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# Review on Genotyping of *Mycoplasma gallisepticum* by Multilocus Sequencing Typing

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**Abstract:** *Mycoplasma gallisepticum* causes chronic respiratory disease and reproductive disorders in many bird species, resulting in considerable economic losses to the poultry industry. It is a cell wall lacking bacterium and contains filaments that allow the bacterium to adhere to the host (Erythrocyte) for colonization. The genome of *Mycoplasma* is relatively small compared to other bacterial genomes and contains genes, including VlhA, also known as pMGA lipoproteins, that make up the largest family of genes. Maintenance of *M. gallisepticum* free flocks is the most adequate method to control infection. There is strong demand for efficient epidemiological investigation tools to distinguish *M. gallisepticum* strains in order to control disease. Monitoring systems and vaccination programs with live vaccine strains are applied worldwide. Multilocus sequence typing (MLST) is currently regarded as gold standard for genotyping bacterial species due to its good reproducibility and high discriminatory power. After analyzing housekeeping genes, six loci (*Atpg*, *dnaA*, *fusA*, *rpoB*, *ruvB* and *uvrA*) were selected for the MLST assay due to their genomic location and high diversity. Considering the large number of sequence types and high diversity index, this MLST method is found to be appropriate to discriminate *M. gallisepticum* strains. In addition, the developed method was shown to be suitable for epidemiological investigations, as it confirmed linkage between related strains from outbreaks in different farms. Furthermore, this method can be used for differentiation among vaccine and field strains.

**Key words:** *M. gallisepticum* · MLST · PCR · Genomes

## INTRODUCTION

*Mycoplasma gallisepticum* (MG) is the most virulent avian *Mycoplasma* species affecting chickens and turkeys worldwide [1]. *M. gallisepticum* can be disseminated horizontally, but the major route of transmission is from infected breeder birds to progeny. The chronic nature of *Mycoplasma* infections demonstrates a failure of the host immune system to deal effectively with these organisms. Antigenic variation of surface proteins allows *M. gallisepticum* to evade the host's immune response through the generation of escape variants [2].

Intracellular invasion and survival within eukaryotic cells by *M. gallisepticum* may contribute to this organism's resistance to the host's immune response and antimicrobial therapy [3]. *M. gallisepticum* affected poultry usually suffer from air sacculitis with or without complications and infections are among the costliest diseases in commercial poultry production due to carcass condemnation and downgrading in processing plants and reduced meat and egg production efficiency [4].

*M. gallisepticum* is a cell wall lacking bacterium. It contains filaments that allow the bacterium to adhere to the host (erythrocyte) for colonization. *M. gallisepticum*'s cell membrane is 110A "unit" membrane containing intramembranous particles approximately 5 to 10nm in diameter. The membrane contains many different membrane-associated proteins: ATP binding proteins, SecA, FtsY and proteases. 24 ATP-binding proteins in the membrane are found associated with the ABC transporter making up the second largest gene family in *M. gallisepticum* [5].

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The genome of *Mycoplasma* is relatively small compared to other bacterial genomes; the average size of *Mycoplasma* genome is 1.0 Mb (Range from 580 kb to 1,380kb) [6]. The genome of *M. gallisepticum* contains genes, including VlhA, also known as pMGA lipoproteins, that make up the largest family of genes [7]. Phase variation of *M. gallisepticum* includes the switching on variable lipoprotein and hemagglutinin (VlhA) gene expression. VlhA genes are organized into 3-5 cassettes, uniting ten genes per cassette [8].

Maintenance of *M. gallisepticum* free flocks is the most adequate method to control *M. gallisepticum* infection. To this end, the commercially available live vaccine strains F(Cevac® MG-F, Ceva Inc.), 6/85(Nobilis® MG 6/85, MSD Animal Health), ts-11 (Vaxsafe® MG, Bio properties Pty Ltd.) and K 5831 B-19 (Vaxxinova Inc.) are used in several countries [9]. Differentiation among vaccine strains and field strains is crucial [10] and molecular differentiation of *M. gallisepticum* field strains is very important in tracing infections by evaluating the degree of relatedness between isolates or comparing isolates from different outbreaks [11].

Previously various DNA fingerprinting techniques have been used for the identification and comparison of *M. gallisepticum* strains [12-16]. However, these techniques are labour intensive, time-consuming and have low levels of reproducibility. Several sequence-based methods were developed to replace these traditional DNA fingerprinting techniques [17, 18]. In addition to the higher reproducibility, the most valuable advantage of these techniques is that they do not require the isolation of the bacteria [19].

