University of Veterinary Medicine
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Genetic studies on *Mycoplasma gallisepticum* and *M. synoviae* and development of molecular assays applicable as diagnostic methods

Ph.D. thesis

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Abbreviations

Ala (A)	alanine
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartate
bp	base pair
CI	confidence interval
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
Gln (Q)	glutamine
Glu (E)	glutamate
Gly (G)	glycine
gyrA, *gyrB*	gene of DNA gyrase subunit A, B
His (H)	histidine
Ile (I)	isoleucine
Lys (K)	lysine
MAMA	mismatch amplification mutation assay
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
parC, *parE*	gene of DNA topoisomerase IV subunit A, B
PCR	polymerase chain reaction
Phe (F)	phenylalanine
Pro (P)	proline
RNA	ribonucleic acid
rrlA/*B*	23S ribosomal ribonucleic acid genes
rRNA	ribosomal ribonucleic acid
Ser (S)	serine
SI	Simpson’s index of diversity
SNP	single nucleotide polymorphism
ST	sequence type
Thr (T)	threonine
Tm	melting temperature
Tyr (Y)	tyrosine
Val (V)	valine
WGS	whole genome sequence

1. Summary

Mycoplasma gallisepticum is a facultative pathogen, which causes chronic respiratory disease and reproductive disorders in chicken and turkey, resulting in considerable economic losses to the poultry industry worldwide. 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 widely applied. There is strong demand for efficient molecular biological techniques for epidemiological investigations to identify related *M. gallisepticum* strains. Differentiating vaccine strains from field isolates is also necessary in the control of vaccination programs and diagnostics. Besides, great attention is given to phylogenetic studies as well in order to better understand the development of different bacterial strains.

Up to now, polymerase chain reaction (PCR)-based multi-locus sequence typing (MLST) has been regarded as a gold standard for genotyping bacteria due to its good reproducibility and high discriminatory power. In this study, an MLST assay has been developed for the first time to genetically characterize *M. gallisepticum* strains. After analyzing numerous housekeeping genes, six loci (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, *uvrA*) were selected for MLST assay due to their genomic location and high diversity. Examination of 131 *M. gallisepticum* strains with the developed MLST method yielded 57 unique sequence types with a 0.96 Simpson's index of diversity. Beside the high discriminatory power of the MLST, this system was found to be able to identify closely related strains too, as the assay could confirm linkage between related strains from outbreaks in different Hungarian poultry farms. The results proved that phylogenetically closely related *M. gallisepticum* strains can infect both domestic fowl and game birds, which provide a possible source of infection for poultry. Besides, the results of the MLST assay suggest high impact of extensive international trade on the spread of different *M. gallisepticum* strains. The developed MLST method can be used for phylogenetic studies and epidemiological investigations as well. Relatively high sensitivity of the assay makes it suitable for examining DNA extracted from clinical samples directly. The developed MLST assay was found to be able to differentiate among vaccine strains and field isolates as well.

To provide even more rapid and cost-effective molecular tools to distinguish the *M. gallisepticum* vaccine strains and field isolates, eight mismatch amplification mutation assays (MAMAs) were developed in the present study. After determining the whole genome sequences (WGSs) of the four commercially available live *M. gallisepticum* vaccine strains (F, 6/85, ts-11 and K strains), primers were designed to detect different vaccine-specific single nucleotide polymorphisms (SNPs). After evaluating preliminary results, mutations in the *hlp2*, *crmA*, *lpd*, *gapA*, *plpA*, *potC*, *glpK* and *fruA* genes were selected to be targeted by the assays.

The new MAMA tests can be performed on DNAs extracted from the clinical samples directly and with basic PCR equipment as well, providing a reliable, time- and cost-effective molecular tool for routine diagnostics and for the monitoring systems of vaccination programs.

The developed MLST and MAMA tests showed high congruency confirming the reliability of the designed assays. In certain cases, isolates which were identified as vaccine strains by some, but not all of the MAMA tests, the MLST system enabled the classification of the isolates as field isolates. Thus, in case of incongruent results of the MAMAs, the use of MLST assay is suggested as a confirmatory test.

