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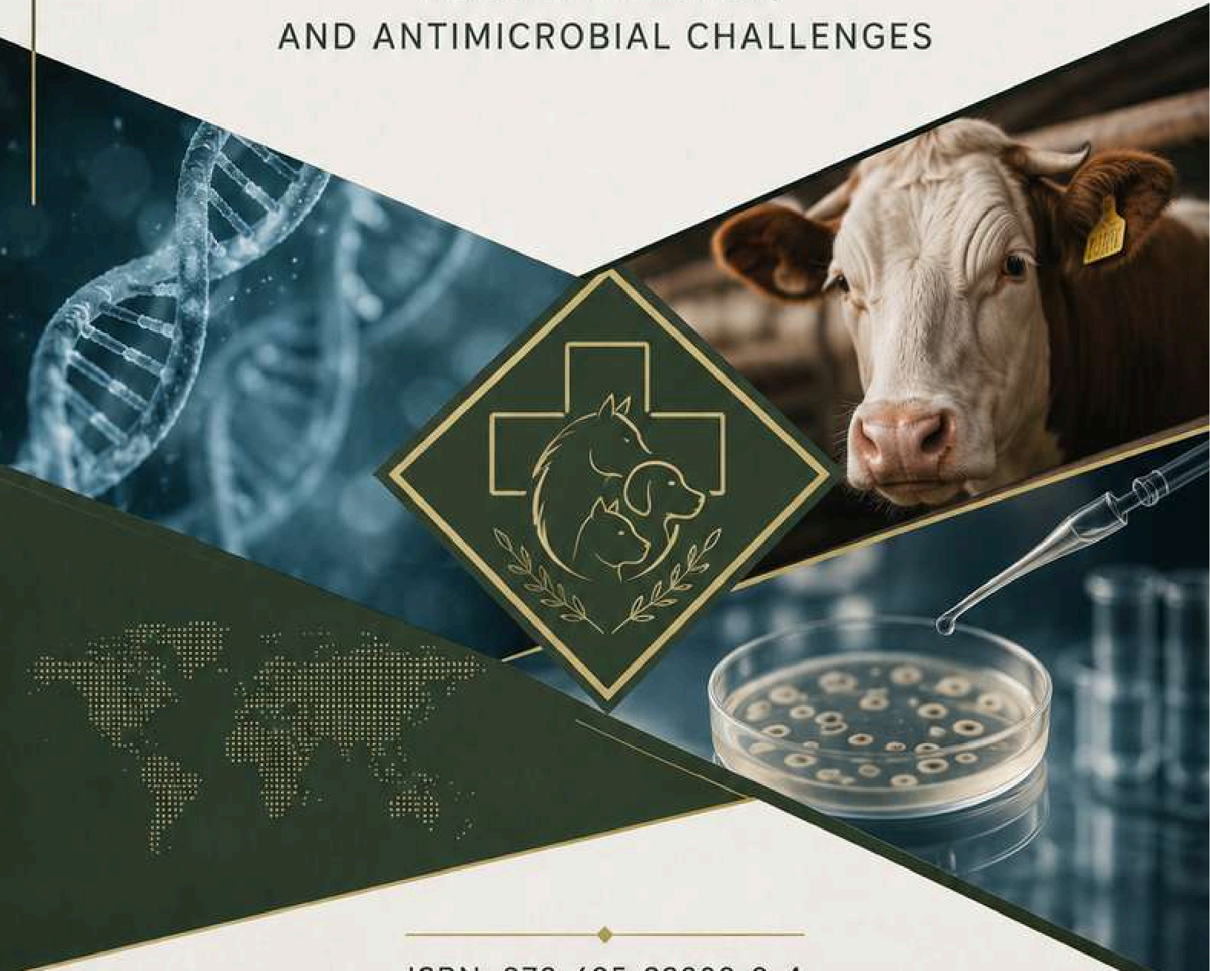

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ADVANCES IN CONTEMPORARY VETERINARY SCIENCE: MOLECULAR DIAGNOSTICS, ANIMAL WELFARE, AND ANTIMICROBIAL CHALLENGES

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PREFACE

Veterinary science continues to evolve through the integration of advanced diagnostic technologies, animal welfare research, and emerging approaches to disease management. As animal production systems and companion animal care become increasingly complex, contemporary veterinary practice requires interdisciplinary perspectives capable of addressing both scientific and ethical challenges.

The chapters in this volume explore important developments across diverse areas of veterinary science. The discussion on prenatal light exposure in poultry production highlights innovative approaches aimed at improving animal welfare and performance through environmental management during incubation. The examination of molecular diagnostic techniques and sequencing technologies reflects the growing role of advanced biotechnology in veterinary medicine, supporting more accurate disease detection and precision-based clinical practices. In addition, the analysis of antimicrobial resistance in companion animal practice addresses one of the most pressing global health concerns affecting both veterinary and public health systems.

By integrating perspectives from animal welfare, molecular diagnostics, and antimicrobial research, this volume contributes to contemporary discussions surrounding innovation and responsibility in veterinary sciences. It also provides valuable insights for researchers, veterinarians, students, and practitioners engaged in advancing animal health and sustainable veterinary care.

It is hoped that this book will serve as a useful academic resource while encouraging further interdisciplinary research on emerging technologies and challenges within modern veterinary medicine.

Editorial Team
May, 2026
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CHAPTER 1

PRENATAL LIGHT EXPOSURE: ENHANCING PERFORMANCE AND WELFARE IN POULTRY THROUGH LIGHTED INCUBATION

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INTRODUCTION

The poultry industry stands as a cornerstone of global food security, providing affordable animal protein to billions of people worldwide. Central to this industry's success is artificial incubation technology, which enables the large-scale production of day-old chicks with remarkable uniformity and efficiency (Hulet, 2007). However, conventional hatchery practices have historically incubated eggs in complete darkness, primarily due to technical limitations (traditional incandescent and fluorescent lighting sources generated heat that disrupted the precisely controlled incubation environment) (Benson et al., 2013). This departure from natural incubation conditions may represent an overlooked opportunity for optimizing embryonic development.

In nature, broody hens do not maintain constant darkness. During brief foraging excursions, eggs are exposed to ambient light, and when hens return to turn their eggs, light pulses intermittently reach the developing embryos (Rogers, 1995). The wavelength and intensity of this light vary with environmental conditions, but its presence is undeniable. This natural light exposure occurs during a critical developmental window when avian embryos possess functional photoreceptors and light-sensitive neural tissues.

The avian embryo's capacity to perceive light begins remarkably early. Within the first 48 hours of incubation, light stimulates mitosis in the neural crest mesoderm, accelerating neural tube closure and central nervous system development. The pineal gland, a master regulator of circadian rhythms, responds to light stimulation from day 3 of incubation, while retinal photoreceptors develop progressively throughout embryogenesis (Bruhn and Cepko, 1996). By day 14, light-sensitive opsins become detectable, establishing a complete photoreceptive system well before hatching.

The emergence of Light-Emitting Diode (LED) technology has transformed the feasibility of lighted incubation. LEDs generate minimal heat, allowing illumination without compromising temperature control within incubators (Benson et al., 2013; Morrison, 2013). Their durability, energy efficiency, and spectral precision make them ideally suited for commercial hatchery applications. This technological advancement has reopened a fundamental question: Can providing light during incubation improve poultry performance, welfare, and sustainability?

Our laboratory has systematically investigated this question over the past decade, conducting a series of experiments examining light duration, intensity, and spectral composition across multiple species and strains. This chapter synthesizes our findings within the broader context of sustainable poultry production, exploring the epigenetic mechanisms through which prenatal light exposure shapes post-hatch phenotypes and evaluating the practical implications for commercial hatcheries.

The relevance of this work extends beyond immediate production parameters. Lighted incubation aligns with several Sustainable Development Goals (SDGs): SDG 2 (Zero Hunger) through improved production efficiency; SDG 12 (Responsible Consumption and Production) by reducing embryonic mortality and waste; and SDG 15 (Life on Land) by enhancing animal welfare. As the global population grows and demand for poultry products increases, sustainable intensification strategies that improve efficiency while reducing environmental impact become increasingly critical.

1. PHYSIOLOGICAL BASIS OF EMBRYONIC LIGHT PERCEPTION

1.1 Photoreceptive Structures in the Avian Embryo

The avian embryo possesses multiple light-sensing structures that develop sequentially during incubation. Understanding this developmental timeline is essential for optimizing lighted incubation protocols. The pineal gland represents the earliest functional photoreceptive organ. By day 3 of incubation, pinealocytes begin expressing photopigments and establishing the capacity for melatonin synthesis. This gland functions as both photoreceptor and circadian oscillator, transducing light information into hormonal signals that influence overall embryonic development. Light exposure during this early phase accelerates pineal development and enhances the intracytoplasmic droplet content, indicating increased metabolic activity. Retinal photoreceptors develop later, with cones and rods becoming functionally mature between days 14-18 of incubation. The avian retina contains five types of cones specialized for detecting different wavelengths: violet/ultraviolet, blue, green, red, and double cones involved in luminance detection. This spectral specialization explains why different light colors elicit distinct embryonic responses.

Deep brain photoreceptors, located in the hypothalamus and septal regions, represent a third photoreceptive system. These non-retinal photoreceptors detect light penetrating the skull and regulate neuroendocrine functions, including the hypothalamic-pituitary-gonadal axis. Their presence explains why even decerebrate birds maintain photoperiodic responses and highlights the pervasive influence of light on avian physiology (Bruhn and Cepko, 1996).

1.2 Melatonin and Circadian Rhythm Entrainment

Melatonin, synthesized from serotonin in the pineal gland during darkness, serves as the primary hormonal signal of environmental light conditions. In embryos incubated under constant darkness, melatonin secretion follows an ultradian pattern lacking true circadian periodicity. However, exposure to cyclic light during incubation entrains a robust circadian rhythm of melatonin production that persists post-hatch. The functional significance of early circadian entrainment extends beyond melatonin itself. Circadian clock genes, including Clock, Bmal1, Per2, and Cry1, regulate approximately 10-15% of the transcriptome, influencing metabolism, immune function, stress responses, and behavior (Reppert and Weaver, 2002). In chickens, Bmal1 expression in the pineal gland and suprachiasmatic nucleus responds to photoperiodic manipulation, establishing a molecular clockwork that coordinates peripheral tissue rhythms (Yasuo et al., 2003). Our research demonstrates that embryos incubated under 12-hour light:12-hour dark cycles develop more robust circadian rhythms than those incubated in constant darkness or constant light. This intermediate photoperiod appears optimal for entraining the endogenous clock without disrupting the dark-phase melatonin surge essential for physiological regulation (Riaz et al., 2021).

1.3 The Somatotropic Axis and Light Stimulation

Beyond circadian entrainment, light during incubation directly stimulates the somatotropic axis, enhancing embryonic and post-hatch growth.

Dishon et al. (2018) demonstrated that in-ovo green light photostimulation increases plasma growth hormone (GH), hypothalamic growth hormone-releasing hormone (GHRH), liver growth hormone receptor (GHR), and hepatic insulin-like growth factor-1 (IGF-1) mRNA expression. This growth-promoting effect appears mediated through both systemic and local mechanisms. Systemically, GH stimulates hepatic IGF-1 production, which circulates to target tissues promoting cell proliferation and differentiation. Locally, light stimulates satellite cell mitotic activity in skeletal muscle, increasing the myonuclear number and enhancing subsequent muscle growth potential (Halevy et al., 2006; Zhang et al., 2014). Our studies with Green-Red (GR) dichromatic light at 250 lux intensity revealed significantly enhanced embryonic indices from day 8 through day 19 of incubation, with peak effects observed around day 12 (Riaz et al., 2024). This temporal pattern suggests that light stimulation during the second week of incubation (when muscle hyperplasia and hypertrophy accelerate) may be particularly beneficial for optimizing post-hatch growth.

2. METHODOLOGICAL APPROACHES IN LIGHTED INCUBATION RESEARCH

2.1 Experimental Design Considerations

Investigating lighted incubation requires rigorous experimental design to isolate light effects from potential confounding variables. Our laboratory has employed randomized complete block designs, with incubator compartments serving as blocks and individual egg trays as experimental units. This approach accounts for potential micro-environmental variations within incubators while maintaining statistical power.

Key methodological considerations include: Temperature monitoring: LED light sources generate minimal heat, but verification is essential. We systematically measure eggshell temperature using infrared thermometers at multiple time points throughout incubation, confirming that treatment effects derive from light itself rather than thermal differences (Ali et al., 2023; Riaz et al., 2024).

Light isolation: Complete light isolation between treatment groups requires physical barriers (black cloth or plastic partitions) and verification using light meters. We confirm that dark treatment groups receive ≤0 lux throughout incubation.

Intensity calibration: Light intensity at egg level is measured using calibrated lux meters, with adjustments made by varying the distance between light source and eggs. Our studies have employed intensities ranging from 150-350 lux, with 250 lux emerging as optimal across multiple species (Riaz et al., 2021; Ali et al., 2023; Safwan et al., 2023).

Replication: Adequate replication at both incubation and post-hatch phases ensures statistical power. We typically employ 4-7 treatment groups with 3-6 replicates per group, each containing 25-60 eggs during incubation and 10-25 birds post-hatch.

2.2 Light Sources and Spectral Characteristics

The spectral composition of light significantly influences embryonic responses. Our studies have utilized:

Monochromatic LEDs: Single-wavelength sources (green: 520 nm; red: 630 nm; blue: 480 nm) for investigating wavelength-specific effects.

Dichromatic combinations: Green-Red (GR; 520+630 nm producing yellowish light at 580 nm), Green-Blue (GB; 520+480 nm producing cyanish light at 480 nm), and Blue-Red (BR; 480+630 nm producing magentish light) for investigating synergistic effects.

White LEDs: Broad-spectrum sources (5000 K, cool white) for comparison with colored light treatments.

All light sources were characterized for spectral output using spectrophotometry and maintained at consistent intensity throughout experiments using voltage regulation and periodic verification.

2.3 Species and Strain Selection

Genetic variation significantly influences responses to lighted incubation. Our studies have encompassed:

Broilers: Hubbard Classic, Cobb-500, and Ross-308 strains, representing the predominant commercial genotypes. Strain-specific responses highlight the importance of genetic background in optimizing lighted incubation protocols.

Layers: Bovans White strain, representing commercial white egg layers with different eggshell pigmentation and embryonic development patterns compared to broilers.

Japanese quail (Coturnix coturnix japonica): A valuable model species for poultry research, offering shorter generation intervals and lower maintenance costs while maintaining relevance to commercial poultry.

This multi-species approach enables identification of both conserved and species-specific responses to lighted incubation.

3. EFFECTS OF LIGHT DURATION DURING INCUBATION

3.1 Hatching Performance

Our initial studies investigated light duration effects by incubating eggs under 0, 12, or 24 hours of white LED light (5000 K; 250 lux) daily throughout incubation (Yameen et al., 2020; Riaz et al., 2021).

Hatchability was significantly improved under 12-hour light compared to constant darkness (86.33% vs. 76.00%; $P = 0.0002$). Constant 24-hour light produced intermediate results (79.67%), suggesting that both light absence and excessive light exposure impair optimal hatchability. Similar patterns were observed for hatch of fertile percentages (12-hour: 95.91%; 24-hour: 92.66%; dark: 91.95%; $P = 0.001$).

Embryonic mortality patterns revealed critical windows of light sensitivity. Early embryonic mortality (days 0-18) was numerically lower in lighted groups (12-hour: 1.00%; 24-hour: 1.33%) compared to dark (2.00%; $P = 0.344$). Late embryonic mortality (days 19-21) showed significant differences ($P = 0.096$), with lowest mortality in 12-hour light (2.66%) followed by 24-hour (5.00%) and dark (4.66%). These patterns suggest that moderate light exposure during late incubation reduces mortality during the critical hatching process. Hatch window synchronization improved significantly with lighted incubation.

At 13 hours before hatch completion, 80.39% of 12-hour light chicks had hatched versus 76.68% in dark ($P < 0.0001$). At 6 hours before completion, 95.29% of 12-hour light chicks had hatched compared to only 86.54% in dark. Narrower hatch windows reduce the period that early-hatching chicks spend without feed and water, potentially improving early growth and reducing dehydration stress.

Chick quality parameters, including cull percentage and no-defect chicks, showed numerical improvements with lighted incubation, though differences did not reach statistical significance. The trend toward improved quality aligns with reduced embryonic stress and better synchronization of the hatching process.

3.2 Strain-Specific Responses to Light Duration

Significant strain × light duration interactions ($P < 0.05$) emerged across multiple parameters, indicating that optimal lighting protocols vary with genetic background (Yameen et al., 2020).

