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# Genotyping *Mycoplasma gallisepticum* by multilocus sequence 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. Maintenance of *M. gallisepticum*-free flocks is the most adequate method to control infection. To this end, monitoring systems and vaccination programs with live vaccine strains are applied worldwide. There is strong demand for efficient epidemiological investigation tools to distinguish *M. gallisepticum* strains in order to control disease. Up to now, multilocus sequence typing (MLST) has been regarded as gold standard for genotyping bacteria due to its good reproducibility and high discriminatory power.

The aim of this study was to develop an MLST assay which can determine phylogenetic distances between *M. gallisepticum* strains. After analysing more than 30 housekeeping genes, six loci (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, *uvrA*) were selected for the MLST assay due to their genomic location and high diversity.

Examination of 130 *M. gallisepticum* strains with this MLST method yielded 57 unique sequence types (STs) with a 0.96 Simpson's index of diversity.

Considering the large number of STs and high diversity index, this MLST method was 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. Besides, MLST also suggested high impact of extensive international trade on the spread of different *M. gallisepticum* strains. Furthermore this method can be used for differentiation among vaccine and field strains.

## 1. Introduction

*Mycoplasma gallisepticum* is a worldwide avian pathogen affecting various bird species. *M. gallisepticum* can be disseminated horizontally, but the major route of transmission is from infected breeder birds to progeny. Infection causes chronic respiratory disease (CRD), infectious sinusitis or reproductive disorders in chickens and turkeys. The consequential reduction in meat and egg production results in considerable economic losses to the poultry industry (Ley, 2008).

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, Bioproperties Pty Ltd.) and K 5831 B-19 (Vaxxinova Inc.) are used in several countries (Ley, 2008). Differentiation among vaccine strains and field strains is crucial (El Gazzar et al., 2011; Kempf, 1998;

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Whithear, 1996; Kleven, 1997, 2008); 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 (De Been et al., 2015). Thus, there is strong demand for efficient and reliable epidemiological investigation tools to distinguish *M. gallisepticum* strains.

Previously various DNA fingerprinting techniques have been used for the identification and comparison of *M. gallisepticum* strains (Kiss et al., 1997; Lysnyansky et al., 2005; Hong et al., 2005; Feberwee et al., 2005; Cherry et al., 2006). 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 (Ferguson et al., 2005; Raviv et al., 2007). In addition to the higher reproducibility, the most valuable advantage of these techniques is that they do not require the isolation of the bacteria (Ghanem and El-Gazzar, 2016).

Among sequence-based methods, multilocus sequence typing (MLST) is currently regarded as gold standard for genotyping bacterial species (Larsen et al., 2012). There are numerous advantages of MLST such as high discriminatory power, good reproducibility, feasibility and accuracy (Fan et al., 1995). It has been already successfully employed in the characterization of several bacteria including avian *Mycoplasma* species, *e.g. Mycoplasma synoviae* (El-Gazzar et al., 2017; Dijkman et al., 2016) and *Mycoplasma iowae* (Ghanem and El-Gazzar, 2016). 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 ST are assumed to have a recent common ancestor (Spratt, 1999).

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 (Maiden, 2006; Pérez-Losada et al., 2006).

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* (Ghanem et al., 2018). WGS-based cgMLST provides an efficient and accurate method for the differentiation among strains of the same bacterial species (Kwong et al., 2016). 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.

The aim of this study was to develop a reproducible, feasible MLST assay that can be used as a standard method to differentiate *M. gallisepticum* strains with high discriminatory power.

## 2. Materials and methods

### 2.1. Sample handling

In total, 130 samples were examined including 19 *M. gallisepticum* WGSs available online in GenBank (strain S6, GenBank Acc. N.: NC_023030.2; strain $R_{low}$, GenBank Acc. N.: AE015450.2; strain $R_{high}$, GenBank Acc. N.: NC_017502.1, house finch isolates, GenBank Acc. N.: NC_018412.1, NC_018409.1, NC_018406.1, NC_018407.1, NC_018408.1, NC_018410.1, NC_018411.1, NC_018413.1 and ts-11 re-isolates, GenBank Acc. N.: MAFU00000000, MAFV00000000, MAFW00000000, MADW00000000, MATM00000000, MATN00000000, MAGQ00000000, MAGR00000000).

