Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Candidate Gene Polymorphisms Associated with Newcastle Disease Outcomes in Chickens: A Systematic Literature Review

Nsiyapnze Katte Yato Katte

yatokatte@gmail.com

University of Dschang https://orcid.org/0009-0005-7453-0914

Shey Ndogmi Yoniwo

University of Dschang https://orcid.org/0000-0002-7982-7021

Blaise Arnaud Hako Touko

University of Dschang https://orcid.org/0000-0001-5841-8811

Julius Awah-Ndukum

University of Bamenda https://orcid.org/0000-0002-0930-2641

Systematic Review

Keywords: Newcastle disease virus, candidate gene polymorphisms, Mx gene, major histocompatibility complex, ROBO2, marker-assisted selection, systematic review, poultry genetics

Posted Date: June 2nd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9868577/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: The authors declare no competing interests.

Abstract

Background

Newcastle disease (ND) is a severe and economically devastating viral disease of chickens caused by avian orthoavulavirus 1 (AOAV-1; formerly APMV-1/NDV), responsible for catastrophic mortality in susceptible flocks globally. Genetic resistance to Newcastle disease virus (NDV) represents a sustainable and complementary approach to conventional control measures. Candidate genes, including the myxovirus resistance (*Mx*) gene, major histocompatibility complex (*MHC*/*B* complex), and roundabout guidance receptor 2 (*ROBO2*), have been implicated in host antiviral immunity. A systematic synthesis of evidence on the associations between polymorphisms in these genes and Newcastle disease outcomes was lacking.

Methods

A systematic literature review was conducted following PRISMA 2020 guidelines. Four databases (Scopus, Google Scholar, PubMed, and Web of Science) were searched for records published between January 2000 and May 2026 using structured, gene-specific queries. Records were screened in two stages using a nine-criterion eligibility framework with pre-specified score thresholds (abstract: ≥4.0; full-text: ≥4.5). Data were extracted on target genes, study design, chicken populations, sample sizes, ND outcomes, and key findings. In the absence of a validated domain-based quality appraisal tool applicable to this heterogeneous evidence base, methodological quality was assessed narratively.

Results

Of 701 identified records, 481 unique records remained after removal of 220 duplicates. Abstract screening excluded 457 records; 24 advanced to full-text assessment, of which five were excluded. Nineteen studies met all eligibility criteria and were included in qualitative synthesis. These encompassed *Mx* gene studies (n = 4), *MHC* polymorphism studies (n = 8), one *ROBO2* study, and six studies examining other candidate genes identified through genome-wide association studies (GWAS) or quantitative trait locus (QTL) mapping. Included studies were geographically diverse (Asia, Africa, North America, Europe) and employed diverse designs including candidate gene association studies, GWAS, QTL mapping, and functional knockout experiments.

Conclusions

Evidence suggests reproducible associations between reported *Mx* G/A polymorphisms and NDV-related antiviral outcomes, and between MHC-linked markers, including B-F, B-LB and LEI0258, and antibody titres or susceptibility outcomes across multiple chicken populations. *ROBO2* and additional GWAS-identified loci (*TIRAP*, *ETS1*, *CAMK1d*, *KIRREL3*) represent emerging targets. NDV resistance is polygenic and shows population-specific effects, underscoring the need for population-level marker validation before implementation in breeding programmes.

1. Introduction

1.1 Rationale

Newcastle disease (ND) is a highly contagious viral disease caused by avian orthoavulavirus 1 (AOAV-1), formerly classified as avian paramyxovirus type 1 (APMV-1), which infects more than 250 avian species worldwide (Dimitrov et al., 2019). In domestic chickens (*Gallus gallus domesticus*), ND ranks among the most economically devastating diseases in poultry production, with velogenic strains capable of causing flock mortality up to 100% (Dimitrov et al., 2017; Reid et al., 2023). Clinical manifestations encompass respiratory, neurological, and enteric disorders, resulting in substantial production losses across both commercial and smallholder poultry operations globally (Luo et al., 2013; Ganar et al., 2014). The disease remains especially important in low-input and smallholder poultry systems, where outbreaks can rapidly reduce household food security, income and access to animal-source protein (Rowland et al., 2018a; Walugembe et al., 2020).

Current ND control relies principally on vaccination programmes and biosecurity measures, but field effectiveness remains constrained by vaccine quality, thermal stability, storage conditions, delivery logistics and flock-level coverage, especially in low-input poultry systems (Kapczynski et al., 2013; Dimitrov et al., 2017). Recent evidence shows that the thermal stability of commercial Newcastle disease vaccines differs substantially by vaccine product and quality, reinforcing the need for careful vaccine handling under tropical conditions (Osman et al., 2021). Similarly, Degefa et al. (2024) demonstrated that storage temperature can affect the efficacy of Newcastle disease vaccines produced under African field-relevant conditions, highlighting the practical importance of cold-chain management and vaccine integrity. In Nepal, Napit et al. (2023) showed that Newcastle disease continues to impose a measurable burden in both commercial and backyard poultry systems, while also demonstrating that appropriately delivered Tablet I2 vaccine can provide effective protection. More recent vaccine-development work has also explored thermostable formulations, including vacuum-foam-dried Newcastle disease vaccines, as potential tools for improving vaccine resilience under challenging storage and distribution conditions (Lyu et al., 2024). These findings do not diminish the central role of vaccination; rather, they indicate that host genetic resistance should be considered as a complementary, cumulative and population-level strategy alongside vaccination, biosecurity and improved vaccine delivery systems (Raut et al., 2025).

Host response to NDV is biologically complex and involves innate antiviral restriction, antigen presentation, cytokine signalling, antibody production, T-cell activation, viral clearance, survival and maintenance of growth under infection or stress conditions (Kapczynski et al., 2013; Rowland et al., 2018a; Saelao et al., 2019). Antibody response to NDV has been shown to vary among chicken populations and to possess a heritable component, supporting the biological plausibility of selective breeding for improved NDV response (Luo et al., 2013; Lwelamira et al., 2009; Hako Touko et al., 2021). However, NDV resistance is not expected to be controlled by a single locus, because genome-wide studies have identified multiple genomic regions and candidate genes associated with antibody response, viral load, growth under challenge and response under heat stress (Luo et al., 2013; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023).

Among candidate genes, the myxovirus resistance (*Mx*) gene has received sustained attention because it encodes an interferon-inducible dynamin-like GTPase implicated in antiviral defence (Ko et al., 2002; Wang et al., 2023). Reported *Mx* polymorphisms, including G/A variants in different chicken populations, have been associated with antiviral activity or NDV-related phenotypes, although the specific variants, allele frequencies

and effect estimates differ across studies (Hassanane et al., 2018; Pagala et al., 2013; Dahiru, 2018). Experimental disruption of *Mx* in DF-1 chicken fibroblast cells further supports a mechanistic role for *Mx* in NDV response, although such knockout evidence should be interpreted as functional support rather than as a natural polymorphism-association result (Wang et al., 2023).

The chicken major histocompatibility complex (MHC), particularly the B-F and B-L regions and the MHC-linked LEI0258 microsatellite, represents another major candidate gene because of its central role in antigen presentation and adaptive immune response (Kaufman, 2022; Liu et al., 2009; Lwelamira et al., 2008). Studies in African, Asian and experimental chicken populations have reported associations between MHC-linked markers and NDV antibody titres, immune-cell responses, embryo susceptibility or related immune traits (Hako Touko et al., 2013; Lestari et al., 2023; Liu et al., 2009; Lwelamira et al., 2008; Mpenda et al., 2020; Norup et al., 2011). Nevertheless, the specific alleles or haplotypes associated with favourable outcomes appear to vary across breeds and populations, indicating that marker effects require population-specific validation before use in breeding programmes (Luo et al., 2013; Mpenda et al., 2020; Walugembe et al., 2020).

Beyond *Mx* and *MHC*, genome-wide association and QTL studies have expanded the genetic architecture of NDV response to include additional loci involved in immune signalling, cell adhesion, transcriptional regulation and stress response (Luo et al., 2013; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020). The *ROBO1*/*ROBO2* region on chicken chromosome 1 has been associated with antibody response to NDV in an F2 resource population, but the functional role of ROBO genes in NDV immunity remains preliminary and requires further validation (Luo et al., 2013; Wang et al., 2014). More recent genomic studies have identified additional candidate genes, including *TIRAP*, *ETS1*, *CAMK1d*, *CCDC3*, *KIRREL3*, *CHORDC1*, *VAV2*, *IL12B*, *DUSP1* and *IL17B*, supporting a polygenic model of NDV response (Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023).

Despite this growing body of evidence, the literature remains fragmented across candidate-gene association studies, MHC-linked marker studies, GWAS, QTL mapping and functional experiments (Luo et al., 2013; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020). Differences in chicken populations, NDV exposure models, vaccination or challenge protocols, genotyping methods, outcome definitions and statistical power complicate direct comparison across studies (Dimitrov et al., 2017; Kapczynski et al., 2013; Rowland et al., 2018a). A systematic synthesis is therefore needed to distinguish well-supported markers from preliminary candidates, evaluate the robustness of the available evidence, and clarify how current findings can inform marker-assisted or genomic selection for NDV resilience in poultry (Page et al., 2021; Saelao et al., 2019; Walugembe et al., 2020).

The present systematic literature review evaluates evidence linking candidate gene polymorphisms and genome-wide loci with Newcastle disease outcomes in chickens, with emphasis on *Mx*, MHC-linked markers, ROBO2 and additional GWAS/QTL-derived genes. It further assesses study design, population specificity, functional support, methodological robustness and breeding relevance to guide future research and implementation in poultry genetic improvement programmes.

1.2 Objectives

The primary objective of this review is to systematically evaluate and synthesise evidence on associations between candidate gene polymorphisms, genome-wide loci and Newcastle disease outcomes in chickens.

