were not permanent residents of the household, below 18 years of age, unable to understand the purpose of the study despite explanation, and who did not provide consent were excluded as household informants.

2.4. Development of Data Collection Tool

The questionnaire was developed to follow a HBM approach to explore key constructs that influence health protection behaviors, namely knowledge, perceived threat, perceived benefits, perceived barriers, readiness to action and self-efficacy [25]. The questionnaire was developed based on the contents of the RA 9482 and its IRR [15].

Perceived threats include perceived susceptibility and severity about the risk of exposure to animal bites and rabies, and concerns about the seriousness of the illness. Perceived benefits relate to the outcome that reduces susceptibility or severity. Perceived barriers identify concerns or negative beliefs about the intended protective behavior. Cues to action are strategies or information sources that promote protective behavior adoption. Self-efficacy measures the pet owner's confidence in their ability to adopt the behavior.

The questionnaire collected socio-demographic data, rabies exposure history, and pet vaccination practices. The first section assessed respondents' knowledge of animal bites and rabies prevention. The questions covered human, animal, and environmental aspects of rabies. The second section measured willingness to

comply with interventions. Questions in both sections were answered with "yes," "no," and "I don't know." The third section covered perceptions and attitudes, while the fourth section focused on practices. The third and fourth sections used a Likert Scale to gauge levels of agreement or disagreement.

Content validation was conducted by a panel of two public health experts. The questionnaire demonstrated excellent relevance (S-CVI/Ave = 1.00; S-CVI/UA = 1.00) and high clarity (S-CVI/Ave = 0.96; S-CVI/UA = 0.96). It was pre-tested among fifty (50) pet owners in Montalban, Rizal to assess its reliability and usability. The instrument showed acceptable internal consistency, with a Cronbach's alpha of 0.76, indicating good reliability.

2.5. Data Collection

Approval was secured from the Office of the Municipal Mayor followed by coordination with the Association of Barangay Captains (ABC) president and barangay health worker (BHW) president.

A one-day workshop was held in August 2024 to orient data collectors and ensure consistent implementation of the survey methodology. Fifteen (15) barangay health workers and the BHW president attended the training. The trained workers conducted a two-week house-to-house survey, interviewing pet owners using a structured Filipino-translated questionnaire. Each interview lasted approximately 15–30 minutes, with written informed consent obtained before participation.

2.6. Study Variables

Dependent variables included the level of rabies prevention and control knowledge, attitudes, and practices. Independent variables included personal factors such as history of pet ownership, history of exposure to animal bites and rabies, and source of information. Confounding variables include socio-economic demographic factors, such as age, educational status, and economic status, among others.

2.7. Data Analysis

Socio-demographic data were encoded and analyzed using descriptive statistics. For the knowledge questions, one point was assigned for each correct answer, while zero was given to incorrect and "I don't know" answers. For perception and practice questions, scores from 1 to 5 were assigned for each option, with 5 being the highest score, indicating positive perception or good practices. Scoring was reversed for items depicting negative perception or poor practice [32].

The KAP scores were categorized using Bloom's cut-off point with scores above 80% score as being good or positive, between 60%-80% score as fair or ambivalent, and scores below 60% as being poor or negative [33].

Statistical analysis was done using R Software ver. 4.4.1. Response frequencies were analyzed for each item. Aggregate scores were analyzed using non-parametric tests due to the skewness of the data. Kruskal-Wallis test was used to associate the aggregated KAP scores and variables with more than two groups, and Mann-Whitney U test was used to associate the aggregated KAP scores and variables with only two groups; while Fisher's exact test examined relationships among pet owner characteristics and methods of controlling pet mobility, and pet characteristics and compliance with government program strategies. These nonparametric tests were chosen due to the highly skewed nature of the aggregated scores. Further analysis using Spearman Rank-Order Correlation assessed relationships among KAP scores.

2.8. Ethical Considerations

The research posed minimal risk to the respondents and the community. It was conducted in accordance with the Declaration of Helsinki and the University of the Philippines Code of Research Ethics with reference code UPMREB 2024-0327-EX.

3. Results

A total of 402 respondents representing households owning pets were interviewed using a structured questionnaire (Table 1). The majority of the respondents were 30 to 50 years of age (65.42%), of female gender (64.93%), at least high school level or higher educational attainment (90.05%), earning less than Php 10,000 per household per month (67.16%) and residing in a detached house structure without yard enclosure (60.95%).

Table 1. Socio-demographic characteristics of respondents representing households owning pets in the Municipality of Alaminos, September 2024 (n=402).

Demographic Variable	Count	Percentage
Age group		
19 years old and below	5	1.24
20 to 29 years old	62	15.42
30 to 39 years old	94	23.38
40 to 49 years old	91	22.64
50 to 59 years old	78	19.40
60 years old and above	72	17.91
Sex		
Male	141	35.07
Female	261	64.93
Highest Educational Attainment		
Elementary Level/Graduate	40	9.95
High School Level/Graduate	233	57.96
College Level/Graduate	129	32.09
Employment Status		
Student	11	2.74
Unemployed	149	37.06
Employed	146	36.32
Self-employed	96	23.88
Monthly Household Income		
Less than Php 10,000	270	67.16
Php 10,001 to 20,000	103	25.62
Php 20,001 to 30,000	21	5.22
Php 30,001 to 40,000	3	0.75
More than Php 40,000	5	1.25
Housing Status		
House with enclosed yard	146	36.32
House with no enclosed yard	245	60.95
Apartment	11	2.74

Barangay health workers conducted the survey on weekdays, as reflected in the predominance of female, middle-aged, stay-at-home respondents. Consistent with rural settings, most families lived in detached houses despite household incomes below the poverty threshold set by the Philippine Statistics Authority (PSA) in 2022 [34].

Table 2 presents pet owners' characteristics, including the number of current pets and duration of ownership. The majority had 1 or 2 pets at present (77.36%), and slightly over half (51.99%) owned pets for two years or less. Among the pet owners, only 8.21% always allowed their pets to roam outside the house, and only 18.91% do not restrain their pets when taken outdoors.

Table 2 also shows that a third (32.59%) of pet owner respondents primarily received their information about animal bites and rabies from local government officials, particularly barangay officials and barangay health workers who are ever present in the community. However, it is also notable that another third (34.33%) relied on social media. Social media has become an integral part of daily life. While it could improve awareness through responsible health promotion, health professionals must remain vigilant in addressing misinformation and strengthening fact-checking mechanisms to ensure accurate online health information [35].

Further analysis of the data found in Tables 1 and 2 showed significant associations between housing status and pet management practices. Housing status was significantly related to the frequency of allowing pets to roam outside ($p=0.005$) and to the total number of pets per household ($p=0.004$). Duration of pet ownership was not significantly associated with the use of cages or leashes inside ($p=0.492$) or outside the house ($p=0.635$), but it was significantly associated with the frequency of allowing pets to roam outdoors ($p=0.037$). These findings indicate that

Table 2. Frequency distribution of characteristics of pet ownership, physical measures on controlling pet movement, and primary source of information pertaining to animal bites and rabies among pet owners in the Municipality of Alaminos, Laguna, September 2024 (n=402).

Description	Frequency	Percentage
Total pets owned		
1	190	47.26
2	121	30.10
3	32	7.96
4	23	5.72
5 or more	36	8.95
Duration of pet ownership		
< 1 year	39	9.70
1 to 2 years	170	42.29
3 to 4 years	71	17.66
5 to 6 years	50	12.44
7 years and above	72	17.91
Frequency of letting the pets roam outside the house		
Always	33	8.21
Often	88	21.89
Sometimes	211	52.49
Never	70	17.41
Ways to control pets inside the house		
Cage	64	15.92
Leash	236	58.71
Free roaming	102	25.37
Ways to control pets outside the house		
Cage	58	14.43
Leash	268	66.67
Free roaming	76	18.91
Primary source of information		
Social media	138	34.33
Government officials	131	32.59
Print and mass media	101	25.13
Private medical practitioners	20	4.98
Friends or relatives	12	2.99

both housing type and ownership experience influence how pet owners manage roaming behaviors and overall pet numbers.

Overall, pet owners showed moderate to strong understanding of general knowledge about animal bites and rabies (Table 3). However, it is notable that there was poor knowledge about livestock being affected by rabies, with only a quarter of the respondents correctly answering the question (26.87%).

On the other hand, a high proportion of respondents correctly identified the symptoms of rabies (Table 4). Interestingly, the negatively phrased item on "frequent thirst" as a symptom yielded the lowest correct response rate (18.16%), while the high accuracy for "tingling at the wound site" (81.84%) suggests respondents gave thoughtful consideration to their answers.

Table 5 indicates that most pet owners expressed positive perceptions toward rabies prevention and control. However, belief in "tandok" as an effective traditional remedy persisted, with only 24.88% of respondents answering correctly by rejecting it. A more concerning misconception was that pets are safe to play with while eating, opposed by only 3.48% of respondents.

For perceived benefits, over 95% agreed on the importance of annual pet vaccination and prompt consultation after animal bites, while 80.10% held positive views on barangay officials' roles.

For perceived barriers, 66.67% agreed that indoor pets still need vaccination, and 63.68% disagreed that rabies vaccines cause pet deaths. Most respondents (88.89%) supported government

Table 3. Frequency distribution on the respondents in terms of the general knowledge pertaining to animal bites and rabies among pet owners in the Municipality of Alaminos, September 2024 (n=402).

Animal Bites and Rabies Knowledge	Correct Answer	
	Frequency (n)	Percentage (%)
A person can get rabies through a scratch from a rabid animal.	390	97.01
A person bitten by an unvaccinated or stray animal should be brought to the health center within 24 hours.	390	97.01
Environmental sanitation plays an important role in preventing incidence of animal bites.	383	95.27
Mass vaccination of dogs and cats are conducted in our community at least once a year.	374	93.03
Owners who allow their pets to roam the streets without leash should be fined.	368	91.54
Animal bites increase the risk of getting rabies infection.	353	87.81
Rabies infection is treated with antibiotics.	304	75.62
Rabies is caused by a bacterium.	302	75.12
Barangay officials are responsible for killing animals suspected to have rabies.	259	64.43
Livestock such as cows or cattle are not affected by rabies.	108	26.87

Table 4. Frequency distribution of the respondents in terms of their knowledge of symptoms of rabies in the Municipality of Alaminos, September 2024 (n=402).

Symptoms of Rabies	Correct Answer	
	Frequency (n)	Percentage (%)
Drooling of saliva	383	95.27
Aggression	376	93.53
Headache	372	92.54
Fatigue or body weakness	370	92.04
Fever	361	89.80
Tingling sensation in the area of the wound bite	329	81.84
Frequent feeling of thirst	73	18.16

funding for human vaccines, and 81.09% agreed that pet owners should share vaccination costs.

Regarding perceived threats, most respondents recognized risks from stray animals (84.08%), open garbage bins (91.04%), failure to restrain suspected rabid animals (76.86%), allowing pets to lick wounds (65.42%), and ignoring proper bite protocols (71.39%).

Table 6 showed that dogs (76.37%) remained the preferred pets over cats (23.63%). Over half (63.43%) of pets were received as gifts, and guarding the home (21.24%) was the most common reason for ownership.

Among reported pets, only 30.94% were registered with the municipal veterinary office, 39.8% had up-to-date rabies vaccinations, and 12.1% were neutered (Table 7).

To better understand low participation in government rabies prevention programs, respondents were asked about their reasons. For pet registration (Table 8), nearly half (44.47%) cited being too busy, while another 13.11% were uninterested. About 24.16% were unaware of registration guidelines, and some reported difficulty restraining or transporting pets.

The association between the pet's characteristics (Table 7) and compliance with government animal control strategies (Table 8) was investigated. Analysis shows that both pet species and acquisition method significantly influence registration and vaccination status. Dogs are more likely to be registered ($p=0.0005$) and vaccinated ($p=0.005$) compared to cats. Neutering is not significantly influenced by the type of pet ($p=0.052$) nor its mode of acquisition ($p=0.065$). Pets adopted from streets or shelters are less likely to be registered or vaccinated. Pets purchased from breeders or shops are more often registered, neutered, and vaccinated. Pets received as gifts follow this trend.

Table 5. Frequency distribution of the respondents in terms of the perceived benefit, barrier, and threats in the implementation of the animal bites and rabies program among pet owners in the Municipality of Alaminos, September 2024 (n=402).

Statements Pertaining to Animal Bites and Rabies	Positive Perception*	
	Frequency (n)	Percentage (%)
Perceived Benefit		
An animal bite victim should be brought to the Animal Bite Treatment Center within 24 hours.	384	95.52
Pet owners should have their pets vaccinated against rabies every year.	382	95.02
I should inform barangay officials when there is a dog biting incident.	322	80.10
I believe that our barangay is efficient in controlling stray animals from roaming freely.	298	74.13
**I believe that Tandok is a traditional remedy that is effective in treating animal bites.	100	24.88
Perceived Barrier		
The human anti-rabies vaccine should be funded by the local government unit.	360	88.89
Pet owners should pay for the cost of vaccination against rabies.	326	81.09
**It is not necessary to have pets vaccinated against rabies if they do not go outside and play with other animals.	268	66.67
**I believe that rabies vaccination has caused the death of some pets that received it.	256	63.68
Perceived Threat		
Garbage storage bins should be properly covered and not accessible to stray animals.	366	91.04
All stray dogs/cats that are freely roaming around should be brought to an animal pound or shelter.	338	84.08
**When an animal is suspected of rabies infection, it should immediately be killed.	309	76.86
**When a dog runs amok biting people, the police should be called to shoot down the dog.	287	71.39
I believe that allowing dogs and cats to lick wounds of humans will increase risk of rabies transmission.	263	65.42
**Pets are usually in a good mood while eating and are willing to play.	14	3.48

Note: *A positive perception indicates agreement or strong agreement to desirable belief, or disagreement or strong disagreement to an undesirable belief.
**Statement reflects a negative health behavior; hence, coding was reversed accordingly.

Table 6. Frequency distribution of respondents in terms of pet characteristics in the Municipality of Alaminos, September 2024 (n=711).

Pet Characteristics	Frequency (n)	Percentage (%)
Type of pet		
Dog	543	76.37
Cat	168	23.63
Origin of pet		
Received as gift	451	63.43
Born at home	97	13.64
Bought from breeders/shops	90	12.66
Adopted from streets/shelter	73	10.27
Reason for keeping pet		
Guard	151	21.24
Passion/ Animal lover	136	19.13
Stress reliever/ Emotional support	50	7.03
Companion/ Family member	24	3.38
Mouse catcher	16	2.25
Other reasons	16	2.25
No reason	318	44.73

Overall, only 46.29% were registered. Also, only 13.11% had been neutered. Most concerning is that only 31.16% had current anti-rabies vaccination. Even among dogs alone, only 33.0% were up-to-

Table 7. Frequency distribution of pet's participation in services of the National Rabies Prevention and Control Program in the Municipality of Alaminos, September 2024 (n=711).

Pet Characteristics	Frequency (n)	Percentage (%)
Registered to the municipal veterinary		
Registered	220	30.94
Not registered	491	69.06
Neutering of pets		
Neutered	86	12.10
Not neutered	625	87.90
Last vaccination status		
Up to date	283	39.80
Not up to date	428	60.20

Table 8. Frequency distribution of reasons for not enlisting their pets to the municipal animal registry among pet owners who responded in the Municipality of Alaminos, September 2024 (n=389).

Reason Why Unregistered	Frequency (n)	Percentage (%)
Too busy	173	44.47
Unaware of guidelines on pet registration	94	24.16
Not interested	51	13.11
Pet is too young/ newly homed	32	8.23
Difficult to restrain or hard to transport pet	26	6.68
Goes to private clinic	13	3.34
Total Responses	389	100.00

date. This contradicts provincial and municipal claims of achieving 70% vaccination coverage.

For non-neutering (Table 9), 21.91% cited lack of familiarity. Many were unaware that pets, especially cats and females, could be neutered. This aligns with the limited neutering capacity at the municipal agriculture office.

For outdated vaccinations (Table 10), 27.65% cited pets being too young, 17.06% reported having difficulty handling aggressive pets, 14.71% faced financial constraints or were waiting free services, and 13.53% missed campaigns due to lack of notice. These were reasons echoed by key informants.

Table 9. Frequency distribution of reasons for not having their pets neutered among pet owners who responded in the Municipality of Alaminos, September 2024 (n=389).

Reason Why Not Neutered	Frequency (n)	Percentage (%)
Unfamiliar with neutering	101	21.91
Too busy	89	19.31
Pet is too young	62	13.45
Afraid it may harm the pet	54	11.71
Unaware of where to avail the service	36	7.81
Pet is for breeding	33	7.16
Not interested	24	5.21
Difficult to restrain/ very aggressive	21	4.56
Financial/ waiting for free service	18	3.90
Pet is always indoors	16	3.47
Other reasons	7	1.52
Total Responses	461	100.00

Table 10. Frequency distribution of reasons for pet not having up-to-date anti-rabies vaccination status among pet owners who responded in the Municipality of Alaminos, September 2024 (n=389).

Reason for Outdated Vaccination Status	Frequency (n)	Percentage (%)
Pet is too young	47	27.65
Difficult to restrain/ very aggressive	29	17.06
Financial/ Waiting for free service	25	14.71
Not informed of vaccination campaign	23	13.53
Not interested/Forgot	14	8.24
Too busy/ Nobody home	12	7.06
Pregnant	9	5.29
Other reasons	11	6.46
Total Responses	170	100.00

Further inquiry revealed a dichotomy in pet owners' practices regarding rabies prevention and control (Table 11). Most respondents reported good practices such as proper pet care (93.78%), controlling pets' outdoors (87.06%), preventing stray access to garbage (82.09%), keeping children away while pets eat (84.82%), and ensuring annual vaccination (85.32%). However, fewer were willing to pay for bite victims' medical expenses (59.20%) or pet vaccination costs (61.44%). Only 63.93% knew that the municipal veterinary office handles suspected rabid animals.

Table 11. Frequency distribution on the self-efficacy, external cue to action and internal cue to action among pet owners in the implementation of the animal bites and rabies program in the Municipality of Alaminos, September 2024 (n=402).

Practice Statement	Good Practice	
	Frequency (n)	Percentage (%)
Self-efficacy		
I make sure that my pet is clean and well-fed every day.	377	93.78
I make sure that my pet always has a leash when it goes out of the household premises.	350	87.06
I make sure that my pets are vaccinated against rabies every year.	343	85.32
I make sure that children do not disturb my pet when it is eating.	341	84.82
I make sure that stray animals do not go through my garbage storage.	330	82.09
I will call the municipal veterinary office in case an animal is suspected of rabies infection.	257	63.93
I pay for the cost of my pet's vaccination against rabies.	247	61.44
I will pay for the medical expenses of the person bitten by my pet.	238	59.20
External Cue to Action		
I report to barangay officials when there are stray animals roaming near my home.	263	65.42
I always hear about an information dissemination campaign regarding animal bites and rabies going around in our barangay.	247	61.44
I attend training or information campaigns on health and wellness in our community.	185	46.02
**I seek traditional healers to treat animal bites.	163	40.55
Internal Cue to Action		
**I eat dog meat.	389	96.77
**I allow my pet to lick the wound caused by their bite to make them feel sorry.	372	92.54
**I don't need to wash or disinfect animal bites when there is no bleeding.	227	56.47

Note: *A positive perception indicates agreement or strong agreement to a good practice, or disagreement or strong disagreement to a poor practice.
**Statements reflect a negative health behavior; hence, coding was reversed accordingly.