Among sequence-based methods, multilocus sequence typing (MLST) is currently regarded as gold standard for genotyping bacterial species [20]. Multilocus sequence typing (MLST) typically refers to the systematic sequencing of six or seven well-conserved, house-keeping (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB* and *uvrA*) genes or loci within the bacterial genome. Allelic variation at each locus is catalogued and a sequence type or lineage is assigned by comparing the set of alleles to other isolate profiles in the database [21].

MLST involves obtaining the sequences of internal fragments of seven house-keeping genes for each strain of a species. The sequences of each fragment are compared with all the previously identified sequences (Alleles) at that locus and, thereby, are assigned allele numbers at each of the seven loci. The combination of the seven allele numbers defines the allelic profile of the strain and each different allelic profile is assigned as a sequence typing, which is used to describe the strain [21].

There are numerous advantages of MLST such as high discriminatory power, good reproducibility, feasibility and accuracy [22]. It has been already successfully employed in the characterization of several bacteria including avian *Mycoplasma* species, *e.g. Mycoplasma synoviae* [23, 24] and *Mycoplasma iowae* [19]. It is based on the nucleotide sequences of internal fragments of housekeeping genes, in which mutations are assumed to be largely neutral. For each gene fragment, the different nucleotide sequences are assigned allele numbers and the sequence type (ST) of each isolate is defined by the alleles present at each distinct locus. Isolates that share the same STs are assumed to have a recent common ancestor [25]. An important advantage of MLST is that sequence data can be transmitted and compared among different laboratories. The data obtained by MLST can be used for epidemiological studies and evolutionary or population biological investigations as well [26, 27].

Recently, a core genome multilocus sequence typing approach (cgMLST) has been proposed for whole genome sequence (WGS)-based strain differentiation and epidemiological investigation of *M. gallisepticum* [28]. WGS based cgMLST provides an efficient and accurate method for the differentiation among strains of the same bacterial species [29]. However, whole genome sequencing is still expensive and time-consuming method which requires the isolation and cultivation of the bacterial strain and needs special laboratory technique and equipment [28]. Therefore, the objective of this seminar is:

- To review on Genotyping *Mycoplasma gallisepticum* by Multilocus Sequencing Typing.

**Mycoplasma Gallisepticum:** *Mycoplasma gallisepticum*, a member of the family Mycoplasmataceae (Class Mollicutes, order Mycoplasmatales), is one of the most important agents of mycoplasmosis in terrestrial poultry [30]. *M. gallisepticum* is a cell wall lacking bacterium. It contains filaments that allow the bacterium to adhere to the host (Erythrocyte) for colonization. *M. gallisepticum*'s cell membrane is a 110A "unit" membrane containing intramembranous particles approximately 5 to 10nm in diameter. The membrane consists of two 30A lines separated by a 50A area [5, 9].

The membrane contains many different membrane-associated proteins: ATP binding proteins, SecA, FtsY and proteases. 24 ATP-binding proteins in the membrane are found associated with the ABC transporter making up the second largest gene family in *M. gallisepticum*. This family constitutes one-third of the total 75 proteins predicted to be involved in biomolecule transport. The membrane proteins SecA, SecE, SecY, YidC and a trigger factor are involved in membrane-associated protein secretion and FtsY and Ffh are involved in signal recognition particle pathway [5].

Several transmembrane domains found from the VlhA family include GDSL motif, zinc metalloproteases and lectin-binding motifs, suggesting that some membrane-associated proteins can bind portions of sugar for the purpose of cytadherence and nutrient uptake. *M. gallisepticum* produces dihydrolipoamide transacetylase and pyruvate dehydrogenate enzymes, part of the multi-enzyme pyruvate dehydrogenase complex (PDHC). With these enzymes, pyruvate oxidation occurs, producing acetyl-CoA and generating ATP, the bacteria's source of energy. Also included is the ATP binding cassette transporters, an important transport system needed to acquire many precursors needed for the bacteria's survival [5].

The fastidious nature of *Mycoplasma* is also reflected in the culture conditions required in *vitro,* with the consequence that isolation of the organism is usually performed in specialist laboratories [31]. In the absence of a cell wall, organisms are highly pleomorphic and are not usually detectable by standard light microscopy of Gram-stained cultures or tissue smears. The typical 'fried egg' morphology of the microscopic colonies results from insinuation of the tiny pleomorphic organisms among the fibers of the *Mycoplasma* agar medium where growth is initiated. In addition, since *Mycoplasmas* are not sensitive to antibiotics that affect cell wall synthesis, these antibiotics may be incorporated in culture media used for isolation of *Mycoplasmas*, in order to inhibit growth of other bacteria present in clinical samples [31].