The economically most significant avian *Mycoplasma* species beside *M. gallisepticum* is *M. synoviae*. It can cause great financial losses to the poultry industry by inducing respiratory diseases, infectious synovitis, or eggshell apex abnormalities. There are different approaches to control *M. synoviae* infection. Although antimicrobial therapy cannot replace long-term solutions, like eradication and vaccination, this strategy can be effective in the short run, as adequate antibiotic treatment can relieve economic losses through the attenuation of clinical signs and reduction of vertical transmission.

After determination of minimal inhibitory concentration (MIC) values of fourteen antibiotics of eight antimicrobial groups against *M. synoviae* strains (n=96), whole genome sequencing (n=94) and sequence analysis revealed mutations potentially associated with decreased susceptibility to fluoroquinolones, macrolides and lincomycin. Molecular markers responsible for the high MICs to fluoroquinolones were found in the *gyrA*, *gyrB*, *parC* and *parE* genes. Besides, amino acid change in the 50S ribosomal protein L22 could be associated with decreased susceptibility to macrolides, while two SNPs identified in genes encoding the 23S rRNA were found to be responsible for high MICs to the 50S inhibitor macrolides and lincomycin as well. The revealed mutations can contribute to the extension of knowledge about the genetic background of antibiotic resistance in *M. synoviae*. Moreover, the explored potentially resistance-related mutations can be targeted by molecular biological assays, thus antibiotic susceptibility profiles of avian *Mycoplasma* strains would be available previous to the laborious and time-consuming isolation.

Összefoglalás

A *Mycoplasma gallisepticum* egy világszerte elterjedt, fakultatív patogén kórokozó, mely krónikus légzőszervi megbetegedést és szaporodásbiológiai zavarokat idéz elő csirkében és pulykában, ezáltal jelentős gazdasági károkat okoz a baromfiágazatban. Legcélravezetőbb védekezési mód ellene a *M. gallisepticum*-mentes állományok fenntartása, e célból széles körben alkalmaznak különböző monitoring programokat és élő vakcinatörzseket. Napjainkban ezért nagy igény mutatkozik hatékony molekuláris biológiai módszerek iránt, melyekkel epidemiológiai vizsgálatok végezhetők és azonosíthatók az egymással közeli rokonságban álló *M. gallisepticum* törzsek. Ugyancsak létfontosságú a vakcinatörzsek vad izolátumoktól való elkülönítése a vakcinázási programok hatékonyságának az ellenőrzéséhez a rutin diagnosztika részeként. Emellett filogenetikai tanulmányokra is nagy szükség van, hogy jobban megérthessük az egyes baktériumtörzsek kialakulásának módját.

Napjainkban a polimeráz-láncreakció (polymerase chain reaction, PCR) alapú multi-lókusz szekvencia tipizálás (multi-locus sequence typing, MLST) tekinthető az egyik legmegbízhatóbb baktérium-genotipizáló módszernek kiemelkedő reprodukálhatósága és felbontóképessége miatt. Csoportunk elsőként fejlesztett *M. gallisepticum* törzsek genetikai jellemzésére alkalmas MLST rendszert. Számos háztartási gén vizsgálata alapján hat lókuszt (*atpG*, *dnaA*, *fusA*, *rpoB*, *ruvB*, *uvrA*) választottunk ki az MLST rendszerhez azok elhelyezkedése és nagyfokú változatossága alapján. Az újonnan fejlesztett MLST rendszer segítségével 131 *M. gallisepticum* törzset vizsgálva összesen 57 egyedi szekvenciatípust tudtunk elkülöníteni 0,96-os Simpson-féle diverzitás indexszel. Az MLST nagy elkülönítő képessége mellett képes volt azonosítani az egymással közeli rokonságban álló *M. gallisepticum* törzseket is, sikerült ugyanis kapcsolatot kimutatnia különböző telepekről izolált törzsek között egy magyarországi járvány alkalmával. Az adatok igazolták, hogy egymással közeli filogenetikai kapcsolatban álló *M. gallisepticum* törzsek képesek megfertőzni házi szárnyasokat és vadon élő madarakat egyaránt, utóbbiak tehát lehetséges fertőzési forrásai a baromfiféléknek. Emellett az MLST vizsgálatok eredményei azt is jelzik, hogy a különböző *M. gallisepticum* törzsek elterjedésében meghatározó szerepet tölt be az intenzív nemzetközi kereskedelem. Az MLST rendszer filogenetikai és epidemiológiai vizsgálatokra egyaránt alkalmas. A teszt viszonylagos nagy érzékenysége és specificitása révén akár közvetlenül a klinikai mintából kivont DNS-en is elvégezhető. Ezenfelül az MLST rendszer vakcinatörzsek vad izolátumoktól való elkülönítésére is alkalmasnak bizonyult.