Few respondents showed "external cues to action." Only 40.55% avoided traditional healers, 65.42% reported strays to barangay officials, 61.44% regularly heard information campaigns, and 46.02% attended health trainings. Despite limited exposure to education campaigns, most respondents practiced safe behaviors, such as avoiding dog meat consumption (96.77%) and preventing pets from licking bite wounds (92.54%). The question about not washing non-bleeding wounds may have been unclear, since only 56.47% of respondents answered it correctly.

Subsequent analysis examined the relationship between pet owners' KAPs and influencing factors. The KAP responses were aggregated into numerical scores for correlation analysis [36]. Table 12 shows a decline from high knowledge to fair attitudes and poor practices among pet owners based on Bloom's cut-off point [33].

Table 12. Descriptive statistics of knowledge, attitudes, and practices (KAP) scores on animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402).

KAP on Animal Bites and Rabies	Mean	SD
Knowledge	83.36	11.14
General knowledge	85.00	11.78
Symptoms of rabies	81.02	18.03
Attitudes	72.40	11.27
Perceived benefits	74.96	13.34
Perceived barriers	76.31	17.67
Perceived threats	67.67	13.22
Practice	53.01	11.07
Self-efficacy	60.45	11.11
External cues to action	60.18	23.38
Internal cues to action	23.61	14.66

Using Spearman Rank-Order correlation analysis, Table 13 reveals a statistically significant positive monotonic correlation between respondents' knowledge and attitudes, and attitudes and practices. However, there is no significant relationship between knowledge and practice.

Table 14 shows that 46.27% of respondents knew a household member, and 62.94% knew a neighbor, who had been bitten or scratched by animals. Only two respondents (0.5%) knew a household member, and four (1.0%) knew a neighbor who had contracted rabies. Since no time frame was specified, these may refer to past cases.

Tables 15 to 18 show that pet owners with household exposure to animal bites or rabies had significantly higher KAP scores. Awareness of neighbors with bites or rabies showed no significant effect on knowledge but did significantly influence attitudes and practices.

Table 19 shows that most residents in apartment-type houses have 2 or fewer pets (90.9%). In households without enclosures, 83.3% have 1 to 2 pets, 11.0% have 3 to 4 pets, and only 1.6% have 6 or more pets. In contrast, households with yard enclosures tend to have more pets, with 66.4% having 1 to 2 pets, 19.2% having 3 to 4 pets, and 6.8% having 6 or more pets.

Table 13. Spearman rank-order correlation analysis between knowledge, attitudes, and practices (KAP) towards animal bites and rabies in the Municipality of Alaminos, September 2024 ($p<0.05$).

Variables	rho	p-value
Knowledge - Attitudes	0.3128	< 0.001*
Knowledge - Practices	0.0647	0.1950
Attitudes - Practices	0.4021	< 0.001*

Table 14. Frequency distribution of exposure to animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402).

Experience with Animal Bites and Rabies	Count	Percentage
Has a household member been bitten or scratched by an animal?		
Yes	186	46.27
No	182	45.27
Don't know	34	8.46
Has a neighbor been bitten or scratched by an animal?		
Yes	253	62.94
No	68	16.92
Don't know	81	20.15
Has a member of the household contracted rabies?		
Yes	2	0.50
No	344	85.57
Don't know	56	13.93
Has a neighbor contracted rabies?		
Yes	4	1.00
No	327	81.34
Don't know	71	17.66

Table 15. Summary of the relationship between exposure of pet owners and their overall knowledge, attitudes, and practices regarding animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Experience with Animal Bites and Rabies	Overall Knowledge		Overall Attitudes		Overall Practices	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
HH member bitten or scratched by an animal	0.002	Significant	0.000	Significant	0.006	Significant
Neighbor bitten or scratched by an animal	0.114	Not Significant	0.001	Significant	0.002	Significant
HH member contracted rabies	0.002	Significant	0.000	Significant	0.008	Significant
Neighbor contracted rabies	0.114	Not Significant	0.001	Significant	0.002	Significant

Table 16. Summary of the relationship between exposure of pet owners and their general knowledge about and symptoms of animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Experience with Animal Bites and Rabies	General Knowledge		Symptoms of Rabies	
	p-value	Interpretation	p-value	Interpretation
HH member bitten or scratched by an animal	0.000	Significant	0.000	Significant
Neighbor bitten or scratched by an animal	0.068	Not Significant	0.000	Significant
HH member contracted rabies	0.000	Significant	0.000	Significant
Neighbor contracted rabies	0.068	Not Significant	0.000	Significant

Table 17. Summary of the relationship between exposure of pet owners and their attitudes toward animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Experience with Animal Bites and Rabies	Perceived Benefits		Perceived Barriers		Perceived Threats	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
HH member bitten or scratched by an animal	0.007	Significant	0.001	Significant	0.148	Not Significant
Neighbor bitten or scratched by an animal	0.057	Not Significant	0.000	Significant	0.206	Not Significant
HH member contracted rabies	0.007	Significant	0.001	Significant	0.148	Not Significant
Neighbor contracted rabies	0.057	Not Significant	0.000	Significant	0.206	Not Significant

As shown in Table 20, there was a statistically significant relationship between the duration of pet ownership and the frequency of allowing pets to roam outside (p=0.037). Across all ownership

Table 18. Summary of the relationship between exposure of pet owners and their practices regarding animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Experience with Animal Bites and Rabies	Self-efficacy		External Cue to Action		Internal Cue to Action	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
HH member bitten or scratched by an animal	0.022	Significant	0.005	Significant	0.005	Significant
Neighbor bitten or scratched by an animal	0.004	Significant	0.002	Significant	0.008	Significant
HH member contracted rabies	0.022	Significant	0.005	Significant	0.005	Significant
Neighbor contracted rabies	0.004	Significant	0.002	Significant	0.008	Significant

Table 19. Association between housing status and total number of pets owned per household in the Municipality of Alaminos, September 2024 (n=402).

Total Pets	Housing Status			p-value
	House with enclosed yard	House with no enclosed yard	Apartment	
1	51 (34.9%)	133 (54.3%)	6 (54.5%)	0.004
2	46 (31.5%)	71 (29.0%)	4 (36.4%)	
3	14 (9.6%)	18 (7.3%)	0 (0.0%)	
4	14 (9.6%)	9 (3.7%)	0 (0.0%)	
5	11 (7.5%)	10 (4.1%)	1 (9.1%)	
6 or more	10 (6.8%)	4 (1.6%)	0 (0.0%)	

Table 20. Relationship between duration of pet ownership and frequency of allowing pets to roam outside in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Duration of Pet Ownership	Frequency of letting the pets roam around				Overall	p-value
	Always	Often	Sometimes	Never		
Less than 1 year	2 (5.1%)	10 (25.6%)	22 (56.4%)	5 (12.8%)	39 (9.7%)	0.037
1 to 2 years	15 (8.8%)	50 (29.4%)	87 (51.2%)	18 (10.6%)	170 (42.3%)	
3 to 4 years	6 (8.5%)	12 (16.9%)	39 (54.9%)	14 (19.7%)	71 (17.7%)	
5 to 6 years	5 (10.0%)	6 (12.0%)	26 (52.0%)	13 (26.0%)	50 (12.4%)	
7 to 8 years	2 (6.5%)	7 (22.6%)	14 (45.2%)	8 (25.8%)	31 (7.7%)	
9 years and above	3 (7.3%)	3 (7.3%)	23 (56.1%)	12 (29.3%)	41 (10.2%)	

durations, "sometimes" allowing pets to roam was the most common practice, while longer durations of pet ownership were associated with a higher proportion of respondents who never allowed their pets to roam. In contrast, households with shorter pet ownership duration more frequently reported allowing pets to roam often. These findings suggest that increased experience in pet ownership may be associated with more responsible and restrictive pet management practices.

Overall, the primary source of information was found to impact the respondent's knowledge, attitudes, and practices (Table 21). Other

Table 21. Summary of the relationship between different sociodemographic factors of pet owners and their overall knowledge, attitudes, and practices regarding animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Sociodemographic Factors	Overall Knowledge		Overall Attitudes		Overall Practices	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
Age	0.004	Significant	0.309	Not significant	0.075	Not significant
Sex	0.045	Significant	0.323	Not significant	0.733	Not significant
Educational attainment	0.093	Not significant	0.136	Not significant	0.504	Not significant
Employment status	0.010	Significant	0.096	Not significant	0.123	Not significant
Monthly income	0.067	Not significant	0.036	Significant	0.015	Significant
Primary source of information	0.470	Significant	0.000	Significant	0.029	Significant

determinants of overall knowledge include age, sex, and employment status. Apart from the information source, only the monthly income is shown to influence overall attitude and practice.

Breaking down the components of knowledge, attitudes, and practices among pet owners shows that general knowledge about animal bites and rabies was significantly influenced by age, sex, education, employment, and the main source of information. Meanwhile, knowledge of rabies symptoms was significantly influenced by age, employment status, and primary source of information (Table 22).

External cues to action, which reflect exposure to government information or training, were affected only by the primary information source. Internal cues to action, which are actions shaped by personal or observed experiences, were linked solely to educational attainment (Table 24).

Discussion

The survey was conducted during weekdays, and this timing is reflected in the respondents' profile, which consisted mostly of middle-aged women who were typically housewives and at home during the day. Typical of a rural community, most families live in a detached home structure despite having a household income below the poverty threshold set by the Philippine Statistics Authority (PSA) in 2022 [34].

Table 22. Summary of the relationship between various sociodemographic factors of pet owners and their knowledge about animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Sociodemographic Factors	General Knowledge		Symptoms of Rabies	
	p-value	Interpretation	p-value	Interpretation
Age	0.007	Significant	0.003	Significant
Sex	0.026	Significant	0.821	Not significant
Educational attainment	0.033	Significant	0.708	Not significant
Employment status	0.024	Significant	0.004	Significant
Monthly income	0.152	Not significant	0.559	Not significant
Primary source of information	0.000	Significant	0.001	Significant

Perceived benefits, which are beliefs that reduce susceptibility to animal bites or rabies, were influenced by education, income, and information source. Perceived barriers, referring to negative beliefs or concerns about preventive behavior, were affected solely by the primary information source. Perceived threats, which are statements that heighten perceived risk, were influenced by employment, income, and information source (Table 23).

The sentiments of the government key informants interviewed regarding the lack of control of pet movement in the community were contradicted by the majority of pet owners surveyed. According to the pet owners, there was a

Table 23. Summary of the relationship between different sociodemographic factors of pet owners and their attitudes toward animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Sociodemographic Factors	Perceived Benefits		Perceived Barriers		Perceived Threats	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
Age	0.855	Not significant	0.423	Not significant	0.302	Not significant
Sex	0.632	Not significant	0.406	Not significant	0.503	Not significant
Educational attainment	0.016	Significant	0.094	Not significant	0.075	Not significant
Employment status	0.054	Not significant	0.519	Not significant	0.020	Significant
Monthly income	0.039	Significant	0.324	Not significant	0.026	Significant
Primary source of information	0.000	Significant	0.036	Significant	0.004	Significant

Self-efficacy statements gauging preventive and control practices were influenced by respondents' income and information source. low proportion among them who allowed their pets to roam outside the house and who neglected to restrain their pets when outside of the house.

Table 24. Summary of the relationship between various sociodemographic factors of pet owners and their practices regarding animal bites and rabies in the Municipality of Alaminos, September 2024 (n=402, p<0.05).

Sociodemographic Factors	Self-efficacy		External Cue to Action		Internal Cue to Action	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
Age	0.392	Not significant	0.062	Not significant	0.432	Not significant
Sex	0.124	Not significant	0.564	Not significant	0.096	Not significant
Educational attainment	0.189	Not significant	0.793	Not significant	0.029	Significant
Employment status	0.279	Not significant	0.120	Not significant	0.806	Not significant
Monthly income	0.001	Significant	0.214	Not significant	0.490	Not significant
Primary source of information	0.002	Significant	0.002	Significant	0.409	Not significant

Nonetheless, the problem of stray animals mostly concerns those that are not adopted by anyone. Responsible pet ownership is important in the success of the program, but it may not be the core reason.

Although it is essential to manage pets' movement to prevent accidental harm to humans, Overgaaw *et al*. (2020) pointed out that pets can develop abnormal behaviors, such as excessive aggression, fear, and anxiety, when exposed to an environment with low stimuli and restricted space [37]. A balance is needed to safeguard both the pet's health and well-being and ensure humans remain protected from unnecessary harm.

Considering that a third of the pet owners mentioned that they received their information about animal bites and rabies from local government officials, particularly from the barangay officials and barangay health workers who are always present in the community, this provides a validation of the government stakeholders' response regarding their roles and responsibilities in the implementation of the program. However, another third of the pet owners mentioned that their source of information comes from social media. Social media has become an integral part of daily life. It is shown to help improve awareness through responsible health promotion; however, health professionals should be vigilant in mitigating the impact of misinformation and should enhance fact-checking to ensure accurate online health information [35].

Knowledge alone often fails to produce protective behavior because people need confidence, social incentives, and clear triggers to act. Self-efficacy and perceived ability to perform preventive steps are critical determinants of whether knowledge becomes practice; interventions that raise confidence and simplify actions close the gap. Empirical KAP studies of rabies and other zoonoses confirm that knowledge-practice gaps are common, driven by low perceived control, competing daily priorities, and weak motivational cues [39,25,40].

These findings show how specific HBM constructs shaped pet owners' behavior. For instance, high knowledge did not translate to practice because perceived susceptibility to rabies remained low, and many pet owners underestimated the consequences of delayed vaccination despite understanding disease severity [13,21,3].

Most of the pets owned by the respondents were dogs and were acquired through receiving them as gifts. This demographic aligns with other Philippine studies showing that dogs are the most commonly owned pets [17] and that more pets are acquired by receiving them as a gift from someone [38].

Overall, pet owners show a strong to moderate understanding of general knowledge about animal bites and rabies, and the symptoms of rabies. There was also a positive perception towards preventing and controlling animal bites and rabies in alignment with the recommendations of the health experts; however, there is still the persistence of belief in "tandok" as an effective traditional remedy for treating animal bites.

The low proportion of pets registered with the municipal veterinary office, having up-to-date anti-rabies vaccination status, and being neutered, reported by the pet owners are congruent with previous studies showing that using 1 dog per 10

people for the standard estimation of dog population undercounts the local dog population by 2 to 3 times. Evidence-based planning for household surveys and tracking vaccination history can increase data accuracy and simplify data compilation [17,29,38].

The low percentage of respondents agreeing or strongly agreeing to statements demonstrating "external cue to action" is a warning sign that there remains a gap in information and education dissemination pertaining to animal bites and rabies. Local government actors (barangay officials, barangay health workers) are highly trusted community messengers and therefore influential for knowledge uptake, but message accuracy and consistent framing must be ensured through training and standardized materials. Social media reaches large audiences quickly and can boost awareness, yet it also magnifies misinformation unless messages are verified, framed clearly, and routed through credible channels [24,35,21].

The weak "cues to action" observed, especially the low proportion of respondents receiving reminders or community prompts, support the model's prediction that individuals rarely act without timely triggers, even when they possess correct information [7,9,14,23,24].

Individuals who do not perceive themselves as vulnerable to a health issue are less likely to adopt recommended preventive behaviors. Furthermore, an individual's belief in their ability to successfully perform a task influence whether they will engage in health-promoting actions, follow health policies, and adopt recommendations [39]. The weak enforcement of health strategies can result from a combination of an individual's perception about the severity of the problem, the benefits of the strategy, and their ability to overcome barriers. Proper education and support systems are critical in overcoming these barriers.

This study demonstrated multiple sociodemographic and experiential factors influencing the knowledge, attitudes, and practices of pet owners regarding animal bites and rabies.

Sociodemographic factors are often shown to influence health beliefs and health-seeking behaviors [25]. Other factors that affect respondents' overall knowledge include age, sex, and employment status. In addition to the source of information, only monthly income is shown to influence overall attitude and practice.

The downward trend from the high level of overall knowledge to the fair level of overall attitude, and further downward to a poor practice among pet owners regarding animal bites and rabies according to Bloom's cut-off point is congruent with the results of similar studies in Los Baños, Laguna [23], and the pre-implementation results of a project conducted in Bicol province [7]. The trend is similar to findings from Bangladesh [40], although all parameters in the Bangladesh study were more concerning.

Upon examining the relationship between knowledge, attitudes, and practices using Spearman Rank-Order correlation analysis, a statistically significant positive monotonic correlation is found between the respondents' knowledge and attitudes, as well as between attitudes and practices. However, there is no significant relationship between knowledge and practice. Knowledge-practice gaps can persist despite high knowledge levels, as social norms, low perceived efficacy, and lack of motivational triggers prevent translation into consistent preventive behaviors [39,25,40].

The positive correlation between attitudes and practices also supports the HBM principle that attitudes shaped by perceived benefits and severity are stronger predictors of behavior than knowledge alone. This helps explain why improving attitudes may influence actions more effectively than simply providing information [21,23,39,40].

Another factor relevant to the health seeking behaviors among individuals is previous experiences with an event or disease, as shown when a household member was bitten, scratched, or infected with rabies. This is most evident with the respondent's knowledge about rabies symptoms and practices, although there are blurred relationships when it comes to their perception of animal bites and rabies. The evidence yielded is congruent with the HBM, further validating the latter as a guiding framework for health behavior [24].

The influence of bite exposure history on higher knowledge and practice also supports the HBM concept that direct experience heightens perceived susceptibility and severity, thereby triggering more protective actions [17,21,25,40].

The association of factors with the knowledge, attitudes, and practices agrees with findings in the studies conducted in the Asia-Pacific and Africa [7,25,26,27,28,29,30]. These studies revealed that socio-demographic factors, cultural beliefs, history of bites, distance from the nearest health center, ownership status of the biting animal, monthly spending, sources of information, level of knowledge, and political initiative are influential in the health-seeking behavior of individuals and willingness to participate in the One Health approach collaboration.

Low turnout for campaigns often reflects top-down delivery and limited community ownership. Participatory communication approaches, including peer learning, barangay-based advocacy champions, and community co-design of campaign timing and locations, increase relevance and uptake by aligning activities with local routines and social norms. Case studies of One Health and grassroots rabies programs show better coverage when communities lead planning and monitoring [22,27,16].

Beyene *et al*. (2018) also found an inverse relationship between education level and willingness to vaccinate [25]. This is echoed by the responses of most pet owners, who cited being too busy or lacking the time to bring their pets for vaccination, indicating a lack of perceived benefit.