Most species of *Mycoplasma* are parasites of animals or plants, including more than a dozen species which are known to infect chickens or turkeys [32]. Of these, *Mycoplasma gallisepticum* and *M. synoviae* are pathogenic for chickens and turkeys, while *M. iowae* affects primarily turkeys and *M. meleagridis* only infects turkeys. *Mycoplasmas* tend to be highly host-specific, although improved diagnostic methods and precise molecular methods for species identification may necessitate some re-evaluation of earlier views [33].

The primary habitats of *Mycoplasmas* are the mucosal membranes of the respiratory tract and/or the urogenital tract, eyes, mammary glands and joints. Most *Mycoplasmas* are considered surface parasites, rarely invading tissues, although spread to other organs strongly suggests a transitory systemic infection, at the least. Adhesion of *Mycoplasmas* to host cells is a prerequisite for successful colonization and ensuing pathogenesis [34].

*Mycoplasma gallisepticum* is one of the species of *Mycoplasma* that, as primary pathogens, can cause acute and chronic diseases at multiple sites, with wide-ranging complications. *M. gallisepticum*, have recently been demonstrated to have the ability to vary the expression of major surface antigens, thus expressing a continually changing 'antigenic profile' to the immune system [35]. Variability occurs not only among strains, but within clones of a single strain [36]. Continual variability in the expression of such surface antigens also occurs *in vivo* and may be a major factor in the development of clinical disease in addition to having a significant impact on the development of serological responses [37]. The marked heterogeneity with respect to presentation of the major surface antigens provides a likely explanation of how *Mycoplasma* infections can persist in birds despite a strong immune response. Resistance to the clinical manifestations of *Mycoplasma* infection and positive response to antibiotic therapy for disease require an intact immune system [31].

**The Genomic structure of Mycoplasma Gallisepticum:** The circular DNA genome of *M. gallisepticum* is 996,422 bp long with low GC content of 31mol%. 742 coding DNA sequences (CDSs), 91% coding density, have been determined of the 996,422 bp. Of the 742 CDSs, functions of 469 coding DNA sequences have been determined, 159 CDSs are conserved hypothetical proteins and the remaining 114 CDSs are hypothetical proteins. 33 tRNA genes were identified in the genome along with a polypeptide release factor (prfA) (Similar to DNA transcription UAA and UAG stop codons). *M. gallisepticum's* genome contains two 16s rRNA genes [38]. The presence of low GC content and only a minimal amount of genetic information accounts for the complex nutritional requirements of these organisms, reflected in an obligate parasitic mode of life, with a high degree of interdependence between the *Mycoplasma* and the host animal [39].

Like *M. pneumoniae* and *M. genitalium*, *M. gallisepticum*'s genes within the OriC region are not conserved. Genes in the OriC region include: gyrA, gyrB,

dnaJ, dnaN, soj (Upstream of dnaA) and ABC transporters, rpl34 and rpnA (Downstream of dnaA). The origin of replication contains an increased number of AT base pairs (Characteristic of prokaryotes) found between the dnaN and soj genes [39].

The genome of *M. gallisepticum* contains genes, including Variable lipoprotein and hemagglutinin A, also known as pMGA lipoproteins, that make up the largest family of genes. This family is noted as the VlhA family that generates an antigenic variation in chickens and avian species and is important in allowing the bacteria to evade the hosts' immune response. The Variable lipoprotein and hemagglutinin A family consists of 43 genes making up a total 43kb of the bacterial genome [7].

Phase variation in *Mycoplasmas* can occur spontaneously or due to an immune attack, it is important for persistence and survival of *Mycoplasmas* in a host [40- 42]. Usually, the mechanisms of variation are species-specific and occur in one species or closely related *Mycoplasmas*. They include DNA slippage, site-specific recombination, reciprocal recombination and gene conversion [43]. Phase variation of *M. gallisepticum* includes the switching on variable lipoprotein and hemagglutinin gene expression. The exact function of Variable lipoprotein and hemagglutinin A proteins is still unknown. They involve in haemagglutination [44] based on data obtained on avian *Mycoplasmas* it can be assumed that Variable lipoprotein and hemagglutinin A proteins participate in host cell adhesion and invasion [45-47]. VlhA genes are organized into 3-5 cassettes, uniting ten genes per cassette [8].