Annak érdekében, hogy lehetővé tegyük a *M. gallisepticum* vakcinatörzsek vad izolátumoktól való elkülönítésének gyorsabb és költséghatékonyabb módját, összesen nyolc, pontmutáció kimutatására alkalmas rendszert (mismatch amplification mutation assay, MAMA)

fejlesztettünk. A négy kereskedelmi forgalomban kapható élő vakcinatörzs (F, 6/85, ts-11 és K törzs) teljes genom szekvenálását követően számos vakcinaspecifikus pontmutáció (single nucleotide polymorphism, SNP) kimutatására terveztünk primereket. Az elővizsgálatok eredményei alapján végül a *hlp2*, *crmA*, *lpd*, *gapA*, *plpA*, *potC*, *glpK* és *fruA* génekben azonosított mutációkra fejlesztettünk MAMA rendszereket.

A MAMA tesztek közvetlenül a klinikai mintából kivont DNS-en és hagyományos PCR vizsgálattal is elvégezhetők, ezáltal megbízható, gyors és költséghatékony eszközként használhatók a rutin diagnosztikában vagy a vakcinázási programok ellenőrzéséhez.

Az MLST és MAMA tesztek eredményei nagyfokú egyezést mutattak, mely a leírt módszerek megbízhatóságát támasztja alá. Mindazonáltal, az MLST rendszer képes volt egyes MAMA tesztek által vakcinatörzsként azonosított izolátumuk elkülönítésére is. A MAMA tesztek eredményeinek kérdésessége esetén ezért az MLST módszer használata javasolt.

A másik, gazdasági szempontból kiemelkedő jelentőséggel bíró madárpatogén *Mycoplasma* faj a *M. gallisepticum* mellett a *M. synoviae*. Az általa előidézett kórképek, úgymint légzőszervi megbetegedések, fertőző synovitis és tojásvég-deformitás, jelentős anyagi veszteségekért tehetők felelőssé a baromfiágazatban. A fertőzés ellen különböző védekezési stratégiák alkalmazhatóak. Bár az antimikrobiális szerek használata nem helyettesítheti a hosszútávú védekezési módokat, mint a mentesítést vagy a vakcinázást, rövidtávon kifizetődő lehet, hiszen a megfelelő antibiotikum-terápia a klinikai tünetek enyhítése és a vertikális terjedés visszaszorítása révén hozzájárul a gazdasági veszteségek csökkentéséhez.

Meghatároztuk nyolc antibiotikum-család tizennégy hatóanyagának minimális gátló koncentráció (minimal inhibitory concentration, MIC) értékeit a *M. synoviae* törzsekkel (n=96) szemben, majd elvégeztük a törzsek teljes genom szekvenálását (n=94). A rezisztenciáért felelős gének szekvenciaanalízisét követően olyan mutációkat azonosítottunk, melyek kapcsolatba hozhatóak a törzsek csökkent fluorokinolon-, makrolid-, vagy linkomicin-érzékenységével. Magas fluorokinolon MIC értékekkel összefüggésbe hozható molekuláris markereket találtunk a *gyrA*, *gyrB*, *parC* és *parE* génekben. Az 50S riboszomális protein L22-ben csökkent makrolid-érzékenységhez kapcsolt aminosavcserét azonosítottunk, míg a 23S rRNS kódoló génekben magas makrolid és linkomicin MIC értékekért felelős pontmutációkat találtunk. A feltárt molekuláris markerek hozzájárulhatnak a *M. synoviae* törzsekben előforduló antibiotikum-rezisztencia genetikai hátterének a megismeréséhez. Ezenfelül, ezek a feltehetően rezisztencia-kapcsolt mutációk célpontként szolgálhatnak olyan molekuláris biológiai tesztek fejlesztéséhez, melyek segítségével a *M. synoviae* törzsek antibiotikum érzékenységi profilja már jóval a meglehetősen munka- és időigényes baktérium izolálás előtt meghatározható.