Effective rabies control requires coordinated communication across animal health, public health, and community sectors, using shared, pre-tested core messages, synchronized campaign calendars, and feedback loops that allow communities to report gaps and officials to respond. Integrated bite-case management and One Health forums show that routine data-sharing and joint messaging improve timeliness and build community trust. Simple feedback systems, such as hotlines or barangay focal persons, and coordinated press materials help ensure consistent, actionable advice [8,16,22].

The responses from pet owners suggest a significant gap between knowledge, attitudes, and practices. Despite having a high level of knowledge, this understanding has not yet led to a change in the health belief of pet owners.

4. Conclusion and Recommendation

The study revealed that while pet owners in the Municipality of Alaminos, Laguna generally possess high levels of knowledge about animal bites and rabies, this cognitive understanding does not consistently translate into positive attitudes and responsible practices. Sociodemographic factors such as age, employment status, primary source of information and history of exposure significantly influenced their knowledge, attitudes, and practices. There is a notable discrepancy between the official anti-rabies vaccination coverage reported by government agencies and the actual data obtained from surveyed pet owners, highlighting the need for improved strategies to estimate pet populations accurately and achieve herd immunity. The outdated municipal resolution on rabies prevention also warrants immediate review and revision to reflect current national strategies, including the integration of the One Health approach. Strengthening the implementation of rabies prevention programs requires more accurate animal surveillance, coordinated information and education campaigns across sectors, and capacity building of local personnel through proper training and adequate staffing.

Taken together, the findings affirm key principles of the Health Belief Model, showing that low perceived susceptibility, weak cues to action, and persistent perceived barriers continue to hinder the translation of knowledge into practice. Enhancing perceived benefits, strengthening self-efficacy, and ensuring timely reminders and supportive community structures are therefore essential to shift pet owners from awareness to consistent preventive behavior.

Behavior change communication strategies should focus on emotional and social motivators, such as protecting children, family, and the community. They should simplify actions into clear, easy-to-follow steps for pet vaccination and bite wound care. Strategies should also build trust in veterinary services by providing transparent schedules and showing visible results, ensuring that the community understands and feels confident in the recommended practices. Messages

should be repeated through multiple channels including barangay talks, posters, and social media, and tested for clarity before rollout. Practical skill building, incentives, and community participation such as peer learning and barangay advocacy champions should be combined with shared messaging across One Health sectors to improve adoption and compliance. Monitoring and adapting messages through community feedback systems will help identify gaps and make interventions culturally relevant, actionable, and trusted. These measures will ultimately reduce rabies transmission and improve the health of both humans and animals in the community.

Availability of Data and Materials

Included as part of the submitted paper.

Author Contributions

Conceptualization, R.R.R. and E.R.G.; Methodology, R.R.R. and E.R.G.; Validation, R.R.R. and E.R.G.; Formal analysis, R.R.R.; Investigation, R.R.R.; Resources, R.R.R.; Data curation R.R.R.; Writing – original draft, R.R.R.; R.R.R.; Writing – review and editing, RRR and ERG; Visualization, R.R.R.; Supervision, E.R.G.; Project administration R.R.R.; Funding acquisition, R.R.R.

Ethics Approval and Consent to Participate

The research posed minimal risk to the respondents and the community. It was conducted in accordance with the Declaration of Helsinki, and the University of the Philippines Code of Research Ethics with reference code UPMREB 2024-0327-EX.

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Conflict of Interest

The authors declare no conflict of interest.

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(Short Communication)

Isolation and Identification of Newcastle Disease Virus from Commercial Layers Farm with Decreased Egg Production in West Java, Indonesia

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Abstract

Background: Newcastle Disease (ND) is an endemic in poultry that can cause high mortality, reduced productivity, and significant economic losses in layer farms. A decrease in hen-day egg production may occur without pathognomonic clinical symptoms. Therefore, supportive diagnostic methods are required to detect active virus infection. **Methods**: This study aimed to determine the presence of the Newcastle Disease Virus (NDV), isolated in embryonated chicken eggs (ECE) from organ samples collected from vaccinated layer hens that still decreased the egg production. This study was conducted on four Isa Brown layer hens from two housing at the ABC Farm, West Java, Indonesia. Organ samples included brain, proventriculus, spleen, lung, caecal tonsils and uterus. Hemagglutination (HA) and hemagglutination inhibition (HI) tests were used to identify NDV. **Results**: The NDV was successfully isolated from the brain, proventriculus, spleen, and uterus organ samples using the *in ovo* method and HA test during the first passage with HA titers ranging from 2^5 to 2^7 HAU. HI test confirmed that the detected virus was NDV. **Conclusions**: *In ovo* isolation method combined with HA and HI test can be utilized to detect active NDV infection in layer hens that experience a drop in egg production. The brain, proventriculus, spleen, and uterus are ideal organs to be used as diagnostic samples for ND.

Keywords

Newcastle Disease, *In ovo* virus isolation, Hemagglutination, Layer hens, Drop production

1. Introduction

The poultry sector plays a pivotal role in global food security by providing high-quality protein, essential nutrients, and economic stability, particularly in developing regions. It is one of the fastest-growing agricultural sub-sectors, driven by increasing population, urbanization, and rising incomes, with significant contributions to rural livelihoods and poverty alleviation [1]. In recent years, the layer hen commodities in Indonesia has experienced rapid growth. The layer hen population in Indonesia has reached approximately 414.8 million chickens in 2024 [2]. However, suboptimal egg production continues to be a recurring issue in layer farms, as laying performance may decrease or fail to reach optimal productivity [3]. This situation was recorded at ABC Farm located in Ciparay, Bandung Regency, West Java that currently houses approximately 200.000 chickens. In several housing productive Isa Brown layers aged 23-28 weeks, hen-day

production (HDP) reached only 78%, which is considerably lower than the performance standard of 90-97% at peak production [4]. This condition may be associated with various factors, including infection disease outbreaks such as Newcastle Disease [5].

Newcastle Disease (ND) is a highly contagious poultry disease caused by virulent strains of Avian Avulavirus-1 [6]. The virus is associated with significant mortality rates, which can reach 100% in infections involving velogenic strains [7]. This disease is classified by the World Organization for Animal Health (WOAH) as a notifiable disease and endemic in Indonesia, where various cases are frequently reported [8]. ND significantly impacts layers performance. Research conducted in Subtropical India indicates that production drops in affected farms with ND can reach up to 29% [9]. Furthermore, a study in 2018 observed that in vaccinated layer flocks, ND infection often manifests without high mortality or severe clinical signs, yet still leads to a decline in egg production [10]. A decrease in hen-day egg production may occur without typical clinical symptoms, therefore, reliable diagnostic methods are required to detect active viral infection.

Newcastle Disease can be detected using various diagnostic techniques. However, the gold standard method recommended by the WOAH for identifying the ND virus involves virus isolation in pathogen-free embryonated chicken eggs, followed by characterization using the HA assay [8]. This method enables the detection of live, infectious viruses using organ tissue samples collected via necropsy [11]. The diagnostic value of tissue samples was highlighted by previous research in Assam, India, which demonstrated that ND detection rates were significantly higher in tissue samples (58.78%) compared to cloacal swabs (22.55%) [12]. However, PCR-based methods, particularly reverse transcription polymerase chain reaction (RT-PCR) and its variants, are highly sensitive tools for detecting the presence of NDV by targeting its genetic material. These methods are widely employed in studies investigating NDV detection in organ samples due to their ability to rapidly identify viral RNA with high specificity and sensitivity [13]. Despite their advantages, PCR-based methods do not directly measure active infection, as they detect viral RNA, which may also originate from non-viable or inactivated viruses. This limitation underscores the importance of combining molecular diagnostics with other methods, such as virus isolation, to confirm the presence of infectious virus particles. While PCR methods are more sensitive and faster, they require specialized equipment, skilled personnel, and are more expensive. In contrast, *in ovo* methods are cost-effective and remain the gold standard for assessing viral infectivity, especially for viruses that cannot be propagated in cell culture [14].

Consequently, the application of the gold standard isolation method remains relatively limited, particularly in studies involving layer chickens. This gap is further compounded by the lack of comprehensive data regarding the optimal target organs for diagnostic sampling in layer flocks specifically experiencing declined egg production. Several studies have identified lesions in organs such as the trachea, spleen, and liver. However, most of this research focuses on general pathology and molecular traits rather than organ-specific details on virus isolation [15]. Therefore, this study is crucial to exploring the capability of the *in ovo* method and HA in detecting NDV active infection isolated from the organs of layer chickens in flocks exhibiting decreased egg production from spleen, brain, uterus, proventriculus, caeca tonsil and lungs.

2. Materials and Methods

2.1 Flock History

Cage house M5 housed a population of 8,000 layer hens aged 28 weeks, recording an average egg weight of 58 g and a HDP rate of 78%. The flock's vaccination regimen included the administration of ND and Avian Influenza (AI) vaccines at 12 weeks of age, followed by ND, Infectious Bronchitis (IB), and Egg Drop Syndrome (EDS) vaccines at 14 weeks. The most recent vaccination, consisting of ND and IB, was administered at 22 weeks. Despite this routine immunization program, Cage house M5 experienced a depletion rate of 12% and a mortality rate of 6%.

Similarly, Cage house M6 contained a population of 8,000 hens aged 23 weeks. This flock also recorded a production rate of 78%, with an average egg weight of 53 g. The vaccination schedule mirrored that of Cage house M5: ND and

AI vaccines were administered at 12 weeks, followed by ND, IB, and EDS at 14 weeks. The final vaccination (ND and IB) was given at 21 weeks of age. Consistent with the findings in Cage house M5, Cage house M6 recorded a depletion rate of 12% and a mortality rate of 6%.

2.2 Sample Collection and Extraction

Specimens were collected via necropsy from four layer hens from housing that have decreased egg production. The two birds were collected from each housing (M5 and M6) as samples and euthanasia was carried out using exsanguination method. Targeted organ samples were collected, such as the brain, trachea, lungs, spleen, uterus, and proventriculus. These samples were immediately submerged in activated viral transport medium (VTM) and preserved at 4°C using ice packs during transport, before being stored in a freezer prior to inoculation process in embryonated chicken egg.

One gram of tissue sample was aseptically collected and homogenized using a three way-stopcock and syringe while gradually adding phosphate-buffered saline (PBS) supplemented with 1% gentamycin. After homogenization, the samples underwent three cycles of freeze-thawing. The tissue suspension was then transferred to a sterile microtube and centrifuged at 2.500 rpm for 10 to 15 minutes to remove debris and separate the supernatant. The resulting supernatant was carefully collected and incubated at 37 °C for 30 minutes prior to inoculation into embryonated chicken eggs with Specific Antibody Negative (SAN) specification. For each organ sample, two eggs were used. Following inoculation, the eggs were incubated for 72 hours. Embryos were monitored via candling every six hours; upon detection of death, the eggs were transferred to a chiller overnight prior to the collection of allantoic fluid.

2.3 Virus Inoculation

Inoculation was performed on 9-day-old embryonated chicken eggs. Prior to inoculation, embryonated chicken eggs were candled to confirm viability and to outline the air sac boundary. A small aperture was drilled above the air sac, through which the viral NDV suspension (0,1 mL) with gentamycin 1% was inoculated into the allantoic cavity via a sterile syringe. The puncture site was then sealed with nail polish, and each egg was labeled according to the respective inoculum. The eggs were subsequently incubated at 37 °C and observed at 6 h intervals [16]. The virus was propagated through a series of sequential blind passages. In this process, harvested material was pooled and used to inoculate one egg per step, which served as the source for two consecutive passages (Passage 1 and Passage 2), as detailed in Table 1. Each step egg per organ samples from M5 and M6 cages and one egg as control.

2.4 Allantoic Harvest and Macroscopic Embryo Observation

The harvesting process began by opening the shell above the air sac with forceps and carefully breaching the membranes to avoid vascular damage. Using a sterile microtip, the allantoic fluid was aspirated from an area free of the embryo and yolk sac. The harvested fluid was clarified by centrifugation, and the supernatant was transferred to sterile tubes. Embryos were subsequently examined in petri dishes for pathological changes, and all observations were photographically documented.

2.5 Hemagglutination Assay (HA) and Hemagglutination Inhibition (HI)

The HA was performed in microtiter plates. Twenty five microliters (0.025 mL) of PBS was dispensed into each of the 12 wells. Organ suspension or harvested allantoic fluid was added 0.025 ml to the first wells, followed by two-fold serial dilutions from the second to the eleventh well. Subsequently, 0.025 mL of a 1% red blood cell (RBC) suspension was added to each well. In the HA test, the twelfth well containing PBS and red blood cells served as a negative control. In the HI test, a standardized antigen was added to wells containing serially diluted antiserum before the addition of red blood cells. All samples were tested in duplicate to ensure result validity and consistency. The plate was gently shaken and allowed to stand until hemagglutination reactions occurred. The HA results were observed at room temperature for approximately 15 minutes or until the control wells showed a definitive reaction [16]. Hemagglutination Inhibition test was performed according to WOAH protocols with harvested allantoic fluid from organ samples as antigens. The NDV and AIV subtype H5N1 anti serum was carried out from Centre for Pharmaceutics and

Veteriner Indonesia (PUSVETMA) Surabaya, Indonesia.

2.6 Ethics Statement

All experimental protocols involving samples from animals in this study have undergone a rigorous review process and received formal approval from the Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran. This authorization is documented under ethical clearance number 982/UN6.KEP/EC/2025, ensuring full adherence to the established ethical standards for scientific research.

3. Results

3.1 Virus Inoculation

The inoculation process of organ suspensions into ECE was carried out through several stages [11]. Harvesting was performed after embryo death was confirmed or after completion of the incubation period for eggs that did not show mortality, by collecting allantoic fluid and embryonic brain tissue. The result of inoculation stage are presented in Table 1.

The viral inoculation process across successive stages demonstrated increasing adaptation and pathogenicity in ECE (Table 1). During the initial blind passage, only 30.7% (4/13) of the embryos showed mortality within 48 hours. However, upon pooling and first passage, mortality rates increased significantly, with 100% of embryos from both flocks (M5-3 and M6-1) dying within 24–48 hours post-inoculation. This consistent mortality pattern was maintained through Passage 2, where 83.3% (10/12) of embryos succumbed within the same timeframe, confirming the successful isolation and stabilization of the virus in the allantoic fluid.

3.2 Macroscopic Embryo

All harvested embryos were examined and compared with control embryos (Fig. 1A). Several pathological lesions were observed during macroscopic examination. One of the notable pathological findings was embryo dwarfism (Fig. 1B), characterized by a smaller embryo size compared with the control. In addition to dwarfism, other pathological lesions included petechiae (Fig. 1D), congestion (Fig. 1E), and hemorrhage (Fig. 1F), which were observed on all

Table 1. Organ sample inoculation in embryonated chicken eggs (ECE).

Inoculation Stage	Specimen	Result
Blind Passage	Supernatants of extracted organs (brain, lungs, proventriculus, caecal tonsils, spleen, and uterus) from one chicken samples cages M5 and M6 inoculated in to one egg per sample organs.	Four ECE died within 24–48 hours post-inoculation, while nine ECE survived beyond 72 hours
Pooling	Pooled organ supernatants from two layer chickens each in groups M5 and M6 were inoculated into embryonated eggs (one egg per pooled sample).	Seven ECE died within 24–48 hours post-inoculation, one ECE died between 48–72 hours, and four ECE survived beyond 72 hours
Passage 1	Allantoic fluid obtained from inoculated pooled samples of cages M5 and M6 inoculated to one egg per sample.	All ECE from flocks M5 and M6 died within 24–48 hours post-inoculation
Passage 2	Allantoic fluid from first passage inoculated to one egg per sample.	Ten ECE died within 24–48 h post-inoculation, while two ECE survived beyond 72 h

or certain areas of the embryo surface. Ascites were also observed in one embryo sample (Fig. 1C).

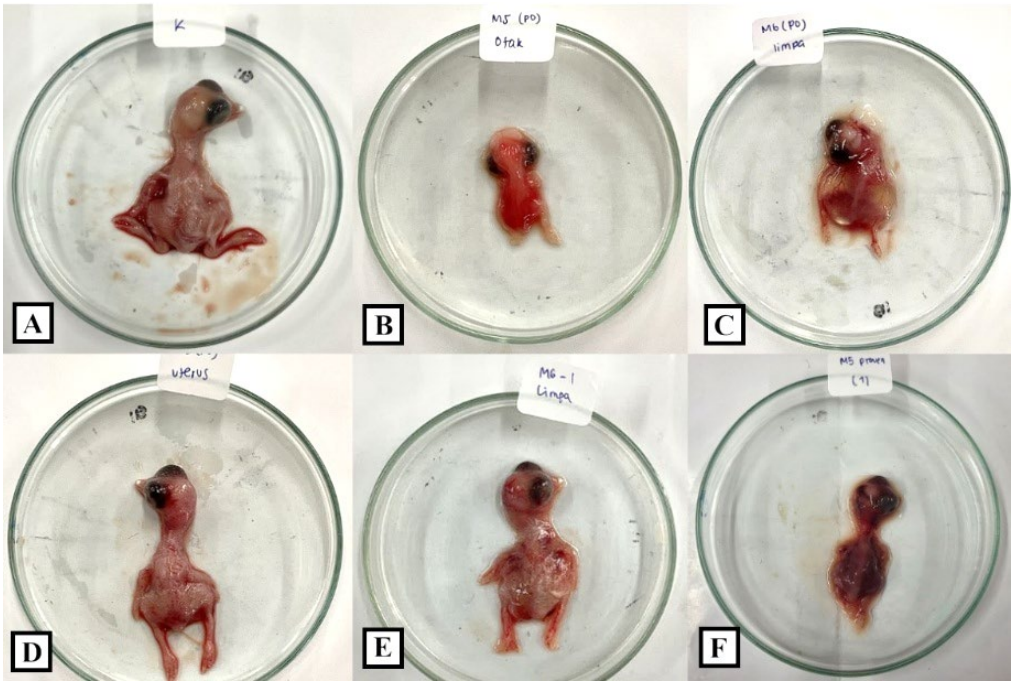

Figure 1. The representative macroscopic lesion of ECE with inoculation from sample organ. A) Normal uninfected chicken embryo (control). B) Embryo dwarfism in inoculated embryo 24 – 36 hours from brain sample. C) Ascites in inoculated embryo 24 – 36 hours from spleen. D) Petechial lesions on the embryo surface death time 48 – 60 hours from uterus. E) Congestion in inoculated embryo death time 48 – 60 hours from spleen. F) Hemorrhage in inoculated embryo observed death time 24 – 36 hours from proventriculus.

Petechial lesions appeared as small red spots distributed in several areas. Congestion was indicated by increased redness of blood vessels, with vessels appearing more prominent beneath the embryonic skin. Hemorrhagic lesions were larger and more extensive than petechiae, presenting as widespread bleeding in all or parts of the embryo. These findings collectively indicate active viral replication following inoculation into embryonated chicken eggs.