The promoter structure of these genes is significantly different from the promoters of the other *M. gallisepticum* genes. VlhA genes lack conserved sigma-70 promoter sequence and often have GTG start codon [48]. They are proposed to employ an alternative sigma factor binding GCGAAAAT sequence [7]. Long regions of GAA repeats are located upstream of Variable lipoprotein and hemagglutinin A genes. In general, the GAA repeats can be considered as short sequence repeats (SSRs). SSRs were found in all eukaryotic and many prokaryotic genomes [49, 50].

In bacteria, SSRs were identified in genes coding for bacterial virulence factors including lipopolysaccharide modifying enzymes or adhesion [51]. So, SSRs provide genetic and, therefore, phenotypic variability. Changes in number of repeated units and/or in the repeat unit itself may arise from recombination processes or polymerase errors including slipped-strand mispairing (SSM), either solely or in combination with DNA repair deficiencies [52, 53]. First experiments showed that *M. gallisepticum* express only one Variable lipoprotein and hemagglutinin A family member at a time and expression depends on the presence of exactly 12 GAA trinucleotide repeats upstream of the gene [54].

Recently it was shown that expression of the gene preceded by 12 GAA exceeds the other Variable lipoprotein and hemagglutinin A genes, but the other genes with a different number of repeats are also expressed and some of them are expressed at a high level [46, 55, 56]. *In vivo* experiments showed the non-stochastic character of Variable lipoprotein and hemagglutinin A switching during infection; Variable lipoprotein and hemagglutinin A expression pattern changes during infection progression and differs between strains [55]. So,Variable lipoprotein and hemagglutinin A expression is determined by GAA repeats, but probably the additional expression control mechanisms exist. An interesting question here is how the cell defines what promoter needs to be activated. One explanation here is the existence of hemagglutinin activator protein (HAP) recognizing 12-GAA repeats [54].

**DNA Extraction of Mycoplasma Gallisepticum:** DNA extraction is performed using the DNeasy blood and tissue kit (Qiagen Inc., Hilden, Germany) according to the manufacturers' instructions for Gram-negative bacteria as cited by Bekõ *et al.* [57]. DNeasy Tissue Kits are designed for rapid isolation of total DNA (e.g., genomic, mitochondrial and viral) from a variety of sample sources including fresh or frozen animal tissues and cells, yeasts, or bacteria. DNeasy purified DNA is free of contaminants and enzyme inhibitors and is highly suited for PCR, Southern blotting, RAPD, AFLP and RFLP applications. DNeasy Tissue Kits use advanced silica-gel-membrane technology for rapid and efficient purification of total cellular DNA without organic extraction or ethanol precipitation. The buffer system is optimized to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. After lysis, the DNeasy procedure can be completed in as little as 20 minutes. Simple centrifugation processing completely removes contaminants and enzyme inhibitors such as proteins and divalent cations and allows simultaneous processing of multiple samples in parallel [58].

**PCR and MLST Sequencing:** Polymerase chain reaction (PCR) has become a valuable supplemental test to aid in the diagnosis of *Mycoplasma gallisepticum* (MG) infection. The primary advantage of PCR is that it is a rapid and sensitive method of direct detection of the organism's nucleic acid. Four generic polymerase chain

reactions(PCRs) (16S rRNA PCR, three newly developed PCR methods that target surface protein genes [mgc2, LP (Nested) and gapA (Nested PCR,)] are compared for analytical specificity and sensitivity and for diagnostic sensitivity(Se) and specificity of detection from tracheal swabs. The presence of *M. gallisepticum* DNA is confirmed by PCR targeting the *mgc2* gene. The mgc2 PCR amplification reaction is 94°C for 3 min followed with 35 cycles of 94°C for 30 sec; 58°C for 30 sec; and 72°C for 60 sec and the sizes of expected amplification products are 236 to 302 bp for mgc2 [59]. The presence of other, contaminant of *Mycoplasmas* is excluded by a universal *Mycoplasma* PCR system targeting the 16S/23SrRNA intergenic spacer region [60] followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA), sequence analysis and BLAST search.

**Development of M. Gallisepticum MLST Assay:** Multilocus sequence typing is based on DNA sequence comparison of internal fragments of housekeeping genes to determine allelic differences and define sequence types (STs). These genes are chosen because their products play a vital function and are present in all isolates [17].