In addition to the 19 published *M. gallisepticum* genomes examined *in silico*, DNA of the *M. gallisepticum* type strain (ATCC 19610), and the 6/85 (Nobilis® MG 6/85, MSD Animal Health), ts-11 (Vaxsafe® MG, Bioproperties Pty Ltd.), F (Cevac® MG-F, Ceva Inc.) and K 5831 B-19 (Vaxxinova Inc.) vaccine strains were also included in the study.

In addition, DNA of 106 *M. gallisepticum* samples including pure *M. gallisepticum* cultures isolated from clinical samples (n = 94) and field samples (n = 12) were investigated. These samples were collected between 1993 and 2017 and originated from 19 countries (Italy, n = 24; UK, n = 23; Spain, n = 19; Israel, n = 10; Australia, n = 6; Hungary, n = 5; USA, n = 3; Romania, n = 2; Portugal, n = 2; Jordan, n = 2; Iraq, n = 2; Slovenia, n = 1; Ukraine, n = 1; Czech Republic, n = 1; Germany, n = 1; France, n = 1; Russia, n = 1; Albania, n = 1; Egypt, n = 1), from seven avian species (chicken, turkey, partridge, pheasant, goose, guinea fowl, quail) and from different production categories of poultry (broiler, layer or breeder chickens, backyard chickens, meat turkeys or turkey breeders).

The 106 samples originated from tracheal swabs or lungs. Ethical approval and specific permission were not required for the study as all samples were collected during routine diagnostic examinations or necropsies with the consent of the owners.

DNA extraction was performed using the DNeasy blood and tissue kit (Qiagen Inc., Hilden, Germany) according to the manufacturers' instructions for Gram-negative bacteria.

The presence of *M. gallisepticum* DNA in the samples was confirmed by polymerase chain reaction (PCR) targeting the *mgc2* gene (García et al., 2005). The presence of other, contaminant mycoplasmas was excluded by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region (Lauerman et al., 1995) followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA), sequence analysis and BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

Background information of the 130 tested *M. gallisepticum* samples is provided in Suppl. Table 1.

### 2.2. Development of M. gallisepticum MLST assay

Target genes of the 19 *M. gallisepticum* WGSs were aligned by Geneious software (Kearse et al., 2012) and analysed 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 suitable for primer design, and amplicon sizes should be in the range of 300–800 bp making them suitable for Sanger sequencing.

The designed primer pairs do not form hairpin, self- or cross-dimers, and have similar melting temperatures, allowing their simultaneous application. Primer design was performed using the NetPrimer software (http://www.premierbiosoft.com/netprimer). The specificity of the primers was analysed *in silico* using BLAST NT algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The developed assays were first tested on ten diverse *M. gallisepticum* samples (*M. gallisepticum* type strain ATCC 19610, strain S6, 95003 (W-5a), SHB-14, MYCAV88, MYCAV228, MYCAV251, MYCAV305, MYCAV388, IZSVE/2012/3057-1d). PCRs were performed with Bio-Rad T100 thermal cycler (Bio-Rad Laboratories Inc., Hercules, USA) in 25 µl total volume containing 13.75 µl nuclease-free water, 5 µl of 5× Green GoTaq Flexi Buffer (Promega, Inc., Madison, WI), 2.5 µl of $MgCl_2$ (25 mM; Promega), 0.5 µl of deoxynucleoside triphosphates (10 mM; Qiagen Inc.), 1 µl of each primer (10 pmol/µl), 0.25 µl of GoTaq FlexiDNA polymerase (5 U/µl; Promega, Inc.) and 1 µl of DNA solution. The initial denaturation/enzyme activation for 2 min at 95 °C was followed by 40 cycles consisting of denaturation step at 95 °C for 30 s, primer annealing at 56 °C for 30 s and extension at 72 °C for 1 min. A final extension step at 72 °C for 5 min was also included. PCR products were visualized by agarose gel electrophoresis and subsequently subjected to Sanger sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

For each locus, all sequences were trimmed and aligned using the Geneious software (Kearse et al., 2012). 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

intervals (CI) (Hunter and Gaston, 1988).