The macroscopic findings observed in the ECE during each inoculation stage are summarized in Table 2. In the blind passage, most organ samples from both flocks (M5 and M6) showed no visible lesions, though isolated instances of congestion and hemorrhages in the head or body were noted in specific samples such as M6 proventriculus and M6 uterus. As the process moved to the pooling stage, more distinct pathological changes emerged, including widespread dwarfism and petechial hemorrhages. By Passage 1 and Passage 2, the severity of the lesions progressed significantly; nearly all specimens exhibited extensive hemorrhages over the entire body or concentrated in the head area, accompanied by consistent dwarfism. Notably, by the second passage, several samples resulted in undeveloped embryos, indicating a high level of viral pathogenicity following adaptation.

3.3 Hemagglutination Assay (HA) and Hemagglutination Inhibition (HI)

The results of the HA assay following the blind passage are presented in Table 3. At this stage, viral activity was detected via the partial agglutination in HA test in only one organ sample from Cage house M5, specifically the brain tissue.

In the pooling phase, supernatants originating from the same organs across all

Table 2. Macroscopic findings in embryonated chicken embryos.

Sample	Inoculation Stage			
	Blind Passage	Pooling	Passage 1	Passage 2
M5 Brain	No lesion	Dwarfism	Hemorrhages all over the body, dwarfism	Hemorrhages in head area, dwarfism
M5 Proventriculus	Hemorrhages all over the body	Dwarfism	Hemorrhages in head area, dwarfism	Hemorrhages in head area, dwarfism
M5 Lungs	No lesion	Petechiae all over the body, dwarfism	Hemorrhages all over the body	Hemorrhages in head area, dwarfism
M5 Caecal tonsils	No lesion	Congestion	Hemorrhages all over the body, dwarfism	Undeveloped embryo
M5 Spleen	No lesion	No lesion	Hemorrhages all over the body	Hemorrhages in head area, dwarfism
M5 Uterus	No lesion	Petechiae	Hemorrhages all over the body	Undeveloped embryo
M6 Brain	No lesion	Dwarfism	Hemorrhages in head area	Hemorrhages all over the body, dwarfism
M6 Proventriculus	Congestion	Dwarfism	Hemorrhages all over the body	Undeveloped embryo
M6 Lungs	Hemorrhages all over the body	Petechiae	Hemorrhages in head area	Undeveloped embryo
M6 Caecal tonsils	No lesion	Dwarfism	Hemorrhages all over the body	Undeveloped embryo
M6 Spleen	Congestion	Ascites, dwarfism	Hemorrhages in head area	Congestion, dwarfism
M6 Uterus	Hemorrhages in head area	No lesion	Hemorrhages all over the body	Hemorrhages in head area, dwarfism

Table 3. Hemagglutination assay results from the blind passage samples from cage houses M5 and M6.

	Organ Tissue	HA Test Results[a]	Titer
M5	Brain*	(+)	2^1
M5	Proventriculus	(-)	-
M5	Lungs	(-)	-
M5	Caecal tonsils	(-)	-
M5	Spleen	(-)	-
M5	Uterus	(-)	-
M6	Brain	(-)	-
M6	Proventriculus	(-)	-
M6	Lungs	(-)	-
M6	Caecal tonsils	(-)	-
M6	Spleen	(-)	-
M6	Uterus	(-)	-

[a]HA positive (+) indicates agglutination; negative (-) indicates RBC button.

individual samples within each cage house were combined. The HA results for the pooled brain samples are presented in Table 4.

The results of the HA and HI assays for samples from Cage house M5 are summarized in Table 5. In the first passage, positive HA activity was observed in all organ samples except the lungs, with titers ranging from 2^2 to 2^7. The identity of the isolate was further confirmed through the HI test, which showed positive results for ND but remained negative for AI,

Table 4. Hemagglutination assay results for pooled samples from cage houses M5 and M6.

Code Sample	Organ Tissue	HA Test Results[a]	Titer
M5	Pooled (embryo brain)	(+)	2^5
M6	Pooled (embryo brain)	(+)	2^4

[a]HA positive (+) indicates agglutination.

Table 5. HA and HI assay results from the first and second passage of samples from Cage house M5.

Organ	First Passage				Second passage	
	HA test result[a]	Titer	HI test result (ND)[b]	HI test result (AI)[c]	HA test result[a]	Titer
Brain	(+)	2^6	(+)	(-)	(+)	2^8
Proventriculus	(+)	2^7	(+)	(-)	(+)	2^9
Lungs	(-)	-	(-)	(-)	(-)	-
Caecal tonsils	(+)	2^2	(+)	(-)	(-)	-
Spleen	(+)	2^5	(+)	(-)	(+)	2^{11}
Uterus	(+)	2^7	(+)	(-)	(-)	-

[a]HA positive (+) indicates agglutination; negative (-) indicates RBC button.
[b]HI (ND) positive (+) indicates hemagglutination inhibition (RBC button); negative (-) indicates agglutination.
[c]HI (AI) negative (-) indicates Agglutination.

demonstrating the specificity of the isolated agent. By the second passage, viral titers increased in the brain, proventriculus, and spleen, reaching a peak titer of 2^{11} in the spleen. These findings indicate successful viral propagation and suggest a high affinity of the isolate for various organ tissues, particularly following serial passage.

As detailed in Table 6, the M6 samples demonstrated a clear pattern of viral adaptation where initially negative tissues became positive after further passage. In the first passage, HA activity was only detectable in the proventriculus and uterus. However, by the second passage, both the brain and spleen—which were previously negative—converted to positive with titers of 2^7 and 2^{11}, respectively. This 'negative-to-positive' shift underscores the necessity of serial passage for the recovery of NDV from field samples where the initial viral load may be below the limit of detection.

4. Discussion

4.1 Isolation and Identification of Newcastle Disease Virus

Based on the isolation and identification results, the *in ovo* isolation method was applicable

Table 6. HA and HI assay results from the first passage of samples from Cage houses M6.

Organ	First Passage				Second passage	
	HA test result[a]	Titer	HI test result (ND)[b]	HI test result (AI)[c]	HA test result[a]	Titer
Brain	(-)	-	(-)	(-)	(+)	2^7
Proventriculus	(+)	2^7	(+)	(-)	(-)	-
Lungs	(-)	-	(-)	(-)	(-)	-
Caecal tonsils	(-)	-	(-)	(-)	(-)	-
Spleen	(-)	-	(-)	(-)	(+)	2^{11}
Uterus	(+)	2^8	(+)	(-)	(+)	2^7

[a]HA positive (+) indicates agglutination; negative (-) indicates RBC button.
[b]HI (ND) positive (+) indicates hemagglutination inhibition (RBC button); negative (-) indicates agglutination.
[c]HI (AI) negative (-) indicates Agglutination.

for detecting NDV from organ samples of layer hens in flocks experiencing decreased egg production. In layer flock, decreased egg production is commonly confirmed using additional diagnostic methods such as PCR as one of the available option [13]. However, *in ovo* virus isolation remains the gold standard for confirming live and infectious NDV [8]. A production rate of 78% in Isa Brown layers aged 23 and 28 weeks was considered below the standard performance, which can reach 90–94% under optimal management conditions [9]. The average egg weights in both flocks were also below the standard, at 58 g and 53 g compared with the reference value of 63.1 g [17]. The 6% difference between depletion (12%) and mortality (6%) indicated that a proportion of birds was culled, likely as a preventive measure to control disease spread or due to declining physiological conditions affecting productivity [18].

Newcastle Disease is one of the diseases known to affect egg quality and production in layer hens, particularly velogenic and mesogenic strains. ND infection can result in reduced egg production and reproductive tract lesions [19,20]. Although routine vaccination is effective in preventing severe clinical signs and deterioration of egg quality, it does not fully prevent production decline in infected layer hens [10].

The partial agglutination observed in this organ sample indicates the presence of NDV with hemagglutinating activity in the brain tissue, albeit at a very low level. This finding aligns with previous study, which reported that partial agglutination signifies a genuine interaction between the virus and erythrocytes [21]. Consequently, while the virus is considered biologically present, the extremely low viral load renders the result diagnostically insignificant (below the positive threshold). Despite the low viral load detected by the HA test, macroscopic examination of inoculated embryos revealed pathological lesions, including hemorrhage and congestion. Similar findings have been reported in NDV-infected embryos, where endothelial viral replication leads to vascular damage and widespread hemorrhagic lesions [22,23].

The low viral load observed in organ samples may be attributed to vaccine-induced immune responses that suppress viral replication [10], resulting in predominantly negative findings during the blind passage. Consequently, organ pooling was performed in subsequent stages to improve the sensitivity of virus isolation and detection, as recommended in the WOAH Terrestrial Manual [8]. While pooling samples optimizes surveillance throughput, the resulting HA titer is an indicator of the virus's replicative success in the embryonated egg's allantoic fluid. Samples are pooled to improve the chances of detecting virus in cases where the viral concentration in a single organ may be insufficient for isolation. This strategy ensures that even minimal viral presence is captured and subsequently amplified during incubation in the allantoic cavity [11].

Initial HA testing of allantoic fluid from the pooled organ samples yielded negative results for both Cage houses M5 and M6. However, viral activity was subsequently detected in the pooled embryo brains harvested following the blind passage. The HA assay revealed titers of 2^5 for Cage house M5 and 2^4 for Cage house M6. The result has met the minimum positivity threshold of 2^4, confirming the success of the isolation procedure. The contrast between the positive tissue and the negative allantoic fluid indicates that viral replication occurred in the embryonic tissue but not yet reached detectable levels in the allantoic fluid [24]. Following these results, the allantoic fluid from the pooled samples was passaged into fresh ECE. This first passage was essential to provide an opportunity for extended viral replication from previous allantoic fluid.

The first passage results showed hemagglutinating virus in five of six organ samples from flock M5 and two of six samples from flock M6, indicating successful viral replication. Haemagglutination inhibition testing using specific antisera was conducted to identify the haemagglutinating agent. The isolates showed positive HI reactions with ND antiserum and negative reactions with AI antiserum, confirming that the haemagglutinating virus was NDV and not AI virus, although further serological characterization of other viral antigens is recommended. Based on the second passage results, HA assays confirmed that the ND virus successfully replicated in fresh ECE. Furthermore, examination of the embryos revealed pathological changes consistent with NDV infection.

4.2 Viral Identification in Organ Samples

Based on the isolation and identification results, NDV was detected in several organ samples collected from layer hens showing decreased egg production, with variable viral titers. During the blind passage, virus detected in the brain sample from flock M5 with titers below 2^4 HAU, indicating the presence of virus at very low levels that could not be considered diagnostically positive. The first passage shows that virus was detected in multiple organs from flock M5, including the brain, proventriculus, spleen, cecal tonsils, and uterus, while in flock M6, positive titers were observed in the proventriculus and uterus. The second passage further confirmed virus presence, with increased titers detected in the brain, spleen, and proventriculus of flock M5 and in the brain, spleen, and uterus of flock M6, indicating successful viral amplification.

Utilizing *in ovo* isolation followed by HA and HI tests provides the distinct advantage of detecting biologically active virus from organ samples. This approach is essential for identifying the primary viral drivers of decreased egg production. While embryo mortality within 60 hours post-inoculation [25] serves as a preliminary indicator of velogenic (wild-type) NDV, RT-PCR remains necessary to differentiate between wild-type and vaccine strains. Furthermore, HI testing is critical to rule out Avian Influenza (H5N1) as a confounding cause of production loss. However, a limitation remains: non-hemagglutinating viruses may cause embryo lethality without detection by HA tests. Therefore, a holistic diagnostic approach integrating both serological and molecular assays is required for definitive pathogen characterization.

5. Conclusions

In conclusion, *in ovo* virus isolation combined with HA and HI tests successfully detected NDV in organ samples collected from layer hens experiencing decreased egg production. NDV was predominantly identified in the brain, spleen, proventriculus, and uterus, with varying viral titers after inoculation, indicating that these organs represent the most reliable and optimal samples for ND diagnosis in layer hens with declined egg production, although further serological and molecular characterization is recommended.

Abbreviations

AI, Avian Influenza; ECE, Embryonated Chicken Eggs; EDS, Egg Drop Syndrome; ND, Newcastle Disease; NDV, Newcastle Disease Virus; HA, Hemagglutination Assay; HDP, Hen Day Production; HI, Hemagglutination Inhibition; IB, Infectious Bronchitis.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, A.D.P.; Methodology, N.S., C., and A.D.P.; Investigation, N.S., and A.D.P.; Writing – Original Draft, N.S.; Review & Editing, C., and A.D.P.; Funding Acquisition, A.D.P.; Supervision, C., and A.D.P.

Ethics Approval and Consent to Participate

All experimental protocols involving animals in this study have undergone a rigorous review process and received formal approval from the Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran. This authorization is documented under ethical clearance number 982/UN6.KEP/EC/2025, ensuring full adherence to the established ethical standards for scientific research.

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Conflict of Interest

The authors declare no conflict of interest.

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(Case Report)

Co-infection of Avian Reovirus, *Mycoplasma synoviae,* and Marek's Disease in a Gamefowl (*Gallus gallus domesticus* L.) Flock in Ibaan-San Jose, Batangas, Philippines

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Abstract

Background: Avian reovirus (ARV) affects nearly all domestic poultry species and commonly associated with severe viral arthritis. This predisposes the birds to other viral and bacterial infections such as Marek's disease (MD), and *Mycoplasma synoviae* (MS). Approximately 25% of gamefowls with swollen shanks, exudation on the keel bone, and nasal discharge were reported by the farm. Hence, this paper aims to describe the clinical presentation of a gamefowl flock infected with ARV, MD, and *M. synoviae*. **Methods:** Blood and organ samples were collected from ten (10) morbid birds for serology, histopathology and qPCR to confirm the diagnosis. **Results:** Necropsy findings showed lesions in the tarsometatarsal, keel, and tracheal regions. Serum samples tested positive for ARV and MS antibodies using ELISA. Real-time RT-PCR detected ARV RNA, while PCR detected MDV DNA in the pooled tissue samples. Follow-up necropsy, conventional PCR, and phylogenetic analysis were also conducted post-vaccination. Phylogenetic analysis classified the MD-positive samples as *Gallid alphaherpesvirus* type 2 and suggested a possible association with VV+ strains. **Conclusion:** To the best of the authors' knowledge, this is the first documented case of ARV with co-infection with MS and MD in gamefowls in the Philippines.

Keywords

Avian reovirus, Marek's disease, *Mycoplasma synoviae*, Gamefowls

1. Introduction

Avian reovirus (ARV), a virus from the family Reoviridae, subfamily Spinareovirinae, and genus Orthoreovirus, affects nearly all domestic poultry species [1]. This virus is associated with malabsorption syndrome, runting-stunting syndrome, hepatitis, myocarditis, immunosuppression, and severe arthritis – the most common clinical sign [2]. The associated arthritis and tenosynovitis can negatively affect

the performance parameters and marketability of the birds due to reduced feed conversion and weight gain. Additionally, Reovirus infections can increase the susceptibility of birds to other bacterial or viral infections [3].

Marek's disease (MD), another major viral threat to poultry worldwide, typically manifests as a neoplastic disease in visceral organs, neurological disorders leading to paralysis, and immunosuppression [4]. The disease is caused by Gallid alphaherpesvirus-2 (Serotype 1/MDV 1), and serotype 1 strains are considered pathogenic [5].

Furthermore, *Mycoplasma synoviae* (MS), a 300–500 nm bacterium lacking a cell wall, is a significant pathogen causing synovitis, airsacculitis, and abnormalities in eggshell and laying patterns in chickens [6]. This bacterial disease can interact with other pathogens, further amplifying its pathogenic effects in chickens [7].

Infections with these pathogens pose significant losses to poultry producers. Studies have reported the occurrence of ARV in backyard chickens in Bangladesh [8], Africa [9], Brazil [10], and Canada [11]. However, the detection of ARV, MS, and MD in the Philippines has not been documented in any published literature. Hence, this paper aims to describe the clinical presentation of a gamefowl flock infected with ARV, MD, and MS.

2. Case Presentation

2.1. History

The farm of interest is a brooding and growing gamefowl farm located in Ibaan-San Jose, Batangas. The facility has a total of 11 open-range pens, and each pen can accommodate up to 200 birds depending on size. The birds are of mixed strains (Sweater, Roundhead, Hatch, Kelso, and Albany), and 30% of the parent stock is imported from Tennessee, USA. The vaccines given are shown in Table 1.

Table 1. Vaccination history of the gamefowl flock.

Age (days)	Vaccine (Type)	Strain	Route
1	*Eimeria* spp. (Live)	*E. acervulina, E. mivati, E. maxima, E. tenella*	Mouthdrop
7	Avian Encephalitis + Fowlpox (Live)	AE virus strain Calnek Live Fowl Pox virus strain Gibbs	Wingweb
	Newcastle Disease (ND) (Live)	LaSota	Intranasal
21	Infectious Bronchitis (IB) (Live) ND (live)	Ma5 Clone 30	Intranasal
31	Avian Encephalitis + Fowlpox (Live)	AE virus strain Calnek Live Fowl Pox virus strain Gibbs	Wingweb
41	Inactivated Coryza (Inactivated)	Serotypes A, B, and C	Intramuscular (IM)
51	Infectious Laryngotracheitis (live)	TCO	Intranasal
61	*Pasteurella multocida* (live)	X-73 (Type 1)	Wingweb
71	Inactivated Coryza (Inactivated)	Serotypes A, B, and C	Intramuscular (IM)
84	IB + Gumboro Disease + ND (Inactivated)	IB M41 Gumboro D78 ND Clone 30	Intramuscular (IM)
120	*Pasteurella multocida* (live)	X-73 (Type 1)	Wingweb
150	IB (Live) ND (Live)	IB ma5 ND Clone 30	Intranasal

On March 22, 2024, the management reported that approximately 25% of birds exhibited swollen shanks, exudation on the keel bone, and nasal discharge. The clinical onset was at around 3.5 to 5 months of age and leg lameness was more apparent in 4-month-old birds. Given these clinical signs, the farm veterinarian initially suspected MS as the causative agent. Hence, Tilmicosin + Florfenicol + Aspirin (Nutribiz Corp.) was administered via drinking water for 7 days to assess treatment response. Unfortunately, the response was slow and only around 40% of the symptomatic birds recovered. Hence, on June 6, 2024, 8-month-old birds (n=30) from Batch 5 were physically examined for further testing. Blood (n=30) and organ samples (n=10) were collected for serology, histopathology, and qPCR.

2.2 Necropsy

Ten (10) morbid birds were humanely euthanized to assess the external and internal lesions. Externally, the hock joints appeared inflamed, with swelling extending downward through the tarsometatarsal region (Fig. 1A). Despite these external signs, gross examination of the joint capsule revealed no significant gross lesions. In contrast, the sternal region of the bird showed poor feathering and erythema (Fig. 1B). Beneath the skin, there was visible accumulation of yellow seromucous exudate around the keel bone and surrounding muscles (Fig. 1C). Mucosal hyperemia was also seen in the tracheal mucosa (Fig. 1D).