The principal element in the design of an MLST scheme is the choice of genetic loci. The selection and number of loci is based on principle, precedent and practice. Since MLST was developed as an updated version of MLEE, which indexes variation of multiple core metabolic or housekeeping genes at the protein level, the selected loci typically correspond to housekeeping genes encoding proteins for core metabolic functions. Furthermore, housekeeping genes are expected to be somewhat conserved and vertically transmitted and thus should reveal genetic relationships among strains without concern for the in?uence of host or environmental factors. For instance, such in?uences might occur when genes encoding hypervariable surface proteins are subject to immune-driven diversifying selection or genes under antibiotic selection. The genes should be physically spaced around the genome in order to minimize genetic linkage of loci [57].

Target genes of the *M. gallisepticum* WGSs were aligned by Geneious software [61] and analyzed manually. Criteria for the selection of genes were that they are present in all published genomes, possess highly diverse internal fragments surrounded by conserved regions suite for primer design and amplicon sizes should be in the range of 300-800bp making them suitable for Sanger sequencing [57]. To improve the MLST target selection process, single copy genes were selected and the number of single nucleotide polymorphisms (SNPs) and conserved regions that could be used for primer design was identified. The remaining target genes were sorted by the number of SNPs they contained and listed by genomic location [17].

Developed MLST assays were first tested on ten diverse *M. gallisepticum* samples (*M. gallisepticum* type strain ATCC19610, strain S6, 95003 (W-5a), SHB-14, MYCAV88, MYCAV228, MYCAV251, MYCAV305, MYCAV388, IZSVE/2012/30571d). PCR were performed with BioRadT100 thermal cycler (BioRad Laboratories Inc., Hercules, USA) in 25µl total volume containing 13.75µl nuclease-free water, 5µl of 5×Green Go Taq Flexi Buffer (Promega, Inc., Madison, WI), 2.5µl of $MgCl_2$ (25mM; Promega), 0.5µl of deoxynucleoside triphosphates (10mM; Qiagen Inc.), 1µl of each primer(10pmol/µl),0.25µl of Go Taq Flexi DNA polymerase (5 U/µl; Promega, Inc.) and 1µl of DNA solution. The initial denaturation/enzyme activation for 2min at 95°C followed by 40 cycles consisting of denaturation's temperature at 95°C for 30s, primer annealing at 56 °C for 30s and extension at 72°C for 1min. The final extension step is at 72 °C for 5 min [57]. PCR products were visualized by agarose gel electrophoresis and subsequently subjected to Sanger sequencing on an ABI Prism3100/automated DNA sequencer (Applied Biosystems, Foster City, CA) as stated by Bekõ *et al.* [57].

For each locus, all sequences were trimmed and aligned using the Geneious software [61]. An allelic number was assigned to each unique allele variant. The discriminatory power for each locus was calculated using Simpson's index of diversity with 95% confidence interval. For the final MLST assay, genes were selected based on the results of the pilot study performed with the ten *M. gallisepticum* samples listed above as stated by Bekõ *et al.* [57].

The specificity of the assays was tested *in vitro* with the following avian *Mycoplasma* species: *M. anatis* (ATCC25524), *M. anseris* (ATCC49234), *M. sp*.1220 ("*M. anserisalpingitis*", ATCC BAA-2147), *M. cloacale* (ATCC 35276), *M. columbinasale* (ATCC33549), *M. columborale* (ATCC 29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC19708), *M. gallopavonis* (ATCC 33551), *M. iners* (ATCC19705), *M. imitans* (ATCC51306), *M. iowae* (ATCC33552), *M. meleagridis*(NCTC10153) and *M. synoviae* (ATCC25204) [57].

In order to test the sensitivity of the assays, tenfold dilution series of the DNA extracted from pure *M. gallisepticum* culture (ATCC19610) were used in the range of 105-100 copy number/µl. Copy number was

Table 1: Data of target genes, primers, amplicons and sensitivity of the designed multilocus sequence typing assay