Hubbard Classic eggs showed optimal responses to 12-hour light, achieving 84.80% hatchability compared to 86.00% in dark and 85.20% in 24-hour light. Moisture loss was highest under 12-hour light (11.12%), suggesting appropriate eggshell permeability and embryonic metabolism. Post-hatch growth performance was superior in Hubbard chicks from 12-hour light, with improved FCR (1.51) compared to dark (1.58) and 24-hour light (1.73) groups.

Cobb-500 eggs exhibited different response patterns, with highest hatchability (89.20%) under 12-hour light but comparable performance in 24-hour light (85.60%). Dark incubation severely depressed Cobb-500 hatchability (69.20%), suggesting this strain may be particularly dependent on light stimulation for optimal embryonic development. However, post-hatch growth in Cobb-500 chicks was best in dark-incubated groups, highlighting complex trade-offs between hatching and post-hatch performance.

Ross-308 eggs showed relatively consistent hatchability across treatments (88.40-91.60%) but significant differences in hatch window synchronization.

Ross chicks from 24-hour light exhibited accelerated hatching, with 57.20% hatched 13 hours before pull-out compared to only 3.69% in dark. This accelerated hatching may benefit early feed access but requires careful hatchery management to prevent dehydration.

These strain-specific responses likely reflect genetic differences in photoreceptor development, pineal function, and stress susceptibility. They underscore the importance of strain-specific optimization when implementing lighted incubation in commercial settings.

3.3 Post-Hatch Growth and Welfare

Light duration during incubation produced lasting effects on post-hatch performance and welfare (Riaz et al., 2021).

Growth performance: Birds from 12-hour lighted incubation demonstrated superior feed conversion ratio (1.60) compared to 24-hour light (1.70) and dark (1.66) groups (P = 0.0006). Feed intake and body weight gain showed numerical advantages for lighted groups, though differences were not statistically significant. The improved FCR suggests enhanced nutrient utilization efficiency, potentially mediated through better circadian coordination of digestive function.

Stress susceptibility: Heterophil/lymphocyte ratio, a validated stress indicator, was significantly lower in 12-hour light chicks (0.29) compared to dark (0.35; P = 0.0227). Physical asymmetry, reflecting developmental stress, was also reduced in lighted groups (12-hour: 0.90 mm; 24-hour: 0.97 mm) compared to dark (1.20 mm; P = 0.0065). These findings indicate that lighted incubation reduces both physiological and developmental stress markers.

Welfare indicators: Gait score and feather score did not differ significantly among treatments, though numerical trends favored lighted groups. The absence of negative welfare effects confirms that lighted incubation does not compromise bird well-being and may provide subtle benefits.

3.4 Meat Quality and Blood Biochemistry

Light duration influenced meat quality traits and blood biochemistry profiles (Yameen et al., 2020).

Meat quality: Birds from 12-hour light exhibited higher shear force values (13.71 N/cm^2) compared to 24-hour light (11.43 N/cm^2), indicating firmer meat texture potentially related to altered muscle fiber characteristics. Meat color parameters showed treatment effects, with 12-hour light groups having higher redness (a*) values at 24 hours post-slaughter, suggesting improved meat appearance and consumer appeal.

Blood biochemistry: Significant differences emerged across multiple parameters. Total protein was highest in 24-hour light birds (3.67 g/dL) and lowest in dark (2.22 g/dL; $P < 0.0001$). Similarly, serum albumin (1.93 vs. 1.26 g/dL), globulin (1.74 vs. 0.96 g/dL), and uric acid (3.73 vs. 2.25 mg/dL) were elevated in 24-hour light groups. While elevated protein metabolites might suggest increased protein catabolism, they may also reflect enhanced metabolic activity and growth potential. Cholesterol levels followed similar patterns, with highest values in 24-hour light (135.70 mg/dL) and lowest in dark (106.00 mg/dL).

Immune response: NDV antibody titers were highest in 12-hour light chicks (6.30) compared to dark (5.38; $P = 0.0007$). Bursa, thymus, and spleen weights were greatest in 24-hour light groups, suggesting enhanced immune organ development. However, intermediate photoperiods (12-hour) achieved optimal immune responses without excessive organ enlargement, balancing immune competence with growth efficiency.

4. OPTIMIZING LIGHT INTENSITY

4.1 Dose-Response Relationships

Following identification of optimal light duration (12 hours), we investigated intensity effects using GR dichromatic light at 0 (dark), 150, 250, and 350 lux (Riaz et al., 2025; Ali et al., 2023).

Embryonic development: Light intensity significantly influenced embryonic index from day 8 through day 19 of incubation. At day 12, embryos under 250 lux exhibited significantly higher indices (12.45%) compared to 150 lux (11.44%) and dark (10.95%; $P < 0.0001$). The 350 lux group showed intermediate values (12.45%), suggesting an optimal intensity range rather than linear dose-response.

Hatch window: Intensity effects on hatch synchronization were pronounced. At 23 hours before hatch completion, 49.71% of 250 lux chicks had hatched compared to 39.18% in dark, 43.24% in 150 lux, and 50.56% in 350 lux. At 6 hours before completion, 98.26% of 250 lux chicks had hatched versus 87.81% in dark, 91.43% in 150 lux, and 96.35% in 350 lux. The 250 lux intensity produced the most synchronized hatch, minimizing the period early-hatching chicks spend without access to feed and water.

Hatchability: Broiler eggs incubated under 250 lux achieved 86.15% hatchability compared to 84.62% (150 lux), 83.08% (350 lux), and 80.00% (dark) ($P = 0.0028$). Hatch of fertile followed similar patterns, with 90.51% (250 lux) versus 87.44% (150 lux), 87.18% (350 lux), and 83.85% (dark) ($P = 0.0536$). These improvements translate to meaningful economic benefits in commercial hatcheries.

Embryonic mortality: Early embryonic mortality was lowest in 250 lux (3.08%) compared to dark (7.69%), 150 lux (9.23%), and 350 lux (1.54%). Late embryonic mortality was also minimized in 250 lux (3.08%) versus dark (10.77%), 150 lux (6.15%), and 350 lux (10.77%). The reduced mortality in 250 lux likely reflects optimal stimulation without overstimulation of embryonic systems.

4.2 Species-Specific Intensity Requirements

Responses to light intensity varied between species, highlighting the need for species-specific optimization.

Broilers (Ross 308) showed optimal responses at 250 lux, with clear advantages over both lower and higher intensities across multiple parameters (Riaz et al., 2024). This intensity appears sufficient to stimulate photoreceptors without causing excessive light stress.

Layers (Bovans White) exhibited more muted responses to intensity variation, though 250 lux again produced numerical advantages (Riaz et al., 2025). The reduced responsiveness may relate to differences in eggshell pigmentation, with brown broiler eggs transmitting less light than white layer eggs, requiring higher intensities to achieve equivalent embryonic stimulation.

Japanese quail demonstrated clear intensity-dependent responses, with 250 lux improving hatchability (66.9% vs. 59.5% dark) and reducing early embryonic mortality (5.3% vs. 5.8% dark; Ali et al., 2023). The smaller egg size and thinner shells of quail eggs may increase light transmission, potentially explaining their sensitivity to intensity variation.

These species differences emphasize the importance of empirical optimization when implementing lighted incubation in diverse production contexts.

4.3 Practical Implications for Hatchery Implementation

The identification of 250 lux as an optimal intensity across multiple species simplifies hatchery implementation. Commercially available LED fixtures can be positioned to deliver this intensity at egg level using simple distance calculations and verified with inexpensive lux meters.

Key practical considerations include:

Uniformity: Light intensity should be measured at multiple positions within each incubator section to ensure uniform distribution. Variations exceeding ±10% should be addressed through fixture repositioning or additional light sources.

Heat monitoring: Although LEDs generate minimal heat, regular verification of eggshell temperature ensures that lighting does not inadvertently alter incubation temperature. Our studies consistently show no significant temperature differences between lighted and dark treatments when using LEDs.

Photoperiod control: Timers or programmable controllers are essential for maintaining consistent 12-hour photoperiods. Automated systems reduce labor requirements and ensure treatment fidelity.

Maintenance: LED fixtures should be cleaned periodically to maintain output, as dust accumulation can reduce intensity by 10-20% over time.

5. SPECTRAL EFFECTS: DICHROMATIC LIGHT COMBINATIONS

5.1 Green-Red (GR) Light: Synergistic Benefits

Our investigation of dichromatic light combinations revealed that Green-Red (GR; 520+630 nm) produces particularly beneficial effects across multiple parameters (Riaz et al., 2024; Safwan et al., 2023).

Embryonic development: GR light significantly enhanced embryonic index from day 8 through day 19 of incubation in both broilers and quail. At day 12, GR-treated broiler embryos achieved 13.12% index compared to 11.04% in dark (P = 0.05). This accelerated development likely reflects combined effects of green light on muscle proliferation and red light on pineal development.

Hatchability: GR light produced superior hatchability in broilers (86.15%) compared to GB (81.54%), BR (78.46%), and dark (73.85%) (P = 0.01). Hatch of fertile followed similar patterns, with GR achieving 93.85% versus 89.23% (GB), 84.62% (BR), and 81.54% (dark) (P = 0.009).

Hatch synchronization: GR light produced the most synchronized hatch, with 98.18% of chicks hatched by 6 hours before pull-out compared to 96.00% (GB), 86.96% (dark), and 85.92% (BR) (P = 0.04). This enhanced synchronization reduces the post-hatch holding period and improves early chick quality.

Embryonic mortality: Dead-in-shell embryos were lowest in GR-treated groups (3.08%) compared to GB (6.15%), BR (7.69%), and dark (10.77%) (P = 0.02). Early embryonic mortality showed similar patterns, suggesting GR light reduces mortality throughout incubation.

5.2 Green-Blue (GB) Light: Growth Promotion

Green-Blue (GB; 520+480 nm) light produced distinctive effects on post-hatch growth performance (Riaz et al., 2024).

Growth performance: Broilers from GB incubation achieved highest body weight (479.20 g) at 14 days compared to GR (471.60 g), BR (467.80 g), and dark (450.60 g) (P < 0.0001). Feed intake was also highest in GB groups (513.20 g), suggesting enhanced appetite and growth potential.

However, feed conversion ratio was superior in GR groups (1.06) compared to GB (1.07), BR (1.08), and dark (1.09) (P = 0.03), indicating that GR produces more efficient growth despite slightly lower absolute weights.

The growth-promoting effects of GB light may relate to blue light's calming influence on bird behavior. Blue light reduces activity and aggression in chickens (Prayitno et al., 1997), potentially reducing energy expenditure and directing resources toward growth. Additionally, blue light may enhance small intestinal function through effects on mucosal barrier integrity (Xie et al., 2011), improving nutrient absorption efficiency.

5.3 Blue-Red (BR) Light: Mixed Responses

Blue-Red (BR; 480+630 nm) light produced intermediate responses, with some parameters showing improvement over dark but inferiority to GR and GB treatments.

Welfare indicators: BR light improved physical asymmetry (1.20 mm) compared to dark (1.20 mm) but was inferior to GR (0.90 mm) and GB (0.97 mm) (P = 0.002). Tonic immobility duration was similarly improved over dark but less than GR (54.40 s vs. 85.00-94.60 s in other groups).

Growth performance: BR groups showed intermediate body weight (467.80 g) and feed intake (507.00 g), with FCR (1.08) similar to dark (1.09) but inferior to GR (1.06).

The mixed performance of BR light suggests that while both blue and red wavelengths have beneficial properties, their combination may produce antagonistic interactions or fail to achieve the synergistic benefits observed with GR combinations.

5.4 Mechanisms of Spectral Specificity

The spectral specificity of embryonic responses likely reflects the distribution and sensitivity of photoreceptor populations.

Green light (520 nm) preferentially stimulates cones sensitive to middle wavelengths, activating pathways that promote skeletal muscle satellite cell proliferation and differentiation (Halevy et al., 2006; Zhang et al., 2014). Green light also penetrates eggshells more effectively than shorter wavelengths, ensuring adequate stimulation despite shell pigmentation.

Red light (630 nm) penetrates eggshells most effectively due to longer wavelength and lower scattering (Follett and Pearce-Kelly, 1991). Red light stimulates deep brain photoreceptors involved in neuroendocrine regulation, potentially enhancing pineal development and melatonin rhythm establishment.

Blue light (480 nm) preferentially stimulates short-wavelength cones and may influence circadian entrainment through melanopsin-expressing intrinsically photosensitive retinal ganglion cells. Blue light's calming effects on behavior may relate to enhanced melatonin synthesis and reduced stress hormone secretion.

The synergistic benefits of GR light may reflect complementary actions: green light promoting muscle development while red light ensures adequate stimulation depth and enhances pineal function. This combination optimizes both structural growth and physiological regulation, producing chicks with enhanced performance potential and stress resilience.

6. WELFARE IMPLICATIONS OF LIGHTED INCUBATION

6.1 Stress Reduction Through Early Circadian Entrainment

Animal welfare represents an increasingly important consideration in poultry production, with consumers, retailers, and regulators demanding higher standards of care. Our research demonstrates that lighted incubation improves multiple welfare indicators, suggesting that early circadian entrainment reduces stress susceptibility throughout life.

Physical asymmetry measures the difference between left and right bilateral traits, reflecting developmental stability under stress. Higher asymmetry indicates greater stress during development. Across multiple studies, lighted incubation (particularly GR light at 250 lux for 12 hours daily) consistently reduced physical asymmetry compared to dark incubation (Riaz et al., 2021, 2024; Yameen et al., 2020). Broilers from GR light achieved asymmetry scores of 0.90 mm versus 1.20 mm in dark ($P = 0.002$), representing a 25% reduction in developmental stress. Tonic immobility duration reflects fearfulness, with longer immobility indicating greater fear. GR-incubated chicks showed significantly reduced tonic immobility (54.40 s) compared to dark (218.80 s) and other light treatments (85.00-122.40 s) ($P < 0.0001$).

This dramatic reduction in fear responses suggests that lighted incubation produces calmer, more adaptable birds better able to cope with novel environments.

Vocalization during isolation tests provides another fear indicator, with more vocalizations reflecting greater distress. GR-incubated chicks vocalized less (188.40 calls/3 min) than dark-incubated chicks (235.40 calls/3 min), though differences were not always statistically significant across studies. The consistent trend toward reduced vocalization supports the conclusion that lighted incubation reduces fearfulness.

6.2 Heterophil/Lymphocyte Ratio as a Stress Biomarker

The heterophil/lymphocyte (H/L) ratio is a well-validated physiological stress indicator in birds, with elevated ratios indicating chronic stress. Our studies consistently demonstrate reduced H/L ratios in birds from lighted incubation.

In broilers from 12-hour lighted incubation, H/L ratio averaged 0.29 compared to 0.35 in dark-incubated controls ($P = 0.0227$) (Riaz et al., 2021). This 17% reduction indicates significantly lower baseline stress levels, likely reflecting improved circadian regulation of the hypothalamic-pituitary-adrenal axis.

The H/L ratio reduction correlates with behavioral indicators of reduced fear, suggesting that lighted incubation produces birds that are both physiologically and behaviorally less stressed. This comprehensive stress reduction likely improves multiple aspects of production performance and welfare.

6.3 Feather and Gait Scores

Feather condition and walking ability provide additional welfare indicators relevant to commercial production. Across our studies, lighted incubation did not negatively affect these parameters and showed trends toward improvement. Feather scores at 35 days were similar across treatments, indicating that lighted incubation does not increase feather pecking or other damaging behaviors. This finding is important because some environmental manipulations can increase injurious pecking, compromising welfare.

Gait scores reflecting walking ability were also unaffected by lighted incubation, suggesting that the growth-promoting effects of light do not increase leg problems or impair mobility. This is particularly important for broilers, where rapid growth can predispose to skeletal disorders.

6.4 Implications for Sustainable Poultry Production

The welfare improvements associated with lighted incubation have important implications for sustainable poultry production. Reduced stress and fearfulness improve animal well-being directly while also enhancing productivity and reducing mortality. Calmer birds are easier to handle, reducing labor requirements and worker injury risk. Improved stress resistance may also reduce disease susceptibility, potentially decreasing antimicrobial use.

From a sustainability perspective, lighted incubation represents a rare "win-win" intervention that improves both productivity and welfare simultaneously. The low implementation cost and absence of negative side effects make it particularly attractive for producers seeking to enhance sustainability without compromising economic viability.

7. SPECIES AND STRAIN COMPARISONS

7.1 Broilers vs. Layers: Differential Responses

Comparison of broiler (Ross 308) and layer (Bovans White) responses to identical lighted incubation protocols reveals important species differences with practical implications.