Specific objectives are:

- To identify and characterise polymorphisms in the *Mx* gene associated with Newcastle disease resistance, viral load, antibody response, and survival in chickens.
- To evaluate associations between *MHC* polymorphisms (B-F, B-LB genes, and the LEI0258 microsatellite) and Newcastle disease susceptibility, antibody titres, cellular immune responses, and clinical outcomes across chicken breeds and populations.
- To assess evidence for associations between *ROBO2* gene polymorphisms and Newcastle disease outcomes, including antibody responses and disease severity.
- To identify additional candidate genes beyond *Mx*, *MHC*, and *ROBO2* associated with Newcastle disease resistance or susceptibility, as revealed by GWAS and QTL approaches.
- To synthesise findings across studies, identifying consistent patterns, evaluating effect sizes where reported, and assessing the reproducibility of genetic associations.
- To discuss biological mechanisms underlying observed associations and their implications for marker-assisted and genomic selection programmes aimed at improving Newcastle disease resistance in poultry.

2. Methods

2.1 Protocol and Registration

This systematic literature review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 statement (Page et al., 2021). The review protocol was registered with the Systematic Reviews for Animal Food (SYREAF) registry in May 2026. The protocol is available from the corresponding author upon request. No amendments to the protocol were made after registration.

2.2 PICOS Framework

The research question was operationalised using the Population, Intervention/Exposure, Comparator, Outcome, and Study design (PICOS) framework:

Table 1
PICOS framework for Systematic Literature Review

Element	Description
Population (P)	Domestic chickens (*Gallus gallus domesticus*), encompassing commercial lines, indigenous/local breeds, inbred *MHC*-defined lines, and crossbreds
Exposure (I/E)	Naturally occurring or experimentally induced polymorphisms, SNPs, haplotypes, or microsatellite variants in candidate genes: *Mx*, *MHC* (B-F, B-LB, LEI0258), *ROBO2*, or other molecularly characterised loci
Comparator (C)	Different genotypes, alleles, or haplotypes at the same locus; contrasts between homozygous and heterozygous individuals or between defined allele-carrying and non-carrying groups
Outcome (O)	Newcastle disease-related phenotypes: antibody titres (HI or ELISA), viral load/titre, viral shedding, clinical scores, mortality/survival, T cell proliferation/activation, body weight change post-infection
Study design (S)	Candidate gene association studies, GWAS, QTL mapping, controlled challenge trials with genotyping, and functional knockout/overexpression studies; narrative reviews, editorials, conference abstracts, and expression-only studies (without polymorphism stratification) were excluded

2.3 Search Strategy

A comprehensive literature search was conducted across PubMed, Scopus, Web of Science and Google Scholar for studies published between January 2000 and May 2026. Search strategies were designed to identify studies investigating genetic determinants of NDV resistance for immune response in chickens, including candidate gene association studies, GWAS, QTL mapping, and functional genomic investigations. Search terms combined NDV-related keywords with terms related to polymorphisms, SNPs, genomic loci, and immune-associated genes, including Mx, MHC, LEI0258 and ROBO2. Database-specific syntax adaptations were applied. The full search strings are provided in the Supplementary Materials.

2.4 Eligibility Criteria

Studies were evaluated against nine pre-defined eligibility criteria, categorised as inclusion or exclusion criteria:

Inclusion criteria:

- Target population: Domestic chickens (*Gallus gallus domesticus*) as the primary species.
- Specified genetic markers: Investigation of polymorphisms, variants, or haplotypes in *Mx*, *MHC* (including B-F, B-LB, LEI0258), *ROBO2*, or other molecularly characterised candidate genes.
- Newcastle disease phenotypes: Measurement of specific NDV-related outcomes (survival, viral load/shedding, clinical signs, antibody responses).
- Genotype–phenotype association: Explicit linkage between genetic variants and measured ND outcomes.
- Acceptable study designs: GWAS, QTL mapping, candidate gene association studies, controlled challenge trials with genotyping, and functional studies (knockout/overexpression). Functional knockout or overexpression studies were included only when they directly tested the contribution of a candidate gene to NDV response; these studies were treated as mechanistic support rather than as evidence of naturally occurring polymorphism–phenotype association.

Exclusion criteria:

- Expression-only data: Studies reporting gene expression (mRNA/transcriptomics) without stratification by specific genetic polymorphisms.
- Other avian diseases: Studies focused on genetic resistance to other poultry diseases (e.g., Marek's disease, avian influenza) unless they also reported specific ND outcome data.
- Lack of genetic variation comparison: Studies evaluating ND responses in different chicken lines without identifying specific polymorphic markers.
- Insufficient study type: Narrative reviews, conference abstracts without full methods/results, editorials, pure polymorphism surveys without NDV phenotype data, and studies where the primary pathogen was not NDV.

2.5 Screening Process

Screening proceeded in two sequential stages, consistent with PRISMA 2020 and the quality of the selected papers for the review was evaluated using previously described critical appraisal checklists (Munn Z. et al., 2014). This exercise was conducted by two reviewers (NKYK and SNY) working independently, with disagreements resolved by consensus or adjudication by the two other reviewers (BAHT and JAN). Full-text assessment was similarly conducted by two independent reviewers.

Stage 1 — Title and abstract screening: All 481 unique records were assessed against the nine eligibility criteria. Each record received a relevance score (scale: 1–5); a threshold of ≥ 4.0 was applied. Records scoring below 4.0 were excluded at this stage.

Stage 2 — Full-text screening

The 24 records passing abstract screening underwent full-text evaluation against all nine eligibility criteria. A more stringent threshold of ≥ 4.5 was applied. Five articles were excluded at this stage. All 24 full-text articles were accessible electronically. Reasons for exclusion are reported in the PRISMA flow diagram (Fig. 1).

The scoring framework operationalised the nine eligibility criteria into a structured relevance assessment used solely to prioritise screening consistency and was not intended as a formal risk-of-bias tool.

2.6 Data Extraction

Data extraction was performed by two reviewers (NKYK and SNY) working independently. Structured data extraction was performed for all 19 included studies using a standardised template. Discrepancies were resolved by discussion or referral to the other reviewers (BAHT and JAN). Six fields were extracted:

- Target gene(s) and specific polymorphisms/markers examined.
- Study design (candidate gene association, GWAS, QTL mapping, functional study, or combination).
- Chicken population/breed (commercial lines, indigenous breeds, inbred MHC lines, crossbreds, or cell lines).
- Newcastle disease outcomes measured (antibody titres, viral load, clinical scores, mortality, immune cell responses, viral shedding, body weight change).

- Key findings (direction of associations, statistical significance, effect sizes where reported).
- Sample size (number of individual chickens or experimental units).

2.7 Quality Assessment

Methodological quality was assessed using a structured critical appraisal adapted from Munn et al. (2014) for genetic association studies, supplemented with domains specific to immunogenetics and livestock genomics. Ten domains were evaluated narratively:

- Representativeness of the study population
- Sample size and statistical power
- Genotyping methodology and validation
- Phenotype measurement standardisation
- Phenotype-genotype association and statistical analysis
- Population stratification and relatedness
- Functional and mechanistic validation
- Reproducibility and replication
- Reporting completeness and transparency
- External validity and practical applicability

Each domain was rated as adequately addressed (✓), not adequately addressed (✗), unclear or not reported (?), or not applicable (N/A). The appraisal was conducted narratively to accommodate the heterogeneity of designs while maintaining analytical rigour.

2.8 Data Synthesis

Given the substantial heterogeneity in study designs, chicken populations, genotyping methods, NDV challenge protocols, and outcome measures, formal statistical meta-analysis was not performed. Results are presented as a narrative synthesis, organised by candidate gene category. Patterns across studies, including consistency of associations, population specificity, pleiotropic effects, and gene-by-environment interactions, are discussed qualitatively. No sensitivity analyses were performed as formal meta-analysis was not undertaken.

3. Results

3.1 Study Selection

The systematic search and screening process is summarised in the PRISMA 2020 flow diagram (Fig. 1). From an initial pool of 701 records, 220 duplicate records were removed, yielding 481 unique records for screening.

During title and abstract screening, 457 records were excluded: 178 for lack of explicit genotype–phenotype association, 112 for reporting expression-only data without polymorphism stratification, 90 for focusing on other avian diseases without NDV-specific data, and 77 for absence of specific genetic markers. 24 articles advanced to full-text assessment; five were excluded (insufficient genotype–phenotype linkage: n = 3; transcriptomics without polymorphism data: n = 2). No articles were unavailable for full-text retrieval.

Nineteen studies met all eligibility criteria and were included in the qualitative synthesis. These were categorised by target gene: *Mx* gene (n = 4), *MHC* polymorphisms (n = 8), *ROBO2* (n = 1), and other candidate genes/GWAS/QTL/broader genomic studies (n = 6).

3.2 Study Characteristics

The 19 included studies exhibited considerable diversity in study designs, chicken populations, sample sizes, and outcome measures.

Study designs

Study designs were classified according to their primary design, while secondary design components such as controlled challenge, QTL mapping or functional validation were described narratively where applicable. Because several studies used more than one approach, some descriptive design categories were not mutually exclusive, and counts by design type may exceed the total number of included studies. Candidate gene association studies (n = 7; Lwelamira et al., 2008; Liu et al., 2009; Li et al., 2013; Lestari et al., 2023; Dahiru, 2018; Wang et al., 2014; Mpenda et al., 2020); Functional study/controlled immune-response study (n = 1; Norup et al., 2011); GWAS (n = 5; Luo et al., 2013; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023); QTL mapping (n = 1; Hako Touko et al., 2013); Functional knockout (n = 1; Wang et al., 2023); combined/multi-approach (n = 7; Walugembe et al., 2020; Ahmed et al., 2007; Pagala et al., 2013; Hassanane et al., 2018; Rowland et al., 2018a ; Rowland et al., 2018b; Saelao et al., 2019).

Chicken populations and breeds

Studies encompassed commercial layers (White Leghorn; Hy-Line Brown), indigenous/local breeds (Chinese Beijing-You and three Chinese breeds; Tolaki chicken [Indonesia]; Nigerian indigenous chickens; Cameroon native chickens including naked-neck ecotype; Tanzanian local chickens and ecotypes; Ghanaian local chickens; Ethiopian Sasso chickens; IPB-D2 chickens [Indonesia]), inbred MHC-defined lines (B12, B13, B130, B201), crossbred populations (Kuroiler, Sasso × local Tanzanian; Nigerian indigenous × NAPRIX), and the DF-1 chicken fibroblast cell line.