2. Introduction

Mycoplasma gallisepticum and *Mycoplasma synoviae* are widespread facultative pathogens and the most economically significant *Mycoplasma* species in the chicken and turkey industry (Kleven and Levisohn, 1996; Moreira *et al.*, 2015). *M. gallisepticum* infection most commonly manifests as respiratory symptoms or reproduction disorders (Ley and Yoder, 1997), while *M. synoviae* usually induces infectious synovitis or rarely respiratory signs and it can be related to eggshell apex abnormalities in chickens as well (Kleven, 1998a; Feberwee *et al.*, 2009; Catania *et al.*, 2010; Jeon *et al.*, 2014; Moreira *et al.*, 2015). Reduced feed intake, weight gain, egg production and hatchability can be observed in the affected flocks which lead to great economic losses (Kleven and Levisohn, 1996; Ley and Yoder, 1997; Moreira *et al.*, 2015).

2.1. History

M. gallisepticum induced respiratory disease of turkeys was first described in 1905 by Dodd, while the first isolation of the agent from chickens with “coryza of slow onset” was in 1935 by Nelson (Dodd, 1905; Nelson, 1936a; 1936b; 1936c; 1936d). Later, *M. gallisepticum* was cultured and classified by Markham and Wong, as well as Van Roekel and Olesiuk (Markham and Wong, 1952; Van Roekel and Olesiuk, 1953).

M. synoviae was first described in 1954 by Olson *et al.* and by Wills as an unidentified agent causing synovitis in chickens (Olson *et al.*, 1954; Wills, 1954a; 1954b). However, the aetiology was not known until 1960, when mycoplasma colonies were isolated from birds with infectious synovitis (Chalquest and Fabricant, 1960).

In Hungary, Bamberger and Csontos reported the occurrence of a multifactorial respiratory disease associated with *M. gallispeticum* infection for the first time in 1953, and in the year of 1957, Bamberger isolated and investigated several *M. gallisepticum* strains from different Hungarian farms affected by chronic respiratory disease (Bamberger and Csontos, 1953; Bamberger, 1957). The first occurrence of the infectious synovitis in Hungary was in 1958 (Derzsi and Tóth Baranyi, 1960). Thereafter, with the global spread of intensive poultry farming, papers discussing avian mycoplasmosis caused by *M. gallisepticum* and *M. synoviae* were published increasingly in Hungary (Mészáros, 1965; Glávits *et al.*, 1986; Stipkovits, 2000; Kőrösi, 2013; Gyuranecz and Horváth-Papp, 2017).

2.2. Etiology

M. gallisepticum and *M. synoviae* belong to the class Mollicutes (named after the cell wall-less characteristic from the Latin words mollis - soft; cutis - skin), to the order Mycoplasmatales, to the family *Myoplasmataceae* and the genus *Mycoplasma* (Raviv and Ley, 2013). Mycoplasmas are the smallest and simplest free-living prokaryotes capable of self-replication with a size of 0.2-0.5 μm (Citti and Blanchard, 2013). In the lack of cell wall, cytoplasm of mycoplasmas is surrounded solely with a thin cell membrane. Therefore, mycoplasmas cannot be stained according to Gram, and their morphology is variable depending on the circumstances (i.e. they are pleomorf) (Razin, 1992; Razin *et al.*, 1998). They show low degree of resistance, commonly used detergents, disinfectants, dry environment and high temperature (55-60 °C) are all effective against them. In contrast, on low temperature they stay viable for a long time. In broth culture *M. gallisepticum* can survive at -30 °C for 2-4 years, at -60 °C for 20 years, while in lyophilized form it can be viable at 4 °C for 7 years (Yoder, 1970). *M. synoviae* can survive in the environment 2-3 days at room temperature, while it can be viable for years at -70 °C in lyophilized form (Marois *et al.*, 2005).