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.

The specificity of the assays was tested *in vitro* with the following avian *Mycoplasma* species: *M. anatis* (ATCC 25524), *M. anseris* (ATCC 49234), *M*. sp. 1220 ("*M. anserisalpingitis*", ATCC BAA-2147), *M. cloacale* (ATCC 35276), *M. columbinasale* (ATCC 33549), *M. columborale* (ATCC 29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC 19708), *M. gallopavonis* (ATCC 33551), *M. iners* (ATCC 19705), *M. imitans* (ATCC 51306), *M. iowae* (ATCC 33552), *M. meleagridis* (NCTC 10153) and *M. synoviae* (ATCC 25204).

In order to test the sensitivity of the assays, tenfold dilution series of the DNA extracted from pure *M. gallisepticum* culture (ATCC 19610) were used in the range of $10^5$–$10^0$ copy number/µl. Copy number was calculated with the help of an online tool (http://cels.uri.edu/gsc/cndna.html; Staroscik, 2004) based on the DNA concentration measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts,USA). The lowest DNA concentrations yielding visible products during agarose gel electrophoresis were considered to be the detection limit for the PCR assays of each loci.

### 2.3. Six loci-based M. gallisepticum MLST assay

MLST assay based on six selected housekeeping genes (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, *uvrA*) was performed on 130 *M. gallisepticum* samples. PCRs were carried out as described above using the oligonucleotide primers listed in Table 1. Sequences of the amplicons were determined by the Sanger method. Novel sequence data of the six individual loci of the MLST scheme for the 106 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 (Kearse et al., 2012). 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 (Suppl. Table 1). The discriminatory power of the method and for each locus was calculated using Simpson's index of diversity with 95% confidence intervals (CI) (Hunter and Gaston, 1988).

Molecular phylogenetic analysis of the concatenated sequences containing the six loci of the 130 *M. gallisepticum* strains was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano (HKY) model with standard error estimated through 1000 bootstrap replicates in MEGA7.0.26 software (Hasegawa et al., 1985; Kumar et al., 2016a; 2016b).

Molecular phylogenetic analysis with the inclusion of an outgroup 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_KI912416.1 - NZ_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 (Volokhov et al., 2012; Kempf, 1998; Bradbury et al., 1993). 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 (Nei and Saitou, 1987; Tamura, 1992; Kumar et al., 2016a; 2016b).

## 3. Results

### 3.1. Development of M. gallisepticum MLST assay

Based on data found in the literature concerning genotyping of different *Mycoplasma* species, more than 30 housekeeping genes were examined during the *M. gallisepticum* MLST target selection (Suppl. Table 2).

Among these housekeeping genes, 15 loci (*adk*, *atpG*, *dnaA*, *dnaN*, *fusA*, *gltX*, *lepA*, *leuS*, *pta*, *rpoB*, *ruvB*, *tpiA*, *tuf*, *ugpA*, *uvrA*) met the requirements described above (see Materials and Methods). After performing a pilot study for these 15 target genes using 10 different *M. gallisepticum* strains, four genes were excluded due to insufficient amplification. Products of the remaining 11 genes were subjected to Sanger sequencing. A further two genes were excluded due to their highly variable sequences, containing insertion/deletion events beside single nucleotide polymorphisms (SNPs). Simpson's diversity index was calculated for the remaining nine loci. Six loci (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, *uvrA*) were selected for the MLST studies based on their genomic location and high diversity. The chromosomal locations of these six genes, as shown in Table 1, suggests that it is unlikely for any of them to be coinherited in the same recombination event as the minimum distance between 2 loci is 97858 bp making them suitable genes for genotyping.

Examination of the assays' specificity revealed cross-reactions in the case of *atpG* with *M.* sp. *1220* („*M. anserisalpinigitis*") and *M. gallinaceum*, and in the case of *dnaA* with *M. iowae* and *M. imitans*. Sequence analysis of the non-specific amplicons clearly distinguished the cross-reacting species from *M. gallisepticum*. The lowest DNA concentration sufficient for the amplification was $10^3$ copy numbers/µl for all selected loci (Table 1). This sensitivity was enough to investigate the 12 tested field samples as well.