Sample sizes

Sample sizes ranged from fewer than 100 individuals (small-scale candidate gene and functional studies) to more than 500 individuals (large-scale GWAS). Precise sample sizes were not uniformly reported across all included studies, particularly in QTL mapping studies with complex population structures.

Outcomes measured

Antibody responses (assessed by ELISA or haemagglutination inhibition [HI] assay following vaccination or challenge) were the most frequently measured outcome, reported in 11 studies. Other outcomes included viral load/titre (n = 3), viral shedding (n = 1), clinical outcomes including mortality and survival (n = 2), T cell proliferation and activation (n = 1), and body weight change post-infection (n = 2).

Geographic distribution: Studies were conducted in Asia (China: Liu et al., 2009; Li et al., 2013; Luo et al., 2013; Wang et al., 2014; Wang et al., 2023; Indonesia: Pagala et al., 2013; Lestari et al., 2023; India: Ahmed et al.,

2007), Africa (Cameroon: Hako Touko et al., 2013; Tanzania: Lwelamira et al., 2008; Mpenda et al., 2020; Egypt: Hassanane et al., 2018 ; Nigeria: Dahiru, 2018; Ghana: Walugembe et al., 2020; Ethiopia: Girma *et al*., 2023), North America (USA: Rowland et al., 2018a; Rowland et al., 2018b; Saelao et al., 2019;), and Europe (Denmark: Norup et al., 2011).

Table 2
Characteristics of included studies

Study	Gene(s)	Population/Breed	Design	ND Outcome	n (approx.)
Ahmed et al. (2007)	IFN-γ (promoter polymorphism, TspEI site)	iNOSLines divergently selected for cutaneous hypersensitivity (high vs. low CH response)	Controlled challenge + immune phenotyping	IFN-γ mRNA expression. Birds with the GG genotype showed enhanced immune capability.	40
Lwelamira et al. (2008)	LEI0258 microsatellite (MHC-linked; alleles 205 bp, 307 bp)	Kuchi & Tanzanian Medium ecotypes (Tanzania)	Candidate gene association study	Ab response to NDV vaccine (HI); body weight	~ 85–88
Liu et al. (2009)	MHC B-F gene (SNPs in exon 2)	Beijing-You & White Leghorn (China)	Candidate gene association study	NDV vaccination Ab titers	NR
Norup et al. (2011)	MHC haplotypes (B12, B13, B130, B201); CD8α polymorphism	Inbred MHC lines: B12, B13, B130, B201 (Denmark)	Functional/controlled challenge (T cell proliferation assay)	Antigen-specific T cell proliferation (CD4+, CD8+) to NDV antigen	NR
Hako Touko et al. (2013)	LEI0258 (4 alleles) + QTL markers: LEI0070, ADL0146, LEI0104, ADL0320, ADL0304	Cameroon native chickens (incl. naked neck ecotype)	QTL mapping	Ab response to NDV (indirect ELISA); NDV seroprevalence	NR
Li et al. (2013)	MHC B-LBII (loci G97A, T138A)	Wenshang Barred, Laiwu Black, Jining Bairi (China)	Candidate gene association study	ND antibody titers (HI)	300
Luo et al. (2013)	GWAS identified *ROBO1* (SNPs rs15354805, rs15355555), *ROBO2*	F2 resource population (fast-growing × slow-growing divergent lines)	GWAS	Ab response to NDV (ELISA, S/P ratio)	~ 511–505
Pagala et al. (2013)	*Mx* gene (AA, AG, GG genotypes; A & G alleles)	Tolaki chicken (Indonesian indigenous breed)	Candidate gene assoc. + controlled challenge trial	Survival/vitality post-NDV challenge; production traits	103 + 30

Study	Gene(s)	Population/Breed	Design	ND Outcome	n (approx.)
Wang et al. (2014)	ROBO2 (SNPs 1418G > A, 2462T > C)	HB × FCYB F_2 (Chinese hybrid)	Candidate gene association study	Immune response to NDV (Ab response)	800
Hassanane et al. (2018)	*Mx* gene (G/A nucleotide dimorphism)	Egyptian indigenous chickens	Candidate gene assoc. + controlled challenge + functional study	Antiviral activity against NDV; NDV resistance	NR
Rowland et al. (2018a)	GWAS-identified: *B3GALNT2*, *GPR137B*, *LACTB*, *LINGO1*, *HMG20A*, *TARDBP*, *CASZ1*, *MAPK6*, *ER81*, *ADAMTS3*	Commercial Hy-Line Brown layers (USA)	GWAS + controlled NDV challenge	Viral load (qPCR, 2 & 6 dpi); anti-NDV Ab levels	~ 600 (145 dams, 16 sires)
Rowland et al. (2018b)	Candidate genes: *TLR3*, *TLR7*, *Mx*, MHC-B, *IFI27L2*, *HSPB1*, *HSPA2*, *HSPA8*, *IFRD1*	Commercial Hy-Line Brown layers (USA)	Candidate gene assoc. + controlled challenge (heat stress model)	Viral load; anti-NDV Ab titer under heat stress conditions	1,080
Dahiru (2018)	Mx gene	Nigerian indigenous chickens × NAPRIX crosses	Candidate gene association study	NDV resistance phenotypes	110
Saelao et al. (2019)	*CAMK1d*, *CCDC3* (chr 1 QTL); *TIRAP*, *ETS1*, *KIRREL3* (chr 24 QTL)	Hy-Line Brown layer chickens (USA)	GWAS + QTL mapping (heat stress model)	Viral titre (2 & 6 dpi); circulating anti-NDV Ab titer; body weight	NR
Mpenda et al. (2020)	LEI0258 marker alleles (MHC-linked haplotypes)	Kuroiler, Sasso & local Tanzanian chicken embryos	Candidate gene assoc. (embryo challenge model)	NDV susceptibility (embryo mortality classification)	75
Walugembe et al. (2020)	*CHORDC1*, *VAV2*, *IL12B*, *DUSP1*, *IL17B*, *TIRAP*, *ETS1*, *KIRREL3*	Ghanaian & Tanzanian local chicken ecotypes; Hy-Line Brown (USA)	GWAS + QTL mapping + controlled challenge	Anti-NDV Ab levels (pre-infection & 10 dpi); viral load (2 & 6 dpi); growth rate	1,440
Lestari et al. (2023)	MHC BLB2 (13 SNPs in exon 2 & 3; AA genotype g.458T > A)	IPB-D2 chickens (Indonesia)	Candidate gene association study	ND Ab titer (ELISA); IgY concentration	100

Study	Gene(s)	Population/Breed	Design	ND Outcome	n (approx.)
Wang et al. (2023)	*Mx* gene (CRISPR/Cas9 knockout)	DF-1 chicken fibroblast cell line	Functional knockout study	Cellular immune response to NDV; effect of *Mx* loss on NDV replication	Cell line
Girma *et al*. (2023)	*FOXP2* (rs316795557), CEP170B (rs313761644) + additional genomic SNPs	Sasso T451A chickens (Ethiopia)	GWAS	Ab response to NDV	935

NB

Ab = antibody; Ch = cutaneous hypersensitivity; dpi = days post-infection; GWAS = genome-wide association study; HI = haemagglutination inhibition; NR = not reported; QTL = quantitative trait locus.

3.3 Synthesis of Evidence

3.3.1 *Mx* Gene Polymorphisms and NDV Outcomes

Four studies investigated associations between *Mx* gene polymorphisms and Newcastle disease outcomes, providing convergent evidence for a role of *Mx* genetic variation in antiviral responses to NDV.

G/A polymorphism and antiviral activity

The most extensively studied *Mx* variant is a G/A SNP. Hassanane et al. (2018) identified this G/A dimorphism in Egyptian chickens and demonstrated its association with differential antiviral activity against NDV, establishing it as a potential marker for antiviral resistance. Pagala et al. (2013) examined Mx genotypes in Tolaki chickens (an Indonesian indigenous breed) and found significant associations between Mx genotypes and both antiviral traits and production characteristics, indicating pleiotropic effects with implications for multi-trait breeding programmes.

Population-specific associations

Dahiru (2018) investigated *Mx* gene associations with Newcastle disease in Nigerian indigenous chickens and their crosses with NAPRIX, a commercial line. Genetic diversity in *Mx* alleles was demonstrated across Nigerian chicken populations, with documented associations with NDV resistance phenotypes. These findings are relevant to smallholder production systems in West Africa, where crossbreeding is commonly practised.

Functional validation

Wang et al. (2023) provided direct functional evidence for *Mx* involvement in NDV resistance by generating an *Mx*-knockout DF-1 chicken fibroblast cell line using CRISPR/Cas9 genome editing. *Mx*-deficient cells exhibited

significantly impaired immune responses against NDV compared with wild-type cells, confirming a functional role for *Mx* in antiviral defence. This study provides mechanistic evidence for *Mx* involvement in NDV response, although it does not test naturally occurring polymorphism-phenotype associations.

Synthesis

Across three population-based association studies in Egyptian, Indonesian and Nigerian chicken populations, reported *Mx* G/A polymorphisms were associated with NDV-related antiviral or resistance phenotypes (Hassanane et al., 2018; Pagala et al., 2013; Dahiru, 2018). Wang et al. (2023), although not a polymorphism-association study, provide mechanistic support by showing that *Mx* disruption in DF-1 cells impairs the cellular response to NDV. Together, these findings support *Mx* as a biologically relevant candidate gene, while also indicating that specific resistance-associated alleles and effect sizes require population-specific validation.

3.3.2 *MHC* Polymorphisms and NDV Outcomes

Eight studies examined *MHC* polymorphisms in relation to Newcastle disease outcomes, constituting the largest body of evidence among candidate gene categories.

B-F gene polymorphisms

Liu et al. (2009) identified six SNPs in exon 2 of the MHC B-F gene significantly associated with NDV antibody responses in White Leghorn chickens, and two SNPs with ND antibody titres in Beijing-You chickens. The specific associated SNPs differed between breeds, indicating breed-specific linkage disequilibrium structures and underscoring the need for population-stratified analysis.