| Gene | Position in the genome of $R_{low}$ (bp) | Primer F (5'-3') | Primer R (5'-3') | Length of amplicon (bp) | Sensitivity (copy number/µl) |
|---|---|---|---|---|---|
| *atpG* | 427048-427917 | TGGAACTAAACTAAATTCGTTTTTAAGA | TAGCATACTCACACACTTTGGATTCA | 395 | $10^3$ |
| *dnaA* | 3163-4548 | GAGCGTCAAAAATTATTCCCAGA | TTACGAATATCGCCTTCATCAAA | 461 | $10^3$ |
| *fusA* | 740849-742930 | CAGTAGCAGTATTAGATGCCCAAATG | TAGTAGGGATCTGTACTTCTTCACCAA | 597 | $10^3$ |
| *rpoB* | 303943-308115 | GTTAATGCTTAAAGAACAACTTGATTTATT | GGTTAATTGGTGCGTGTTAAAGAA | 562 | $10^3$ |
| *ruvB* | 846984-847904 | CAACGACAATGTATGGCAGGAT | AAACAATCAATTCCACTTATTAGTGAAA | 388 | $10^3$ |
| *uvrA* | 102406-105264 | TTTACCAATCTTAATGTGAATAAAGCC | CCGTTCCCTGGGTGGAGTT | 536 | $10^3$ |

Source: [57]

calculated with the help of an online tool based on the DNA concentration measured by Nano drop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The lowest DNA concentrations yielding visible products during agarose gel electrophoresis were the detection limit for the PCR assays of each locus [62].

**Six loci-based *M. gallisepticum* MLST Assay:** MLST assay is based on six selected housekeeping genes (atpG, dnaA, fusA, rpoB, ruvB and uvrA) of *M. gallisepticum* as stated by Bekõ" *et al.* [57]. Targeted sequencing of selected *M. gallisepticum* genomic regions has been demonstrated to facilitate very good strain differentiation, with sequencing of multiple genetic targets allowing better discriminatory power analysis [17]. Despite the extraordinary point substitution rate of *M. gallisepticum* [63] selected genomic targets have proven sequence stability [18]. Targeted genetic sequencing is also a highly reproducible strain differentiation method, allowing the development of a reference database and global comparisons between laboratories [17].

PCRs were carried out using the oligonucleotide primers. Sequences of the amplicons were determined by the Sanger sequencing method. Novel sequence data of the six individual loci of the MLST scheme for the tested *M. gallisepticum* isolates and field samples and the vaccine strains 6/85, ts-11 and K 5831 B-19 have been deposited in Genbank (atpG sequences correspond to accession numbers MK288880 MK288986, MK289522; dnaA to MK288987 MK289093, MK289523; fusA to MK289094 MK289200, MK289524; rpoB to MK289201 MK289307, MK289525; ruvB to MK289308- MK289414, MK289526; and uvrA to MK289415-MK289521, MK289527). Trimmed sequences were concatenated in alphabetical order (atpG-dnaA-fusA-rpoB-ruvB-uvrA) and aligned with all published sequences using the Geneious software [61].

For each locus, all sequences were compared and an allelic number was assigned to each unique allele variant. The strains were grouped in sequence types (STs) according to their allelic numbers of the six loci. The discriminatory power of the method and for each locus was calculated using Simpson's index of diversity with 95% confidence intervals (CI) [64].

**Differentiation among Field and Vaccine Strains by using MLST:** M.gallisepticum lacks a cell wall and has only a plasma membrane, small size and small genome [65]. These pathogens are evolutionarily related to the low-%GC Gram-positive Clostridiales [66] and they are highly host specific and tend to inhabit mucosal surfaces within their host [65].

To manage the disease, *M. gallisepticum* strain F [67] ts-11[10] and 6/85 [68] live vaccines were developed. The F strain is a naturally attenuated field isolate that was first discovered in the 1950s, while ts-11 and 6/85 were commercially produced using serial passage or chemical mutagenesis [10]. These vaccine strains exhibit various degrees of effectiveness and safety [69] and there is evidence that some of these *M. gallisepticum* vaccine strains can revert to virulence [70].

In order to identify *M. gallisepticum* ts-11-specific marker alleles, whole-genome sequencing was used. *M. gallisepticum* DNA was sequenced using both Illumina and 454 sequencing methods and compared to the sequence of *M. gallisepticum* strain R ($R_{low}$), a well-documented reference strain that is virulent in chickens [71].

The gene sequences of gapA and mcg2 were conserved in *M. gallisepticum* ts-11 isolates, except for the presence of a 20-bp insertion in gapA of the vaccine strain ts-11. Although, no major genomic differences that could differentiate ts-11 virulent from ts-11 avirulent isolates were identified, the *M. gallisepticum* ts-11 vaccine and ts-11 isolates obtained from the field and from ts-11 animal passage experiments, exhibited genomic differences compared to the *M. gallisepticum* $R_{low}$ genome in the form of insertions/deletions of sequences. In some cases, entire gene is lets (Consisting of up to four genes) were seen in ts-11 isolates that are absent in $R_{low}$. Variable lipoprotein and hemagglutinin A locus 1 contained many Variable lipoprotein and hemagglutinin A

genes that were shown to contain genotypic differences among the strains. This was essential in allowing for the identification of sequences that distinguish the *M. gallisepticum* ts-11 strain from field isolates [6].