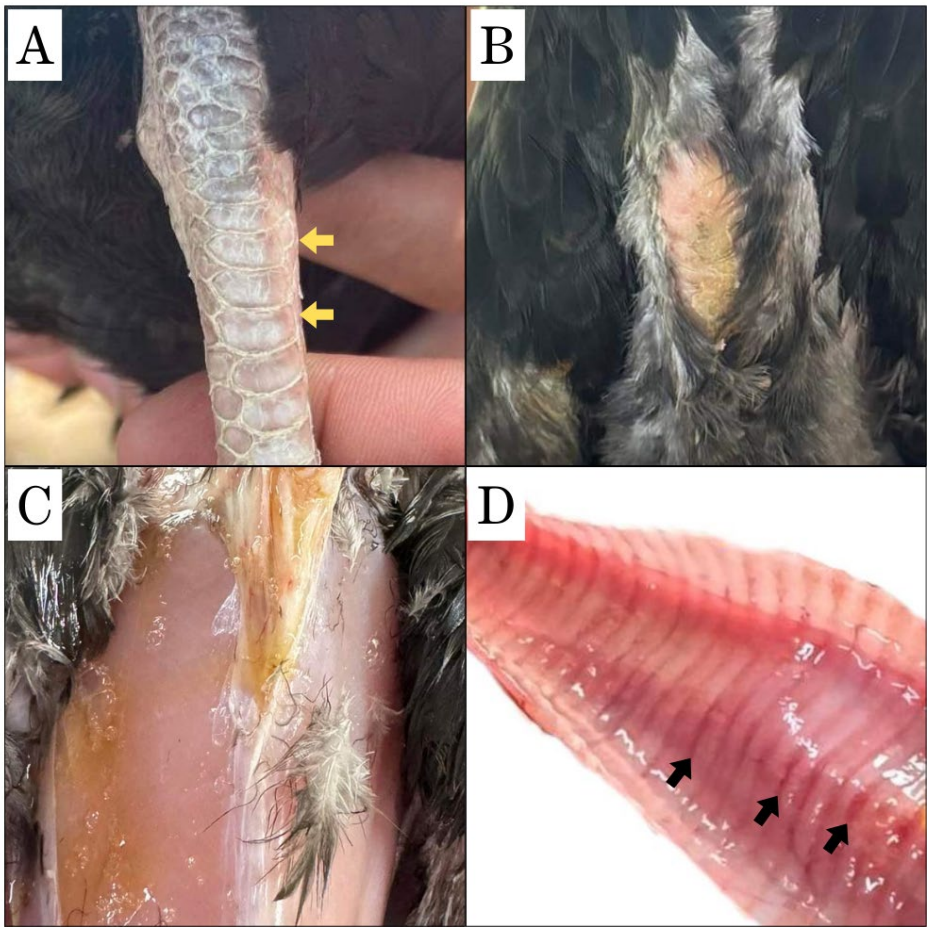

Figure 1. Necropsy findings in a gamefowl flock. (A) Swollen and erythematous tarsometatarsal region (yellow arrows). (B) and (C) Sternal bursitis marked by accumulation of yellowish exudate in the keel region. (D) Mucosal hyperemia in the tracheal mucosa (black arrows).

2.3. Histopathology

The liver, lung, spleen, bone marrow and tracheal samples were fixed using 10% buffered formalin, sectioned and stained using the standard hematoxylin-eosin stain (Fig. 2). Histopathological examination of the liver revealed moderate to severe, multifocal infiltration of inflammatory cells was observed in the perivascular regions of the periportal vessels and hepatic sinusoids. The portal vessels and sinusoidal capillaries also showed mild to moderate congestion. The lungs revealed severe, multifocal to diffuse infiltration of heterogeneous lymphoid cells within the interstitial areas surrounding the air capillaries. Additionally, there was moderate to severe congestion of the pulmonary blood vessels. The spleen showed mild, diffuse lymphoid depletion in the central areas of the splenic parenchyma, which appeared as slightly pale zones upon sub gross examination. In the bone marrow, there was a moderate, diffuse reduction in overall cellular density, with moderate to severe replacement of hematopoietic elements by adipocytes, indicative of marrow suppression or atrophy. The tracheal mucosa exhibited moderate to severe, multifocal to diffuse infiltration of inflammatory cells within the lamina propria. The normal epithelial and mucosal glandular architecture was disrupted and replaced by an irregular arrangement of lymphoid cells, interspersed with disorganized goblet cell-like structures.

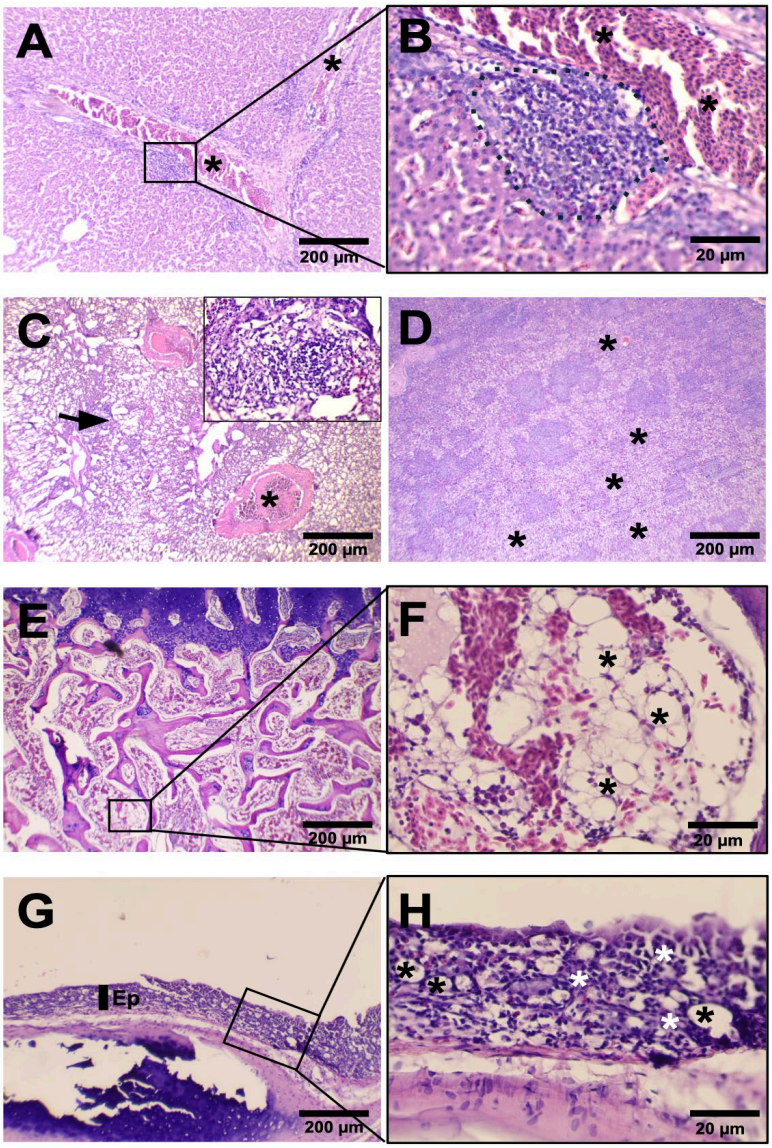

Figure 2. Histopathological images of lesions in the liver, lungs, spleen, bone marrow and trachea. (A) low and (B) high magnification images of the liver showing focal infiltration of inflammatory cells (demarcated with broken lines) in the perivascular area and moderate congestion in the portal vessel (asterisks). (C) low magnification images of the lung showing focal infiltration of heterogenous lymphoid cells in the interstitium of air capillaries (arrow and inset) and severe congestion within pulmonary vessels (asterisk). (D) low magnification image of the spleen showing pale areas of the parenchyma (asterisks), with mild depletion of lymphoid cells. (E) low and (F) high magnification images of the bone marrow show adipocyte replacement of hematopoietic cells (asterisk). (G) low and (H) high magnification images of the tracheal mucosa showing severe infiltration of inflammatory cells in the lamina propria (white asterisk) and disorganized goblet cells (black asterisk); Ep: epithelium.

2.4. Serology

Thirty (30) serum samples were sent to the Precisione International Research and Diagnostic Laboratory Inc. in Marilao, Bulacan for antibody titer assessment against ARV and MS using ELISA. Results are shown in Table 2. All samples tested positive for ARV antibodies, and the mean titer exceeds the expected range for vaccinated birds (7,000-20,000). The coefficient of variation (CV) for ARV is low, which suggests that the antibody titer is relatively uniform across all samples. Five (5) out of 30 samples also tested positive for MS antibodies, with a CV (%) of 187. Since the birds were unvaccinated for these diseases, the results show clear evidence of exposure.

Table 2. Antibody titer results for ARV and MS using ELISA.

Disease Agent	Mean Titer	Min-Max Titer	ELISA standard (Biochek)	CV (%)	Remarks
ARV	27790	11845-37000	live vaccine- 2 000 - 6 000 3 - 5 wks post vaccination; inact. vaccine 7 000 - 20 000 5 - 8 wks post vaccination; Suspect titer >6,000	23	Positive (No ARV vaccination in the farm)
MS	359	1-3247	live MS-H (eye drop)- 500 - 3 000 6 -12 wks post vaccination 30- 70% Pos; Suspect titer > 5000 and > 90% Pos	187	Positive (No MS vaccination in the farm)

2.5 Qualitative Polymerase Chain Reaction (qPCR) and sequencing

Liver, spleen, cecal tonsil, feather, and bone samples from each bird were pooled (n=5) via Flinders Technology Associates cards (Qiagen) and were sent to the ANICON Veterinary Diagnostics and Services Laboratory in Höltinghausen, Germany for REO and MD PCR screening and sequencing. The samples tested positive for both ARV and MD (Table 3). All CT values below 30 were processed for sequencing.

2.6. Consensus sequence alignment and BLAST Search

Nucleotide sequence analyses were conducted using MEGA 11 (Molecular Evolutionary Genetics Analysis) software (Philadelphia, PA, USA). The quality of all newly obtained gene sequences was assessed by visual inspection of chromatograms. Forward and reverse sequences were aligned to generate consensus sequences. Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) against the NCBI GenBank database to identify the most closely related sequences. Representative nucleotide sequences generated from each organ in this study were deposited in GenBank (Accession Numbers PX833342 – PX833346).

2.7. Phylogenetic analysis

Analysis of aligned multiple *Meq* gene sequences was also done using MEGA 11 software. Alignments were visually inspected and trimmed to ensure comparable sequence lengths prior to phylogenetic analysis. A phylogenetic tree was reconstructed for the *Meq* gene sequences, with *Gallid gammaherpesvirus* 2 used as the outgroup. The best-fit nucleotide substitution model was selected based on the lowest corrected Akaike Information Criterion (AICc) value.

The Kimura 2-parameter (K2P) model was identified as the optimal substitution model for the *Meq* gene, with no rate heterogeneity among sites supported (AICc = 6369.160). A maximum-likelihood tree was constructed with 1,000 bootstrap replicates, and branches with bootstrap support below 70% were collapsed to improve the reliability of the tree.

2.8. Treatment and Follow-up

On June 23, 2024, live MS and killed ARV emergency vaccines were given to apparently healthy birds. Three hundred (300) out of 500 birds were culled from Batch 5 to prevent further disease transmission. The vaccination program of the younger batches was also adjusted accordingly to include MS and ARV.

The post-vaccination status of the birds was assessed on August 26, 2024. Following vaccination, 318 out of 354 cockerels (90%) from the succeeding batches no longer showed signs of leg lameness. Necropsy was once again conducted in four morbid birds. Splenomegaly was observed in all birds with the spleen size comparable to that of the heart. In addition, corneal opacity and iris discoloration was also observed in a 6-month-old bird. Tissue samples from the tendon, spleen, heart, liver, lungs, and feathers, along with oropharyngeal and cloacal swabs, were screened for ARV, MS, and MD using real time PCR. The feather samples tested positive for MD.

3. Discussion

The gamefowl industry is an economically important poultry sector in the Philippines, where birds are raised for entertainment, specifically cockfighting. Despite its economic importance, epidemiological data on key avian pathogens affecting gamefowls such as ARV, MDV, and MS, remain limited and underreported.

To the authors' knowledge this is the first documented case of ARV, MS, and MD detection in gamefowls in the Philippines. Diagnosing avian diseases is often challenging since many pathogens present similar clinical signs. To optimize the use of resources, the testing procedures focused on the pathogens most likely to cause the infection based on the clinical signs, necropsy findings, and vaccination history. This list included ARV, MD, and MS. *Mycoplasma gallisepticum* (MG) and Newcastle disease (ND) were initially considered in the differential diagnoses. MG is known to cause chronic respiratory disease in chickens and the tracheal lesions observed could also be attributed to this pathogen [12]. However, tenosynovitis, the chief complaint for this case, is more commonly associated with MS infections in chickens. Hence, MG was excluded from the diagnostic testing. Similarly, ND, which is linked to leg paralysis and respiratory signs [13], was ruled out due to the vaccination history of the birds against this disease.

Avian Reovirus infections are usually asymptomatic. Tenosynovitis/arthritis is the predominant clinical manifestation among all genotypic clusters of ARV [3]. In clinical cases, this is characterized by swelling and redness of the hock joint [13-15]. Several ARV strains previously reported in Asia include ARV LY383 China 2016, HeN130728, LN160607-1, GX150816, SD150806, JS170705-1, A15-48/Wild bird/ Korea 2015, A18-205/Wild bird/Korea2018, ARV2IR018, and ARV1IR019. These strains have been identified from China, Korea, and Iran [3]. Further virus isolation and propagation are recommended prior to sequencing.

Similar to ARV, most MS infections are subclinical. Symptomatic cases primarily affect the synovial structures and upper respiratory tract [6,16]. The clinical signs in this case are consistent with the typical ARV and MS symptoms. Experimental infection of chickens with both ARV and MS showed that the tibiotarsal-metatarsal joint is the main predilection site of dual-infection [17]. As a result, erythema is often observed in the synovial membrane and tendon sheaths.

Sternal bursitis and tracheitis are primarily associated with MS [18], while most reported ARV infections are focused on arthritic lesions and stunted growth [9,13-14]. In this particular case, aside from bacterial infection, repeated contact of the keel region with the ground may have induced trauma, which could also be linked with the apparent leg lameness. Leg lameness can also be induced by the enlargement of peripheral nerves due to MD. Typical MD lesions are lymphoproliferative and neoplastic. The most consistent gross lesion involves the enlargement of the sciatic nerve [5,19]. However, the

histopathological findings for this case only revealed mild to moderate congestion of the visceral organs and presence of inflammatory cells in bone sections (Fig. 2). Additionally, it is possible that the enlargement of the sciatic nerve was not observed due its bilateral nature and the lack of comparison with the sciatic nerve of normal chickens.

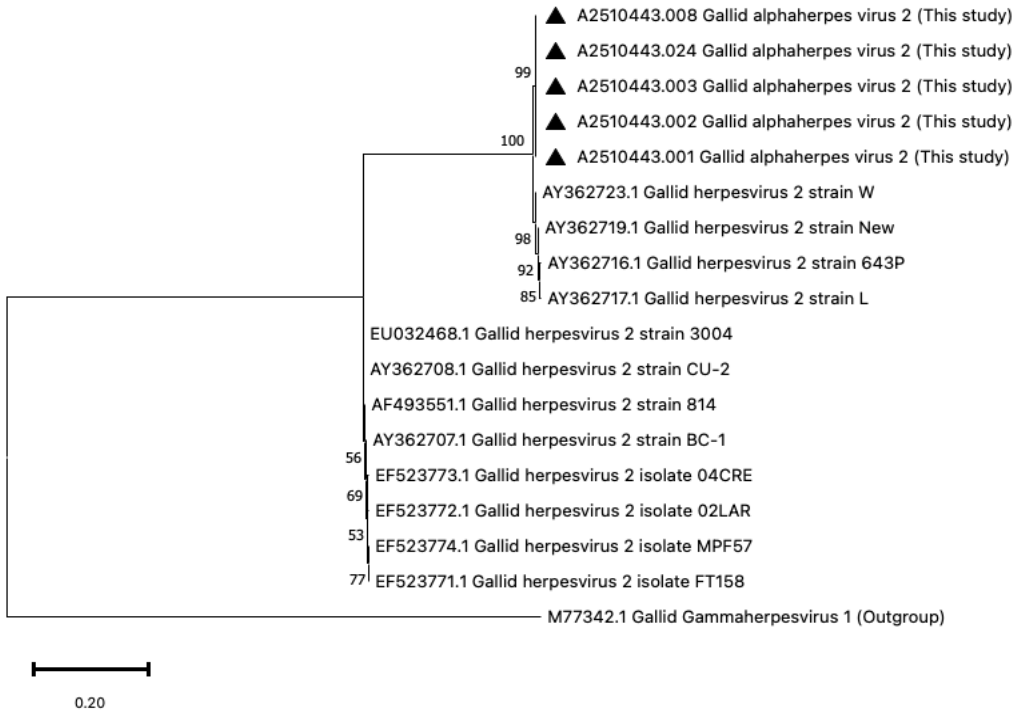

Figure 3. Phylogenetic reconstruction of *Gallid alphaherpes virus 2* in gamefowls from Ibaan-San Jose Batangas based on *Meq* gene.

The positive PCR result for MD in the follow-up suggests that the ocular discoloration represents lymphoid infiltration of MDV in the iris [12]. REO detection via qPCR was not sequenced due to high CT value. Factors such as timing of sample collection, low viral load, or viral shedding variability may explained the results [20-22].

The qPCR results indicated that some samples had a low viral load, rendering further sequencing inadvisable, whereas all MD-positive samples with higher viral loads were used for subsequent analysis to determine the specific MDV strain. Sequencing analysis confirmed that all analyzed samples showed 99.88% homology to *Gallid alphaherpesvirus* type 2 isolated from the feather follicle of chickens in India (GenBank accession number: MK639178.1). Phylogenetic analysis showed that the identified sequences clustered closely with VV+ strains (Fig. 3), with a strong bootstrap support of 100, suggesting possible association with VV+ strains. Unlike ARV and MS, which are both transmitted horizontally and vertically [14,6], MDV is only limited to horizontal transmission [12]. MDV can survive in poultry dust and feather dander for extended periods [23]. Hence, even without vertical transmission, MDV can still proliferate in the farm. Given that this is a brooding to growing farm with an open-range set-up, contaminated sources can easily transfer from the growing to the brooding area and vice-versa. The occurrence and transmission of ARV and MS are influenced by farm setup and production practices. ARV is primarily spread through vertical and fecal–oral transmission, whereas MS is transmitted vertically and through the respiratory route. Factors such as housing conditions and biosecurity measures can affect both horizontal and vertical transmission, contributing to the persistence and broader transmission of these viruses within poultry operations [6,14].