B-LB gene polymorphisms

Li et al. (2013) investigated MHC B-LBII genetic variation in three Chinese chicken breeds (Wenshang Barred, Laiwu Black, Jining Bairi) and identified two loci (G97A and T138A) significantly associated with ND antibody titres, with particularly strong effects in the Jining Bairi breed. Lestari et al. (2023) identified novel polymorphisms in the MHC BLB2 gene in IPB-D2 chickens and demonstrated associations with both total immunoglobulin Y (IgY) concentration and NDV-specific antibody titres, suggesting that MHC class II variation influences both broad immune capacity and pathogen-specific humoral responses.

LEI0258 microsatellite marker

Hako Touko et al. (2013) identified four LEI0258 alleles exerting favourable effects on NDV antibody response in Cameroon native chickens, including the naked-neck ecotype. Notably, naked-neck chickens exhibited higher mean antibody responses (1,816–2,228 U/mL) compared with other chickens (1,409–1,843 U/mL), though no MHC or QTL allele was unique to this phenotype, suggesting polygenic or environmental contributions. Six additional QTL marker alleles from linked loci (LEI0070, ADL0146, LEI0104, ADL0320, ADL0304) were also associated with enhanced antibody titres. Lwelamira et al. (2008) found significant associations between LEI0258 alleles, NDV antibody response, and body weight in Tanzanian chicken ecotypes, demonstrating pleiotropic effects relevant to multi-trait selection. Mpenda et al. (2020) employed an embryo challenge model with Kuroiler, Sasso, and local Tanzanian chickens to demonstrate LEI0258 allele associations with differential susceptibility to virulent NDV strains in the absence of maternal antibody interference.

MHC haplotype effects

Ahmed et al. (2007) studied immune responses to NDV in lines divergently selected for cutaneous hypersensitivity (CH), which differ in *MHC* composition, revealing that selection for one immune trait affects NDV responses, evidence of pleiotropic MHC effects. Norup et al. (2011) assessed NDV-specific T cell proliferation in inbred MHC lines (B12, B13, B130, B201), finding that CD4 + and CD8 + T cell responses were generally low in B12 chickens while B13, B130, and B201 showed intermediate responses. A second experiment revealed significant differences in CD4 + T cell proliferation between B13 and B130 haplotypes with no corresponding difference in CD8 + responses, indicating that MHC haplotypes differentially modulate the magnitude and composition of cellular immune responses to NDV.

Synthesis

Eight studies collectively demonstrate that MHC polymorphisms, across B-F and B-LB genes, the LEI0258 microsatellite, and defined haplotypes, are major determinants of NDV-related phenotypes in chickens. Associations encompass multiple immune parameters (antibody titres, T cell responses, disease susceptibility) and are reproducible across Africa, Asia, and Europe. However, the specific resistance-conferring alleles or haplotypes vary across populations, reflecting differences in linkage disequilibrium and allele frequencies. Pleiotropic effects on production traits and susceptibility to other pathogens must be explicitly modelled in multi-trait breeding programme designs.

3.3.3 *ROBO2* and Other Candidate Genes

Seven studies identified candidate genes beyond *Mx* and *MHC* through GWAS, QTL mapping, or targeted candidate gene approaches.

***ROBO2* gene**

Wang et al. (2014) investigated genetic variation in the *ROBO2* gene, originally characterised for axon guidance roles, and demonstrated significant associations between *ROBO2* polymorphisms and immune traits in chickens, including NDV antibody responses. This finding broadens the genetic architecture of NDV resistance beyond classical immune genes and raises the hypothesis that *ROBO2* influences immune cell trafficking and activation during NDV infection. Given that only one study examined *ROBO2* in this context, these findings should be considered preliminary and in need of replication.

GWAS-identified candidate genes

Luo et al. (2013) conducted a GWAS of NDV antibody response and identified multiple genomic regions associated with this polygenic trait. Rowland et al. (2018a) performed genetic analysis of a challenged commercial egg-laying line and identified genomic regions associated with viral titre and antibody responses, demonstrating the utility of unbiased genome-wide approaches for discovery. Rowland et al. (2018b) subsequently examined associations of GWAS-identified candidate genes with NDV responses under heat stress, revealing gene-by-environment interactions with practical implications for breeding programmes targeting performance under production-relevant environmental challenges. Saelao et al. (2019) conducted the most detailed genomic dissection, identifying a SNP on chromosome 1 associated with viral titre at two days

post-infection and 30 SNPs spanning a QTL on chromosome 24 associated with viral titre at six days post-infection. Candidate genes in these regions included *CAMK1d* and *CCDC3* (chromosome 1) and *TIRAP*, *ETS1*, and *KIRREL3* (chromosome 24), functionally implicated in Toll-like receptor signalling, immune transcriptional regulation, and lymphocyte adhesion, respectively. Walugembe et al. (2020) extended these findings through GWAS and QTL mapping in Ghanaian and Tanzanian local chicken ecotypes, alongside the Hy-Line Brown commercial line, under controlled NDV challenge. They reported associations involving genes such as *CHORDC1*, *VAV2*, *IL12B*, *DUSP1*, *IL17B*, *TIRAP*, *ETS1*, and *KIRREL3*, with measured traits including anti-NDV antibody levels (pre-infection and 10 days post-infection), viral load (at 2 and 6 days post-infection), and growth rate.

Genomic markers in African populations

Girma *et al*. (2023) identified genomic markers associated with NDV antibody response in Sasso chickens in Ethiopia, contributing evidence for the genetic basis of NDV resistance in African chicken populations where disease burden is high, and vaccination coverage is incomplete.

Synthesis

Emerging evidence demonstrates that NDV resistance is governed by a broader genetic network than *Mx* and *MHC* alone. Novel candidates identified through GWAS, including genes in Toll-like receptor signalling (*TIRAP*) and transcriptional regulation of immune responses (*ETS1*), provide a biologically plausible expanded framework. The polygenic architecture, with loci of small-to-moderate individual effect sizes distributed across the genome, favours genomic selection approaches over single-gene selection strategies for capturing the full complement of genetic variation.

3.3.4 Patterns Across Studies

Consistency of associations

Despite heterogeneity in designs and populations, *Mx* G/A polymorphisms and MHC markers show reproducible association in the direction of available association studies with NDV outcomes, strengthening inference of genuine genetic effects.

Population specificity

The specific alleles or haplotypes conferring resistance frequently differ across breeds and geographic regions, reflecting differences in linkage disequilibrium, allele frequencies, and epistatic contexts. Population-specific validation is therefore a prerequisite before translating genetic markers into breeding programmes.

Polygenic architecture

GWAS and QTL studies consistently demonstrate that NDV resistance is polygenic, with *Mx* and MHC as major genes and numerous additional loci contributing smaller effects. Genomic selection, integrating information across the entire genome, is likely to capture more genetic variation than single-gene selection.

Pleiotropic effects: Multiple studies documented pleiotropic effects of resistance loci on production traits (Pagala et al., 2013: *Mx* and production traits; Lwelamira et al., 2008: LEI0258 and body weight), and on immune responses to other pathogens (Ahmed et al., 2007). These pleiotropic relationships must be incorporated into selection index design to avoid unintended consequences.

Gene-by-environment interactions

Rowland et al. (2018b) and Saelao et al. (2019) demonstrated that genetic effects on NDV responses differ under heat stress, a practically important finding because heat stress is prevalent in tropical poultry production systems. Breeding programmes targeting NDV resistance should evaluate genetic markers under the environmental conditions prevailing in the target production context.

Methodological quality

Larger GWAS studies with standardised phenotyping and genome-wide significance thresholds provide more robust evidence than small candidate gene studies with undisclosed correction for multiple testing. Only one study (Wang et al., 2023) employed gene knockout to establish causality. Reporting of effect sizes (percentage of variance explained, odds ratios, or genotypic means) was inconsistent, limiting quantitative comparison across studies.

3.4 Quality Assessment Results

The structured critical appraisal revealed a clear quality gradient across study designs (see Supplementary Material). Large-scale GWAS studies (Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023) and the functional knockout study (Wang et al., 2023) demonstrated the strongest methodological rigour, with adequate sample sizes (> 500 for GWAS), appropriate correction for multiple testing, control for population stratification, and either functional validation or standardised challenge protocols. Candidate gene association studies occupied the middle ground: methodologically adequate but limited by small samples (n < 200 in seven studies), inconsistent multiple testing correction, and absence of independent replication. The *ROBO2* study (Wang et al., 2014) was the weakest, with an unclear population definition (sampling method within breeds not described, despite running an association analysis on an 800-bird HB x FCYB F_2 population, and no statement on random selection) and no replication. The statistical rigour expected by modern association-study standards was largely absent. Based on this appraisal, we categorise methodological robustness as high (6 studies), moderate (12 studies), or low (1 study).

4. Discussion

4.1 Principal Findings and Evidence Hierarchy

This systematic review synthesised evidence from 19 studies, spanning four continents, six study designs, and 17 years of research, on the genetic determinants of Newcastle disease outcomes in chickens. The findings reveal a tiered evidence base for genetic determinants of Newcastle disease outcomes in chickens. The strongest and most replicated evidence concerned MHC-linked markers, including B-F, B-LB and LEI0258, which were associated with antibody titres, cellular immune responses or susceptibility outcomes across several chicken populations (Liu et al., 2009; Hako Touko et al., 2013; Li et al., 2013; Lwelamira et al., 2008; Mpenda et

al., 2020; Norup et al., 2011). Evidence for reported *Mx* polymorphisms was moderate and biologically plausible, supported by candidate-gene association studies in Egyptian, Indonesian and Nigerian chickens, and strengthened by functional knockout evidence showing that *Mx* contributes to the cellular response to NDV (Hassanane et al., 2018; Pagala et al., 2013; Dahiru, 2018; Wang et al., 2023). In contrast, *ROBO2* and several GWAS/QTL-derived loci remain promising but preliminary candidates because they are supported by fewer studies and require independent replication and functional validation (Luo et al., 2013; Wang et al., 2014; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023). Overall, the available evidence supports a polygenic model of NDV response in which marker effects are likely to be population-specific and influenced by environmental context.