### 3.2. Results of the six loci-based M. gallisepticum MLST assay

Based on concatenated sequences of the six loci, the 130 *M. gallisepticum* samples yielded 57 unique MLST sequence types (STs). Single gene analysis resulted in 18 unique alleles (corresponding to allele numbers) for *atpG*, 18 for *dnaA*, 20 for *fusA*, 21 for *rpoB,* 20 for *ruvB* and 17 for *uvrA*. The classification of the 130 *M. gallisepticum* samples into 57 STs resulted in a Simpson's index of diversity of 0.958.

Numerous SNPs were detected in the examined loci, with *dnaA* possessing the highest number of variable nucleotide positions (36/415; 8.67%). Besides nucleotide diversity, a 9 bp nucleotide deletion was

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

| Genes | Position in the genome of Rlow (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 | $10^3$ |

**Table 2**
Number of single nucleotide polymorphisms (Nr. of SNPs) and the Simpson's index of diversity for each locus based on the sequence analysis of 130 *M. gallisepticum* samples.

| Loci | Nr. of SNPs/length of the examined locus (bp)* | Simpson's index of diversity |
|---|---|---|
| *atpG* | 19/341 (5.57%) | 0.833 |
| *dnaA* | 36/415 (8.67%) | 0.874 |
| *fusA* | 24/544 (4.41%) | 0.891 |
| *rpoB* | 34/508 (6.69%) | 0.913 |
| *ruvB* | 28/338 (8.28%) | 0.884 |
| *uvrA* | 37/490 (7.55%) | 0.856 |

* Proportions of the variable nucleotides/genes are in brackets.

detected in the *ruvB* gene of sample 96022 (6-3a). The Simpson's index calculated for each locus showed that *rpoB* had the highest diversity (0.913) (See Table 2).

A phylogenetic tree created by the analysis of the concatenated sequences containing the six loci of the 130 *M. gallisepticum* samples revealed two major clades (Fig. 1). Clade A contained the type strain ATCC 19610 (ST1), strains $R_{high}$, $R_{low}$ (ST2), S6 (ST17), and the vaccine strains ts-11 (ST49) and F (ST5). ATCC 19610 and the R strains were found to be closely related, they differed only in one allele (4/544 nucleotides in *fusA*). Strain S6 and F vaccine strain were located close to each other on the phylogenetic tree, but differed from each other in five of the six loci (at 14 nucleotide positions). Vaccine strains 6/85 (ST14) and K 5831 B-19 (ST57) were classified in Clade B.

Most frequently found sequence types (at least five samples) were ST9 (n = 6), ST14 (n = 15), ST22 (n = 5), ST24 (n = 5), ST29 (n = 15), ST34 (n = 5) and ST49 (n = 13).

Samples in ST9, ST10 and ST11 were all house finch-derived strains from the USA. ST9 was detected in isolates between 1994 and 2006 originating mainly from the eastern part of the US, but occurred on the west coast as well. The closely related ST10 was detected in 2006 in North Carolina, followed by the isolation of a strain with ST11 in 2008 originating from the same state. ST9 differed in three nucleotides (1/508 in *rpoB* and 2/338 in *ruvB*) from ST10 and two nucleotides (1/415 in *dnaA* and 1/338 in *ruvB*) from ST11, while ST10 and ST11 differed from each other at all of these five nucleotide positions.

ST22 contained samples from chickens originating from three different neighbouring countries (Israel, Egypt and Jordan), all of them located in the Middle East. During this study this sequence type was detected in samples from 2006 and 2009 (Israel), 2013 (Jordan) and 2016 (Egypt) as well.

Samples belonging to the ST24 were isolated from chickens originating from Italy in 2013, (n = 2), from Spain in 2015 (n = 2) and from Ukraine in 2016 (n = 1).