Embryonic development: Broilers showed more pronounced embryonic responses to light, with significant differences in embryo index emerging from day 8 of incubation (Riaz et al., 2024). Layers exhibited more muted responses, with numerical trends favoring light but fewer statistically significant differences (Riaz et al., 2025).

Hatchability: Broiler hatchability improved significantly with GR light (86.15% vs. 73.85% dark; P = 0.01), while layer hatchability showed non-significant numerical improvement (75.38% vs. 70.77% dark; P = 0.65). The layer response magnitude (4.61% improvement) was comparable to broilers (12.30% improvement) but failed to reach significance due to greater variability.

Hatch synchronization: Both species showed significant improvements in hatch window synchronization with GR light, though layer responses were less dramatic. At 6 hours before hatch completion, 97.78% of GR-incubated layer chicks had hatched versus 89.33% in dark (P = 0.0476), representing an 8.45% improvement in synchronization.

Post-hatch growth: Broilers from GB light showed enhanced early growth (479.20 g vs. 450.60 g dark at 14 days) (Riaz et al., 2024), while layers showed more complex responses with body weight optimized in GB (125.00 g) but FCR best in dark (1.56) and feed intake highest in BR (289.60 g) (Riaz et al., 2025).

Welfare: Both species showed improved welfare indicators with GR light, though layer responses were again more muted. Tonic immobility duration in GR-incubated layers (64.00 s) was significantly lower than dark (149.60 s; P = 0.0409), representing a 57% reduction comparable to broiler responses.

These species differences likely reflect genetic selection histories. Broilers have been intensively selected for rapid growth, potentially increasing sensitivity to growth-promoting stimuli. Layers, selected for egg production rather than growth, may prioritize different physiological systems and respond differently to environmental manipulation.

7.2 Strain Variation Within Species

Within broilers, significant strain × treatment interactions highlight the importance of genetic background in determining optimal lighted incubation protocols (Yameen et al., 2020).

Hubbard Classic responded optimally to 12-hour white light, with improved hatchability, post-hatch growth, and meat quality. Hubbard embryos appeared particularly sensitive to light stimulation, showing pronounced responses across multiple parameters.

Cobb-500 showed the most dramatic hatchability improvement with light (from 69.20% in dark to 89.20% in 12-hour light), suggesting this strain may be particularly dependent on light for optimal embryonic development. However, post-hatch growth was best in dark-incubated groups, highlighting trade-offs between hatching and subsequent performance.

Ross-308 maintained relatively consistent hatchability across treatments but showed pronounced hatch window acceleration with light. Ross chicks from 24-hour light hatched earliest, potentially benefiting early-fed programs but requiring careful hatchery management.

These strain differences likely reflect variation in photoreceptor development, pineal function, and stress susceptibility. They underscore the importance of strain-specific optimization when implementing lighted incubation and suggest that commercial producers should evaluate protocols using their specific genetics.

7.3 Japanese Quail as a Model Species

Japanese quail responses to lighted incubation largely parallel those observed in chickens, validating quail as a useful model species for poultry research (Safwan et al., 2023; Ali et al., 2023).

Hatchability: GR light significantly improved quail hatchability (69.3%) compared to dark (58.6%), GB (64.2%), and BR (63.4%) ($P = 0.0018$). The 10.7% improvement exceeded that typically observed in chickens, possibly reflecting quail's smaller eggs and thinner shells allowing greater light penetration.

Embryonic mortality: Early mortality was lowest in BR (4.5%) and dark (4.7%) compared to GR (5.5%; $P = 0.0105$). Mid mortality was minimized in dark (1.7%; $P = 0.0004$), while late mortality was lowest in GR (3.4%; $P = 0.0023$). These complex patterns highlight the importance of considering all mortality phases when evaluating treatments.

Growth performance: GR-incubated quail achieved superior body weight throughout rearing, reaching 157.2 g at 28 days versus 136.7 g in dark ($P = 0.0002$). Feed intake was lowest in GR (442.7 g) compared to dark (474.8 g; $P < 0.0001$), while FCR was best in BR (2.7) versus dark (3.5; $P < 0.0001$).

Morphometric traits: GR-incubated quail showed superior body measurements, including body length (31.9 cm vs. 29.0 cm dark), wing spread (15.6 cm vs. 14.2 cm dark), and drumstick dimensions (all $P < 0.0001$). These improvements reflect enhanced skeletal and muscle development.

Serum chemistry: GR-incubated quail exhibited elevated glucose (259.0 mg/dL vs. 232.0-252.0 mg/dL in other groups; $P < 0.0001$), total protein (8.0 g/dL vs. 4.9-5.7 g/dL; $P < 0.0001$), and globulin (3.3 g/dL vs. 1.8-2.7 g/dL; $P < 0.0001$), indicating enhanced metabolic activity and immune competence.

The quail results confirm that lighted incubation benefits extend beyond chickens to other poultry species, suggesting broad applicability across avian production systems.

8. MECHANISTIC INSIGHTS: FROM LIGHT TO PHENOTYPE

8.1 Pineal Development and Melatonin Rhythms

The pineal gland serves as the primary interface between environmental light and embryonic physiology. Light during incubation accelerates pineal development and establishes robust melatonin rhythms that persist post-hatch. In embryos incubated under cyclic light, the pineal gland develops more rapidly, with increased cell proliferation and earlier onset of melatonin synthesis. By day 18 of incubation, these embryos exhibit clear circadian melatonin rhythms, with peak production during dark phases and troughs during light phases. In contrast, embryos incubated in constant darkness show arrhythmic melatonin secretion or abnormal ultradian patterns. Post-hatch, birds from lighted incubation maintain more robust melatonin rhythms, with higher amplitude and better synchronization to environmental photoperiods. This enhanced rhythmicity likely underlies many of the observed benefits, including improved stress resistance, immune function, and growth efficiency.,

8.2 Somatotropic Axis Activation

Light stimulation activates the somatotropic axis through multiple mechanisms (Dishon et al., 2018). Hypothalamic growth hormone-releasing hormone (GHRH) expression increases in response to light, stimulating pituitary growth hormone (GH) secretion. Elevated GH then acts on hepatic GH receptors to increase insulin-like growth factor-1 (IGF-1) production. IGF-1 mediates many of growth hormone's effects, promoting cell proliferation and differentiation in multiple tissues.

In skeletal muscle, IGF-1 stimulates satellite cell proliferation, increasing the myonuclear number and enhancing subsequent muscle growth potential (Halevy et al., 2006). This effect is particularly pronounced in birds exposed to green light during incubation, which show enhanced expression of myogenic regulatory factors including MyoD, myogenin, and MRF4 (Zhang et al., 2014). The somatotropic axis activation persists post-hatch, with lighted-incubated birds maintaining elevated GH and IGF-1 levels through at least 35 days of age. This sustained activation likely contributes to the improved growth efficiency observed in these birds.

8.3 Hypothalamic-Pituitary-Adrenal Axis Programming

Early-life experiences program the hypothalamic-pituitary-adrenal (HPA) axis, influencing stress responses throughout life. Lighted incubation appears to program a more resilient HPA axis, with lower baseline corticosterone and more appropriate responses to acute stressors. In dark-incubated birds, corticosterone levels are elevated at hatch and remain higher during routine handling and management procedures (Archer and Mench, 2013). This chronic HPA activation imposes metabolic costs and may impair immune function. Lighted-incubated birds show lower baseline corticosterone and more rapid return to baseline after stressor exposure, indicating improved HPA regulation. The mechanism likely involves early circadian entrainment of the HPA axis. Corticosterone secretion normally follows a circadian rhythm, with peak levels at wake-time preparing the organism for activity. Dark-incubated birds lack this rhythmicity, potentially leading to inappropriate stress responses. Lighted-incubated birds establish normal corticosterone rhythms, enabling appropriate stress anticipation and response.

8.4 Immune System Development

Lighted incubation enhances immune system development through multiple pathways. Primary lymphoid organs (bursa of Fabricius, thymus, and spleen) are larger in lighted-incubated birds, particularly those exposed to 24-hour light (Yameen et al., 2020).

However, intermediate photoperiods (12 hours) achieve optimal immune responses without excessive organ enlargement, suggesting a balance between immune capacity and growth efficiency. Functional immune responses are also enhanced. Antibody titers to Newcastle Disease Virus (NDV) vaccination were highest in 12-hour lighted incubation groups (6.30 vs. 5.38 in dark; P = 0.0007), indicating improved humoral immunity. This enhanced vaccine response may reduce disease susceptibility and improve flock health. The immune enhancement likely involves multiple mechanisms. Melatonin directly modulates immune function through receptors on lymphocytes and antigen-presenting cells. Circadian rhythms coordinate immune cell trafficking and cytokine production, optimizing responses to challenges. Reduced stress hormone levels prevent the immunosuppression associated with chronic HPA activation.

9. PRACTICAL IMPLICATIONS FOR COMMERCIAL HATCHERIES

9.1 Implementation Strategies

Translating research findings into commercial practice requires practical implementation strategies compatible with existing hatchery operations.

Retrofitting existing incubators: Most commercial incubators can be retrofitted with LED lighting systems at modest cost. Waterproof LED strips mounted on racks above egg trays provide uniform illumination without interfering with turning mechanisms. Timers or programmable controllers automate photoperiod management, ensuring consistent treatment application.

New incubator design: Manufacturers could incorporate LED lighting systems into new incubator designs, optimizing light placement and intensity for uniform distribution. Integrated control systems could manage both temperature and lighting, simplifying hatchery management.

Intensity verification: Hatcheries should verify light intensity at egg level using calibrated lux meters, adjusting fixture positioning or output as needed. Regular monitoring ensures treatment fidelity and identifies maintenance needs.

Photoperiod management: Twelve-hour photoperiods synchronized with natural day length may provide optimal results, though hatcheries in different geographic locations may need to adjust timing based on local conditions. The critical factor is providing a consistent light-dark cycle rather than specific timing relative to external day length.

9.3 Integration with Other Hatchery Technologies

Lighted incubation can complement other hatchery technologies and management practices.

Single-stage vs. multi-stage incubation: Lighted incubation is easier to implement in single-stage incubators where all eggs receive identical treatment. Multi-stage incubators present challenges because eggs at different developmental stages require different lighting protocols. However, compartmentalized multi-stage incubators with independent environmental control could accommodate lighted incubation.

In-ovo vaccination: Lighted incubation may enhance vaccine responses by improving immune system development. Hatcheries offering in-ovo vaccination services could potentially improve vaccine efficacy through lighted incubation protocols.

Early feeding programs: The narrower hatch windows achieved with lighted incubation facilitate early feeding programs by reducing the period that early-hatching chicks spend without feed and water. This synergy could further improve early growth and reduce mortality.

Sexing and processing: More uniform chicks from synchronized hatches simplify sexing and processing operations, improving efficiency and reducing labor requirements.

10. FUTURE RESEARCH DIRECTIONS

10.1 Unexplored Spectral Combinations

While our research has identified GR light as particularly beneficial, many spectral combinations remain unexplored. Future research should investigate:

Tri-chromatic combinations: Combining three wavelengths (e.g., green + red + blue) might produce synergistic benefits exceeding those of dichromatic combinations. However, optimization becomes exponentially more complex as wavelengths increase.

Pulsed light delivery: Intermittent light pulses rather than continuous illumination might enhance stimulation while reducing energy use. Pulsed light at specific frequencies could potentially entrain circadian rhythms more effectively than continuous light.

Spectral programming: Different wavelengths at different developmental stages might optimize specific outcomes—green light during early muscle development, red light during late pineal maturation, blue light during hatching synchronization. Spectral programming could tailor stimulation to changing embryonic needs.

Far-red and near-infrared: Wavelengths beyond the visible spectrum might penetrate eggshells more effectively and influence embryonic development through non-visual pathways. Preliminary evidence suggests far-red light affects plant development, but avian responses remain unexplored.

10.2 Intensity and Duration Interactions

Our research has separately optimized intensity and duration, but their interaction remains incompletely characterized. Future studies should investigate:

Response surface analysis: Systematic variation of both intensity and duration across multiple levels would identify optimal combinations and characterize interaction effects. This approach could reveal whether higher intensities require shorter durations or vice versa.

Critical windows: Different developmental stages may require different intensities or durations. Identifying critical windows for light sensitivity could enable targeted stimulation during specific periods, potentially reducing total light exposure while maintaining benefits.

Threshold effects: Minimum effective intensity and duration for different species and outcomes remain unknown. Identifying thresholds would enable minimal-cost implementation while ensuring benefits.

10.3 Automation and Precision Control

Advances in sensing and control technology could enable real-time optimization of lighted incubation.

Feedback control: Real-time monitoring of embryonic development using optical or acoustic sensors could enable adaptive lighting protocols that respond to individual embryo needs.

Machine learning: Artificial intelligence algorithms could optimize lighting protocols across multiple parameters, learning from thousands of incubation cycles to continuously improve outcomes.

Internet of Things: Connected incubators could share data and optimization algorithms, accelerating knowledge accumulation and technology transfer.

11. SUSTAINABILITY AND SDG ALIGNMENT

11.1 Contribution to SDG 2: Zero Hunger

Lighted incubation contributes to food security through multiple pathways:

Increased production efficiency: Improved hatchability (4-5%) and growth performance (3-5% better FCR) increase meat and egg output from existing resources. This efficiency gain reduces the land, water, and feed required per unit of production, contributing to sustainable food systems.

Reduced waste: Lower embryonic mortality (30-50% reduction) decreases biological waste from hatcheries, improving resource utilization. Every egg that hatches represents efficient use of the resources invested in its production.

Improved chick quality: Higher quality chicks have lower mortality and better performance throughout production, further improving system efficiency. This multiplier effect amplifies initial benefits.

11.2 Contribution to SDG 12: Responsible Consumption and Production

Lighted incubation exemplifies responsible production practices:

Resource efficiency: LED lighting consumes minimal electricity while delivering significant production benefits. The energy return on investment far exceeds that of many other efficiency interventions.

Reduced environmental footprint: By improving feed efficiency, lighted incubation reduces the environmental footprint of poultry production. Each kilogram of meat or dozen eggs requires less feed, reducing land use, water consumption, and greenhouse gas emissions.

Waste reduction: Lower embryonic mortality reduces biological waste requiring disposal. Synchronized hatching may also reduce chick culling by improving uniformity.

11.3 Contribution to SDG 15: Life on Land

Animal welfare improvements from lighted incubation align with SDG 15's focus on responsible stewardship:

Reduced stress: Lower H/L ratios, reduced physical asymmetry, and improved tonic immobility indicate better welfare throughout production. Birds experience less fear and stress, improving their quality of life.

Enhanced natural behavior: Lighted-incubated birds show more appropriate fear responses and better adaptability, suggesting improved behavioral competence. They may be better able to express natural behaviors and cope with environmental challenges.

Reduced mortality: Lower mortality throughout production reduces suffering and improves welfare outcomes. Every bird that survives represents successful welfare management.

CONCLUSION

This chapter synthesizes a decade of systematic research from our laboratory demonstrating that lighted incubation (providing appropriate light during embryonic development) significantly improves poultry performance, welfare, and sustainability. Key findings include:

- Optimal light duration: Twelve hours of light daily (12L:12D) improves hatchability by 4-5%, reduces embryonic mortality, and synchronizes hatch windows compared to constant darkness or constant light.

- Optimal light intensity: Two hundred fifty lux provides sufficient stimulation without overstimulation, improving embryonic development, hatchability, and post-hatch growth across multiple species.
- Optimal spectral composition: Green-Red (GR) dichromatic light produces superior outcomes, enhancing hatchability to 86.15% in broilers, improving stress resistance, and reducing fear responses.
- Strain-specific responses: Genetic background significantly influences responses to lighted incubation, with Hubbard, Cobb-500, and Ross-308 broilers showing distinct optimal protocols.
- Species differences: Broilers show more pronounced responses than layers, while Japanese quail validate findings and suggest broad applicability across avian species.
- Mechanistic understanding: Lighted incubation activates the somatotropic axis, establishes robust circadian melatonin rhythms, programs resilient HPA axis function, and enhances immune system development.
- Welfare improvements: Reduced physical asymmetry, H/L ratios, and tonic immobility duration demonstrate that lighted incubation produces birds with better stress resistance and welfare.
- Sustainability alignment: Lighted incubation contributes to SDG 2 (Zero Hunger), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land) through improved efficiency, reduced waste, and enhanced welfare.

The implementation of lighted incubation in commercial hatcheries represents a rare opportunity to simultaneously improve productivity, welfare, and sustainability. With minimal investment and no negative side effects, this technology offers immediate benefits to producers while contributing to long-term sustainability goals. As the global population grows and demand for poultry products increases, such sustainable intensification strategies become increasingly critical for meeting human needs while preserving environmental resources and ensuring animal welfare.