Free-range birds may be predisposed to co-infections due to lack of physical barriers and risk

of contact with wild birds [24]. Immunosuppression induced by stress and exposure to other pathogens also increases the likelihood of co-infections in a gamefowl flock [4,24]. Therefore, regular monitoring and surveillance should be conducted using sensitive diagnostic tests, such as qPCR. If possible, the testing procedures should also include all possible differentials.

Culling of infected birds is recommended since there is no well-established treatment for ARV, MDV, and MS [12]. Vaccination should be implemented at the breeding, brooding, and growing sections of the farm to prevent horizontal and vertical transmission. Improved biosecurity practices such as addition of footbaths can help mitigate the spread of infectious diseases.

For MD vaccination, considerations should be made regarding its potential side effects. All MD vaccines consist of live viruses administered to 18-day-old embryos or post-hatch [23]. It is important to note that vaccinated flocks can still be infected by virulent MD. Marek's Disease Virus transmission to other chickens occurs via the feather follicle epithelium [25]. As such, post-vaccination infections typically occur in immunosuppressed birds. Birds may develop the clinical signs of the virulent form which includes ocular changes [12]. Ocular lesions may significantly affect the performance and economic value of gamefowls. Hence, possible adverse effects should always be relayed to the client prior to vaccine administration. Additionally, it is recommended to formulate a vaccination program for gamefowls to minimize the financial losses caused by field challenges. Proper vaccine handling and administration is also crucial in preventing vaccination failure [25]. The implementation of immunoprophylaxis and strict biosecurity measures among poultry farms in the Philippines is essential in effective controlling the introduction and transmission dynamics of ARV, MDV, and MS infections. These strategies can also effectively reduce and limit virus transmission within poultry farms and limit the spread of infections within and between poultry operations.

4. Conclusion

This paper reports and confirms the presence of MD, MS, and ARV in a gamefowl flock in the Philippines. Moreover, immunosuppressed birds can be predisposed to secondary bacterial infections. The clinical signs of these pathogens may overlap, further complicating the diagnosis and treatment.

This underscores the need for an integrated approach that combines tailored immunoprophylaxis programs and strict biosecurity implementation among farms in the Philippines to limit the transmission dynamic of MS, MD, and ARV. The addition of sensitive, specific, and cost-effective diagnostic tests into surveillance and control programs is essential to enable an effective and sustainable evidence-based interventions against emerging avian diseases.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, S.I.C., and M.L.P.P; Methodology, S.I.C., F.P.D.V., M.J.C.A., and M.L.P.P.; Resources, S.I.C.; Writing - Original Draft, M.L.P.P.; Data Analysis, S.R.E; Writing - Review & Editing, S.I.C and F.P.D.V.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Effects of Dietary α-Lipoic Acid and Selenium Supplementation on Sperm Motility, Morphology and Viability in Philippine Native Chickens (*Gallus gallus domesticus*)

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Abstract

Background: Previous studies show that α-lipoic acid and selenium supplements independently enhance semen quality. This study evaluated their combined and individual effects on avian semen quality. **Methods**: Seventeen (17) sexually mature Banaba Roosters at 5-8 months and average weight of 1.68 kg, were used to evaluate the effects of dietary supplementation of antioxidants, α-lipoic acid and selenium, on semen quality that were randomly assigned to four groups: Control (no antioxidant supplement), Treatment 1 or T1 (100 mg of α-lipoic acid), Treatment 2 or T2 (100 µg of selenium-enriched yeast), and Treatment 3 or T3 (combination of both 100 mg α-lipoic acid and 100 µg commercial selenium-enriched yeast). Semen collected from each rooster was pooled and assessed for volume, color, and consistency. Samples were diluted with Lactated Ringer's Solution (LRS), and those with ≥70% initial sperm motility were further processed and were evaluated for %motility and %morphology using Computer-Assisted Sperm Analysis (CASA), while viability was assessed at 0, 4, 8, 12, and 24 hours using staining after storage at 4–9°C. **Results**: Semen from T1, T2, and T3 groups had significantly better sperm motility after 12 hours ($p=0.05$). Treatments shown significant effects of the sperm characteristics except on sperm cell morphology. **Conclusion:** Dietary α-lipoic acid and selenium yeast significantly enhance sperm quality and storage longevity in Banaba cockerels, improving their suitability for artificial insemination and cryopreservation.

Keywords

α-lipoic acid, Selenium, Semen quality, Banaba native chicken

1. Introduction

Native or improved chickens continue to comprise roughly 43% of the total chicken inventory in the Philippines, serving as a crucial, hardy, and low-input poultry resource. Often raised under free-range systems by smallholder farmers, they provide meat, eggs, and supplemental income and are prized for their resilience to harsh conditions. According to the latest report from the Philippine Statistics Authority in September 2023, Central Luzon and CALABARZON are the top-producing regions. Most of these chickens are raised in the backyard to small-scale operations for meat, eggs, or game. Known for their adaptability to the Philippine climate, these chickens are resistant to common local poultry diseases, reducing input costs [1]. However, emerging avian diseases, climate change, and the influx of imported breeds pose threats, prompting researchers to study the conservation of these native breeds [1,2].

Native chickens are vital to food security in provincial and far-flung areas because they are well adapted to local conditions, require minimal inputs, and provide a dependable source of meat and eggs for rural households. The Banaba breed, believed to have originated in Batangas province, is a recognized Philippine native chicken used for meat, eggs, and as a game fowl. Hens exhibit protective behavior and efficient maternal care toward their offspring [3]. The Banaba breed also exhibits superior baseline semen characteristics, such as higher ejaculated semen volume compared to Joloano and Paraoakan, and better sperm concentration compared to Joloano [2]. These traits make the Banaba breed ideal for semen processing, including extension and cryopreservation, and suitable for artificial insemination. Enhancing and preserving their reproductive performance supports both the conservation of native chicken genetics and the sustainability of food and livelihood systems in underserved communities.

Antioxidants like α-lipoic acid and selenium improve sperm motility and other seminal parameters in roosters by scavenging oxidative radicals, like the effects of vitamins C and E [4,5]. Specifically, α-lipoic acid and selenium enhance seminal characteristics when supplemented through feed. α-Lipoic acid (ALA) inhibits oxygen-free radicals in both lipid and aqueous environments, chelates transition metals, and prevents protein oxidation and membrane lipid peroxidation [6]. Selenium, particularly in its organic form, has shown significant advantages over traditional sodium selenite, including better improvements in sperm motility [4,7].

Previous studies have demonstrated that ALA and selenium supplementation can independently improve semen quality. However, the combined and potentially synergistic effects of these antioxidants, particularly when incorporated into basal commercial feeds, remain poorly understood. This study aims to evaluate both the individual and interactive effects of dietary ALA and selenium supplementation on sperm motility, morphology, and viability in Banaba native chickens. By improving semen quality in this indigenous breed, the study offers a practical nutritional tool to enhance reproductive efficiency and contribute to the conservation and long-term preservation of native chicken genetic resources.

2. Materials and Methods

2.1 Time and Place of Study

The study was conducted from June to July 2024 at the Animal Physiology Laboratory of the Institute of Animal Science, College of Agriculture and Food Science, University of the Philippines Los Baños (UPLB). It was carried out with the protocol approved (UPLB-2024-038a) by the UPLB Institutional Animal Care and Use Committee.

2.2 Management of the Philippine Native Chickens

Seventeen (17) mature Banaba roosters, aged 5-8 months with an average weight of 1.68 kg, were housed in 1m-by-1m cages for eight weeks at the area beside the Advanced Animal Science Research and Training Laboratory. At the start of the experiment, the roosters were weighed and randomly assigned to four treatment groups. All groups were provided with commercial grower feeds with 14% crude protein content and fresh, clean water *ad libitum*. Treatments were given to each of the roosters orally. The facility where the cages are housed were cleaned weekly by soaking and brushing the floors with soapy water and rinsed with flowing water and disinfected after.

2.3 Experimental Design

The experiment utilized a completely randomized design (CRD) with four experimental groups: Control (no antioxidant supplement), T1 (given with 100 mg of commercial ALA), T2 (given with 100 µg of commercial selenium-enriched yeast), and T3 (given with 100 mg of commercial ALA and 100 µg of commercial selenium-enriched yeast). The dose was based on the available dosage of table and the weight of the roosters. Semen samples were collected and pooled by group, with a total of six independent collections performed for each group. Only semen samples with an initial motility of ≥ 70% were selected for further processing and analysis.

2.4 Semen Collection

Semen collection was performed using the Burrow and Quinn (1937) method, with samples pooled by group after two weeks of dietary treatment [8]. Collections were made twice a day,

with a rest day between collection days. The volume of the semen samples was measured using collecting tubes and calibrated commercial syringes. Fresh semen samples were extended with Lactated Ringer's Solution (LRS, 1:15) and analyzed immediately after extension. Computer Assisted Sperm Analysis (CASA) was used to assess sperm motility and normal morphology. Only semen samples with initial motility of ≥ 70% were processed further.

2.5 Semen Quality Evaluation

The semen quality parameters measured in the study are motility (%), normal morphology (%) and viability (%). Sperm motility and normal morphology were evaluated using the Gallus module of CASA (Ceros II, IMV Technologies, China) operating at a frame capture rate of 60 hertz and a camera exposure time of 4 milliseconds. About 4 µl of each sample was transferred to a glass slide and covered with a cover slip. Five different frames under 3 minutes were captured for each observation. The motility and normal morphology measurements are the average of these five fields. Sperm viability was determined by evaluating the acrosome integrity using the Trypan Blue-Giemsa staining method. About 5 µl trypan-blue solution and 5 µl semen sample was mixed in glass slides and were allowed to air dry vertically. Formaldehyde-neutral red solution was used as the fixative agent wherein the slides were soaked for approximately 2-3 minutes. The slide was then rinsed with distilled water and air-dried. The slides were then covered with Giemsa stain for 2.5 hours and incubated at 37°C. Lastly, the slide was rinsed with distilled water and air-dried. A total of 200 spermatozoa were evaluated in each observation by counting sperm having purple acrosome and white head which were viable while sperm having pale lavender acrosome and blue head were non-viable. All semen quality parameters were observed at different time intervals (0h, 4h, 8h, 12h, and 24h) until motility dropped to 20%.

2.6 Statistical Analyses

Data was summarized using mean and standard error. Test of significant difference was performed to compare sperm percent motility, percent normal morphology and percent viability. To identify the most appropriate statistical test, assumptions such as normality and variance homogeneity were assessed. The Shapiro-Wilk test was employed to check the normality of the data, while Levene's test was used to determine variance homogeneity. For data meeting these assumptions, One-Way ANOVA followed by Tukey HSD Test (post-hoc) was used. If the assumptions were not met, the Kruskal-Wallis H-test and Dunn's Test (post-hoc) were applied.

3. Results and Discussion

Fresh semen of seventeen (17) Banaba cockerels assigned to different groups were evaluated based on semen volume, semen color, and semen consistency before initial dilution with LRS extender. Other sperm characteristics such as percent motility, percent normal morphology and percent viability of sperm were also examined after dilution. These three parameters were observed at 0, 4, 8, 12, and 24 hours until motility dropped to 20% and were analyzed. No observation was made beyond 24 hours of extension since the motility already dropped to 20%.

3.1 Fresh Semen Evaluation

Initial evaluation of semen color and consistency was conducted on samples from a total of 69 observations. Forty-eight ejaculates were white, 15 were creamy, six were milky, and three were clear, with varying consistencies ranging from thick to thin or watery. The consistency of the fluid released by domestic poultry's reproductive organs varies, ranging from a clear, watery liquid to a dense, opaque suspension [9]. The color differences among the ejaculates also indicate sperm concentration, with creamy samples generally showing higher sperm concentrations, while clear samples may suggest lower concentrations or the presence of younger Banaba cockerels [10].

3.2 Sperm Motility

Sperm motility describes the sperm that can fertilize an egg cell after being deposited to the female reproductive tract either by natural mating or via artificial insemination. In this experiment, observations were done to check on the shelf life of the collected samples until 20% motility was reached. After 24 hours of observation, samples from the control group have gone below 20% motility which halts the observations to be done across all treatments.

Figure 1 shows the mean % sperm motility observed across different observation hours. Initial % sperm motility observed across different treatment groups all reached above the 70% mark which is ideal for sperm samples to be used for cryopreservation and artificial insemination. Several replicates of the control group were observed to have reached lower than the 20% mark after 24 hours of storage in low temperature.

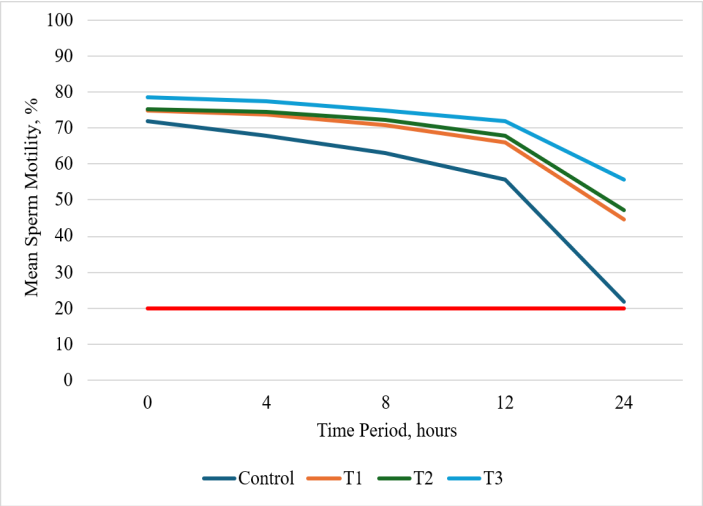

Figure 1. Mean % sperm motility of Banaba native chicken semen across different observation hours.

Table 1 presents the sperm motility percentages of extended semen samples from different experimental groups, observed at various time intervals. At 0 hours, the control group showed the lowest motility (71.95±2.04%), while the T3 group (ALA and organic selenium) exhibited the highest motility (78.42±1.47%). At 4, 8, and 12 hours, motility in the control group was significantly lower compared to the supplemented groups (T1, T2, and T3). By 24 hours, motility in the control group had drastically decreased to 21.65±2.52%, whereas the T3 group maintained relatively high motility at 55.75±2.78%. Additionally, T1, T2, and T3 had significantly higher motility than the control group, with T2 and T3 demonstrating significantly higher motility than T1.

Table 1 also demonstrates that T1 (ALA) and T2 (organic selenium) are effective dietary supplements for improving sperm motility in roosters compared to the control group. The findings for T1 are consistent with Behnamifar *et al.* (2021) and Ye *et al.* (2021), which reported that ALA improves rooster sperm motility, with optimal results observed after two weeks of supplementation [11,12]. Similarly, Shanmugam *et al.* (2015), Chauychu-Noo *et al.* (2021) and

Table 1. Sperm motility (%) in extended semen samples from roosters in each experimental group and observed at different time intervals.

Period	Control	T1	T2	T3	Test statistic	p-value
	Mean±SEM					
0H	71.95±0.83^{b}	74.72±1.61^{ab}	75.05±1.20^{ab}	78.42±1.47^{a}	4.082	0.021*
4H	67.72±2.05^{b}	73.82±1.41^{a}	74.51±1.13^{a}	77.38±1.35^{a}	14.624	0.002*
8H	63.12±2.11^{b}	70.98±1.47^{a}	72.22±1.27^{a}	74.75±1.54^{a}	14.951	0.002*
12H	55.50±2.58^{b}	65.98±2.06^{a}	67.67±2.06^{a}	71.93±1.63^{a}	15.980	0.001*
24H	21.65±2.52^{c}	44.43±2.89^{b}	47.25±2.37^{ab}	55.75±2.78^{a}	17.307	0.001*

*Control – No Supplementation, T1 - α-lipoic acid, T2 – organic selenium, T3 - α-lipoic acid and organic selenium Statistical test used: One-Way ANOVA, Tukey HSD Test (post-hoc) for 0H; Kruska-Wallis H-Test, Dunn's test (post-hoc) for 4H, 8H, 12H, and 24H *significant at 0.05; different superscripts indicate significant difference between two groups.*

Sabzian-Melei *et al.* (2022) support the effectiveness of T2, showing that organic selenium also enhances sperm characteristics [13,4,14]. T3, which combined both ALA and selenium, yielded the highest sperm motility among the treatment groups. This highlights the synergistic effect of both treatments on sperm motility. Although all groups experienced a general decline in sperm motility over time, the decrease was significantly less pronounced in the supplemented groups, particularly in T3.

3.3 Sperm Morphology

Figure 2 shows a comparison between a damaged avian sperm cell and normal avian sperm cell. Only morphologically normal spermatozoa can travel from the hen's vagina to the sperm storage tubules to reach the egg cells [15,16]. Sperm morphology percentage was measured using CASA, calculated by subtracting the percentage of sperm with bent or coiled tails from the total number of sperm cells observed. Notably, previous studies have reported that the percentage of normal sperm morphology does not directly correlate with sperm motility percentage and were considered as independent parameters [17,18,19].

Table 2 presents the sperm normal morphology percentages of extended semen collected from each experimental group, observed at various time intervals. The results show no significant differences in sperm morphology among the groups at any time, as indicated by p-values greater than 0.05. At 0 hours, sperm morphology was high across all groups, with the control group at 96.62±0.50% and the T2 group (organic selenium) showing the highest value at 97.58±0.45%. Similarly, at subsequent time periods (4, 8, 12, and 24 hours), no statistically significant differences were observed among the groups. By 24 hours, all groups exhibited a decrease in sperm morphology, with values ranging from 85.20±1.68% in the T3 group (ALA and organic selenium) to 85.78±1.44% in the T2 group. These findings are consistent with previous studies by Behnamifar *et al.* (2021) on ALA and Sabzian-Melei *et al.* (2022) on organic selenium [11,4].

Normal sperm morphology remained relatively stable and high across all groups and time periods,

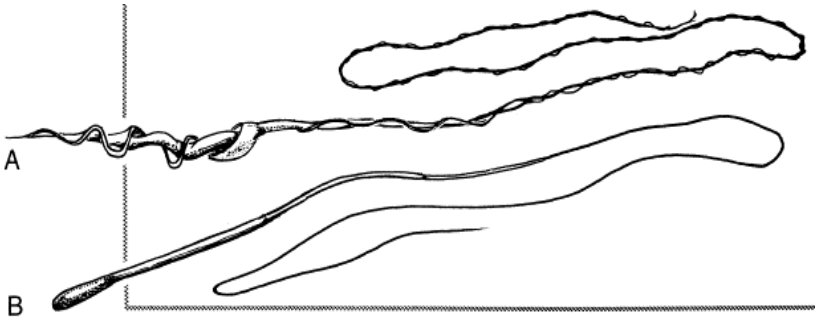

Figure 2. (A) Damaged avian sperm cell and (B) Normal avian sperm cell (Pollock & Orosz, 2002)

Table 2. Normal sperm morphology (%) in extended semen samples from roosters in each experimental group and observed at different time intervals.