ST29 contained 15 samples, most of them were isolated in the UK in 2016 and 2017 from pheasants (n = 8) and partridges (n = 5). The same sequence type was observed for strains isolated earlier, in 2012: a partridge-derived sample originated from France, and a chicken sample from Italy.

ST34 isolates were exclusively Italian samples from 2013 and 2014,

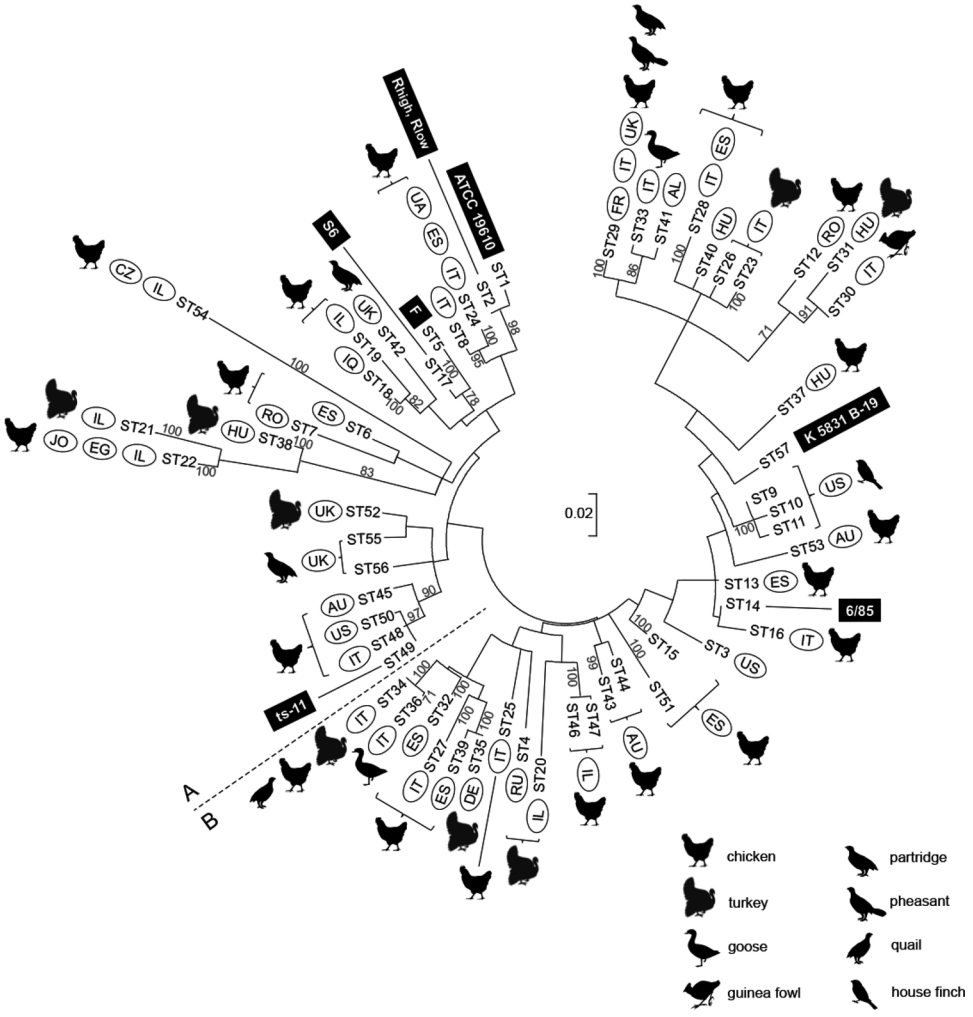


**Fig. 1.** Maximum Likelihood phylogenetic tree showing relationships between the 57 unique sequence types (STs) derived from the 130 examined *M. gallisepticum* strains based on multilocus sequence typing. Origins of the strains (country and host species) belonging to certain STs are indicated, except for sequence types of the vaccine strains. Countries are indicated according to the following abbreviations: AL-Albania, AU-Australia, CZ-Czech Republic, DE-Germany, EG-Egypt, ES-Spain, FR-France, UK-United Kingdom, HU-Hungary, IL-Israel, IQ-Iraq, IT-Italy, JO-Jordan, RO-Romania, RU-Russia, UA-Ukraine, US-United States. The letters A and B indicate the two major clades on the dendrogram. Bootstrap values of neighbor-joining (1000 replicates) of > 70 are shown. The scale bar represents the average number of substitutions per site.

but from different host species (turkey, chicken, quail).