Future research should explore additional spectral combinations, optimize protocols for specific genetics and production systems, characterize molecular mechanisms, and investigate transgenerational effects. Continued innovation in lighting technology and hatchery automation will further enhance the feasibility and benefits of lighted incubation. By embracing this simple yet powerful intervention, the poultry industry can take a significant step toward more sustainable and welfare-friendly production.

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CHAPTER 2

MOLECULAR DIAGNOSTIC TECHNIQUES AND SEQUENCING TECHNOLOGIES IN VETERINARY MEDICINE

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INTRODUCTION

Accurate infectious disease management in veterinary practice is critical, with timely diagnosis being one of its key components. There are different approaches by which a clinician initially diagnoses a case, ranging from tentative diagnosis based on history, clinical signs and laboratory findings to confirmatory diagnosis using culture, microscopy, serological and molecular techniques. However, these techniques vary in sensitivity and specificity and have their own limitations. Serological tests are generally employed when chronic or subclinical infections are suspected or when assessing immune status; however, they are not very useful in detection of per-acute and acute phase of infection. Molecular techniques are necessary for the detection of acute infections, as they provide high accuracy even with minimal concentrations of pathogen's genetic material. They play a key role in differentiation of infectious organisms and enable to study the mutation patterns, isolate's similarity & genetic variability and evolutionary dynamics of pathogens.

These techniques are especially important in the context of emerging and re-emerging diseases, as well as zoonotic and outbreak-associated infections, highlighting their significance in both human and veterinary medicine. With the effective implementation and continuous advancement of molecular techniques, conventional diagnostic boundaries are gradually diminishing, thereby broadening the scope of disease investigation and intervention. Furthermore, the development of rapid, point-of-care molecular assays has significantly improved field-level diagnostics, particularly in resource-limited settings. Therefore, it is essential for every veterinarian, regardless of their field of specialization, to possess a fundamental understanding of conventional, current and advanced diagnostic techniques due to their increasing necessity and wide applicability.

1. EVOLUTION OF MOLECULAR DIAGNOSTIC TECHNIQUES IN VETERINARY MEDICINE

The evolution of diagnostic techniques in veterinary medicine has progressed from purely empirical approaches to highly advanced molecular methodologies.

Initially, diagnosis was largely based on clinical observation and empirical judgment. With the advent of microbiology in the late 19th century, particularly the introduction of agar-based culture techniques in the early 1880s, the isolation and identification of bacterial pathogens became possible. These culture methods remained the cornerstone of diagnostic microbiology until the mid-20th century. However, they are often non-specific, time-consuming and may require days to weeks, or even months, for certain organisms to grow in specialized media. Serological and histopathological techniques subsequently emerged as important diagnostic tools, contributing significantly to disease detection and understanding of pathological changes.

Despite their utility, these methods also have inherent limitations, including reduced sensitivity in early infections and challenges in differentiating closely related pathogens. Early attempts at nucleic acid-based diagnostics relied on labour-intensive and time-consuming biological methods, primarily involving molecular cloning in bacterial systems using plasmids, bacteriophages and cosmid vectors. A major breakthrough occurred in 1983 with the invention of the polymerase chain reaction (PCR) by Kary Mullis, which revolutionized molecular diagnostics by enabling rapid amplification of specific nucleic acid sequences. This advancement significantly reduced the time required for pathogen detection and improved diagnostic sensitivity.

Conventional PCR, although highly valuable, presented certain limitations, including the need for post-amplification analysis through agarose gel electrophoresis. This step is time-consuming, increases the risk of contamination and requires additional processing. To overcome these drawbacks, several PCR variants have been developed.

Real-time PCR (qPCR) allows simultaneous amplification and detection of nucleic acids using fluorescent dyes, thereby enhancing speed, sensitivity and quantitative capability. Similarly, multiplex PCR enables the simultaneous detection of multiple pathogens in a single reaction, improving efficiency and reducing turnaround time. Despite these advancements, PCR-based methods require sophisticated equipment such as thermocycler, which may limit their use in field conditions. This limitation has led to the development of isothermal amplification techniques, which operate at a constant temperature and eliminate the need for thermal cycling.

Techniques such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) offer rapid, sensitive and cost-effective alternatives suitable for point-of-care and field-level diagnostics. While PCR-based methods rely on prior knowledge of target sequences for primer design, next-generation sequencing (NGS) has introduced a paradigm shift by enabling unbiased, high-throughput detection of nucleic acids. This technology allows comprehensive analysis of genetic material, facilitating the identification of known and novel pathogens without prior assumptions.

Currently, molecular diagnostics is entering a phase of increased precision and sensitivity with the development of advanced technologies such as digital PCR (dPCR), which allows absolute quantification of nucleic acids and CRISPR-based diagnostic platforms, including SHERLOCK and DETECTR, which provide rapid, highly specific detection of target sequences. The continuous evolution of these diagnostic technologies is expanding the scope and capability of veterinary diagnostics and further advancements are expected to enhance disease detection, surveillance and control in the future.

2. LIMITATIONS OF CONVENTIONAL DIAGNOSTICS

Despite their advantages, molecular diagnostic techniques have several limitations. Non-specific amplification may occur due to improper primer design or reaction conditions, leading to false-positive results. Molecular assays can also amplify nucleic acids from non-viable pathogens, resulting in misinterpretation of active infection (viability misinterpretation). Amplification of carrier or commensal organisms unrelated to the clinical disease may further complicate diagnosis. In addition, sample contamination can lead to false positives, while the presence of inhibitors such as enzymes in samples may cause false negatives.

Improper handling and instability can affect the result accuracy. The establishment of molecular diagnostic laboratories requires high initial investment and operational costs, along with technical expertise and skilled personnel. Mutations at the primer binding sites may result in false-negative results even when the pathogen is present.

Conventional molecular techniques are often target-specific, detecting only known organisms and potentially missing other causative agents due to lack of appropriate primers or regional strain variations. Although real-time PCR (qPCR) provides quantitative data, the correlation between nucleic acid load and clinical severity is often inconsistent. Furthermore, lack of standardization across protocols may affect reproducibility and interpretation of results can be complex.

3. SCOPE AND SIGNIFICANCE IN ANIMAL HEALTH AND ZOONOSIS

Molecular diagnostics has significantly improved the management of outbreaks and epidemics, particularly those of zoonotic origin. It is estimated that nearly 50-60% of human diseases are zoonotic, highlighting the complexity of disease dynamics and the importance of effective diagnostic approaches during outbreaks. As these diagnostic tools target pathogen-specific genetic material, they serve not only in detection but also as important tools for monitoring and surveillance. They play a key role in molecular epidemiology, enabling rapid identification of pathogens, wildlife and environmental surveillance and tracking the spread of diseases. In addition, molecular techniques facilitate the detection of antimicrobial resistance through resistance profiling, offering advantages over conventional culture-based methods.

The significance of molecular diagnostics is further enhanced by the integration of the One Health approach, particularly in monitoring disease spill-over at animal-human-environment interfaces. Field-based tools such as loop-mediated isothermal amplification (LAMP) support early detection and rapid management of diseases, especially in resource-limited settings. These techniques contribute to precision-based care, providing high accuracy and speed in diagnosis, thereby improving animal health outcomes. Furthermore, molecular diagnostics has expanded its role in veterinary medicine beyond infectious diseases. Applications such as genetic testing for hereditary disorders and genomic selection for improved livestock productivity demonstrate its broader impact.

Genetic analysis also enables tracing the origin of infections or contamination, aiding in outbreak investigation, understanding disease dynamics and improving control measures. This ultimately contributes to enhanced food safety and security by minimizing contamination risks.

4. FUNDAMENTALS OF MOLECULAR DIAGNOSTICS

4.1 Nucleic Acids as Diagnostic Targets

Nucleic acids, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), serve as the primary diagnostic targets in molecular diagnostics, as they carry the genetic information of organisms, including specific regions unique to particular pathogens or species, forming the basis of molecular diagnostic techniques. These nucleic acids act as highly specific biological markers for the detection of pathogens, genetic mutations and neoplastic conditions with minimal cross-reactivity. DNA and RNA are preferred targets over proteins due to their high specificity and reliability; DNA enables identification of pathogens and detection of structural genetic changes and is relatively stable, allowing extraction from various sample types such as blood, tissues, hair, or saliva for diagnostic and somatic studies. In contrast, RNA reflects ongoing cellular activity and is particularly useful for detecting RNA viruses and studying gene expression through transcriptomics.

Among RNA types, messenger RNA (mRNA) is used to assess gene expression, ribosomal RNA (rRNA) is widely utilized in pathogen detection and microbiological studies, while transfer RNA (tRNA) has limited application and is mainly studied in mutation-related research, predominantly in humans. Additionally, exosomal RNA has emerged as a valuable biomarker for the identification and monitoring of tumour progression. Nucleic acid preparation is a critical step in molecular testing, as the quality and integrity of DNA or RNA directly influence assay performance; therefore, high-quality nucleic acids must be obtained, with particular emphasis on preserving RNA integrity and removing inhibitory substances to ensure accurate results (Chandler, 2013).

4.2 Sensitivity, Specificity and Accuracy

- Analytical sensitivity is defined as the ability of a diagnostic test to correctly identify true positives; that is, if the pathogen or gene of interest is present in the sample, the test accurately reports it as positive.
- Analytical specificity is defined as the ability of a diagnostic test to correctly identify true negatives; that is, if the pathogen or gene of interest is absent in the sample, the test accurately reports it as negative.
- Analytical accuracy refers to the overall correctness of a diagnostic test and is defined as the proportion of all correctly identified results among the total number of samples tested. It is expressed as: Accuracy = (True Positives + True Negatives) / Total Population.

4.3 Amplification of Target

Nucleic acid amplification is a technique used to increase the quantity of a specific target nucleic acid sequence using enzymatic reactions, followed by detection through methods such as polymerase chain reaction (PCR), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), among others (Wang et al., 2024). In this process, the target molecules are amplified into millions of copies, enabling sensitive and accurate detection even when the initial concentration is very low.

4.4 Hybridization Principles

Molecular hybridization is based on the specific pairing of complementary nucleotide bases between a target nucleic acid sequence and a labelled probe, a process known as complementary base pairing. The probe is designed to bind specifically to its complementary sequence and may be labelled with radioactive isotopes such as sulphur (^{35}S) or phosphorus (^{32}P), or with non-radioactive markers such as fluorophores or biotin. The process involves denaturation of double-stranded nucleic acids into single strands, followed by hybridization through the binding of the complementary probe to the target sequence and subsequent washing steps to remove any unbound or non-specifically bound material.

4.5 Analytical Performance

According to CLSI (2004) and Forootan et al., 2017, the limit of detection (LoD) is defined as the lowest amount of analyte (measurand) in a sample that can be reliably detected with a stated probability, although it may not be quantified as an exact value. The limit of quantification (LoQ) is defined as the lowest amount of analyte in a sample that can be quantitatively determined with acceptable precision and accuracy under stated experimental conditions (CLSI, 2004). The limit of blank (LoB) is the highest apparent analyte concentration expected to be observed when multiple replicates of a blank sample containing no analyte are tested (Armbruster and Pry, 2008).

Armbruster and Pry (2008) provided the following formulas:

- $\text{LoB} = \text{mean}_{\text{blank}} + 1.645\ (\text{SD}_{\text{blank}})$
- $\text{LoD} = \text{LoB} + 1.645\ (\text{SD}_{\text{low concentration sample}})$

These parameters are essential for evaluating the analytical sensitivity and performance of molecular diagnostic assays.

4.6 Primers and Probes

Primers and probes are essential synthetic oligonucleotides used in molecular diagnostic assays, particularly those based on nucleic acid amplification. Primers are short DNA sequences that initiate the amplification of target nucleic acids, while probes are designed to bind specifically to target sequences and enable detection. Molecular probes are specialized tools that bind selectively to specific molecules or biomarkers within cells, tissues, or biological samples, allowing precise detection and analysis (Ye et al., 2023; Cheng and Lu., 2023). Probes are utilized not only in hybridization-based techniques but also in amplification-based methods such as real-time PCR (qPCR), where fluorescently labelled probes enable real-time detection and quantification of amplified products. In techniques such as qPCR and fluorescent in situ hybridization (FISH), both primers and probes are employed; initially, the target sequence is amplified by primers, after which the probe binds to the target and produces a fluorescent signal for detection.

The design of primers and probes must be specific to the target organism and requires careful optimization of reaction conditions, including temperature, to minimize cross-reactivity and prevent primer-dimer formation.

Key properties of primers include specificity, melting temperature (Tm) and the absence of intra- or inter-primer homology. Primer specificity is largely determined by the sequences at the 3′ end (Yang et al., 2006).

4.7 Nucleic Acid Quantification

Nucleic acid quantification is an essential step to determine the concentration and purity of DNA or RNA in a given sample, particularly for downstream applications such as sequencing. Various methods are available to assess nucleic acid concentration, among which UV spectrophotometry is widely used and is based on Beer-Lambert’s law. In this method, absorbance is measured at 260 nm and 280 nm and the ratio (A260/A280) is used to evaluate purity. A ratio of 1.7-2.0 indicates high-quality DNA, while a ratio of approximately 2.0 indicates high-quality RNA. Proteins show maximum absorbance at 280 nm and elevated values at this wavelength indicate protein contamination. Absorbance at 230 nm is also important for detecting contaminants such as phenol and other organic compounds.

Nucleic acids exhibit maximum absorbance at 260 nm and the intensity of absorbance is directly proportional to their concentration (Chang-Hui Shen, 2023). Standard conversion factors are used for estimation: double-stranded DNA (dsDNA), 50 µg/mL per absorbance unit; single-stranded RNA (ssRNA), 40 µg/mL; and single-stranded DNA (ssDNA), 33 µg/mL. The concentration of nucleic acids is calculated using the formula: conversion factor × A260 × dilution factor and the total yield is determined by multiplying the concentration by the sample volume. Fluorometric methods provide higher sensitivity compared to spectrophotometry and utilize fluorescent dyes such as SYBR Green and Hoechst 33258, enabling detection of nucleic acids even at nanogram levels using a fluorometer. Additionally, techniques such as real-time PCR (qPCR) and agarose gel electrophoresis can also be used for qualitative and semi-quantitative assessment of nucleic acids.

4.8 Restriction Endonucleases

Restriction endonucleases are molecular enzymes (DNases) that cleave DNA at specific nucleotide sequences known as restriction sites, either within or near the target gene, producing blunt or sticky ends.

These enzymes are highly useful in molecular biology, particularly in cloning techniques and the resulting DNA fragments can be visualized using electrophoresis. An example includes the enzyme HindIII. Recognition sites for restriction enzymes typically range from 4 to 13 base pairs in length and are often palindromic. A unit of restriction enzyme is defined as the amount required to cleave all recognition sites in 1 µg of a specific DNA sample under optimal conditions (Frank, 2016). Digestion of purified genomic DNA with restriction enzymes produces a collection of fragments with identical end sequences and discrete sizes, corresponding to the distances between recognition sites within the DNA sequence (Diaz and Kurt, 2004).

5. TYPES OF MOLECULAR DIAGNOSTIC ASSAYS

All molecular diagnostic assays are based on a fundamental principle: each organism possesses unique nucleic acid sequences and detection relies on complementary base pairing (adenine with thymine/uracil and guanine with cytosine). Although the underlying principle remains the same, assays differ in how they detect target nucleic acids and generate measurable outputs, which may be visualized through fluorescence, colour change, or electrical signals. In most assays, initial denaturation of nucleic acids is essential to allow probe or primer binding through hybridization.

Molecular diagnostic assays are broadly classified into four main categories:

- Probe-based or hybridization-based assays,
- Amplification-based assays
- Signal-based assays
- Sequencing-based assays.

Hybridization-based assays involve denaturation of the target nucleic acid followed by binding of complementary, specifically designed and labelled probes for detection and quantification. Common examples include fluorescence in situ hybridization (FISH) and DNA microarray technology.

Amplification-based assays, also known as nucleic acid amplification tests (NAATs), use primers or complementary sequences to detect and amplify target nucleic acids. Amplification may occur through thermal cycling or at a constant temperature (isothermal amplification).

Key techniques include polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA) and rolling circle amplification (RCA).

Signal-based assays rely on amplification of the detection signal rather than the target nucleic acid itself. In these methods, target recognition triggers a cascade of events that produces an amplified signal for detection and quantification (Maura Pieretti, 2010). Examples include branched DNA (bDNA) assays, hybrid capture assays (HCA), gold nanoparticle-based assays and padlock probe systems.