Multiple sequence alignments allowed for the rapid identification of genomic differences between the *M. gallisepticum* ts-11 isolates and the virulent $R_{low}$ strain. One such difference was the identification of a 20-bp insertion in gapA of the vaccine strain ts-11. This insertion has been predicted to disrupt the protein and affect the adherence capability of *M. gallisepticum* and consequently, this insertion in GapA affects the colonization of chickens by the vaccine strain. The insertional mutation was lost in the *M. gallisepticum* ts-11 field isolates, suggesting that the vaccine can revert to wild type. Studies have reported reversion of $GapA^-$ to $GapA^+$ when ts-11 is passaged in chickens, resulting in an increased ability of the bacteria to colonize. However, $GapA^-$ ts-11 isolates are not pathogenic, indicating that additional changes are necessary for full reversion to the virulent phenotype [72].

Most of the other genes identified as candidates for ts-11-specific detection were vlhA genes. These encode variable surface lipoproteins and contain significant interstrain sequence variation. This genetic diversity is not surprising, as *Mycoplasmas* have been known to have high mutation rates. This variability is introduced by various mechanisms, such as insertions, deletions, or rearrangements [73].

VlhA locus 1 is the main locus in which many Variable lipoprotein and hemagglutinin A genes exhibited significant sequence diversity. The vlhA3.04a, -3.04b and -3.05 genes from this locus were chosen because they contained sequences that were unique to the *M. gallisepticum* ts-11 isolates and highly conserved within this group. The vlhA3.05 gene possessed significant differentiating power, identifying all ts-11 isolates (All of these were positive), while only 5% of the field isolates tested positive. To achieve optimal discriminatory power, a combination of vlhA3.05, mg0359 and vlhA3.04a PCR tests was effective in differentiating *M. gallisepticum* ts-11 isolates from natural field isolates [6].

**Epidemiological Investigation of Mycoplasma Gallisepticum Strains:** Recently, a core genome multilocus sequence typing approach (cgMLST) was proposed as a standard reproducible method for WGS- based strain differentiation and epidemiological investigation. cgMLST provides an efficient, accurate and reproducible method for differentiation among strains and field isolates of the same species with stable and expandable nomenclature. This allows for comparing isolates from different outbreaks and sharing the typing results between different laboratories worldwide through web-based databases [11].

Moreover, it could provide enough discriminatory power for outbreak investigations and a more reliable evaluation for the degree of relatedness between isolates. Here, we are describing the development and evaluation of a cgMLST scheme for typing *M. gallisepticum* strains and field isolates. This is the first application of the cgMLST typing approach on an important poultry pathogen. This newly developed assay could improve applied avian *Mycoplasma* epidemiology and serve as an example for other important animal pathogens [1].

The cgMLST typing results were compared to core genome SNP typing results and a high degree of agreement was detected between the two methods. Both of them had high reliability and discriminatory power and matched the related epidemiological information for all samples. The same closely related groups of samples (House finch samples, including Alabama samples, TS-11vaccine samples, TS-11 vaccine-like samples, UK samples, Israel samples, 6/85 vaccine-like samples, F vaccine samples and F vaccine-like samples) were observed in both trees with similar degrees of relatedness [1].

**Phylogenetic Analysis of Mycoplasma Gallisepticum Strains:** Molecular phylogenetic analysis of the concatenated sequences containing the six loci of the *M. gallisepticum* strains was inferred by using the Maximum Likelihood method based on the Hasegawa Kishino-Yano (HKY) model with .0.26 software [74]. Molecular phylogenetic analysis with the inclusion of an out group was also prepared. For this purpose, corresponding sequences of *M. imitans* type strain ATCC 51306 (GenBank Acc.N.:NZ_JADI00000000.1 NZ_JADI01000029.1;NZ_KI912416.1NZ_KI912419.1) were used. Among the avian *Mycoplasma* species, *M. imitans* is the most similar microorganism to *M. gallisepticum* according to the nucleotide sequence data of their 16S rRNA or rpoB gene [75]. The evolutionary history was inferred using the Neighbor-Joining method, evolutionary distances were computed using the Tamura 3-parameter method with standard error estimated through 1000 bootstrap replicates [74].