Several samples belonged to the same ST as the vaccine strain 6/85 or ts-11. Vaccine strain 6/85 was assigned to ST14 along with 14 other samples originating from five different countries and isolated between 2007 and 2014. The closest sequence types for ST14 are ST13 and ST16, both of them differed in only one allele from ST14 (2/338 in *ruvB* of ST13 and 7/544 in *fusA* of ST16). These three sequence types showed unique allele variant of the *rpoB* gene with only one not ST-specific SNP compared to the type strain.

Strain ts-11 belonged to ST49 with 12 other samples including the WGSs of ts-11 (K2966) and its re-isolates (K5322C, K6112B, K6208B, K6222B, K6356 and K6372). Samples in this ST derived from three different countries (Australia, USA and Italy) isolated between 1985 and 2016. K6216D which is a ts-11 vaccine re-isolate showed unique sequence type (ST50), which differed in only one nucleotide (1/508 in *rpoB*) from ST49. Similarly, ST48 which contained a chicken sample isolated from Italy in 2013 also differed in one nucleotide (1/544 in *fusA*) from ST49. These three sequence types showed unique allele variant of the *atpG* gene with seven SNPs compared to the type strain, but only one of these SNPs was specific to these sequence types.

Besides the vaccine strain F, only one isolate was assigned to ST5. This sequence type showed unique allele variant of the *dnaA, fusA* and *rpoB* genes with several SNPs compared to the type strain. Among these, only one SNP located in the *dnaA* gene was found to be specific to ST5.

In this study no other samples have been found to share ST57 with the vaccine strain K 5831 B-19. None of its SNPs or allele variants was found to be specific for this sequence type.

Concatenated sequences of the six examined loci of *M. imitans* type strain (ATCC 51306) had 380/2636 point mutations and a 3 bp length nucleotide deletion when aligned to the *M. gallisepticum* type strain (ATCC 19610). Accordingly, the neighbor joining tree prepared with the inclusion of *M. imitans* showed relatively high phylogenetic distances between these two *Mycoplasma* species (Suppl. Fig. 1).

## 4. Discussion

*M. gallisepticum* can cause considerable economic losses to the poultry industry by inducing respiratory and reproductive disorders. The most viable method to control the infection is the maintenance of *M. gallisepticum*-free flocks. Efficient monitoring systems and epidemiological investigations are crucial and require reliable genotyping tools. The MLST method described in this study was able to discriminate the tested *M. gallisepticum* strains with high diversity index.

The high number of STs may have been partially induced by the high diversity of the tested samples, as they originated from 19 different countries and seven various avian species, isolated between 1985 and 2017. However, common features of the examined samples did not correlate with identical STs in each case. Certain sequence types were demonstrated in larger number of samples. Nevertheless, as sample collections were not performed systematically, the detected tendency concerning the prevalence of these sequence types might be biased. In general, mycoplasmas are characterized by high genetic variability including their housekeeping genes as confirmed in the current study.

Partly due to this instability, samples belonging to a certain ST were isolated most commonly in the same year, or within a few consecutive years. However, in some cases strains with identical STs were detected in a longer time period. Presumably in the absence of inhibitory factors occurring in industrial poultry flocks, such as antibiotic treatment or vaccination, virulent *M. gallisepticum* strains can survive for a longer period of time in wild birds and also in semi-wild birds such as game birds. This lesser selection pressure may contribute to the subsistence of certain STs in wild populations as observed in the house finch-derived strains from the USA. For example, ST9 was isolated first in 1994 and persisted for twelve years in the population, being found even in 2006. Likewise, ST29 was first detected in 2012 in a chicken and a partridge and then found again five years later, in 2017 in UK game birds. This evidence suggests that closely related *M. gallisepticum* strains can infect both game birds and poultry, thus providing a possible source of infection for chickens and turkeys. Transmission of the pathogen from wild- and semi-wild birds to poultry might be a possible explanation for the phenomenon that identical STs were isolated over a longer time period in poultry as well.