This tiered interpretation is important because not all genetic evidence carries the same translational weight. MHC-linked associations are supported by multiple studies and by a well-established biological mechanism involving antigen presentation and adaptive immunity (Kaufman, 2022). Reported *Mx* associations are biologically plausible because *Mx* encodes an interferon-inducible dynamin-like GTPase implicated in antiviral defence, but the precise functional relevance of specific naturally occurring *Mx* polymorphisms remains incompletely resolved (Ko et al., 2002; Benfield et al., 2010; Wang et al., 2023). GWAS and QTL studies provide broader insight into polygenic architecture, but most candidate loci emerging from these studies still require replication across breeds, challenge conditions and production environments before they can be considered robust markers for selection (Luo et al., 2013; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020).

4.2 *Mx* polymorphisms and antiviral response

Reported *Mx* G/A polymorphisms were associated with NDV-related outcomes in several candidate-gene studies, including studies in Egyptian indigenous chickens, Tolaki chickens and Nigerian indigenous chickens or crosses (Hassanane et al., 2018; Pagala et al., 2013; Dahiru, 2018). These studies suggest that *Mx* variation may contribute to differences in antiviral activity, survival or resistance-related phenotypes. However, the evidence should be interpreted cautiously because the exact variant positions, allele frequencies, population backgrounds, experimental designs and reported effect sizes differed across studies. Therefore, it is safer to refer to “reported *Mx* G/A polymorphisms” rather than assuming that all studies evaluated the same causal polymorphism.

The biological relevance of *Mx* is strengthened by experimental evidence showing that targeted knockout of *Mx* in DF-1 chicken fibroblast cells impaired cellular responses to NDV infection (Wang et al., 2023). This provides mechanistic support for the role of *Mx* in the host response to NDV. Nevertheless, this knockout study should be interpreted as functional evidence for the gene, not as direct proof that a specific naturally occurring *Mx* SNP is causative. This distinction is critical for breeding applications because a gene may be functionally relevant while individual markers within or near the gene may differ in predictive value across populations.

The *Mx* literature also contains complexity that should not be ignored. Benfield et al. (2010) reported that chicken *Mx* alleles tested in their assays did not inhibit NDV, indicating that *Mx* activity may depend on allele background, viral strain, experimental system or broader host genetic context. This does not invalidate later evidence supporting *Mx* involvement in NDV response, but it cautions against oversimplifying *Mx* as a universally predictive single-gene marker. Future studies should therefore combine sequencing of the full *Mx* gene, haplotype reconstruction, allele-specific expression analysis, protein functional assays and controlled

NDV phenotyping to determine whether specific *Mx* variants have reproducible effects across chicken populations.

4.3 MHC-linked markers and adaptive immune response

The MHC emerged as the most consistently supported candidate system in this review. Associations were reported across MHC B-F SNPs, B-LB or BLB2 polymorphisms, LEI0258 microsatellite alleles and defined MHC haplotypes (Liu et al., 2009; Li et al., 2013; Lestari et al., 2023; Hako Touko et al., 2013; Lwelamira et al., 2008; Mpenda et al., 2020; Norup et al., 2011). These findings are biologically coherent because MHC molecules are central to antigen presentation, T-cell activation and adaptive immune response (Kaufman, 2022). In the context of NDV, MHC-linked variation may influence how efficiently viral antigens are presented to the immune system, thereby affecting antibody titres, cellular immune responses and susceptibility outcomes.

For LEI0258-based studies, associations should be interpreted as MHC-linked marker effects rather than direct causal effects of the microsatellite itself. LEI0258 is useful because it is widely used as a proxy for MHC-B haplotypic variation, but its predictive value depends on linkage disequilibrium patterns within each chicken population. This explains why favourable alleles or haplotypes may differ across Tanzanian, Cameroonian, Indonesian and experimental populations (Lwelamira et al., 2008; Hako Touko et al., 2013; Mpenda et al., 2020; Lestari et al., 2023). Consequently, LEI0258 may be practical for low-resource marker screening, but it should not be treated as a universal causal marker without population-specific validation.

The reviewed studies also suggest that MHC-linked variation may have pleiotropic effects. Lwelamira et al. (2008) reported associations between LEI0258 alleles, NDV antibody response and body weight, while Norup et al. (2011) showed that MHC-defined lines differed in NDV-specific T-cell proliferation. These findings are relevant for breeding because selection for disease response must not compromise growth, reproductive traits or responses to other pathogens. In practical terms, MHC-linked markers are among the best candidates for further validation. Still, their use should be embedded within multi-trait selection indices rather than applied as isolated resistance markers.

4.4 *ROBO2* and genome-wide candidate loci

The identification of *ROBO2* and other GWAS/QTL-derived loci broadens the genetic architecture of NDV response beyond classical immune genes (Luo et al., 2013; Wang et al., 2014; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023). *ROBO2* was initially characterised for axon guidance, but its association with immune traits in chickens raises the possibility that genes involved in cell migration, tissue organisation or signalling may also influence immune response to NDV (Wang et al., 2014). However, because *ROBO2* is supported by limited evidence in this specific context, it should be regarded as an emerging candidate requiring independent replication and mechanistic validation.

The GWAS and QTL studies reviewed here are particularly important because they show that NDV response is not adequately explained by *Mx* and MHC alone. Candidate loci identified in genome-wide studies include genes involved in Toll-like receptor signalling, immune transcriptional regulation, lymphocyte adhesion, inflammatory regulation and growth response under challenge conditions (Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023). For example, *TIRAP* is biologically plausible given its role in Toll-like receptor signalling, whereas *ETS1* is relevant to lymphocyte differentiation and immune regulation. Nevertheless, GWAS signals should be interpreted as discovery-level evidence unless replicated and

functionally validated. Many loci of small-to-moderate effect may contribute cumulatively to NDV response, supporting genomic selection approaches over reliance on single-marker selection.

4.5 Population specificity and marker transferability

A recurrent pattern across the included studies is population specificity. Favourable alleles or haplotypes often differed across breeds, ecotypes or experimental populations, even when the same broad candidate gene was implicated (Liu et al., 2009; Lwelamira et al., 2008; Hako Touko et al., 2013; Mpenda et al., 2020; Walugembe et al., 2020). This pattern may reflect differences in allele frequencies, linkage disequilibrium structure, genetic background, prior selection, pathogen exposure, vaccination history and production environment. It also explains why markers identified in commercial or experimental lines may not transfer directly to indigenous or crossbred populations without validation.

This point is particularly important for African and smallholder poultry systems. Local chicken populations are often genetically diverse and managed under heterogeneous environmental conditions, including heat stress, nutritional limitation, mixed infections and variable vaccination access. These conditions may modify the expression of genetic resistance or resilience traits. The heat-stress challenge studies by Rowland et al. (2018b) and Saelao et al. (2019) are therefore highly relevant because they show that genetic effects on NDV response can be environment-dependent. For breeding programmes in tropical systems, markers should be evaluated under production-relevant conditions rather than only under controlled or temperate experimental settings.

4.6 Breeding implications

The evidence supports further evaluation of complementary genetic strategies for improving NDV resilience in chickens. In the short term, marker-assisted selection could focus on the best-supported candidate systems, especially MHC-linked markers and reported *Mx* polymorphisms, but only after validation in the target population. In resource-limited settings, LEI0258 may offer a relatively accessible marker for MHC-linked screening, although its predictive value should be confirmed locally. Reported *Mx* variants may also be useful, but the specific polymorphisms, haplotypes and effects should be verified before selection decisions are made.

In the medium to long term, genomic selection may be more appropriate than single-gene selection because NDV response appears polygenic. GWAS and QTL studies indicate that antibody titres, viral load, growth response and heat-stress-modified NDV outcomes are influenced by multiple loci distributed across the genome (Luo et al., 2013; Rowland et al., 2018a; Saelao et al., 2019; Walugembe et al., 2020; Girma *et al*., 2023). Genomic selection could capture both major and small-effect loci, while multi-trait selection indices could balance disease resilience with growth, reproductive performance, survival and adaptation to local production environments. This is especially important because several candidate markers may have pleiotropic effects on production traits or immune responses to other pathogens.

Genetic improvement should also be framed as complementary to vaccination, not as a replacement. Vaccination remains essential for ND control, but recent studies continue to show that vaccine effectiveness may be influenced by storage conditions, thermal stability, field delivery and vaccine quality (Osman et al., 2021; Degefa et al., 2024; Raut et al., 2025). Thermostable vaccine technologies and improved vaccine delivery systems remain important for smallholder settings, while host genetic improvement may provide cumulative, generation-to-generation gains in resilience. An integrated strategy combining vaccination, biosecurity,

surveillance and locally validated genetic improvement is therefore more realistic than reliance on any single intervention.

4.7 Methodological limitations of the evidence base

Several limitations of the evidence base should be considered when interpreting the findings of this review. First, substantial heterogeneity in chicken populations, NDV strains, vaccination or challenge protocols, outcome measures and time points precluded formal meta-analysis. Some studies measured antibody titres after vaccination, others measured viral load after challenge, and others assessed survival, embryo mortality, T-cell proliferation or growth response. These phenotypes are related but not interchangeable, and they may reflect different components of resistance, tolerance or immune responsiveness.

Second, many candidate-gene studies had modest sample sizes and incomplete reporting of effect sizes, genotyping quality control, multiple-testing correction, Hardy–Weinberg equilibrium, population stratification and replication. These limitations are common in genetic association studies and can increase the risk of false-positive findings. Future studies should align more closely with reporting frameworks such as STREGA, which extends STROBE for genetic association studies and emphasises transparent reporting of genotyping methods, population structure, haplotypes, Hardy–Weinberg equilibrium, missing data and replication (Little et al., 2009). Quality assessment tools such as Q-Genie could also strengthen future systematic reviews by providing a more standardised appraisal of genetic association studies (Sohani et al., 2015).