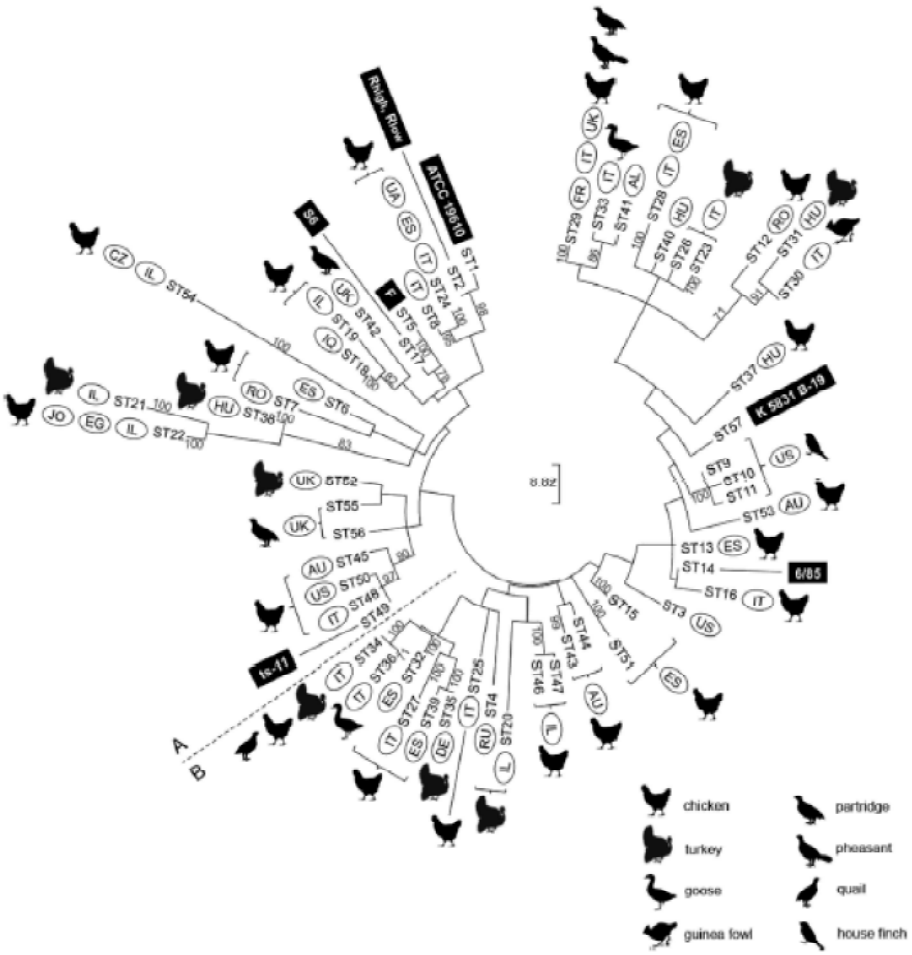


Fig. 1: Maximum Likelihood phylogenetic tree showing relationships between unique sequences types (STs) derived from *M. gallisepticum* strains based on multilocus sequence typing [57].

## CONCLUSIONS

*Mycoplasma gallisepticum* is the most economically significant *Mycoplasma* pathogen of poultry and has a worldwide distribution. It causes chronic respiratory disease and reproductive disorders in many avian species, resulting in considerable economic losses to the poultry industry. The genome of *Mycoplasma* is relatively small and contains genes, including VlhA, also known as pMGA lipoproteins. Maintenance of *M. gallisepticum*-free flocks is the most adequate method to control infection. Multilocus sequence typing (MLST) assay, currently regarded as gold standard among genotyping techniques, which can differentiate between *M. gallisepticum* strains developed by Bekõ *et al* in 2019 was revised. The method can be a useful genotyping tool for differentiation among vaccine strains and field strains, for phylogenetic studies and for epidemiological investigations.

Based on the above conclusion the following recommendations were forwarded:

- Because *Mycoplasma gallisepticum* is the most economically significant *Mycoplasma* pathogen of poultry and has a worldwide distribution, *M. gallisepticum*-free flocks must be maintained by giving live vaccines.
- Government officials should fulfill all facilities to conduct Multilocus sequence typing (MLST) assay for differentiation of *M. gallisepticum*
- Endogenous research centers should create awareness to commercial poultry farm and farmers about the crisis that is caused by *M. gallisepticum*.

- Due to scarcity of resources, further review must be under taken focusing in detail on the use and limitation of Multilocus sequence typing (MLST) assay.

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