Sequence types 21 and 22 have been isolated from countries of the Middle East and derived from a common ancestor according to the phylogenetic tree. The first detected incidence of ST21 in Israel was from a turkey in 1997, and the next emergence was seven years later, in 2004. Likewise, ST22 was first detected in chickens in Israel in 2006, and then ten years later in Egypt. Interestingly, no STs were found to persist for such a long time in other geographic regions. The reason for this persistence could be due to the greater survival skills of these strains or to lower standards of biosecurity or management. *M. gallisepticum* can be passed from infected breeder birds to progeny. If monitoring systems fail to reveal chronic infection of breeders, then *M. gallisepticum* strains can emerge from time to time in the stock. Furthermore, due to the tendency that different companies in different countries fill their farms with birds derived from common parent stock, the same strain can spread worldwide. This intensive international trade could explain the occurrence of identical STs in distant regions.

Besides the good capability for discriminating different *M. gallisepticum* strains, the developed MLST was shown to be suitable for epidemiological investigations, as the same STs were identified in strains originating from geographically distinct outbreaks in farms with epidemiologic links. For instance, two samples (MYCAV387 and MYCAV419) isolated from different Hungarian turkey farms belonging to the same integration showed identical sequence type (ST38) by MLST.

Analysing the degree of relatedness between *M. gallisepticum* isolates and vaccine strains is also important in the control of the infection and in epidemiological investigations. Vaccine strain ts-11 and the published WGSs of its re-isolates were classified to ST49, independently of the virulence of these strains (Ricketts et al., 2017). Both ST50, which contained the ts-11 re-isolate K6216D, and ST48 differed in only one nucleotide from ST49. Based on these results, field samples in these STs are likely to be re-isolations of the ts-11 vaccine strain. These isolates originated from countries (Italy, USA and Australia) in which ts-11 vaccine is commercially available and used against *M. gallisepticum* infection. ST45, which is also located relatively close to ST49 on the phylogenetic tree, varied from the sequence of ts-11 at ten nucleotide positions. This number of SNPs is high enough to consider this strain (99179 (A-UTa)) as potentially wild type. Close relatedness can be explained with the common country of origin, because both 99179 (A-UTa) and the parent strain of ts-11 originated from Australia.

As with ts-11, numerous clinical samples were grouped into the ST of the vaccine strain 6/85 (ST14). According to the classification of ts-11 re-isolates, field samples in ST14 are also presumably re-isolations of the 6/85 vaccine strain. ST13 (differed in two SNPs) and ST16 (differed in seven SNPs) seem to be closely related to 6/85.

As mycoplasmas possess high genetic variability, reliable differentiation of wild strains from vaccine strains with genotyping is particularly difficult. Nevertheless, evaluating genetic distances between strains using MLST offers a feasible opportunity for this purpose, as analysing more genes provides more established results, with the careful interpretation of the data in ambiguous cases.

In summary, the designed MLST assay was able to differentiate *M. gallisepticum* strains with high discriminatory power and identify closely related strains as well. Moreover, relatively high sensitivity of the assay makes it suitable for examining clinical samples directly after the exclusion of the presence of some other *Mycoplasma* species (*e.g. M. gallinaceum*). It can be used in practice for phylogenetic studies, epidemiological investigations of *M. gallisepticum* strains and as a confirmatory method to differentiate wild-type and vaccine strains. Development of this genotyping method can contribute to better understanding and, in the future, to elimination of this avian pathogen from poultry industry.

## 5. Conclusion

In this study an MLST assay, currently regarded as gold standard among genotyping techniques, which can differentiate between *M. gallisepticum* strains was developed. This MLST assay was found to be an adequate method to discriminate between *M. gallisepticum* strains from a wide range of host and geographical locations and to identify closely related strains. The method can be a useful genotyping tool for differentiation among vaccine strains and field strains, for phylogenetic studies and also for epidemiological investigations.

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## Conflict of interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.03.016.

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