Third, the credibility of candidate-gene associations should ideally be judged using criteria that consider the amount of evidence, replication and protection from bias. Frameworks such as the Venice interim guidelines for cumulative genetic association evidence are useful in this regard because they distinguish between biological plausibility and robust epidemiological support (Ioannidis et al., 2008). Applying such frameworks in future reviews would help separate strongly supported markers from preliminary candidates.

Fourth, functional validation remains limited. Wang et al. (2023) provide important experimental evidence for *Mx*, but comparable functional work is lacking for ROBO2 and many GWAS/QTL-derived loci. Functional validation through gene editing, overexpression, allele-specific expression, transcriptomic integration and controlled challenge experiments will be essential for confirming causal mechanisms. Without such validation, many candidate loci should remain classified as discovery signals rather than implementation-ready breeding markers.

Finally, publication bias and selective reporting remain possible. Studies reporting positive associations are more likely to be published, while null results may remain inaccessible. Because the number of studies per gene category was small, formal assessment of publication bias using funnel plots was not feasible. This limitation reinforces the need for preregistered replication studies, publication of null findings and open reporting of genotype–phenotype datasets where possible.

4.8 Future research priorities

Future research should prioritise large-scale, multi-population studies using harmonised NDV phenotyping protocols. Standardisation is especially needed for antibody titre measurement, viral load quantification, challenge models, clinical scoring, survival analysis and post-infection growth response. Multi-country studies

involving indigenous, commercial and crossbred chickens would help determine which markers are population-specific and which have broader predictive value.

Functional validation should be expanded beyond *Mx* to include ROBO2 and GWAS/QTL-derived candidates such as *TIRAP*, *ETS1*, *CAMK1d*, *CCDC3*, *KIRREL3*, *CHORDC1*, *VAV2*, *IL12B*, *DUSP1* and *IL17B*. These studies should combine genotyping, gene expression, protein function and NDV challenge phenotypes to connect statistical associations with biological mechanisms. Multi-omics approaches integrating genomics, transcriptomics, proteomics and immunophenotyping would also help clarify how innate antiviral response, antigen presentation and adaptive immunity interact during NDV infection.

Longitudinal designs are also needed. Most included studies measured outcomes at one or a few time points, but NDV response is dynamic. Future studies should track early innate response, antibody kinetics, viral clearance, clinical progression, survival and recovery over time. This would help distinguish genetic effects on resistance, tolerance, immune responsiveness and resilience.

Finally, economic and implementation studies are required before marker-assisted or genomic selection can be translated into smallholder breeding programmes. The practical value of a marker depends not only on statistical association but also on genotyping cost, effect size, compatibility with production traits, ease of deployment, farmer adoption and integration with vaccination programmes. In low-resource settings, the most realistic pathway may involve locally validated low-cost markers for short-term selection, combined with gradual development of genomic selection infrastructure for long-term improvement.

4.9 Synthesis

Taken together, the reviewed evidence indicates that genetic variation contributes meaningfully to Newcastle disease outcomes in chickens, but the strength of evidence differs by gene category. MHC-linked markers currently represent the most consistently supported candidate system, reported *Mx* polymorphisms are biologically plausible and moderately supported, and ROBO2 and several GWAS/QTL-derived loci remain emerging candidates. The most defensible interpretation is therefore not that any single marker can universally predict NDV resistance, but that NDV response is a polygenic, population-dependent and environment-sensitive trait. Breeding applications should proceed through population-specific validation, standardised phenotyping, functional confirmation and integration into multi-trait selection strategies.

5. Conclusions

This systematic review indicates that MHC-linked markers and reported *Mx* polymorphisms are the best-supported candidate systems for Newcastle disease-related outcomes in chickens, while ROBO2 and several GWAS/QTL-derived loci remain emerging candidates requiring replication and functional validation. The synthesis of 19 studies demonstrates:

Genetic variation in *Mx*, MHC-linked markers and additional candidate loci appears to contribute to inter-individual differences in Newcastle disease-related outcomes, although the strength of evidence differs across gene categories. Candidate-gene associations and functional validation of *Mx* provide biological support for its involvement in NDV response, but naturally occurring *Mx* polymorphisms still require population-specific validation before breeding implementation.

NDV resistance is polygenic. While *Mx* and *MHC* exert relatively large effects, GWAS and QTL studies identify numerous additional loci of smaller effect that collectively contribute substantial genetic variation, favouring genomic selection approaches.

Genetic associations show population specificity. Different alleles or haplotypes confer resistance in different breeds, necessitating population-specific marker validation as a prerequisite for selection programme implementation.

Pleiotropic effects and gene-by-environment interactions must be explicitly modelled. Multi-trait selection indices and evaluation under production-relevant environments are essential for sustainable and context-appropriate genetic improvement.

Integration of validated genetic markers into breeding programmes is scientifically justified and promising. Still, implementation should follow population-specific validation, assessment of pleiotropic effects and evaluation under the target production system context. Both MAS for major genes and genomic selection for polygenic traits offer practical strategies.

Abbreviations and Acronyms

ADAMTS3: ADAM Metallopeptidase with Thrombospondin Type 1 Motif 3

AOAV-1: Avian orthoavulavirus 1

APMV-1: Avian paramyxovirus 1

B3GALNT2: Beta-1,3-N-acetylgalactosaminyltransferase 2

CAMK1d: Calcium/calmodulin-dependent protein kinase 1D

Cas9: CRISPR-associated protein 9

CASZ1: Castor Zinc Finger 1

CCDC3: Coiled-coil Domain Containing 3

CEF: Chicken embryo fibroblast

CHORDC1: Cysteine and Histidine Rich Domain Containing 1

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DF-1: Chicken embryo fibroblast cell line

DNA: Deoxyribonucleic Acid

DUSP1: Dual Specificity Phosphatase 1

ELISA: Enzyme-Linked Immunosorbent Assay

ER81: ETS Variant Transcription Factor 1

ETS1: ETS proto-oncogene 1

FOXP2: Forkhead Box Protein 2

GPR137B: G Protein-Coupled Receptor 137B

GWAS: Genome Wide Association Studies

HMG20A: High Mobility Group 20A

HSPA2: Heat Shock Protein Family A Member 2

HSPA8: Heat Shock Protein Family A Member 8

HSPB1: Heat Shock Protein Beta-1

IFI27L2: Interferon Alpha Inducible Protein 27 Like 2

IFRD1: Interferon-related Developmental Regulator 1

IL12B: Interleukin 12B

IL17B: Interleukin 17B

KIRREL3: Kin of IRRE-like protein 3

LACTB: Lactamase Beta

LEI0258: LEI0258 microsatellite marker

LINGO1: Leucine Rich Repeat and Immunoglobulin-like domain-containing protein 1

MAPK6: Mitogen-Activated Protein Kinase 6

MAS: Marker-assisted Selection

MHC: Major Histocompatibility Complex

Mx: Myxovirus resistance gene

ND: Newcastle Disease

NDV: Newcastle Disease Virus

qPCR: Quantitative Polymerase Chain Reaction

QTL: Quantitative Trait Loci

RNA: Ribonucleic Acid

ROBO2: Roundabout Guidance Receptor gene 2

SNP: Single Nucleotide Polymorphism

TARDBP: Transactive response DNA-binding protein 43

TIRAP: Toll/interleukin-1 receptor domain-containing adapter protein gene

TLR3: Toll-like Receptor 3

TLR7: Toll-like Receptor 7

VAV2: Vav Guanine Nucleotide Exchange Factor 2

Declarations

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing interests

The authors declare no competing interests.

Data availability

The original contributions made in this study are contained within the article and supplementary material; any additional questions can be addressed to the corresponding author.

References

1. Ahmed, K. A., Saxena, V. K., Saxena, M., Singh, K. B., & Chauhan, H. V. S. (2007). Immune response to Newcastle disease virus in chicken lines divergently selected for cutaneous hypersensitivity. *International Journal of Immunogenetics*, 34(6), 445–455. https://doi.org/10.1111/j.1744-313X.2007.00722.x
2. Benfield, C. T. O., Lyall, J. W., & Tiley, L. S. (2010). The Cytoplasmic Location of Chicken Mx Is Not the Determining Factor for Its Lack of Antiviral Activity. *PLoS ONE*, 5(8), e12151. https://doi.org/10.1371/journal.pone.0012151
3. Dahiru, M. G. (2018). *Association of Mx gene with Newcastle disease and the genetic diversity in Nigerian indigenous chickens and their crosses with NAPRIX* [Master's dissertation, Ahmadu Bello University]. Ahmadu Bello University Institutional Repository. https://kubanni.abu.edu.ng/handle/123456789/12129
4. Degefa, T., Birehanu, M., Mulugeta, D., Ferede, H., Girma, E., Alemu, A., Muleta, D., Aga, A. M., Shimeket, D., & Woldemariyam, F. T. (2024). Evaluation of the efficacy of three Newcastle disease vaccines produced at the

National Veterinary Institute, Bishoftu, Ethiopia, at different temperature storage conditions. *Acta Microbiologica Hellenica, 69*(4), 212–223. https://doi.org/10.3390/amh69040020