Sequencing-based assays are advanced, high-throughput molecular techniques that simultaneously detect and determine the exact nucleotide sequence of genetic material with the support of bioinformatics tools for analysis and interpretation. They include first-generation sequencing methods such as Sanger sequencing and Maxam-Gilbert sequencing, second-generation or next-generation sequencing (NGS) platforms such as Illumina, pyrosequencing & Ion Torrent and third-generation sequencing technologies such as single-molecule real-time (SMRT) sequencing and Oxford Nanopore sequencing. Applications include whole genome sequencing (WGS), whole exome sequencing (WES), RNA sequencing and chromatin immunoprecipitation sequencing (ChIP-seq). Additionally, CRISPR-based assays utilize Cas enzymes (Cas9, Cas12, Cas13) and guide RNAs for highly specific detection.

5.1 Hybridization-Based Assays

5.1.1 Blotting Techniques

Blotting is a well-established technique used in molecular diagnostics for the detection of specific nucleic acids or proteins in a given sample. It involves the separation of DNA, RNA, or proteins often treated with restriction endonucleases or denaturing agents such as sodium dodecyl sulfate (SDS), followed by their transfer onto a stable membrane such as nitrocellulose or nylon. The immobilized molecules are then detected through hybridization with labelled probes.

Based on the type of target molecule, blotting techniques are classified as Southern blotting (DNA), Northern blotting (RNA), Western blotting (proteins) and Eastern blotting (post-translational modifications).

Southern Blotting

Southern blotting, developed by Edwin Southern, is used for DNA detection. The process begins with digestion of DNA using restriction endonucleases, followed by separation of fragments through agarose gel electrophoresis. The DNA is then denatured into single strands and transferred onto a nylon membrane by capillary action. The transferred DNA is immobilized, typically using UV radiation and subsequently hybridized with labelled probes, often fluorescent, for detection.

Northern Blotting

Northern blotting, a modified technique from Southern Blotting is used for RNA detection and analysis of gene expression. RNA samples are initially treated with formaldehyde to maintain linear structure and then separated by agarose gel electrophoresis. The separated RNA fragments are transferred onto a nylon membrane using saline sodium citrate (SSC) buffer through capillary action. The RNA is then immobilized, commonly by vacuum oven treatment and hybridized with labelled DNA or RNA probes for detection.

Western Blotting

Western blotting, or immunoblotting, is used to detect specific proteins in a sample and to confirm protein expression, such as in recombinant studies. The process begins with extraction of proteins from cells, typically by cell lysis, with precautions taken to prevent protease-mediated degradation. The extracted proteins are then separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the separated proteins are transferred onto a nitrocellulose membrane using a transfer buffer containing approximately 10% methanol. Methanol facilitates the removal of SDS from proteins and enhances their binding to the membrane, thereby improving transfer efficiency (Mozdzanowski and Speicher, 1992; Pettegrew et al., 2009).

The immobilized proteins are then detected using labelled antibodies based on antigen-antibody interactions, rather than nucleic acid probes, enabling specific identification of the target protein.

Eastern Blotting

Eastern blotting is an immunoblotting technique used for the detection of post-translational modifications of proteins. In this method, components are initially separated by silica gel thin-layer chromatography (TLC) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane is treated with sodium periodate ($NaIO_4$) to facilitate conjugation and the blotted components are subsequently detected using monoclonal antibodies (MAb) (Tanaka et al., 2012). This technique is considered an extension or modification of Western blotting and is particularly useful for studying post-translational modifications such as glycosylation.

5.1.2 FISH (Fluorescence In-Situ Hybridization)

Fluorescence in situ hybridization (FISH) is a quantitative molecular technique that utilizes fluorescently labelled probes to detect complementary nucleic acid sequences within chromosomes, cells, or tissue sections under a fluorescence microscope. The emission of fluorescence indicates the presence of the target sequence, thereby confirming a positive result. In this technique, probes are labelled either with fluorophores or haptens. When haptens are used, detection requires additional immunological or enzymatic steps to visualize the target. During hybridization, the probe penetrates the sample, binds to the complementary nucleic acid sequence and forms a stable DNA duplex with the target strand (Tönnies, 2017).

FISH has been further advanced to enable whole-genome analysis through multicolour techniques such as multiplex FISH (M-FISH) and spectral karyotyping, as well as array-based approaches like comparative genomic hybridization (CGH) (Shakoori, 2017). Common fluorescent dyes used in FISH include fluorescein isothiocyanate (FITC), fluorescein diacetate, carboxyfluorescein (FAM) and cyanine dyes such as Cy3 and Cy5 (Nishi et al., 2015).

Chromogenic in situ hybridization (CISH) is a cost-effective alternative to FISH that combines in situ hybridization with chromogenic detection methods similar to immunohistochemistry. Unlike FISH, which requires specialized fluorescence microscopy, CISH signals can be visualized using a standard light microscope (Tina et al., 2022).

5.2 Amplification Based Assays

5.2.1 Polymerase Chain Reaction (PCR) and Its Variants

Among target amplification techniques, polymerase chain reaction (PCR) is the most commonly used molecular diagnostic method. PCR operates on the principle of thermocycling, involving controlled temperature changes required for three main steps: denaturation, annealing and extension. Denaturation occurs at high temperatures (approximately 95°C), separating the DNA strands. This is followed by annealing, where primers bind to the target sequence at lower temperatures (52-60°C). Finally, elongation (72°C) occurs through the action of Taq polymerase, which synthesizes new DNA strands by extending from the primers. These steps collectively constitute the basic PCR cycle.

Over time, several modifications of conventional PCR have been developed to improve specificity, sensitivity and efficiency:

Conventional PCR: Follows the standard thermocycling process for amplification of nucleic acids.

Asymmetric PCR: Uses unequal primer concentrations, resulting in preferential amplification of a single DNA strand. It is useful for generating templates for sequencing and microarray applications.

- COLD-PCR (Co-amplification at Lower Denaturation Temperature PCR): Enriches mutant or variant sequences by exploiting slight differences in melting temperature. Denaturation is performed at a critical temperature ($Tc \approx Tm-1$), enabling selective amplification of variant alleles (Milbury et al., 2011).
- Hot Start PCR: Involves withholding one or more reaction components until the temperature reaches 60-80°C, thereby reducing non-specific amplification and primer-dimer formation (Chou et al., 1992).

- Colony PCR: Used for rapid screening of bacterial or yeast colonies to confirm the presence of a desired genetic construct without prior DNA extraction (Bergkessel and Guthrie, 2013).
- Rapid PCR: Designed to reduce amplification time, with rapid cycle PCR completing in 10-30 minutes and extreme PCR in under 1 minute. It requires precise and rapid temperature control and optimized reagent conditions (Wittwer, 2023).
- Touchdown PCR: Enhances specificity by initially using a higher annealing temperature (5-10°C above primer Tm), followed by gradual reduction to improve target amplification while minimizing non-specific binding (Green and Sambrook, 2018).
- Solid-Phase PCR: One or both primers are immobilized on a solid surface such as beads or agarose, allowing amplification to occur on the surface. This reduces contamination and is widely used in next-generation sequencing.
- Nested PCR: Involves two rounds of amplification, where the second set of primers binds within the first PCR product, increasing both sensitivity and specificity (Chang-Hui, 2019).
- Multiplex PCR: Enables simultaneous amplification of multiple target sequences in a single reaction. It requires careful optimization of primer design, concentrations and reaction conditions (Markoulatos et al., 2002).
- Inverse PCR: Used for introducing mutations or amplifying unknown regions flanking known sequences in circular DNA using inversely oriented primers.
- Suicide PCR: Employs primers designed for single use to prevent contamination, particularly useful in ancient DNA or low-copy-number samples.
- Ligation-Mediated PCR: Requires knowledge of only one end of the target sequence, with the second end introduced through ligation of a DNA linker, enabling amplification of unknown regions (Mueller and Wold, 1991).

- Nanoparticle-Assisted PCR (NanoPCR): Utilizes nanoparticles to enhance heat transfer, enzyme stability and primer binding, thereby improving amplification efficiency, especially in degraded or low-quality samples (Vajpayee et al., 2025).
- Miniprimer PCR: Uses ultra-short primers (approximately 10 nucleotides) along with engineered polymerases to amplify highly divergent sequences, particularly useful in environmental and microbial studies (Isenbarger et al., 2008).

5.2.1a Real-Time PCR (qPCR)

Real-time PCR (qPCR) is an advanced amplification technique in which the amount of product formed is monitored during the course of the reaction by measuring fluorescence emitted by dyes or probes incorporated into the system. The fluorescence intensity is directly proportional to the quantity of amplified product and the number of amplification cycles required to reach a detectable level is recorded (Kubista et al., 2006). Real-time quantitative PCR enables sensitive, specific and reproducible quantification of nucleic acids (Arya et al., 2005). Most real-time PCR chemistries are based on the hybridization of an oligonucleotide probe to its complementary sequence within the amplicon and increased primer efficiency can enhance fluorescent signal generation (Gyllensten and Erlich, 1988). Based on their chemistry, real-time PCR methods are broadly classified into two groups: (1) double-stranded DNA intercalating dyes, such as SYBR Green I and EvaGreen and (2) fluorophore-labeled oligonucleotides, which include primer-probe systems (e.g., Scorpions, Amplifluor, LUX), hydrolysis probes (e.g., TaqMan, MGB-TaqMan) and hybridization probes (e.g., molecular beacons, FRET-based probes) (Navarro et al., 2015).

Among these, SYBR Green and TaqMan are the most widely used. SYBR Green is a cost-effective and simple dye that binds to double-stranded DNA but has lower specificity, whereas TaqMan uses dual-labelled probes and exonuclease activity, offering higher specificity at a greater cost (Tajadini et al., 2014).

An additional advantage of qPCR is post-amplification melting curve analysis, which allows immediate verification of amplification specificity, thereby reducing the need for post-PCR processing and minimizing the risk of contamination.

5.2.1b Reverse Transcriptase PCR (RT-PCR)

Reverse transcription is the process of synthesizing complementary DNA (cDNA) from RNA using an RNA-dependent DNA polymerase (Mo Y et al., 2012). In reverse transcription PCR (RT-PCR), the synthesized cDNA serves as a template for subsequent amplification, enabling the detection and analysis of RNA targets. RT-PCR is also widely used for cloning cDNA for further applications in molecular biology, including analysis of RNA ends and poly(A) tails (Bachman, 2013). Using random hexamer primers, representative cDNA copies can be generated from the entire length of mRNA and pre-mRNA populations. This cDNA can then be used as a template for PCR amplification, allowing comprehensive analysis of gene expression (Rio, 2014).

5.2.2 Isothermal Amplification Techniques

Isothermal amplification (IA) is a nucleic acid amplification technology (NAAT) that operates at a constant temperature, eliminating the need for thermocycling. This characteristic allows the reaction to be performed using simple equipment such as a water bath or even basic heat sources with temperature monitoring, including non-electric setups, making it highly suitable for field and resource-limited conditions (Van Ness et al., 2003; Sukanya et al., 2024). IA has significantly contributed to advancements in molecular diagnostics by enabling rapid, sensitive and specific detection of nucleic acids. When combined with appropriate detection platforms, isothermal amplification techniques provide efficient and timely diagnosis of diseases, enhancing their applicability in clinical and veterinary settings (Boonbanjong et al., 2022).

Strand Displacement Amplification (SDA)

Strand displacement amplification (SDA) is an in vitro isothermal nucleic acid amplification technique that serves as an alternative to PCR.

It is based on the coordinated action of a restriction endonuclease and a strand-displacing DNA polymerase. In SDA, the restriction enzyme (commonly HincII) nicks the unmodified strand at a hemiphosphorothioate recognition site, allowing exonuclease-deficient (exo⁻) Klenow polymerase to extend the 3′ end at the nick while displacing the downstream DNA strand. Exponential amplification occurs through coupled sense and antisense reactions, where displaced strands serve as templates for further amplification (Walker et al., 1992).

In a typical SDA reaction, two sets of primers are used: bumper primers, which function similarly to conventional PCR primers and SDA primers, which bind adjacent to the bumper primers. The reaction mixture includes HincII restriction enzyme and exo⁻ Klenow polymerase and is carried out at a constant temperature, usually around 37°C. The incorporation of phosphorothioate-modified nucleotides such as dATPαS protects newly synthesized strands from complete digestion while allowing selective nicking. Amplification proceeds through repeated cycles of nicking, extension and strand displacement, resulting in rapid exponential amplification. SDA can achieve amplification levels of up to 10^{10}-fold within a short time frame, often as little as 15 minutes under optimized conditions (Hellyer and Nadeau, 2004), with substantial product yields obtained within approximately 2 hours. Additionally, SDA combined with in situ hybridization (SDA-ISH) has been shown to be as sensitive as PCR followed by in situ hybridization and more sensitive than conventional in situ hybridization coupled with catalysed signal amplification (Nuovo, 2000).

Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a rapid, efficient and highly specific nucleic acid amplification technique performed under constant temperature conditions, typically between 60-65°C. It utilizes a DNA polymerase with strong strand displacement activity, commonly Bst polymerase and a set of four to six specially designed primers that recognize six to eight distinct regions of the target DNA (Notomi et al., 2000; Yang et al., 2024).

The primer set includes two outer primers (F3 and B3), two inner primers (forward inner primer [FIP] and backward inner primer [BIP]) and optional loop primers (loop forward and loop backward), which accelerate the reaction (Wong et al., 2018). The amplification process begins when inner primers initiate DNA synthesis on both sense and antisense strands. Outer primers then displace these newly synthesized strands, generating single-stranded DNA that forms stem-loop structures at one end. These structures serve as templates for subsequent rounds of amplification.

During cycling, inner primers bind to loop regions, leading to continuous strand displacement and elongation, resulting in exponential amplification. Each cycle increases the stem length, producing large amounts of DNA with complex structures, including stem-loop DNAs and cauliflower-like formations with multiple inverted repeats. This mechanism enables LAMP to generate up to 10^9 copies of the target DNA within less than one hour (Notomi et al., 2000). The amplification products can be detected by various methods, including agarose gel electrophoresis (characteristic ladder-like pattern), turbidity measurement due to magnesium pyrophosphate formation, or direct visual observation through colour change using dyes such as calcein in the presence of manganese ions (Ragavan et al., 2018; Rishi Shanker et al., 2020). These features make LAMP particularly suitable for rapid, field-level and point-of-care diagnostics.

Recombinase Polymerase Amplification (RPA)

Recombinase polymerase amplification (RPA) is a highly sensitive and selective isothermal amplification technique that operates at a constant temperature of 37-42°C. The amount of sample required for assay is less and within 20 minutes RPA exponentially amplifies the target DNA. RPA is based on the activity of recombination proteins that facilitate the insertion of primers into double-stranded DNA. Recombinase enzymes bind to primers to form nucleoprotein complexes, which scan the target DNA for homologous sequences. Upon recognition, strand invasion occurs, forming a displacement loop (D-loop) and a strand-displacing polymerase extends the primer to synthesize new DNA strands (Sharma et al., 2014).

The amplification process depends on a dynamic balance of proteins, including T4 UvsX recombinase, which binds primers in the presence of ATP to form active filaments and subsequently dissociates after ATP hydrolysis. Single-stranded DNA-binding protein (SSB), such as T4 gp32, stabilizes the displaced DNA strands, while UvsY enhances recombinase activity by preventing competition between UvsX and SSB for primer binding sites (Piepenburg et al., 2006; Hueso et al., 2025). RPA reactions also rely on viscous crowding agents to enhance amplification efficiency. An intermediate mixing step, typically performed after 3-6 minutes of incubation, helps redistribute reaction components and improves overall performance (Lillis et al., 2016). The use of forward and reverse primers enables exponential amplification within a short time frame, making RPA a powerful tool for rapid, point-of-care molecular diagnostics.

Transcription-Mediated Amplification (TMA)

Transcription-mediated amplification (TMA) is an isothermal nucleic acid amplification technique that, unlike PCR, can directly utilize both RNA and DNA as templates (Huggett et al., 2003). It is particularly effective for amplifying RNA targets, such as ribosomal RNA (rRNA), which is first reverse transcribed into complementary DNA (cDNA) and then amplified into multiple RNA copies by RNA polymerase. TMA employs two primers, one of which contains a T7 promoter sequence, along with reverse transcriptase (RT) and T7 RNA polymerase. Initially, RT extends the T7-containing primer on the RNA target, forming an RNA-cDNA hybrid. RNase H then degrades the RNA strand, exposing the cDNA. A second primer binds to the cDNA and reverse transcriptase synthesizes a complementary strand to produce double-stranded DNA containing the T7 promoter. This dsDNA then serves as a template for T7 RNA polymerase, which generates multiple RNA amplicons complementary to the detection probe (Thwe et al., 2024).

The newly synthesized RNA molecules re-enter the amplification cycle, leading to rapid and exponential amplification. TMA can produce 100-1000 copies per cycle, resulting in up to a 10^9 to 10^{10} fold increase in target nucleic acid within 15-30 minutes (Wernecke and Mullen, 2014).