Period	Control	T1	T2	T3	Test statistic	p-value
	Mean±SEM					
0H	96.62±0.50	97.25±0.42	97.58±0.45	97.47±0.50	0.841	0.488
4H	92.12±1.58	94.25±1.43	94.25±1.44	95.62±1.01	1.095	0.374
8H	93.58±1.95	92.27±1.08	96.43±0.54	96.22±0.45	6.191	0.103
12H	95.87±0.50	96.10±0.53	96.60±0.34	95.82±0.44	0.605	0.619
24H	85.53±2.18	85.53±2.05	85.78±1.44	85.20±1.68	1.787	0.618

Control – No Supplementation, T1 - α-lipoic acid, T2 – organic selenium, T3 - α-lipoic acid and organic selenium
Statistical test used: One-Way ANOVA, Tukey HSD Test (post-hoc) for 0H, 4H, and 12H; Kruska-Wallis H-Test, Dunn's test (post-hoc) for 8H and 24H *significant at 0.05; different superscripts indicate significant difference between two groups.

with only a slight decline observed at 24 hours. This stability suggests that supplementation with ALA, organic selenium, or their combination did not significantly affect sperm morphology compared to the control group. However, it is important to note that sperm morphology percentage is not directly related to sperm motility percentage, as it only reflects the proportion of sperm cells that are not structurally damaged, as described by Bakst *et al.* (1994) and Mohan *et al.* (2018) [15,16]. Only morphologically normal spermatozoa can travel from the vagina of the hen to the sperm storage tubules to fertilize the egg. These findings indicate that while supplementation may offer other reproductive benefits, it does not significantly impact sperm morphology over time in native chickens.

3.4 Sperm Viability

Sperm viability was assessed by evaluating acrosome integrity after staining. A total of 200 sperm with ideal morphology were counted manually. Sperm with a purple acrosome and white head were classified as viable, while sperm with a pale lavender acrosome and blue head were deemed non-viable. Viable sperm are crucial for determining the fertilizing capability of sperm during natural mating and artificial insemination, as well as for calculating the appropriate sperm dose for artificial insemination procedures.

Table 3 presents the sperm viability percentages in extended semen samples from roosters across different experimental groups, observed at various time intervals. At the 0-hour mark, the control group, which received no supplementation, showed a significantly lower sperm viability of 72.50±2.31% compared to the supplemented groups, where T1 (ALA), T2 (organic selenium), and T3 (ALA) and organic selenium) had higher viability rates of 85.42±1.20%, 86.58±1.23%, and 90.50±1.45%, respectively. This pattern of higher viability in the supplemented groups continued at four (4) hours, with the control group's viability (65.00±2.29%) still significantly lower than that of T1 (77.42±1.52%), T2 (78.42±2.13%), and T3 (83.50±1.18%). At eight (8) hours, the control group's viability decreased further to 59.75±2.45%, which remained significantly lower compared to T1 (72.25±1.73%), T2 (75.00±2.01%), and T3 (81.42±1.68%), with T1 showing significantly lower viability than T3. By 12 hours, the control group continued to exhibit significantly lower sperm viability (57.75±2.40%) compared to the supplemented groups, with T1 (69.33±1.32%), T2 (68.58±2.44%), and T3 (76.08±1.54%) showing higher values. At 24 hours, while the viability percentages for all groups decreased, with the control group at 45.33±4.71%, T1 at 55.75±5.32%, T2 at 56.25±5.39%, and T3 at 62.50±5.70%, the differences between groups were not statistically significant (p=0.060).

Over time, a general trend of decreasing sperm viability was observed in all groups. However, the decline was markedly less severe in the supplemented groups, particularly in the T3 group. By 24 hours, the control group's viability had dropped to 45.33±4.71, whereas the T3 group maintained relatively higher viability at 62.50±5.70, though this difference was not statistically significant (p=0.060). These findings suggest that supplementation, particularly with the combination of ALA and organic selenium (T3), significantly improves and sustains sperm viability in native chickens over time compared to the control group, indicating potential benefits for reproductive performance in poultry.

Table 3. Sperm viability (%) in extended semen samples from roosters in each experimental group and observed at different time intervals.

Period	Control	T1	T2	T3	Test statistic	p-value
	Mean±SEM					
0H	72.50±2.31^{b}	85.42±1.20^{a}	86.58±1.23^{a}	90.50±1.45^{a}	15.900	0.001*
4H	65.00±2.29^{b}	77.42±1.52^{a}	78.42±2.13^{a}	83.50±1.18^{a}	18.237	<0.001*
8H	59.75±2.45^{c}	72.25±1.73^{b}	75.00±2.01^{ab}	81.42±1.68^{a}	20.909	<0.001*
12H	57.75±2.40^{b}	69.33±1.32^{a}	68.58±2.44^{a}	76.08±1.54^{a}	14.538	<0.001*
24H	45.33±4.71	55.75±5.32	56.25±5.39	62.50±5.70	7.425	0.060

Control – No Supplementation, T1 - α-lipoic acid, T2 – organic selenium, T3 - α-lipoic acid and organic selenium; Statistical test used: One-Way ANOVA, Tukey HSD Test (post-hoc) for 4H, 8H, and 12H; Kruska-Wallis H-Test, Dunn's test (post-hoc) for 0H and 24H *significant at 0.05; different superscripts indicate significant difference between two groups.

In line with the findings of Gee *et al.* (2004), which highlight factors affecting sperm viability, the results of this study corroborate previous research by Behnamifar *et al.* (2021), Haghighian *et al.* (2015), and Ye *et al.* (2021) demonstrating the efficacy of ALA as a dietary supplement in enhancing sperm viability [11,20]. Additionally, the results of the current study align with the work of Shanmugam *et al.* (2015), Zoidis *et al.* (2018), Surai *et al.* (2018), and Chauychu-Noo *et al.* (2021) which explored the benefits of selenium as an antioxidant for improving seminal characteristics in cockerels [13,14,7,21]. Both ALA and selenium have proven effective in enhancing sperm quality, supporting their use in dietary supplementation.

4. Conclusion

The use of antioxidants as dietary supplements effectively enhances sperm quality, making it more suitable for artificial insemination and cryopreservation. This is particularly important for preserving valuable breeds and meeting the increasing demands for meat and eggs. The findings from this study offer valuable insights for experts in artificial insemination and cryopreservation laboratories, highlighting how antioxidant supplementation can improve the quality of cockerel semen.

The study demonstrated that cockerels treated with ALA and selenium yeast exhibited superior sperm motility, morphology, and viability compared to those on a standard diet. Immediate processing, extending, and storing of semen samples were emphasized, from antioxidant-dietary supplemented roosters serving as semen donors and showing better semen preservation over time. Specifically, extended semen from cockerels not receiving supplementation could only be stored for less than 24 hours, while extended semen from antioxidant-dietary supplemented roosters remained viable for a longer duration.

The study confirmed that both ALA and selenium yeast, at 100 mg and 100 µg, respectively, significantly improve sperm quality in Banaba cockerels. The combination of these antioxidants yielded the best results in terms of sperm motility, morphology, and viability even after 24 hours. The supplementation did not induce toxicity and effectively reduced oxidative stress on sperm.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, K.P.P., and P.P.S.; Methodology, K.P.P.; Experiment, K.P.P.; Writing – Original Draft, K.P.P.; Writing – Review & Editing, K.P.P., and P.P.S.

Ethics Approval and Consent to Participate

The current research has followed the accepted principles of ethical conduct by the Institutional Animal Care and Use Committee of the University of the Philippines Los Baños, under protocol review number CAFS-2023-029.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

PCR-based Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Philippine Broiler Poultry Flocks

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Abstract

Background: Two major bacterial poultry pathogens that cause chronic respiratory disease and infectious synovitis are *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). To date, no nationwide MG and MS molecular detection data are available in the Philippines. This study aimed to detect MG and MS in broiler poultry flocks. **Methods:** From 2020 to 2023, 2,127 broiler flocks with clinical or subclinical respiratory signs were purposively sampled from 13 Philippine regions. Oropharyngeal swabs and selected tissues were screened by conventional PCR targeting the *mgc2* (cytadhesin protein gene) and *16S rRNA* genes of MG and MS, respectively. Positivity estimates with 95% confidence intervals were calculated, and differences were assessed using the chi-square test. **Results:** PCR amplicons yielded 302-bp (MG) and 211-bp (MS) products. Overall, MG and MS were detected in 13.31% (283/2,127) and 7.33%

(156/2,127) of flocks, respectively. Higher detection levels were observed in CAR, Regions IVA, VII, and III. Regional differences in MG and MS detection were statistically significant ($p < 0.001$). **Conclusion:** This study confirmed the molecular detection of MG and MS among clinically affected broiler poultry flocks in the Philippines. Findings reflect detection in sampled flocks and not a population-level prevalence due to purposive sampling. Given the limitations of this PCR-based design, future surveillance should consider rapid, field-adapted, and strain-discriminative approaches, including advanced AI- or machine learning-assisted LAMP assays, to improve pathogen differentiation and control.

Keywords

Mycoplasma gallisepticum, Mycoplasma synoviae, PCR, Philippine poultry

1. Introduction

Two major economically significant bacterial respiratory pathogens that cause chronic respiratory disease (CRD) and infectious synovitis are *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), respectively. These organisms are known to cause major economic losses by reducing egg production, decreasing feed conversion efficiency, increasing embryo mortality, and increasing carcass condemnations [1,2,3].

Variable respiratory lesions, including airsacculitis, sinusitis, and tracheitis, can be seen with MG and MS infections. It has been reported that concurrent infections with bacterial *Escherichia coli* and *Avibacterium paragallinarum,* and viral infections with infectious bronchitis, are associated with severe clinical outcomes [1,4,5]. Although commonly reported to cause less severe respiratory conditions, infection with MS often progresses to synovitis. This results in joint swelling and clinical lameness, and, with its chronic progression, transmission between and within poultry farms becomes evident [3,6].

Poultry mycoplasma infection continues to be a challenging problem despite the availability of vaccines and efforts to improve farm biosecurity and management. Disease expressions may vary depending on surveillance, production systems, and geographical settings. Prevalence estimates ranging from 7% to 70% have been reported in continental Asia, Europe, and Africa. High prevalence levels are most observed in areas with intensive poultry farming [2,6,7]. Of particular importance in interpreting prevalence data are the intensity of sampling and the diagnostic procedures or tools used. A study conducted in Myanmar, which detected concurrent infections with MG and MS, has demonstrated that these pathogens can indeed co-circulate on poultry farms [2]. In contrast, lower infection prevalence was observed in poultry and wild birds in Belgium [7]. Such findings suggested that migratory birds may be responsible for transmissions of poultry Mycoplasma infections.

In the Philippines, there is limited information on poultry mycoplasma infections. Most studies used serologic rather than molecular methods. An early local study in the country on MG has confirmed the presence of chronic respiratory disease (CRD) using cultural and serologic methods [8]. A recent study involving broilers in South Luzon detected MG antibodies using hemagglutination-inhibition and enzyme-linked immunosorbent assay [9]. Obviously, no large-scale molecular-based detection data is available in the country.

In detecting MG and MS, the PCR method is considered the gold standard for obtaining reliable diagnostic and surveillance data [2,5,7,10]. This is understandable given its rapid, sensitive, and specific outcomes compared with traditional isolation and the common reliance on markers of immune responses. The *mgc2* gene, encoding a cytadhesin involved in attachment to host epithelial cells, is a well-established molecular target for MG detection, while the *16S rRNA* gene provides a reliable marker for MS identification [2,5,6].

With the continually expanding broiler poultry industry in the Philippines, which is a major contributor to national animal protein supply and commercial livestock production, and the growing focus on One Health initiatives, it is imperative that molecular data on Mycoplasma infections in the country be established. This study determined the molecular positivity detection of MG and MS across major poultry-producing regions, which constituted the first large-scale PCR-based screening of Mycoplasma infections

nationwide. This was done to improve diagnostic innovation beyond the usual levels for Philippine poultry flocks [11,12].

2. Materials and Methods

2.1 Ethical Consideration

All procedures for sample collection were approved by the Institutional Animal Care and Use Committee (IACUC) of each collaborating institution of the University of the Philippines, Los Baños - College of Veterinary Medicine (Protocol No. 2019-0027); the Cavite State University (with IACUC Exemption Certification); and the University of Eastern Philippines – College of Veterinary Medicine (Protocol No. 2020-001). Informed consent was obtained prior to sampling from poultry operators/owners. An official letter from the Director of the Bureau of Animal Industry, encouraging poultry farm operators/owners to participate in the study, was made available prior to the actual field sampling.

2.2 Sample Collection

A total of 2,127 poultry flocks from 13 Philippine regions (Fig. 1) sampled between 2020-2023 were included in this study. Each flock represented a farm unit with 25 sampled birds. Within each selected flock, birds were purposively sampled, prioritizing individuals exhibiting respiratory or other clinical signs consistent with MG/MS infection, with additional birds sampled from the same housing unit to achieve a minimum of 25 birds per flock. Overall, this corresponded to a total of 53,175 birds sampled across all flocks included in the study. Flock-level classification was based on pooled PCR results, with a flock considered positive if the pooled sample tested positive; individual bird infection status could not be determined due to pooling. No Mycoplasma vaccination was practiced in the broiler flocks included in this study. Sampling was conducted only with the consent and cooperation of poultry operators/owners.

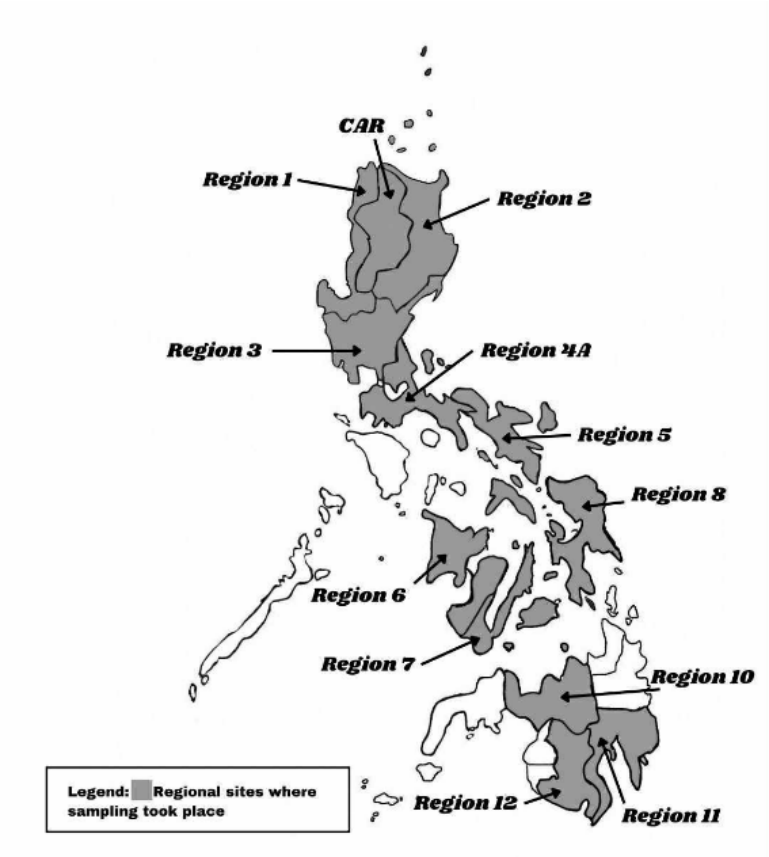

Figure 1. Map of the Philippines showing sampling sites from various poultry farms.

2.3 Sampling Methods

Oropharyngeal swabs were obtained using sterile cotton applicators following the standard procedures [10]. Each swab tip was placed in isotonic phosphate-buffered saline (PBS) containing penicillin (2,000 U/mL), streptomycin (2 mg/mL), gentamicin (50 µg/mL), and mycostatin (1,000 U/mL), transported at 4°C, and subsequently stored at −20 °C until DNA extraction. For each flock, tissue samples from the trachea, lungs, and air sacs were collected from selected birds suspected of MG/MS infection, pooled at the flock level, placed in airtight sterile containers, and stored at −20 °C until analysis.

2.4 DNA Extraction

Approximately 5–10 g of homogenized pooled tissue and swab samples were mixed with 30% normal saline solution containing penicillin (10,000 U/mL) and streptomycin (10 mg/mL). The mixture was centrifuged at 6,000 rpm for 10 min, and the resulting supernatant was stored at −80 °C. DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol.

2.5 Polymerase Chain Reaction (PCR)

Conventional PCR assays were performed to amplify species-specific gene targets for MG (*mgc2*) and MS (*16S rRNA*) using the primer sets listed in Table 1. The *mgc2* gene encodes a cytadhesin protein involved in adhesion to the host respiratory epithelium and contains conserved regions that enable reliable species-specific detection of MG. In contrast, the *16S rRNA* gene region provides highly conserved sequences commonly used for bacterial species-level identification. These loci were selected according to the protocol for PCR detection of poultry Mycoplasma [2,15,16]. Positive control DNA was derived from commercially available live attenuated poultry vaccines used as positive controls for MG and MS PCR assays. A no-template control was included as a negative control in each run. The positive controls consistently produced amplicons of the expected sizes (302 bp for *mgc2* and 211 bp for *16S rRNA*), while the negative controls showed no amplification. [2,13,14,15].

PCR reactions were prepared in 25 µL total volume containing 2.5 µL 10× PCR buffer, 2 µL $MgCl_2$ (50 mM), 0.2 µL dNTPs (10 mM each), 0.1 µL of each primer (10 µM), 0.1 µL Taq DNA polymerase (5 U/µL), 19 µL nuclease-free water, and 1 µL of extracted DNA template. Amplification was performed using an ABI SimpliAmp™ Thermal Cycler (Applied Biosystems, Thermo Scientific) under the following thermocycling conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s, with a final extension step at 72 °C for 5 min.

2.6 Analysis of Data

The proportion of poultry flocks that tested positive for MG and MS by PCR was determined for each region and overall. The PCR-detected

Table 1. Primer sequences used for PCR detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae.*

Target species	Gene target	Primer name	Sequence (5′–3′)	Target Amplicon	References
M. gallisepticum	*mgc2*	mgc2-F	CGCAATTTGGTCCTA ATCCCCAAC	302 bp	[10, 13, 14]
		mgc2-R	TAAACCCACCTCCAG CTTTATTTC		
M. synoviae	16S rRNA	MSL1 (F)	GAGAAGCAAAATAGT GATATCA	211 bp	[10, 15]
		MSL2 (R)	CAGTCGTCTCCGAAG TTAACAA		

positivity rate for each pathogen was computed using the formula [10,16]:

$$\text{PCR Positivity Rate (\%)} = \frac{\text{Number of PCR-positive flocks}}{\text{Total number of flocks tested}} \times 100$$

Ninety-five percent confidence intervals (95% CI) for flock-level positivity estimates were calculated using Wilson's score method for binomial proportions [17]. All analyses treated the flock as the epidemiological unit. Results are presented in tables and figures to summarize spatial variation in MG and MS molecular detection across regions.