Mycoplasmas typically carry minimal genetic information as their non-essential genes has been lost during evolution. Size of their circular genome is small, it is only between 500-1350 kilobase pairs. Due to the minimal genetic information, mycoplasmas are usually characterized by relatively simple metabolic pathways and consequently complex nutrient requirements (Fraser *et al.*, 1995; Sirand-Pugnet *et al.*, 2007). Hence, mycoplasmas depend largely on their host (Kleven *et al.*, 1996).

2.3. Epidemiology

Several *Mycoplasma* species can occur in poultry, including saprophytes as well, but the most important avian mycoplasmas from economic aspects are *M. gallisepticum* and *M. synoviae* (Kleven, 2008). Mycoplasmas are usually considered to be stenoxen microorganisms, however, avian mycoplasmas can cause symptoms in several bird species (Ley and Yoder, 1997). Birds belonging to the order Galliformes (e.g. chicken, turkey, pheasant, partridge, quail) have the largest susceptibility to *M. gallisepticum*, but pigeons, ducks, geese and various pet and wild bird species can be affected, too (Raviv and Ley, 2013). Birds of any age can be infected, however, young birds are more susceptible. *M. synoviae* can be isolated most commonly from meat-type turkey or layer chickens with reduced egg production, but it also can infect other bird species (e.g. guinea fowl, pheasant, partridge, quail, pigeon, duck, goose) as well (Gerchman *et al.*, 2008).

M. gallisepticum and *M. synoviae* are facultative pathogens. Transmission can be horizontal and vertical as well (Carnaghan, 1960; Levisohn and Kleven, 2000). In the vertical route, infected breeding stocks are of particular importance. In this case, infection to progeny occurs *in ovo*: the bacteria presumably spread in the infected layer from the abdominal air sack to the oviduct (Roberts and McDaniel, 1967). In chronic infection, *in ovo* transmission is much less typical (Carnaghan, 1960; Sasipreeyajan *et al.*, 1987). In case of *M. gallisepticum*, the peak of shedding is 3-6 weeks after vertical transmission, affecting even 50 % of the eggs, while only 3-5 % of the eggs proved to be infected between the 8th and 25th week in experimental conditions, and this rate can be even lower in case of natural infection (Raviv and Ley, 2013). In case of *M. synoviae*, egg-transmission rate appears to be the highest during the first 4-6 weeks after infection (Ferguson-Noel and Noormohammadi, 2013).

Horizontal infection can occur through direct contact with infected animals, or it can happen indirectly by contaminated objects, feed, water or dust. The main portals of entry for the organism is the upper respiratory tract and sometimes the conjunctiva (Levisohn and Kleven, 2000; Noormohammadi, 2007). The infection is maintained by animals shedding the bacteria. Subclinically infected backyard stocks and game birds can be a source of infection as well (Bradbury and Levisohn, 1996; Michiels et al., 2016).

2.4. Pathogenesis

The most important proteins of *M. gallisepticum* and *M. synoviae* for pathogenicity are the hemagglutinins and adhesins (Noormohammadi *et al.*, 2000). Mycoplasma synovial protein A (MSPA) is a phase- and size-variable hemagglutinin of *M. synoviae*, expressed by a single gene named variable lipoprotein hemagglutinin A (*vlhA*). The *vlhA* gene of *M. synoviae* has a high degree of identity with the *vlhA* 4.10 (pMGA1.7 gene) of *M. gallisepticum* (Ferguson-Noel and Noormohammadi, 2013). Cultures of the hemagglutinin-negative phenotype are found to be less pathogenic than hemagglutination positive cultures. Mycoplasmas are able to periodically change their main surface antigens encoded by the *vlhA* gene family, even *in vivo* in the infected host (Razin *et al.*, 1998). Protein PvpA can also contribute to this antigen variability of *M. gallisepticum*, as its subunits are exchanged due to the immune responses of the host (Szczepanek *et al.*, 2010). This continuously changing antigen-profile is constantly challenging the immune system of the host. This can be a possible explanation of the phenomenon that *M. gallisepticum* can persist in the infected animals with strong immune response as well (Benčina *et al.*, 1994; Garcia *et al.*, 1994; Rosengarten and Yogev, 1996).