Detection of amplified products is typically achieved using chemiluminescent or fluorescent probes, based on hybridization with complementary oligonucleotides. This high sensitivity and rapid amplification make TMA a valuable tool in molecular diagnostics, particularly for RNA-based pathogens.

Nucleic Acid Sequence-Based Amplification (NASBA)

Nucleic acid sequence-based amplification (NASBA), which is also known as self-sustained sequence replication (3SR), is a sensitive, isothermal, transcription-based amplification technique specifically designed for the detection of RNA targets. It is a primer-dependent method with probe-based detection and follows specific primer and probe design requirements (Deiman et al., 2022). NASBA is highly efficient for RNA amplification, whereas DNA is amplified inefficiently unless present in large excess compared to RNA.

The technique operates through the coordinated activity of three enzymes: reverse transcriptase, RNase H and T7 RNA polymerase, along with two specific oligonucleotide primers (Rosemarie and Peter, 2012). Initially, a primer binds to the target RNA and reverse transcriptase synthesizes a complementary DNA (cDNA) strand. RNase H then degrades the RNA strand in the RNA-DNA hybrid, allowing the second primer to bind to the cDNA. Reverse transcriptase subsequently generates double-stranded DNA containing a T7 promoter sequence. This double-stranded DNA serves as a template for T7 RNA polymerase, which produces multiple RNA copies of the target sequence. These RNA amplicons re-enter the amplification cycle, resulting in exponential amplification. NASBA can achieve amplification levels of approximately 10^9-fold within 60-100 minutes at a constant temperature of around 41°C (Alexandra et al., 2022). Although NASBA is mechanistically similar to transcription-mediated amplification (TMA), it differs in the use of a separate RNase H enzyme. Due to its high sensitivity and specificity for RNA targets, NASBA is widely used for the detection of RNA viruses and gene expression analysis (Moorchung et al., 2009).

Rolling Circle Amplification (RCA)

Rolling circle amplification (RCA) is an isothermal enzymatic process in which a short DNA or RNA primer is extended on a circular DNA template to generate long single-stranded DNA or RNA molecules using specialized polymerases (Ali et al., 2014). RCA is unique because it can function as both a target amplification and a signal amplification technique. This method produces long concatemeric strands consisting of tandem repeats complementary to the circular template (Reiss et al., 2011). In RCA, the template sequence is first designed or ligated into a circular structure, followed by primer annealing. A thermostable polymerase (Ex// Phi29 DNA polymerase) then continuously extends the primer along the circular template, resulting in repetitive oligonucleotide sequences of the target region (Yoshimura et al., 2006). RCA operates under isothermal conditions, typically around 37 °C and can achieve amplification of up to 10^3-fold within one hour (Maria and Spoto, 2017). Various RCA-based strategies have been developed for generating repetitive DNA sequences, including aptamers and DNAzymes, which are used as detection platforms for small molecules and proteins. This makes RCA a versatile tool in genomics and molecular diagnostics (Zhao et al., 2008). The linear amplification process of RCA can be converted into exponential amplification by introducing additional primers that bind to the newly synthesized strands. Furthermore, RCA products can be visualized using fluorescence-based methods, including real-time fluorescence microscopy. The long single-stranded products can hybridize with complementary oligonucleotides and can be manipulated for applications spanning from nanoscale bio-molecular assemblies to larger-scale detection systems (Reiss et al., 2009).

5.3 Probe Amplification Techniques

Probe amplification techniques were developed to overcome the limitations of traditional hybridization methods. Unlike target amplification methods such as PCR, these techniques amplify the detection signal or probe-associated products rather than the target nucleic acid itself.

They are based on advances in molecular biology and the understanding of in vivo nucleic acid processes such as ligation, polymerization, transcription and cleavage (Zhang et al., 2006). These methods serve as alternatives to PCR in certain diagnostic applications.

5.3.1 Ligase Chain Reaction (LCR)

Ligase chain reaction (LCR) is a highly sensitive molecular technique, often considered comparable or, in some contexts, more specific than PCR for detecting known mutations or pathogens. Similar to PCR, LCR utilizes oligonucleotides that anneal to complementary sequences on the target DNA (Weiss and Cowan, 2004). However, unlike PCR primers, LCR probes are designed to bind adjacent to each other and fully cover the target sequence rather than flank it (Drevon, 1995). In LCR, two pairs of oligonucleotides hybridize to each strand of the target DNA. If the sequences are perfectly complementary, a thermostable DNA ligase (such as Taq ligase) joins the adjacent probes by sealing the nick between them, forming a continuous strand. These ligated products then serve as templates for subsequent cycles, resulting in exponential amplification (Griffiths, 1999).

LCR typically involves four oligonucleotides and proceeds through key steps including enzyme activation (ATP/NAD$^+$ dependent), substrate adenylation and nick sealing. Multiplex LCR can be performed using mutation-specific oligonucleotides, often incorporating poly(A) tails to generate products of varying sizes, allowing identification through size-based separation (Catherine and Anthony, 2001). Overall, LCR offers advantages such as high specificity, multiplexing capability and rapid detection, making it particularly useful in mutation analysis and pathogen detection.

5.3.2 Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA®) is a high-throughput, PCR-based technique for detecting copy numbers of up to 50 genomic DNA sequences in one reaction. It uses about 20 ng of DNA and distinguishes single-nucleotide differences (Eijk-Van and Schouten, 2011).

In addition to copy number variation, MLPA can also detect DNA methylation changes and certain point mutations (Hömig-Hölzel and Savola, 2012). Initially developed for identifying disease-causing genomic mutations, MLPA has been widely applied in the detection of various genetic alterations (Kozlowski et al., 2008). Each MLPA probe consists of two synthetic oligonucleotides, the left and right probes flanked by universal primer sequences from the M13 bacteriophage. Genomic DNA is denatured to allow probe hybridization to adjacent target sequences; upon perfect hybridization, probes are ligated into a complete probe and amplified by PCR with universal primers. The resulting unique-length products are separated and identified via capillary electrophoresis (Yang et al., 2013). MLPA enables the screening and mapping of deletions or duplications within specific genomic regions, particularly at the exon level. Compared to broader techniques such as array comparative genomic hybridization (array CGH) or SNP arrays, MLPA is more suitable for targeted analysis of specific regions, allowing precise detection and fine mapping of copy number alterations (Alicia and Bruce, 2018; Vijay, 2016).

5.3.3 Qβ Replicase and RNA-Based Amplification

Qβ replicase is an RNA-dependent RNA polymerase derived from bacteriophage Qβ that uses single-stranded RNA as a template to synthesize complementary RNA strands (Tomita et al., 2018). It is capable of amplifying RNA molecules to very high levels, producing up to 10^9 copies under suitable conditions. The enzyme exhibits high template specificity, preferentially replicating Qβ RNA and certain synthetic RNA sequences, while most natural RNAs are not efficiently utilized unless specific conditions, such as the presence of Mn^{2+}, are provided (Blumenthal, 1980). All RNA synthesis is initiated with GTP and the structure of the template plays a crucial role in determining replication efficiency and specificity. Qβ replicase is a multi-subunit enzyme complex composed of four non-identical subunits: the phage-encoded polymerase (subunits I and II), ribosomal protein S1 and elongation factors EF-Tu and EF-Ts (subunits III and IV). The stability and activity of the enzyme complex are influenced by ionic conditions (Blumenthal et al., 1976).

Studies have shown that Qβ replicase can perform template-free RNA synthesis under certain conditions, possibly due to trace RNA contamination and that RNA molecules can undergo spontaneous recombination (Chetverin, 2018). The enzyme preferentially utilizes specific regions of RNA templates, particularly the 3′ terminal region and can generate internal duplications during replication (Biebricher and Luce, 1992).

The structural conformation of RNA also affects replication efficiency; metastable RNA forms can serve as effective templates, whereas more stable structures, such as hairpin loops, may inhibit replication. Additionally, when initiation is altered (e.g., substitution of GTP with ITP), the enzyme may extend RNA chains at the 3′ end instead of initiating new synthesis (Biebricher et al., 1986). Overall, though old Qβ replicase represents a unique RNA amplification system with high specificity and efficiency, contributing to the understanding of RNA replication mechanisms and nucleic acid amplification technologies.

5.4 Signal Based Amplification Techniques

Signal-based amplification methods enhance the detection signal rather than amplifying the target nucleic acid itself, thereby reducing the limit of detection (LOD) by improving the signal-to-noise ratio. Unlike target-based amplification techniques, which rely on increasing the quantity of nucleic acids, signal-based approaches focus on amplifying the measurable output through engineered sensor or transducer components. These methods are particularly useful in situations where direct target amplification is not feasible or when detecting non-nucleic acid targets (Zhou et al., 2022). Key technologies include Branched DNA (bDNA) assay, Tyramide Signal Amplification, Enzyme-Free Nucleic Acid Amplification.

5.4.1 Branched DNA (bDNA) Assay

The branched DNA (bDNA) assay is a signal amplification technique used for quantitative detection of nucleic acids without amplifying the target itself, thereby reducing errors associated with target amplification.

It utilizes oligonucleotide probes and branched DNA structures to detect targets typically in the range of 500 to 10^7 molecules, with enhanced sensitivity (up to ~50 molecules) achieved using preamplifiers and modified nucleotides (Urdea and Wuestehube, 2000). The method is based on cooperative sandwich hybridization, where the target nucleic acid is captured on a solid surface and hybridized with multiple probe layers. Branched DNA amplifiers carrying enzyme labels, commonly alkaline phosphatase (ALP), generate amplified signals, allowing direct quantification without requiring highly purified samples (Kolberg et al., 1998). Detection is achieved by measuring enzyme activity, for example using substrates such as 1-naphthyl phosphate, with signal output quantified through techniques like square-wave voltammetry (Lee et al., 2008).

5.5 Sequencing-Based Molecular Techniques

Sequencing-based molecular techniques are used to determine the exact order of nucleotides (A, T/U, G, C) in a nucleic acid sample. These methods are essential for genomics, pathogen identification and mutation analysis. Different sequencing platforms vary in read length, throughput and accuracy. The term “generation” refers to the underlying chemistry and technology used in sequencing (Anuj Kumar et al., 2014). First-generation sequencing methods are primarily based on chemical cleavage or chain termination (see Table3).

5.5.1 First-Generation Sequencing

Maxam-Gilbert Method (Chemical Cleavage Method)

This method involves chemical cleavage of DNA at specific bases. Double-stranded DNA is first labelled at the 5′ end with radioactive phosphorus (^{32}P), denatured into single strands and divided into separate reactions with base-specific chemicals. Each reaction produces fragments cleaved at particular nucleotides. These fragments are then separated by electrophoresis and visualized using autoradiography. Due to its complexity, use of hazardous chemicals and technical limitations, this method is no longer commonly used (Dorado et al., 2019; Aniket et al., 2020).

Sanger Sequencing (Chain Termination Method)

Sanger sequencing is the most widely used first-generation sequencing technique due to its high accuracy and reliability and is often considered a gold standard for detecting specific mutations. The method involves amplification and purification of the target DNA, followed by primer annealing and extension using DNA polymerase. During synthesis, incorporation of fluorescently labelled dideoxynucleoside triphosphates (ddNTPs) randomly terminates chain elongation. This generates DNA fragments of varying lengths, each ending with a labelled nucleotide. These fragments are separated by capillary electrophoresis and the sequence is determined based on the fluorescence emitted by the terminal nucleotide. Automated systems detect and interpret the sequence as color-coded peaks corresponding to each base (A, T, G, C) (Sophie et al., 2020; Bayrak and Wooderchak, 2014). Although Sanger sequencing has lower throughput compared to next-generation sequencing methods, it remains widely used for validation studies and precise mutation identification due to its high accuracy (Dard et al., 2020).

5.5.2 Second-Generation Sequencing (Next-Generation Sequencing, NGS)

Second-generation sequencing, commonly referred to as next-generation sequencing (NGS), enables rapid, high-throughput sequencing of entire genomes. These technologies allow simultaneous sequencing of millions of DNA fragments, significantly increasing speed and data output compared to first-generation methods (Eren et al., 2022). Next generation term reflects the sequencing technology advancement.

Roche 454 Sequencing (Pyrosequencing)

Pyrosequencing was the first commercially successful NGS technology. It is based on the detection of pyrophosphate (PPi) released during nucleotide incorporation at the 3′ end of a growing DNA strand. The released PPi is converted into ATP by ATP sulfurylase in the presence of adenosine 5′ phosphosulfate (APS) and the ATP subsequently drives luciferase to produce a chemiluminescent signal.

This light emission is proportional to the number of nucleotides incorporated, allowing sequence determination (Harrington et al., 2013; Twyman, 2009). A major advantage of pyrosequencing is its ability to perform real-time sequencing without electrophoresis, with hundreds of thousands of reactions occurring simultaneously on a single chip. It also provides relatively longer read lengths and is useful for locus-specific applications such as DNA methylation analysis, where bisulfite-converted DNA is used to detect CpG methylation patterns (Patricia et al., 2021; Langaee and Ronaghi, 2005).

Illumina Sequencing (Sequencing by Synthesis)

Illumina sequencing is one of the most widely used NGS platforms and is based on sequencing by synthesis using reversible dye-terminators. It supports applications such as whole-genome sequencing, targeted sequencing, transcriptomics and epigenomics (Anuj Kumar and UD Gupta, 2020). In this method, DNA is fragmented and adapters are ligated to both ends. These fragments are then immobilized onto a flow cell containing oligonucleotide anchors and amplified to form clusters. During sequencing, fluorescently labelled nucleotides are incorporated one at a time and after each incorporation, imaging is performed to detect the emitted fluorescence signal corresponding to the specific base. Each sequencing cycle involves image capture, where the intensity and colour of fluorescence identify the incorporated nucleotide. This process is repeated in cycles, enabling accurate determination of the DNA sequence from millions of clusters simultaneously (Kartz et al., 2018; Alser et al., 2022).

Sequencing by Oligonucleotide Ligation Detection (SOLiD)

Sequencing by oligonucleotide ligation detection (SOLiD) is a next-generation sequencing technique based on ligation rather than polymerase-mediated extension. It utilizes a two-base encoding system, which enhances sequencing accuracy by reading each base twice (Liu et al., 2012). In this method, DNA libraries are sequenced using short, labelled oligonucleotide probes (typically 8 bases long). Each probe contains a ligation site at the first base, a cleavage site around the fifth base and a fluorescent dye attached to the terminal base.

DNA ligase facilitates the binding of these probes to the complementary template strand. After ligation, fluorescence is detected and the probe is cleaved to allow the next cycle of ligation (Mardis, 2008). This cyclic process continues, generating sequence information through repeated rounds of ligation and detection. The two-base encoding system increases accuracy by minimizing sequencing errors. Due to its high precision, SOLiD sequencing is particularly useful in applications requiring accurate variant detection (Satam et al., 2023).

Ion Torrent (Ion Semiconductor Sequencing)

Ion Torrent sequencing is a next-generation sequencing technique based on sequencing-by-synthesis, where nucleotide incorporation is detected through changes in pH rather than fluorescence. In this method, template DNA is fragmented (≈200-400 bp), ligated with adapters and clonally amplified onto beads using emulsion PCR. These beads are then loaded into millions of micro-wells on a semiconductor chip (Nicole, 2023). During sequencing, DNA polymerase incorporates nucleotides complementary to the template strand. Each incorporation releases hydrogen ions (H^+), leading to a change in pH. This pH change is detected by an ion-sensitive layer beneath each well and converted into an electrical signal by the semiconductor sensor. The magnitude of the signal corresponds to the number of nucleotides incorporated; for example, incorporation of two identical bases produces a doubled signal (Gayathiri et al., 2023; Goswami and Neeti, 2022). Unlike fluorescence-based methods, Ion Torrent technology does not require optical detection systems such as cameras or lasers, making it faster and cost-effective. It directly converts chemical signals into digital data, enabling rapid and high-throughput sequencing using platforms such as the Ion Personal Genome Machine (PGM™) (Anuj and Gupta, 2020).

5.5.3 Third Generation Sequencing

Third-generation sequencing (TGS) includes advanced, long-read sequencing technologies that enable real-time analysis of native DNA without fragmentation or prior amplification, allowing faster and more accurate genome characterization.

PacBio Sequencing (SMRT Sequencing)

PacBio (Single Molecule Real-Time, SMRT) sequencing uses circular SMRT-bell templates formed by ligating hairpin adapters to double-stranded DNA. These templates are loaded into Zero-Mode Waveguides (ZMWs), where a single DNA polymerase synthesizes DNA in real time (Rhoads and Au, 2015). Fluorescently labelled nucleotides emit signals upon incorporation, which are detected and converted into sequence data. Circular Consensus Sequencing (CCS/HiFi) improves accuracy by repeatedly sequencing the same molecule.