Differences in PCR-detected flock-level positivity between pathogens (MG vs. MS) and across regions were evaluated using the chi-square test of independence. Flocks were considered the unit of analysis. Statistical significance was set at $\alpha = 0.05$, and exact p-values are reported.

3. Results

3.1 PCR-Detection of Mycoplasma in Philippine Poultry

PCR-detected amplicons of MG and MS using gel-electrophoresis are presented in Fig. 2 and 3. The figure shows the distinct presence of the target

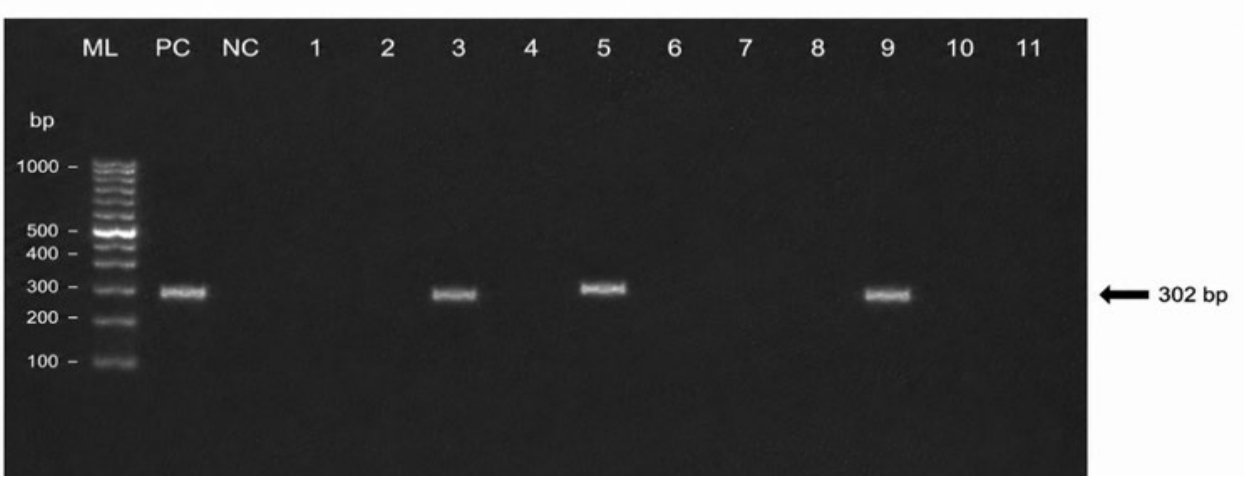

Figure 2. Electrophoretogram of PCR-amplified products of the mgc2 gene of *Mycoplasma gallisepticum* showing a 302 bp product; Lane ML: 100 bp DNA Ladder; lane PC: positive control (*M. gallisepticum* vaccine strain); lane NC: negative control; lanes 1-11: isolates from Luzon, Philippine commercial broiler farms.

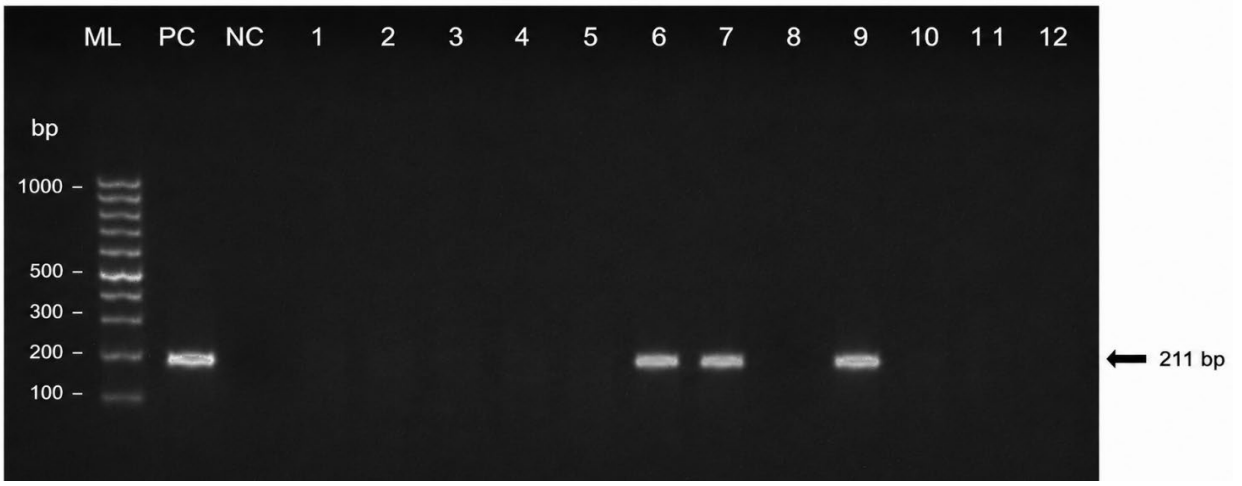

Figure 3. Electrophoretogram of PCR-amplified products of the 16S rRNA gene of *Mycoplasma synoviae* showing a 211 bp product; Lane ML: 100 bp DNA ladder; lane PC: positive control *(M. synoviae* vaccine strain); lane NC: negative control; lanes 1-12: isolates from Luzon, Philippine commercial broiler farms.

MG organisms using polymerase chain reaction (PCR) of the cytadhesin-encoding *mgc2 gene* (Fig. 2), which produced 302 bp products, and the *16S rRNA* genes of MS, producing 211 bp products (Fig. 3).

3.2 PCR-Based Detection of MG and MS in Philippine Broiler Poultry

Poultry broiler farms in 13 geographic regions demonstrated the presence of MG and MS in varying proportions from 2020 to 2023 (Table 2). Nationally, MG was detected in 13.31% of flocks (95% CI: 11.93–14.81), whereas MS was detected in 7.33% (95% CI: 6.30–8.52).

MG positivity varied across regions, ranging from 0% to 17.65%. Higher detection rates were observed in CAR (17.65%; 95% CI: 6.19–41.03), Region IVA (17.55%; 95% CI: 15.21–20.16), and Region VII (16.67%; 95% CI: 5.84–39.22). Intermediate levels were observed in Regions VI (14.29%; 95% CI: 7.95–24.34), Region X (14.29%; 95% CI: 2.57–51.31), Region XII (12.50%; 95% CI: 4.97–28.07), and Region II (12.12%; 95% CI: 4.82–27.33). Lower detection rates were observed in Region III (10.81%; 95% CI: 8.91–13.07), Region VIII (10.00%; 95% CI: 2.79–30.10), Region I (3.33%; 95% CI: 0.59–16.67), and Region XI (2.36%; 95% CI: 0.81–6.72), while no MG-positive flocks were detected in Region V. These regional differences were statistically significant based on Chi-square analysis ($\chi^2 = 36.39$, df = 11, $p < 0.001$).

MS detection ranged from 0% to 17.65% across regions. Higher MS detection rates were

Table 2. PCR-detected positivity rates of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) among poultry flocks in the Philippines (2020–2023).

Region	Total flocks examined	MG Positive (n)	% MG Positive (95% CI)	MS Positive (n)	% MS Positive (95% CI)
CAR	17	3	17.65 (6.19–41.03)	3	17.65 (6.19–41.03)
Region IVA	906	159	17.55 (15.21–20.16)	94	10.38 (8.55–12.53)
Region VII	18	3	16.67 (5.84–39.22)	0	0.00 (0.00–17.59)
Region VI	70	10	14.29 (7.95–24.34)	1	1.43 (0.25–7.66)
Region X	7	1	14.29 (2.57–51.31)	0	0.00 (0.00–35.43)
Region XII	32	4	12.50 (4.97–28.07)	0	0.00 (0.00–10.72)
Region II	33	4	12.12 (4.82–27.33)	0	0.00 (0.00–10.43)
Region III	860	93	10.81 (8.91–13.07)	54	6.28 (4.84–8.10)
Region VIII	20	2	10.00 (2.79–30.10)	1	5.00 (0.89–23.61)
Region I	30	1	3.33 (0.59–16.67)	0	0.00 (0.00–11.35)
Region XI	127	3	2.36 (0.81–6.72)	3	2.36 (0.81–6.72)
Region V	7	0	0.00 (0.00–35.43)	0	0.00 (0.00–35.43)
TOTAL	**2,127**	**283**	**13.31 (11.93–14.81)**	**156**	**7.33 (6.30–8.52)**

observed in CAR (17.65%; 95% CI: 6.19–41.03) and Region IVA (10.38%; 95% CI: 8.55–12.53). Intermediate levels were noted in Region III (6.28%; 95% CI: 4.84–8.10) and Region VIII (5.00%; 95% CI: 0.89–23.61), whereas the remaining regions showed low to zero detection. Regional differences in MS detection were also statistically significant ($\chi^2 = 34.82$, df = 11, $p < 0.001$).

MS was detected in 7.33% (156/2,127) of flocks, whereas MG was detected in 13.31% (283/2,127). MS-positive flocks represented 55.12% of MG-positive flocks at the population level. MS detection occurred only in regions where MG was also identified.

4. Discussion

4.1 PCR Detection Targets

The existing MG and MS detection protocol uses the target genes *mgc2* for MG and *16S rRNA* for MS as PCR targets [10,13,14]. Extensively used in both conventional and advanced PCR protocols, the *mgc2* gene, a conserved region that encodes the cytadhesin protein, is essential for respiratory epithelial attachment [13,14]. High-throughput MS identification in diverse clinical samples targets the *16S rRNA* locus, a highly conserved ribosomal region that contains species-variable sequences, enabling accurate, high-throughput identification [15]. In this study, detected bands were interpreted based on the expected amplicon sizes, species-specific PCR targets, and appropriate positive and negative controls. Although minor differences in band migration may occur during gel electrophoresis, as observed in lane 5 of Figure 2, the positive bands remained within the expected target region. Sequencing of PCR-positive amplicons was not included in the present study as the primary objective was large-scale molecular surveillance rather than strain characterization. Species-specific, internationally validated PCR targets and appropriate positive and negative controls were used to ensure detection specificity. Future studies will incorporate sequencing and strain-level analyses.

While both targets are recommended [10] for confirming avian mycoplasmosis, it is noteworthy that neither gene could offer an absolute "*Differentiating Infected from Vaccinated Animals*" (DIVA) capability. In commercial broiler production in the Philippines, MG and MS vaccination is not routinely practiced; therefore, vaccine-derived detection is unlikely in the broiler flocks included in this study. However, in layer operations where vaccination may occur, conventional PCR assays lack DIVA capability and cannot distinguish between field and vaccine strains. Hence, vaccination history should be considered when interpreting PCR-positive results. Moreover, since this study focused on purposive sampling of birds exhibiting clinical signs of MG/MS, it can be inferred that the birds detected as positive may have been suffering from active MG/MS infections. Future studies should incorporate detailed production and health management data to strengthen epidemiological interpretation.

With advances in molecular diagnostics, LAMP-based platforms are increasingly being explored as rapid and field-adapted tools to support pathogen detection. Previous LAMP simulation and machine learning studies reported modified F1-scores ranging from 71.19% to 72.10%, with specificity reaching 85.45% at optimized reaction conditions [11,12]. These findings suggest that advanced AI- or machine learning-assisted LAMP approaches may complement conventional PCR by improving primer evaluation, amplification prediction, and pathogen differentiation. However, these methods should be further validated for MG and MS detection before routine diagnostic application.

4.2 Nationwide Detection of MG and MS

The current overall molecular detection of MG/MS (13.3% and 7.3%, respectively) has demonstrated the endemic presence of poultry mycoplasma infections in the Philippines. This finding is consistent with molecular positivity rates for MG/MS across Asia and Europe. Molecular surveys of MG/MS have reported positivity rates of 6-20% in Myanmar, 5-10% in Belgium, and as high as 25% in Egypt [2,6,7]. Also aligned with global trends is the predominance of MG over MS in Philippine poultry flocks. MG is considered to be the more infectious and economically important poultry respiratory pathogen [3].

Managing poultry respiratory mycoplasmas, particularly on dense, multi-aged rearing farms, may be cumbersome in Central Luzon and

Calabarzon regions, where co-circulation of MG and MS has been documented. Disease persistence and horizontal spread of infections are commonly observed in poultry operations with overlapping production cycles [7,10]. In the present study, MG was observed in all regions except in Region V (Bicol). Future studies should include larger clinical samples in this area, as a previous serologic study covering South Luzon and Bicol areas reported MG-seropositive flocks [9]. Aside from this, a far-improved diagnostic platform must be explored, such as the use of advanced, machine-learning-enhanced LAMP that can offer better detection accuracy than conventional PCR methods [11, 12]. More importantly, it is well to note that the currently observed detection rates reflect PCR positivity among clinically affected flocks and should not be interpreted as a true national prevalence due to purposive sampling.

In this study, region-specific positivity estimates with 95% confidence intervals were used to provide a clear representation of spatial variation in MG and MS detection [17]. Significant regional variation in MG and MS positivity was observed across regions in the Philippines (χ^2 test, $p < 0.001$), suggesting that pathogen distribution may not be random and may be influenced by region-specific factors. However, given the purposive sampling design, these results should be interpreted as representative only of the sampled flocks and not of the general poultry population.

The relatively high detection in the Cordillera Administrative Region (CAR) (17.65% for both MG and MS) may be attributed to its environmental condition. Findings from low-temperature, high-altitude zones in other Asian regions indicate that cold stress often exacerbates the expression of respiratory disease [19]. The lowest MG PCR positivity rates (2–3%) were observed in Regions XI and I, respectively, while the lowest MS positivity rate was observed in Region VII. These findings are consistent with the statistically significant regional variation identified in this study (χ^2 test, $p < 0.001$). These observations may be attributed to multifactorial poultry-raising management and geographic separation across the country.

The reported MG detection rates in neighboring Asian countries — Japan (14.7%), Malaysia (15.3%), and China (12.6%) [1, 2]—have aligned with the current overall Philippine MG PCR-positive rate of 13.3%. The co-detection of MG with MS in the same geographic regions has highlighted the potential for synergistic poultry respiratory disease outbreaks. Chronic colonization with MG and MS is known to increase antimicrobial resistance and exacerbations of tissue inflammatory lesions [10, 18,19].

Despite antimicrobial use to combat poultry mycoplasmosis, the persistence of MG and MS suggests the possibility of inconsistent/improper antimicrobial administration, or the emergence of resistant field strains of the organisms. These observations are likewise noted in other Asian poultry farms [18,19]. Considering this, an integrated surveillance approach is recommended, including strain identification and monitoring of resistance patterns. This strategy could help assess emerging trends and, thereafter, support targeted immunological and antimicrobial treatments. The risk of prolonged poultry mycoplasma infection could be mitigated by addressing the underlying resistance strains.

4.3. MG and MS Co-circulation Patterns

The observation that MS detection occurred only in regions where MG was also identified suggests overlapping circulation of these pathogens in broiler production systems. This pattern is consistent with their shared transmission ecology and recognized involvement in respiratory disease complexes [1,3,7]. In this study, MG was significantly higher than MS as indicated by non-overlapping 95% confidence intervals [17].

At the population level, MS-positive flocks represented 55.12% of MG-positive flocks in this study, indicating substantial epidemiological overlap between MG and MS in affected broiler populations. Such co-occurrence is biologically plausible because both organisms colonize the respiratory tract and share similar vertical and horizontal transmission pathways [2, 3]. Mycoplasma exposure has been associated with more severe respiratory compromise, higher incidence of airsacculitis, poorer growth performance, and greater susceptibility to secondary bacterial infections [3,6,10]. Although broilers have a relatively short production cycle,

these effects may still contribute to subclinical production losses and increased antimicrobial usage [18,19].

The regional co-distribution of MG and MS may also reflect gaps in biosecurity and source flock control, as both pathogens can be transmitted vertically and horizontally [3,10]. These findings support the need for integrated Mycoplasma monitoring rather than single-pathogen surveillance, particularly in production systems where vaccination against Mycoplasma is not routinely implemented in broilers [1,10].

From an epidemiological perspective, the co-circulation pattern observed in this study underscores the complexity of respiratory disease ecology in poultry and highlights the value of molecular surveillance strategies capable of detecting multiple pathogens simultaneously [10,11,12,20,21].

A limitation of this study is that summarized pathogen detection did not permit direct confirmation of flock-level MG/MS co-infection within the same pooled samples. Although the regional distribution suggests epidemiological overlap, precise quantification of concurrent infection would require paired pathogen testing within the same individual flocks. Future studies incorporating integrated molecular detection approaches, including advanced AI- or machine learning-assisted LAMP assays [11,12], may help clarify co-infection dynamics and their potential impact on production performance and disease severity.

5. Conclusions

This study provides nationwide molecular evidence of MG and MS positivity in Philippine broiler flocks. Because sampling was purposive and focused on clinically affected flocks, the results represent molecular detection rather than population-level prevalence. The predominance of MG highlights its comparatively greater pathogenic significance and potential economic impact on commercial production. The co-detection of MG and MS further underscores the ongoing risk of concurrent infections, which may exacerbate respiratory disease severity and compromise flock performance. Future investigations should incorporate antimicrobial resistance profiling and well-designed intervention trials, alongside the evaluation of newer PCR-based diagnostic platforms, including AI- or machine learning-assisted LAMP assays, to enable more precise pathogen discrimination and improved field-level surveillance.

Availability of Data and Materials

All data are presented in the manuscript.

Author Contributions

Conceptualization, G.A.C., M.C.R.D.C., R.D.D., and D.V.U.; Methodology, G.A.C., M.C.R.D.C, G.M.G., R.D.D., M.L.G.A., and D.V.U.; Investigation, G.A.C., M.C.R.D.C., G.M.G., M.L.G.A., P.L.D.R., E.J.T.S., E.J.A., L.A.D.L., Y.R.M.T., D.B.R.P., C.C.P., C.M.D.P., J.P.R., and D.V.U.; Writing – Original Draft, G.A.C.; Writing – Review & Editing, G.A.C., M.C.R.D.C., G.M.G., R.D.D. and D.V.U.; Funding Acquisition, G.A.C., M.C.R.D.C., R.D.D., and D.V.U.; Resources, G.A.C., M.C.R.D.C., R.D.D., and D.V.U.; Supervision, G.A.C., M.C.R.D.C., and D.V.U.; Project Administration, G.A.C., M.C.R.D.C., R.D.D., and D.V.U.

Ethics Approval and Consent to Participate

All procedures for sample collection were approved by the Institutional Animal Care and Use Committee (IACUC) of each collaborating institution of the University of the Philippines, Los Baños - College of Veterinary Medicine (Protocol No. 2019-0027); the Cavite State University (with IACUC Exemption Certification); and the University of Eastern Philippines – College of Veterinary Medicine (Protocol No. 2020-001). Informed consent was obtained prior to sampling from poultry operators/owners. An official letter from the Director of the Bureau of Animal Industry, encouraging poultry farm operators/owners, was made available prior to the actual field sampling.

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Conflict of Interest

The authors declare no conflict of interest.

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