5. Dimitrov, K. M., Abolnik, C., Afonso, C. L., Albina, E., Bahl, J., Berg, M., Briand, F.-X., Brown, I. H., Choi, K.-S., Chvala, I., Diel, D. G., Durr, P. A., Ferreira, H. L., Fusaro, A., Gil, P., Goujgoulova, G. V., Grund, C., Hicks, J. T., Joannis, T. M., Torchetti M. K., Kolosov, S., Lambrecht, B., Lewis, N. S., Liu, H., Liu, H., McCullough, S., Miller, P. J., Monne, I., Muller, C. P., Munir, M., Reischak, D., Sabra, M., Samal, S. K., Servan de Almeida, R., Shittu, I., Snoeck, C. J., Suarez, D. L., Van Borm, S., Wang, Z., & Wong, F. Y. K. (2019). Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infection, Genetics and Evolution*, 74, 103917. https://doi.org/10.1016/j.meegid.2019.103917
6. Dimitrov, K. M., Afonso, C. L., Yu, Q., & Miller, P. J. (2017). Newcastle disease vaccines—A solved problem or a continuous challenge? *Veterinary Microbiology*, 206, 126–136. https://doi.org/10.1016/j.vetmic.2016.12.019
7. Ganar, K., Das, M., Sinha, S., & Kumar, S. (2014). Newcastle disease virus: Current status and our understanding. *Virus Research*, 184, 71–81. https://doi.org/10.1016/j.virusres.2014.02.016
8. Girma, M., Morris, K. M., Sutton, K., Esatu, W., Solomon, B., Dessie, T., Psifidi, A., Vervelde, L., Hanotte, O., Banos, G., & Malatji, D. P. (2024). Genomic markers associated with antibody response to Newcastle disease virus of Sasso chickens raised in Ethiopia. *South African Journal of Animal Science*, 53(5), 728–736. https://doi.org/10.4314/sajas.v53i5.12
9. Hako Touko, B. A., Kong Mbiydzenyuy, A. T., Tumasang, T. T., & Awah-Ndukum, J. (2021). Heritability Estimate for Antibody Response to Vaccination and Survival to a Newcastle Disease Infection of Native chicken in a Low-Input Production System. *Frontiers in Genetics*, 12. https://doi.org/10.3389/fgene.2021.666947
10. Hako Touko, B.A., Keambou, T.C., Han, J.M., Bembide, C., Cho, C.Y., Skilton, R.A., Djikeng, A., Ogugo, M., Manjeli, Y., Tebug Tumassang, T., Zoli, P.A. and Osama, S. 2013. The Major Histocompatibility Complex B (MHC-B) and QTL Microsatellite Alleles of Favorable Effect on Antibody Response against the Newcastle Disease. *International Journal of Genetic Research* 1(1): 1 – 8 https://hdl.handle.net/10568/35169
11. Hassanane, M. S., Hassan, A. A. M., Abdou, F., El-Kader, E. M., Ramadan, K. M., & Helal, N. H. A. (2018). Identification of Mx gene nucleotide dimorphism (G/A) as genetic marker for antiviral activity in Egyptian chickens. *Journal of Genetic Engineering and Biotechnology*, 16(1), 41–47. https://doi.org/10.1016/j.jgeb.2017.11.002
12. Ioannidis, J. P. A., Boffetta, P., Little, J., O'Brien, T. R., Uitterlinden, A. G., Vineis, P., Balding, D. J., Chokkalingam, A., Dolan, S. M., Flanders, W. D., Higgins, J. P. T., McCarthy, M. I., McDermott, D. H., Page, G. P., Rebbeck, T. R., Seminara, D., Khoury, M. J., & Manolio, T. A. (2008). Assessment of cumulative evidence on genetic associations: Interim guidelines. *International Journal of Epidemiology, 37*(1), 120–132. https://doi.org/10.1093/ije/dym159
13. Kapczynski, D. R., Afonso, C. L., & Miller, P. J. (2013). Immune responses of poultry to Newcastle disease virus. *Developmental & Comparative Immunology*, 41(3), 447–453. https://doi.org/10.1016/j.dci.2013.04.012
14. Kaufman, J. (2022). Innate immune genes of the chicken MHC and related regions. *Immunogenetics*, 74(1), 167–177. https://doi.org/10.1007/s00251-021-01229-2

15. Ko, J.-H., Jin, H.-K., Asano, A., Takada, A., Ninomiya, A., Kida, H., Hokiyama, H., Ohara, M., Tsuzuki, M., Nishibori, M., Mizutani, M., & Watanabe, T. (2002). Polymorphisms and the Differential Antiviral Activity of the Chicken *Mx* Gene. *Genome Research*, 12(4), 595–601. https://doi.org/10.1101/gr.210702
16. Lestari, D., Murtini, S., Ulupi, N., Gunawan, A., & Sumantri, C. (2023). Novel MHC BLB2 gene polymorphism and its association with IgY concentration and Newcastle disease antibody titer in IPB-D2 chickens. *Archives Animal Breeding*, 66(3), 275–283. https://doi.org/10.5194/aab-66-275-2023
17. Li, F., Chen, Y.-P., Lan, Q.-H., Wu, X.-S., Liu, Y.-B., Zhang, X.-Y., Bi, Y.-L., & Zhu, Q. (2013). Relationships among immune traits and MHC B-LBII genetic variation in three chicken breeds. *Chinese Journal of Biotechnology*, 29(7), 887–898. https://doi.org/10.13345/j.cjb.2013.07.010
18. Little, J., Higgins, J. P. T., Ioannidis, J. P. A., Moher, D., Gagnon, F., von Elm, E., Khoury, M. J., Cohen, B., Davey Smith, G., Grimshaw, J., Scheet, P., Gwinn, M., Williamson, R. E., Zou, G. Y., Hutchings, K., Johnson, C. Y., Tait, V., Wiens, M., Golding, J., van Duijn, C., McLaughlin, J., Paterson, A., Wells, G., Fortier, I., Freedman, M., Zecevic, M., King, R., Infante-Rivard, C., Stewart, A., & Birkett, N. (2009). STrengthening the REporting of Genetic Association Studies (STREGA): An extension of the STROBE statement. *Genetic Epidemiology, 33*(7), 581–598. https://doi.org/10.1002/gepi.20410
19. Liu, L. B., Wu, C. M., Wen, J., Chen, J. L., Zheng, M. Q., & Zhao, G. P. (2009). Association of SNPs in exon 2 of the MHC B-F gene with immune traits in two distinct chicken populations: Chinese Beijing-You and White Leghorn. *Acta Agriculturae Scandinavica Section A—Animal Science*, 59(1), 4–11. https://doi.org/10.1080/09064700902988905
20. Luo, C., Qu, H., Ma, J., Wang, J., Li, C., Yang, C., Hu, X., Li, N., & Shu, D. (2013). Genome-wide association study of antibody response to Newcastle disease virus in chicken. *BMC Genetics*, 14, 42. https://doi.org/10.1186/1471-2156-14-42
21. Lwelamira, J., Kifaro, G. C., & Gwakisa, P. S. (2009). Genetic parameters for body weights, egg traits and antibody response against Newcastle Disease Virus (NDV) vaccine among two Tanzania chicken ecotypes. *Tropical Animal Health and Production*, *41*(1), 51–59. https://doi.org/10.1007/s11250-008-9153-2
22. Lwelamira, J., Kifaro, G. C., Gwakisa, P. S., & Msoffe, P. L. M. (2008). Association of LEI0258 microsatellite alleles with antibody response against Newcastle disease virus vaccine and body weight in two Tanzania chicken ecotypes. *African Journal of Biotechnology*, 7(6), 714-720. https://doi.org/10.4314/ajb.v7i6.58502
23. Lyu, F., Zhao, Y.-H., Zuo, X.-X., Nyide, B., Deng, B.-H., Zhou, M.-X., Hou, J., Jiao, J.-J., Zeng, M.-Q., Jie, H.-Y., Olaniran, A., Lu, Y., & Khoza, T. (2024). Thermostable vacuum foam dried Newcastle disease vaccine: Process optimization and pilot-scale study. *Applied Microbiology and Biotechnology, 108*, Article 359. https://doi.org/10.1007/s00253-024-13174-7
24. Mpenda, F. N., Keambou Tiambo, C., Waiho, K., Maharudin, M. N., Prayoga, G., Ismail, N. F., & Ilham, Z. (2020). Association of LEI0258 marker alleles and susceptibility to virulent Newcastle disease virus infection in Kuroiler, Sasso, and local Tanzanian chicken embryos. *Journal of Pathogens*, 2020, Article 5187578. https://doi.org/10.1155/2020/5187578
25. Munn, Z., Moola, S., Riitano, D., & Lisy, K. (2014). The development of a critical appraisal tool for use in systematic reviews addressing questions of prevalence. *International Journal of Health Policy and Management*, *3*(3), 123–128. https://doi.org/10.15171/ijhpm.2014.71
26. Napit, R., Poudel, A., Pradhan, S. M., Manandhar, P., Ghaju, S., Sharma, A. N., Joshi, J., Tha, S. S., Dhital, K., Rajbhandari, U., Basnet, A., Schwind, J. S., Rajbhandari, R. M., & Karmacharya, D. B. (2023). Newcastle

disease burden in Nepal and efficacy of Tablet I2 vaccine in commercial and backyard poultry production. *PLOS ONE, 18*(3), e0280688. https://doi.org/10.1371/journal.pone.0280688