Nanopore Sequencing

Nanopore sequencing is a third-generation sequencing technology that enables real-time analysis of DNA or RNA without prior amplification. It is based on the movement of nucleic acid strands through a nanoscale pore (~1 nm) embedded in a membrane under an applied electric field (Bharagava et al., 2019). As a single-stranded DNA or RNA molecule passes through the nanopore, each nucleotide causes characteristic disruptions in the ionic current. These changes in current are measured and used to identify the nucleotide sequence (Tomasz, 2020). The movement of the strand through the pore is driven by an electrical potential difference, similar to electrophoresis (Michal et al., 2016). This method allows direct, long-read sequencing with rapid data generation and is widely used in platforms developed by Oxford Nanopore Technologies (Anuj Kumar and U.D. Gupta, 2014). Some authors classify nanopore sequencing as a fourth-generation sequencing technology owing to its capability for direct, real-time sequencing of native nucleic acids without prior amplification.

Table 1. This table presents a combined overview of DNA- and RNA-based sequencing assays, covering techniques used for genetic variation analysis and gene expression profiling

Technique	
DNA Sequencing based Assays	**RNA Sequencing based Assays**
Whole-Genome Sequencing (WGS)	RNA-Seq (Total RNA-Seq)
Whole-Exome Sequencing (WES)	mRNA-Seq
Targeted Panel Sequencing, Amplicon Sequencing	Small RNA-Seq
Liquid Biopsy (ctDNA / cfDNA sequencing)	Targeted RNA-Seq
Copy Number Variation (CNV) Sequencing	Isoform Sequencing (Iso-Seq)
Structural Variant Sequencing	Fusion Gene Sequencing
Bisulfite Sequencing / Methyl-Seq	Single-Cell RNA-Seq (scRNA-Seq)
Whole-Genome Bisulfite Sequencing (WGBS)	Ribosome Profiling (Ribo-Seq)
Reduced Representation Bisulfite Sequencing (RRBS)	Spatial Transcriptomics
ChIP-Seq, ATAC-Seq	CITE-Seq

Table 2. This table summarizes sequencing approaches across three domains: pathogen and microbiome analysis, immune repertoire profiling, and multi-omics integration

Technique	
Pathogen and Microbiome Sequencing Assays	**Immune Repertoire Sequencing**
16S rRNA Gene Sequencing	T-cell Receptor (TCR) Sequencing
Metagenomic Next-Generation Sequencing (mNGS)	B-cell Receptor (BCR) Sequencing
Metatranscriptomic Sequencing (mtNGS)	
Viral Genome Sequencing	
Multi-Omics and Emerging Approaches: Integrated Multi-Omics Sequencing, Single-Cell Multi-Omics Sequencing (RNA + ATAC)	

Table 3. This table enlists all sequencing technologies

Technology
Conventional - Maxam-Gilbert Method, Sanger Sequencing
Sequencing by Synthesis (SBS) - Illumina
Sequencing by Ligation (SBL) - Applied Biosystems SOLiD
Pyrosequencing - Roche 454
Semiconductor Sequencing - Ion Torrent
Short-Read Sequencing - (e.g., Illumina)
Long-Read Sequencing - (e.g., Pacific Biosciences, Oxford Nanopore Technologies)
Single-Molecule Real-Time (SMRT) Sequencing - Pacific Biosciences
Nanopore Sequencing - Oxford Nanopore Technologies

CONCLUSION

Understanding molecular diagnostic techniques is essential for advancing diagnostic accuracy and their effective application in veterinary medicine. Although many of these techniques are based on complex principles, a clear understanding of their mechanisms is crucial for proper utilization. In veterinary practice, the availability of molecular diagnostic facilities is often limited due to financial constraints and a lack of trained personnel. Despite these challenges, molecular techniques play a critical role in the detection of infectious diseases, genetic mutations and outbreak surveillance, particularly those with zoonotic potential. They enable precise characterization of pathogens at the genetic level, which is essential for understanding disease dynamics, preventing spill-over events and improving overall animal and public health.

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CHAPTER 3

ANTIMICROBIAL RESISTANCE IN COMPANION ANIMAL PRACTICE

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INTRODUCTION

Antimicrobial resistance (AMR) is a complex and evolving phenomenon in which microorganisms, particularly bacteria, develop adaptive mechanisms that enable them to survive exposure to antimicrobial agents. These mechanisms include enzymatic degradation of drugs (e.g., β-lactamases), target site modification, alternation of membrane permeability and efflux pumps activation. As a result, pathogens can evade therapeutic action, which are meant to kill them. The emergence and spread of AMR are driven by interconnected factors across three major sectors: humans, animals and the environment. While human and veterinary medicine have received considerable attention, the environmental component remains relatively underexplored despite playing a crucial role in the persistence and dissemination of resistance genes. Environmental reservoirs such as soil, water systems and effluents from hospitals, households and agricultural practices act as key interfaces for the circulation of resistant microorganisms.

AMR is now recognized as a global public health emergency, contributing to approximately 5 million human deaths annually. Without effective intervention, this burden is expected to be doubled in the coming decades. Resistance rates are increasing worldwide, with some pathogens exhibiting up to 40% resistance, leading to the emergence of multidrug-resistant organisms (MDROs), often termed “superbugs,” including extended-spectrum β-lactamase (ESBL)-producing Escherichia coli and methicillin-resistant Staphylococcus aureus (MRSA). In veterinary and livestock sectors, AMR is further accelerated by indiscriminate antimicrobial use, including their application as growth promoters, inappropriate dosing and empirical therapy without proper diagnosis. These practices favour the selection of resistant strains, which can spread to humans through direct contact, food products and environmental contamination. Companion animals, due to their close association with humans, serve as important reservoirs and potential sources of zoonotic transmission. Therefore, implementation of the One Health approach, integrating human, animal and environmental health, along with robust antimicrobial stewardship is necessary.

1. SIGNIFICANCE

Antimicrobial resistance has become one of the most pressing health challenges of the 21st century. It arises when microorganisms develop the ability to withstand antimicrobial agents that were once effective against them. This is largely driven by the misuse and overuse of antimicrobials in clinical practice, veterinary medicine, agriculture, and environmental settings. Often referred to as a "silent pandemic," AMR threatens the effectiveness of modern medicine and complicates the management of infectious diseases. If current trends continue, AMR could become a leading cause of global mortality by 2050 (Ahmed et al., 2024). The increasing loss of effective first-line drugs forces reliance on more expensive and potentially toxic alternatives, placing additional burden on healthcare systems (Luepke et al., 2017; BMJ, 2013).

2. MECHANISMS

Microorganisms develop resistance through exposure to antimicrobial agents, which create selective pressure favouring resistant strains. These adaptations arise through genetic mutations or acquisition of resistance genes and are further propagated through horizontal gene transfer mechanisms. As a result, infections become harder to treat, often requiring prolonged therapy and increasing morbidity and mortality (Ahmed et al., 2024). Resistance can be classified as intrinsic or acquired. Intrinsic resistance is a natural property of certain bacterial species, often due to structural features such as reduced membrane permeability or the presence of efflux systems. In contrast, acquired resistance results from genetic changes, including mutations or the acquisition of resistance genes via transformation, transduction, or conjugation, with plasmid-mediated transfer being particularly significant (Reygaert, 2018).

The major mechanisms of AMR include limiting drug uptake, modification of drug targets, enzymatic drug inactivation and active efflux. Gram-negative bacteria commonly utilize all these mechanisms due to their outer membrane barrier, whereas gram-positive bacteria rely more on target modification and enzymatic inactivation (Reygaert, 2018).

Enzymatic degradation is one of the most important mechanisms, particularly through β-lactamases, which hydrolyse antibiotics and render them ineffective.

Other enzymes modify drugs by adding chemical groups such as acetyl or phosphoryl groups, thereby reducing their activity (Wright, 2005). Efflux pumps represent another major resistance mechanism. These membrane proteins actively expel antibiotics from bacterial cells, reducing intracellular drug concentrations. They belong to several families, including ABC, MFS, MATE, RND, SMR and PACE are capable of transporting a wide range of substrates, contributing to multidrug resistance (Webber and Piddock, 2003).

3. MULTIDRUG, EXTENSIVE AND PANDRUG RESISTANCE

Multidrug-resistant organisms (MDROs) are bacteria resistant to one or more classes of antimicrobial agents. Clinically important examples include MRSA, vancomycin-resistant enterococci (VRE) and ESBL-producing gram-negative bacteria. These organisms are difficult to treat and pose significant challenges in healthcare settings (Siegel et al., 2007). Extensively drug-resistant (XDR) bacteria exhibit resistance to nearly all available antimicrobial agents, leaving very limited treatment options. The term was initially used in the context of Mycobacterium tuberculosis but is now applied more broadly (Magiorakos et al., 2012). Pandrug-resistant (PDR) organisms represent the most severe form, being resistant to all available antimicrobial agents. Their emergence signals a critical threat, as even routine infections may become untreatable (Magiorakos et al., 2012).

4. GLOBAL OVERVIEW

AMR is a multifactorial issue affecting both human and animal health. The widespread use of antibiotics in healthcare and livestock production has accelerated the emergence of resistant strains, reducing the effectiveness of standard treatments (Luepke et al., 2017). This has major implications for modern medical procedures, including surgery, organ transplantation and chemotherapy, which rely heavily on effective antimicrobial therapy (BMJ, 2013). The role of animals in AMR transmission is significant. The use of antimicrobials in livestock contributes to the development of resistant pathogens, which can spread to humans through food chains, direct contact, or environmental exposure (Doyle, 2015; Hussein et al., 2023).

These interactions highlight the importance of a One Health approach, integrating human, animal and environmental health strategies to effectively combat AMR (Littmann and Viens, 2015). The economic burden of AMR is substantial, affecting healthcare systems, agriculture and global trade. Without coordinated global action, the impact of AMR will continue to escalate. Therefore, strengthening antimicrobial stewardship, surveillance systems and interdisciplinary collaboration is essential for sustainable control of this growing threat (Ahmed et al., 2024).

5. COMPANION ANIMALS AND AMR

Antimicrobial resistance (AMR) in companion animal medicine has emerged as a significant clinical and public health concern. The increasing integration of pets into human households has reshaped epidemiological dynamics, facilitating the circulation of resistant organisms between humans and animals. This interconnected relationship reflects the One Health concept, which emphasizes that human, animal and environmental health are interdependent (Daireaux & Nobrega, 2025). Recent surveillance studies indicate that antimicrobial resistance in companion animal isolates exceeds 75%, with multidrug-resistant (MDR) organisms accounting for a substantial proportion of infections. These trends are largely driven by repeated antimicrobial exposure, empirical prescribing practices and inadequate diagnostic stewardship.

6. COMMON RESISTANT PATHOGENS IN DOGS AND CATS

6.1 *Staphylococcus pseudointermedius* (MRSP)

Staphylococcus pseudintermedius is the primary pathogen associated with canine skin infections. Methicillin-resistant strains (MRSP) have become increasingly prevalent, significantly limiting therapeutic options. Methicillin resistance is mediated by the mecA gene, which encodes PBP2a, thereby rendering β-lactam antibiotics ineffective (Dewulf et al., 2025). Recent studies have identified novel SCCmec variants with integrated resistance determinants.

MRSP strains frequently exhibit multidrug resistance and possess biofilm-associated genes (e.g., ica operon), which contribute to chronic and recurrent infections. Emerging clones such as ST551 and ST2853 demonstrate increased transmissibility, including spread within household environments (Menezes J et al., 2026).

6.2 *Escherichia coli* (ESBL-producing strains)

Escherichia coli is the predominant pathogen in urinary tract infections in companion animals. The emergence of ESBL-producing strains has significantly reduced the efficacy of commonly used antimicrobials. The blaCTX-M gene family remains the dominant resistance mechanism, particularly blaCTX-M-15 (Daireaux & Nobrega, 2025). However, the recently identified blaTEM-158 variant represents a critical development, as it confers resistance even to β-lactamase inhibitor combinations. Household transmission plays a major role in dissemination. Longitudinal studies have demonstrated persistent sharing of identical ESBL-producing strains between pets and their owners, indicating that households function as reservoirs for resistant bacteria (Menezes et al., 2024).

6.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae is increasingly associated with complicated infections, particularly recurrent urinary tract infections. Recent evidence indicates the emergence of multidrug-resistant hyper-virulent strains (MDR-hvKp). These strains possess virulence factors such as capsule synthesis genes and siderophores (e.g., aerobactin), enabling systemic dissemination. Concurrently, they harbour resistance genes including blaNDM-1 and blaOXA-48, making infections difficult to treat.

6.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a highly resistant opportunistic pathogen associated with chronic infections such as otitis and wound infections. Intrinsic resistance mechanisms include efflux pumps (MexAB-OprM, MexXY), low membrane permeability and AmpC β-lactamase production.

Recent genomic studies have identified extensively drug-resistant (XDR) strains carrying multiple resistance genes. Biofilm formation further complicates treatment by limiting antimicrobial penetration and promoting persistence.

7. DISEASE-WISE PATTERNS

7.1 Skin Infections

Skin infections are the most common indication for antimicrobial use in veterinary practice. MRSP prevalence is particularly high in recurrent cases. The updated ISCAID (2025) guidelines emphasize a shift toward topical therapy as the primary treatment for superficial pyoderma. Cytology is recommended prior to antimicrobial use and systemic antibiotics should be reserved for deep infections (Loeffler et al., 2025).

7.2 Urinary Tract Infections

Urinary tract infections are predominantly caused by E. coli, with increasing involvement of ESBL-producing strains. A key issue in clinical practice is the limited use of culture and susceptibility testing due to cost constraints. However, studies have demonstrated that removing financial barriers significantly reduces empirical antimicrobial use.

7.3 Respiratory Infections

Respiratory infections are often multifactorial in nature. Bordetella bronchiseptica and Pasteurella multocida are common primary pathogens. Resistance in Bordetella is mediated by genes such as blaBOR-1, along with emerging resistance to tetracyclines and macrolides (Kadlec & Schwarz, 2007). While Pasteurella multocida generally retains high susceptibility to β-lactam antimicrobials, the sporadic emergence of β-lactamase-producing strains harboring the blaROB-1 gene warrants continued genomic surveillance (Zhang et al., 2024). Doxycycline remains the first-line treatment for bortodellosis, with fluoroquinolones reserved for severe cases.

8. RISK FACTORS

8.1 Antimicrobial Exposure

Prior antimicrobial use is the strongest predictor of MDR infections. Studies have shown that penicillin use increases the risk by 7.4-fold, while clindamycin increases the risk by 6.17-fold (Allegretti et al., 2025). Repeated antimicrobial exposure disrupts normal microbiota and promotes the selection of resistant strains.

8.2 Immunosuppressive Therapy

The use of corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with an increased risk of MDR infections due to impaired immune function (Allegretti et al., 2025).

8.3 Empirical Therapy

Empirical use of broad-spectrum antibiotics remains common but contributes substantially to resistance development. Greater emphasis on diagnostic-guided therapy is essential to improve outcomes and reduce resistance selection pressure, short-term use of these drugs also plays a key role in development of resistance.

Antimicrobial resistance in companion animals represents a growing challenge driven by microbial adaptation and clinical practices. The emergence of multidrug-resistant pathogens such as MRSP, ESBL-producing E. coli and MDR Klebsiella sp. underscores the urgent need for improved antimicrobial stewardship. Adopting evidence-based prescribing practices, increasing the use of diagnostic testing and adhering to updated clinical guidelines are essential steps in mitigating resistance within the One Health framework.

9. THERAPEUTIC CHALLENGES

AMR significantly compromises the effectiveness of commonly used antimicrobials, leading to therapeutic failure, prolonged illness and increased transmission risk. In veterinary practice, these challenges are compounded by limited drug availability, diagnostic delays and close human-animal interactions.

The effectiveness of first-line antibiotics such as β-lactams and fluoroquinolones is declining due to increasing resistance, necessitating the use of higher-tier antimicrobials that may be less accessible or associated with greater risks (Weese et al., 2021). The emergence of multidrug-resistant (MDR) pathogens further complicates treatment, often requiring combination therapy or the use of critically important antimicrobials (Papich, 2022). Veterinary-specific antimicrobial options are limited, leading to reliance on human-use drugs, which may not always be optimal (Papich, 2022). Biofilm-associated infections further complicate treatment, as bacteria within biofilms exhibit increased tolerance to antimicrobials. Diagnostic delays often result in empirical therapy, contributing to inappropriate drug selection and resistance development (Weese et al., 2021).