GapA and CrmA cytadhesins are also important virulence factors helping the adhesion of *M. gallisepticum* on the surface of the epithelium in the respiratory tract. PlpA and OsmC-like

protein help the binding of the mycoplasma to the molecules in the extracellular matrix (Szczepanek *et al.*, 2010).

As facultative pathogens, mycoplasmas usually cause persistent subclinical infection. After passing through the upper respiratory tract, mycoplasmas adhere with membrane proteins to the epithelial cells of the tracheal mucosa tightly, which is important for the colonization (Razin, 1985). Occasionally, the epithelial cells of the trachea can partially incorporate the bacteria, which can help the microorganisms to escape from the host immune defenses or antimicrobial agents (Razin *et al.*, 1998). The variable expression of surface proteins can contribute to this effect as well.

The number of bacteria in the upper respiratory tract reaches its maximum during the acute phase, and the most intensive shedding can be observed in this period (Yagihashi and Tajima, 1986; Levisohn and Dykstra, 1987; Sasipreeyajan *et al.*, 1987; Hyman *et al.*, 1989). In contrast to *M. synoviae*, the copy number of *M. gallisepticum* can significantly decrease in the later stages of the infection, but small amount of bacteria can persist in the respiratory tract for a long time. This may lead to difficulties in the diagnosis and in the protection with vaccination as well, as the copy number of the live vaccine strains colonizing the respiratory tract are also reduced in the chronic stage (Soeripto *et al.*, 1989; Feberwee *et al.*, 2017; Noormohammadi and Whithear, 2019). In case of attenuated immune status, *M. gallisepticum* infection can exacerbate, the number of the bacteria on the mucosa increases again and the trachea serves as a reservoir, from where mycoplasmas can spread to other parts of the respiratory system, developing the characteristic respiratory symptoms (Yagihashi and Tajima, 1986; Levisohn and Kleven, 2000).

Predisposing factors can lead to the generalization of the disease. Metabolites produced by mycoplasmas cause local tissue damage, and the bacteria can invade the mucosa, enter into the bloodstream and bacteremia leads to the infection of other organs. Individual *Mycoplasma* strains show different affinity to certain tissues (Buim *et al.*, 2011; Ferguson-Noel and Noormohammadi, 2013). In case of *M. gallisepticum*, some strains can invade the cloacal mucosa as well, inducing permanent low immune response (MacOwan *et al.*, 1983). Other strains can grow on the cornea and conjunctiva, especially in finch species (Ley *et al.*, 1996). In turkeys, *M. gallisepticum* has been already isolated from the cerebrum several times (Thomas *et al.*, 1966; Chin *et al.*, 1991).

The predisposing factors are inappropriate housing technology including crowded hutches, disadvantageous climate conditions, hygiene deficiencies, inadequate feeding, other stress factors and most frequently different co-infections. *M. gallisepticum* and *M. synoviae* alone rarely cause respiratory symptoms, they more often participate in multifactorial diseases (Jordan, 1972; Kleven, 1998b). Numerous other bacteria (e.g. *Escherichia coli*, *Staphylococcus aureus, Avibacterium paragallinarum, Ornithobacterium rhinotracheale*) or

viruses (e.g. Reoviruses; infectious laryngotracheitis virus, ILTV; Newcastle disease virus, NDV; infectious bronchitis virus, IBV) can be in the background of respiratory diseases and these symptoms can be exacerbated by mycoplasmal superinfection (Bradbury and Garuti, 1978; Gross, 1990; Naylor *et al.*, 1992; Kleven, 1998b; Jones, 2000; Landman and Feberwee, 2004; Raviv *et al.*, 2007). Likewise, primer mycoplasmosis can exert immunosuppressive effect as well, offering an opportunity for other pathogens. Moreover, even vaccination against viruses with attenuated vaccine strains can play a role in the development of the respiratory symptoms (e.g. vaccination against IBV or NDV), which can be pronounced in birds infected with mycoplasmas (Nakamura *et al.*, 1994; Ley and Yoder, 1997). Besides, co-infection with different *Mycoplasma* species (*M. gallisepticum*, *M. synoviae*, *M. meleagridis*) is observed frequently as well (Rhoades, 1977; Kleven, 1998b; 1998c; Kang *et al.*, 2002).