27. Norup, L. R., Dalgaard, T. S., Pedersen, A. R., & Juul-Madsen, H. R. (2011). Assessment of Newcastle disease-specific T cell proliferation in different inbred MHC chicken lines. Scandinavian *Journal of Immunology*, 74(1), 23–30. https://doi.org/10.1111/j.1365-3083.2011.02534.x
28. Osman, N., Goovaerts, D., Sultan, S., Salt, J., & Grund, C. (2021). Vaccine quality is a key factor to determine thermal stability of commercial Newcastle disease (ND) vaccines. *Vaccines, 9*(4), 363. https://doi.org/10.3390/vaccines9040363
29. Pagala, M. A., Muladno, Sumantri, C., & Murtini, S. (2013). Association of Mx gene genotype with antiviral and production traits in Tolaki chicken. *International Journal of Poultry Science*, 12(12), 735–739. https://doi.org/10.3923/ijps.2013.735.739
30. Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., Shamseer, L., Tetzlaff, J. M., Akl, E. A., Brennan, S. E., Chou, R., Glanville, J., Grimshaw, J. M., Hróbjartsson, A., Lalu, M. M., Li, T., Loder, E. W., Mayo-Wilson, E., McDonald, S., ... Moher, D. (2021). The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ*, 372, n71. https://doi.org/10.1136/bmj.n71
31. Raut, R., Shrestha, R., Adhikari, A., Fatima, A., & Naeem, M. (2025). Revolutionizing Veterinary Vaccines: Overcoming Cold-Chain Barriers Through Thermostable and Novel Delivery Technologies. *Applied Microbiology*, *5*(3), 83. https://doi.org/10.3390/applmicrobiol5030083
32. Reid, S. M., Skinner, P., Sutton, D., Ross, C. S., Drewek, K., Weremczuk, N., Banyard, A. C., Mahmood, S., Mansfield, K. L., Mayers, J., Thomas, S. S., Brookes, S. M., & Brown, I. H. (2023). Understanding the disease and economic impact of avirulent avian paramyxovirus type 1 (APMV-1) infection in Great Britain. *Epidemiology and infection*, *151*, e163. https://doi.org/10.1017/S0950268823001255
33. Rowland, K., Tizard, M., Sutton, K. M., Miller, M. M., & Delany, M. E. (2018b). Association of candidate genes with response to heat and Newcastle disease virus. *Genes*, 9(11), 560. https://doi.org/10.3390/genes9110560
34. Rowland, K., Tizard, M., Sutton, K. M., Witter, R. L., Marthaler, D., de Geus, E., Pijlman, G. P., Miller, M. M., & Delany, M. E. (2018a). Genetic analysis of a commercial egg laying line challenged with Newcastle disease virus. *Frontiers in Genetics*, 9, 326. https://doi.org/10.3389/fgene.2018.00326
35. Saelao, P., Wang, Y., Chanthavixay, G., Gallardo, R. A., Wolc, A., Dekkers, J. C. M., Lamont, S. J., Kelly, T., & Zhou, H. (2019). Genetics and Genomic Regions Affecting Response to Newcastle Disease Virus Infection under Heat Stress in Layer Chickens. *Genes*, 10(1), 61. https://doi.org/10.3390/genes10010061
36. Sohani, Z. N., Meyre, D., de Souza, R. J., Joseph, P. G., Gandhi, M., Dennis, B. B., Norman, G., Anand, S. S., & Kooner, J. S. (2015). Assessing the quality of published genetic association studies in meta-analyses: The quality of genetic studies (Q-Genie) tool. *BMC Genetics, 16*, 50. https://doi.org/10.1186/s12863-015-0211-2
37. Walugembe, M., Amuzu-Aweh, E. N., Botchway, P. K., Naazie, A., Aning, G., Wang, Y., Saelao, P., Kelly, T., Gallardo, R. A., Zhou, H., Lamont, S. J., Kayang, B. B., & Dekkers, J. C. M. (2020). Genetic Basis of Response of Ghanaian Local Chickens to Infection With a Lentogenic Newcastle Disease Virus. *Frontiers in Genetics*, 11. https://doi.org/10.3389/fgene.2020.00739
38. Wang, L., Xu, Z., Wang, S., Zhang, W.-X., & Zhang, T. (2023). Targeted knockout of *Mx* in the DF-1 chicken fibroblast cell line impairs immune response against Newcastle disease virus. *Poultry Science*, 102, Article 102855. https://doi.org/10.1016/j.psj.2023.102855

39. Wang, Y., Wang, J., Li, B. H., Qu, H., Luo, C. L., & Shu, D. M. (2014). An association between genetic variation in the roundabout, axon guidance receptor, homolog 2 gene and immunity traits in chickens. *Poultry Science*, 93(1), 31–38. https://doi.org/10.3382/ps.2013-03512

Figures

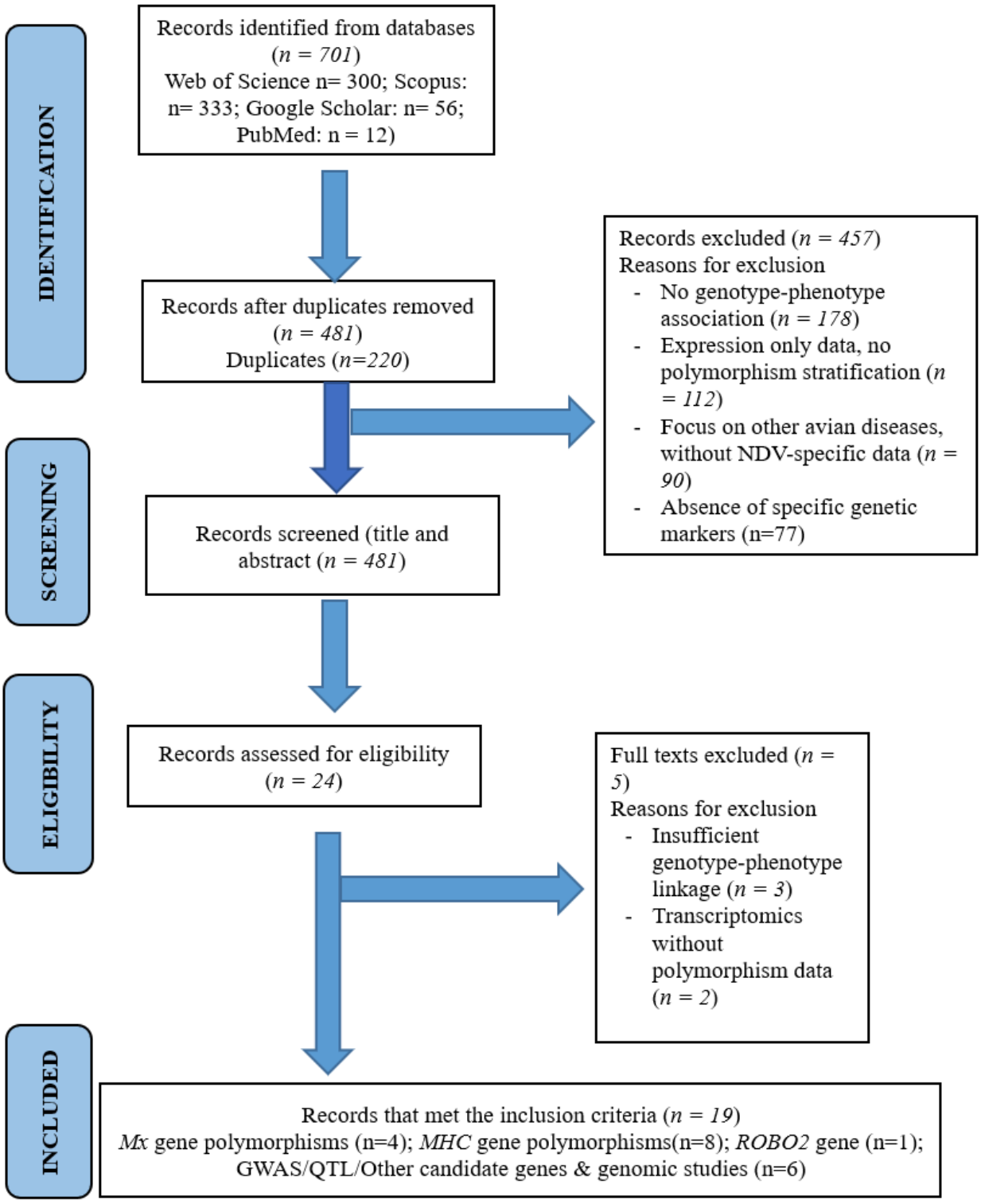


Figure 1

PRISMA 2020 flow diagram illustrating the systematic study selection process.

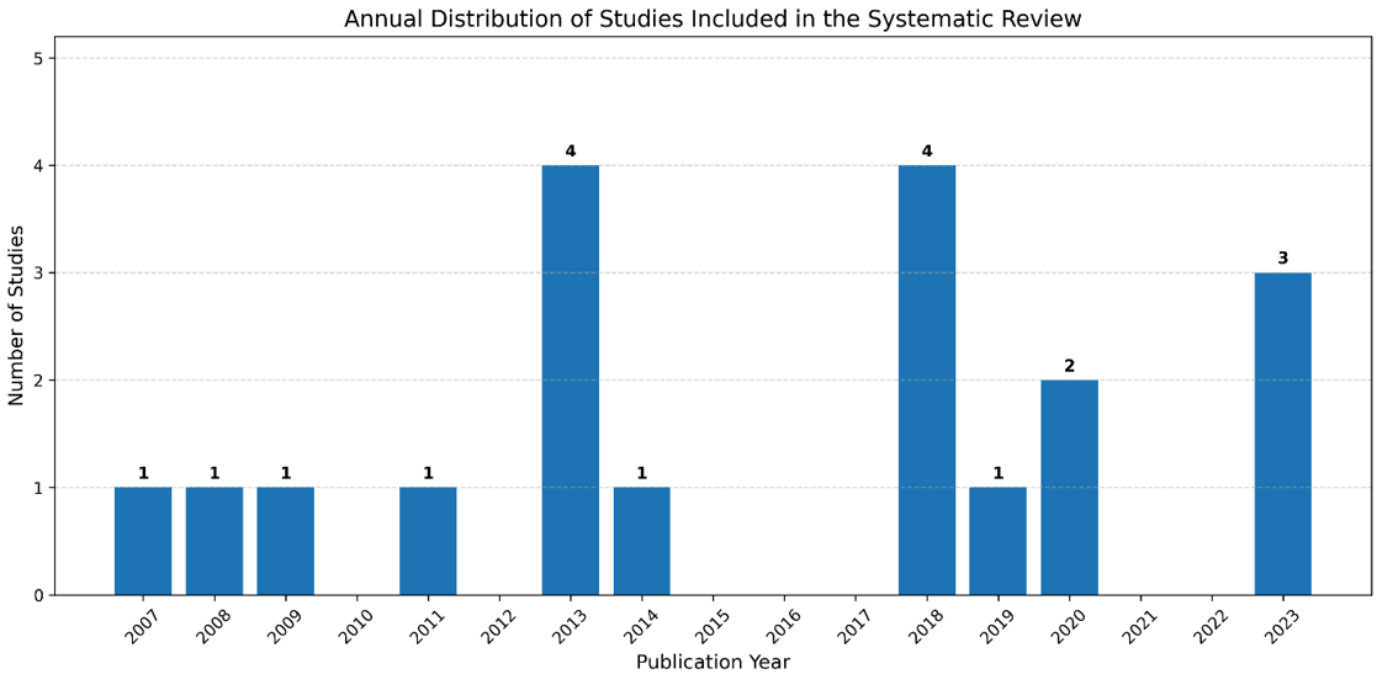


Figure 2

Annual distribution of studies included in the systematic review

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialsKatteetal2026.docx