9.1 Treatment Failure

Treatment failure is a major clinical consequence of AMR, characterized by persistence of infection despite therapy. In companion animals, this leads to prolonged disease, recurrence and increased transmission risk. Factors contributing to treatment failure include resistant pathogens, empirical therapy without susceptibility testing, poor drug penetration into certain tissues (e.g., bone, prostate, CNS), host factors such as immunosuppression and reinfection from contaminated environments (Papich, 2022; Weese et al., 2021). Polymicrobial infections and laboratory errors in susceptibility interpretation may also result in ineffective therapy (Monteiro et al., 2025). Additionally, bacteria may develop secondary resistance during treatment due to antimicrobial selective pressure (Weese et al., 2021). Thus, treatment failure is a multifactorial issue involving microbial, pharmacological and clinical factors.

9.2 Biofilms

Biofilm formation plays a critical role in AMR and is commonly associated with chronic and recurrent infections in companion animals. Biofilms reduce antimicrobial penetration and can increase bacterial tolerance by 10-1000 times compared to planktonic cells.

Common biofilm-associated infections include chronic otitis externa (often involving Staphylococcus pseudintermedius), recurrent urinary tract infections (commonly Escherichia coli) and wound or implant-associated infections.

9.3 Limited Drug Options

The effectiveness of commonly used antimicrobials such as β-lactams, tetracyclines and fluoroquinolones has declined due to widespread resistance among pathogens in companion animals (Weese et al., 2021). This limits their use as first-line therapies and necessitates alternative treatment strategies. The emergence of MDR pathogens further reduces therapeutic options, as resistance mechanisms such as efflux pumps and enzymatic degradation can confer cross-resistance across multiple antimicrobial classes (Weese et al., 2021).

10. DIAGNOSTIC APPROACHES

Accurate and timely diagnostic approaches is essential for effective treatment and control. Diagnostic methods for AMR in companion animals have evolved considerably, ranging from conventional culture-based techniques to advanced genomic and artificial intelligence-driven technologies. Despite these advancements, challenges such as increasing multidrug resistance, zoonotic transmission and limited accessibility to advanced diagnostics continue to persist.

10.1 Culture and Sensitivity

Culture-based methods remain the gold standard for detecting bacterial infections and determining antimicrobial susceptibility (Marco-Fuertes et al., 2022). Techniques such as the Kirby–Bauer disk diffusion method and broth micro-dilution are widely used to classify organisms as susceptible, intermediate, or resistant (Scarpellini et al., 2025). However, these methods are time-consuming, typically requiring 24-72 hours, which often leads to the initiation of empirical antimicrobial therapy (Ojasanya et al., 2025).

Samples are cultured on selective and differential media such as MacConkey agar and blood agar, followed by identification using colony morphology, Gram staining and biochemical tests (Marco-Fuertes et al., 2022; Scarpellini et al., 2025). Antimicrobial susceptibility testing (AST) results are interpreted based on standardized guidelines provided by organizations such as the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Monteiro et al., 2025).

Minimum inhibitory concentration (MIC) determination provides a more precise assessment compared to disk diffusion and aids in selecting appropriate drugs and dosage regimens (Ojasanya et al., 2025; Monteiro et al., 2025). Automated systems such as VITEK improve the speed and standardization of testing; however, their high cost limits widespread use in routine veterinary practice (Scarpellini et al., 2025; Monteiro et al., 2025). Additionally, phenotypic methods may fail to detect underlying genetic resistance mechanisms and are limited in identifying fastidious organisms or biofilm-associated infections.

10.2 Molecular Diagnostic Methods

Molecular diagnostic approaches have significantly enhanced the accuracy and speed of AMR detection. Polymerase chain reaction (PCR) assays enable targeted detection of resistance genes such as mecA and β-lactamase genes with high specificity (Marco-Fuertes et al., 2022). Real-time PCR further allows quantification and rapid processing of clinical samples (Scarpellini et al., 2025).

Whole genome sequencing (WGS) provides comprehensive insights into microbial genomes, enabling identification of resistance determinants, mutations and mobile genetic elements involved in horizontal gene transfer (Monteiro et al., 2025). This technique is particularly valuable for epidemiological investigations and understanding transmission dynamics between animals and humans. DNA microarray platforms allow simultaneous detection of multiple resistance genes, making them suitable for large-scale screening and surveillance (Marco-Fuertes et al., 2022).

Advances in proteomics have led to the development of rapid identification systems such as MALDI-TOF mass spectrometry, which can identify bacterial species within minutes and is increasingly explored for resistance detection (Scarpellini et al., 2025). Immunological assays and lateral flow devices offer rapid detection of specific resistance enzymes and show promise for point-of-care diagnostics (American Society for Microbiology, 2025). Emerging biosensor technologies aim to provide real-time detection, although their routine application in veterinary medicine is still evolving (Monteiro et al., 2025).

11. LIMITATIONS

Despite significant advancements, several limitations restrict the routine application of AMR diagnostic techniques. Conventional culture-based methods require extended time (24-72 hours), delaying targeted therapy and promoting empirical antibiotic use, which contributes to resistance development (Ojasanya et al., 2025). Phenotypic tests reflect observable resistance but may fail to detect underlying genetic mechanisms. Additionally, some resistance genes may remain unexpressed under laboratory conditions, leading to underestimation of resistance (Marco-Fuertes et al., 2022). Advanced molecular methods such as PCR and WGS require substantial financial investment, specialized infrastructure and technical expertise, limiting their accessibility in routine veterinary settings (Monteiro et al., 2025; Scarpellini et al., 2025). These techniques are also limited to detecting known resistance genes and may fail to identify emerging or novel mechanisms.

Diagnostic facilities are often centralized, requiring sample referral and increasing turnaround time (Daireaux and Nobrega, 2025). Furthermore, companion animals remain underrepresented in coordinated AMR surveillance programs, resulting in fragmented data and lack of standardized diagnostic protocols (Marco-Fuertes et al., 2022). Certain pathogens require specialized culture conditions and may not be detected using routine methods, while biofilm-forming bacteria exhibit resistance patterns that are not accurately reflected in standard in vitro tests (Scarpellini et al., 2025). Overall, current diagnostic approaches are constrained by delayed results, limited detection capability, variability and restricted accessibility.

12. MITIGATION OF AMR

12.1 Surveillance Systems

Surveillance systems are essential for monitoring AMR trends, identifying emerging resistance patterns and guiding antimicrobial stewardship strategies within a One Health framework (Daireaux and Nobrega, 2025). Global AMR surveillance is primarily guided by the tripartite collaboration between the World Health Organization (WHO), Food and Agriculture Organization (FAO) and World Organisation for Animal Health (WOAH). This collaboration promotes integrated surveillance across human and animal health sectors, although inclusion of companion animals varies across regions (Daireaux and Nobrega, 2025). In Europe, the European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet) aims to standardize methodologies and improve comparability of AMR data across countries, covering both livestock and companion animals (Marco-Fuertes et al., 2022). Collaborative efforts by agencies such as the European Food Safety Authority (EFSA), European Medicines Agency (EMA) and European Centre for Disease Prevention and Control (ECDC) also contribute to surveillance, although historically focused more on food-producing animals. The Global Action Plan on AMR emphasizes strengthening surveillance systems across all sectors, including companion animals and advocates international collaboration and standardized data collection (American Society for Microbiology, 2025).

In the United Kingdom, the Companion Animal Veterinary Surveillance Network (SAVSNET) collects clinical data to monitor antimicrobial use and resistance patterns, supporting stewardship practices (Monteiro et al., 2025). In the United States, surveillance is coordinated through the National Antimicrobial Resistance Monitoring System (NARMS), with increasing recognition of the need to include companion animal data (Marco-Fuertes et al., 2022). China has established a comprehensive surveillance system integrating laboratory data to monitor AMR trends in companion animals (Daireaux and Nobrega, 2025). Canada is also progressing toward integrated surveillance systems incorporating companion animals to improve cross-sectoral data sharing. However, in many low- and middle-income countries, surveillance systems remain underdeveloped or fragmented.

12.2 Antimicrobial Stewardship in Veterinary Practice

Antimicrobial stewardship (AMS) is a critical strategy aimed at preserving the efficacy of existing antimicrobial agents while minimizing the emergence and spread of antimicrobial resistance (AMR) (Weese et al., 2015; McEwen and Collignon, 2018). The growing burden of resistance is largely driven by inappropriate and excessive use of antimicrobials across human, animal and environmental sectors (Robinson et al., 2016; Antimicrobial Resistance Collaborators, 2022). In companion animal practice, antimicrobial use closely resembles that in human medicine, where drugs are primarily administered for therapeutic purposes in individual patients, with occasional prophylactic use (Weese et al., 2015). The misuse and overuse of antimicrobials exert strong selective pressure on microorganisms, enabling them to acquire and express resistance genes. These genes can subsequently spread between bacterial populations, contributing to the persistence and dissemination of resistant pathogens. Therefore, rational prescribing practices, including appropriate drug selection, dosage and duration of therapy, form the cornerstone of effective antimicrobial stewardship (Weese et al., 2015). Global recommendations emphasize reducing unnecessary antimicrobial usage and promoting evidence-based prescribing practices (O'Neill, 2016). Strengthening diagnostic support is equally important, as empirical treatment without confirmation often leads to inappropriate antimicrobial use. Thus, enhancing stewardship practices in veterinary medicine is essential not only for animal health but also for protecting public health (Weese et al., 2015; McEwen and Collignon, 2018).

12.3 Prevention and Control Stratergies

Infection prevention and control (IPC) measures are fundamental in reducing infection incidence and, consequently, the need for antimicrobial use (Weese et al., 2015). Effective IPC strategies include proper hygiene, sanitationand biosecurity practices, all of which help limit the transmission of infectious agents. Improved hygiene and sanitation have long been recognized as essential tools for controlling infectious diseases and remain equally important in combating AMR (O'Neill, 2016).

By reducing infection rates, the reliance on antimicrobial therapy is minimized, thereby limiting the development of resistance (Weese et al., 2015). Basic practices such as hand hygiene, appropriate waste disposal and environmental sanitation play a crucial role in controlling pathogen spread.

Environmental contamination also contributes significantly to AMR. Antibiotic residues and resistant bacteria may enter the environment through waste from hospitals, farms and pharmaceutical industries, creating reservoirs of resistance genes (Robinson et al., 2016). These reservoirs facilitate the persistence and spread of resistance across ecosystems. Therefore, infection control should extend beyond clinical settings to include environmental management. A comprehensive approach integrating hygiene, sanitation and environmental control is essential to effectively reduce the burden of AMR (McEwen and Collignon, 2018).

12.4 Alternative Approaches

The increasing prevalence of AMR has necessitated the exploration of alternative strategies that can either replace or complement conventional antimicrobial therapy (O'Neill, 2016). These approaches aim to reduce reliance on antibiotics and thereby limit the selective pressure driving resistance. The declining effectiveness of existing drugs, combined with the slow development of new antimicrobials, further highlights the need for innovative therapeutic options.

12.4.a Phage Therapy

Bacteriophage therapy has emerged as a promising alternative, particularly for multidrug-resistant infections. Bacteriophages are viruses that specifically infect bacterial cells, replicate within them and ultimately cause bacterial lysis (Abedon et al., 2017). Their high specificity allows targeted action against pathogens without significantly affecting normal microbiota. Renewed interest in phage therapy is driven by the increasing inadequacy of antibiotics against resistant pathogens (O'Neill, 2016). Advantages include self-amplification at the infection site and reduced likelihood of cross-resistance with antibiotics (Abedon et al., 2017).

However, challenges such as regulatory limitations, narrow host specificity and potential bacterial resistance to phages must be addressed before widespread clinical application. Despite these limitations, phage therapy represents a promising tool in combating AMR.

12.4.b Ethnoveterinary Practice

Ethnoveterinary practices represent traditional yet valuable approaches, particularly in resource-limited settings. These involve the use of plant-based remedies and indigenous knowledge systems for disease management (Vardhan, 2026). Medicinal plants such as neem (Azadirachta indica), turmeric (Curcuma longa), tulsi (Ocimum sanctum) and Phyllanthus amarus possess antimicrobial, anti-inflammatory, antioxidant and immunomodulatory properties. These contribute to improved immunity, reduced disease incidence and better overall health in animals.

Preparation methods typically involve fresh plant materials processed into extracts or pastes and administered orally or topically. Observational evidence suggests improved health outcomes, reduced mortality and enhanced resistance to infections. Despite their benefits, ethnoveterinary practices require scientific validation, standardization of dosages and quality control to ensure safety and efficacy. Their integration into modern veterinary medicine should be evidence-based.

12.4.c Other Emerging Alternatives

In addition to phage therapy and herbal medicine, several alternative strategies are being explored to combat AMR by targeting bacterial survival and virulence rather than directly killing pathogens (O’Neill, 2016).

Probiotics and Prebiotics

Probiotics are beneficial microorganisms that help maintain gut microbial balance and inhibit pathogenic bacteria through competitive exclusion and antimicrobial substance production (Bisht et al., 2024). Prebiotics promote the growth of beneficial microbes. Together, they improve gut health, enhance immunity and reduce infection rates (Biswaro et al., 2018).

Antimicrobial Peptides (AMPs)

Antimicrobial peptides are components of the innate immune system with broad-spectrum activity against bacteria, fungi and viruses. They act by disrupting microbial cell membranes (Gavrilov et al., 2025). Their rapid action and lower risk of resistance development make them promising alternatives.

Nanotechnology-Based Approaches

Nanoparticles such as silver and zinc oxide exhibit antimicrobial properties and can enhance drug delivery, improve efficacy and reduce required dosages (Hayat et al., 2025). Nanotechnology also enables targeted therapy and controlled drug release (Pathak, 2026). Global recommendations emphasize investment in research for such alternative strategies as part of a comprehensive response to AMR (O'Neill, 2016). Additionally, improved diagnostics and preventive measures support the success of these alternatives by reducing unnecessary antimicrobial use (Weese et al., 2015).

Immunomodulators and Host-Directed Therapies

Enhancing host immunity is an effective strategy to reduce reliance on antimicrobials. Immunomodulators and host-directed therapies strengthen the immune response, enabling animals to combat infections more effectively (Weese et al., 2015). Vaccination is one of the most effective preventive tools, reducing disease incidence and antimicrobial usage. By preventing infections, vaccines indirectly limit the development and spread of resistance (O'Neill, 2016). Other approaches, including nutritional supplementation and immune-enhancing compounds, also contribute to improved disease resistance and reduced dependency on antibiotics.

12.5 One Health Approach

AMR is a complex issue that spans human, animal and environmental health (McEwen and Collignon, 2018). The One Health approach recognizes these interconnections and promotes collaborative strategies to address AMR (Robinson et al., 2016). The misuse of antimicrobials in both human and veterinary sectors contributes significantly to resistance.

Resistant bacteria and genes can transfer across humans, animals and the environment. Environmental factors such as pollution and waste further amplify resistance reservoirs.

Animal agriculture also plays a major role, particularly through prolonged use of antimicrobials at sub-therapeutic doses, which promotes resistance selection and transmission. Given the global nature of AMR, international collaboration is essential. The One Health approach integrates stewardship, surveillance, infection control and policy measures, requiring coordination among veterinarians, physicians, environmental scientists and policymakers (McEwen and Collignon, 2018).

12.6 Future Directions

Addressing AMR requires coordinated efforts across policy, research and clinical practice:

- Reducing unnecessary antimicrobial use through stricter regulations and responsible prescribing (McEwen and Collignon, 2018).
- Strengthening surveillance systems to monitor resistance trends and guide interventions (Antimicrobial Resistance Collaborators, 2022).
- Developing rapid diagnostic tools to support evidence-based treatment and reduce empirical therapy (Weese et al., 2015).
- Investing in research for new antimicrobials and alternative therapies (O’Neill, 2016).
- Enhancing public awareness among veterinarians, pet owners and the general public to promote responsible antimicrobial use.

CONCLUSION

Antimicrobial resistance (AMR) represents a major and growing challenge in veterinary medicine, particularly in companion animal practice. Its impact extends beyond animal health, posing significant risks to human health due to the close interaction between pets and their owners. The development and spread of AMR are driven by multiple interconnected factors, including inappropriate antimicrobial use, environmental contamination and microbial adaptation. Addressing this issue requires strict control of antimicrobial misuse, along with improved prescribing practices and diagnostic support.

A collaborative, multi-sectoral approach is essential, as emphasized by the One Health concept, which recognizes the link between human, animal and environmental health. With coordinated efforts involving veterinarians, researchers, policymakers and pet owners, it is possible to slow the spread of resistance. Strengthening antimicrobial stewardship, enhancing surveillance and promoting responsible use will be key to preserving the effectiveness of existing antimicrobials and ensuring the sustainability of modern veterinary and medical practices.

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