2.5. Clinical signs

The incubation period is 6-21 days for *M. gallisepticum*, and 2-20 days for *M. synoviae* infection (Reis and Yamamoto, 1971; Naylor *et al.*, 1992; Ferguson-Noel and Noormohammadi, 2013). Manifestation of the mycoplasmal infection is affected by the route of transmission, the number of infecting bacteria, the virulence of the particular *Mycoplasma* strain and the internal predisposing and environmental factors (Lockaby *et al.*, 1998).

2.5.1. Clinical signs of *M. gallisepticum* infection

M. gallisepticum infection mainly causes respiratory symptoms, especially when predisposing factors are existing (Levisohn and Kleven, 2000). The leading clinical aspects are the chronic respiratory disease (CRD) of chickens and the infectious sinusitis of turkeys (Raviv and Ley, 2013).

In chicken, respiratory symptoms usually occur after the 4th week of age. The disease is often mild, usually turns into chronic infection. Most typical symptoms are serous, then serous-purulent nasal discharge and cough (Raviv and Ley, 2013). Beside tracheitis, the inflammation of the cornea and conjunctivitis can occur with intensive discharge, especially around the age of 30 days. Besides, unilateral enlargement of the eyeball has been already described (Power and Jordan, 1976; Nunoya *et al.*, 1995). However, subclinical infection occurs frequently, i.e. serological response can be observed without clinical signs (Kleven, 2008).

Turkeys are more susceptible for the disease with more serious clinical signs occurring usually between the age of 8-15 weeks. Beside tracheitis, air sacculitis and sinusitis are the most characteristic symptoms. Infraorbital sinuses are filled with serous or serous-fibrinous exudate, or dry, friable material, and swelling can be very spectacular (Figure 1). Besides, intensive

eye- and nasal discharge, sneezing, cough, tracheal rales and wheezing can be observed (Raviv and Ley, 2013). The nervous system of turkeys also can be affected, usually between the age of 12-16 weeks. This *M. gallisepticum* induced encephalitis is manifested in the typical signs of torticollis or opisthotonus (Chin *et al.*, 1991).

Figure 1. *M. gallisepticum* induced sinusitis in turkey (photo taken by Miklós Gyuranecz)

Due to *M. gallisepticum* infection, feed intake, feed conversion ratio and weight gain are decreased, while condemnation and downgrading are increased, leading to economic losses even without visible clinical signs (Ley and Yoder, 1997). Moreover, *M. gallisepticum* infection causes salpingitis and other reproduction disorders leading to reduced egg production. This is most conspicuous in case of commercial layers at the peak of the laying period (Domermuth *et al.*, 1967; Glisson and Kleven, 1984; Nunoya *et al.*, 1997). Due to embryonal mortality, reduced hatchability can be observed, hatched chicks are weak and often suffer from air sacculitis (Power and Jordan, 1973; Levisohn *et al.*, 1985). Maternal immunity can reduce these effects and early infection of the chicks can protect them from serious disease in a subsequent infection (Fabricant, 1975; Levisohn *et al.*, 1985).

Without rapid and adequate treatment, morbidity in the infected chicken stocks can come up to 100 %. In case of infectious sinusitis of turkeys, morbidity can change between 1-70 %, while in respiratory disease, this index can be even 80-90 %. Mortality can reach 30 %, mainly in broilers, especially in the winter period, when resistance of the animals is the weakest (Mohammed *et al.*, 1987).

Beside chickens and turkeys, *M. gallisepticum* can infect other bird species as well. Pheasants and partridges show decreased feed intake, serous nasal discharge and lachrymation. The birds are constantly shaking their heads, while abnormal sounds of breathing and swelling of the infraorbital sinuses can be observed (Cookson and Shivaprasad, 1994; Ganapathy and Bradbury, 1998). In some species of songbirds, especially finches, *M. gallisepticum* infection manifests as conjunctivitis, and occasionally this disease can even become epizootic (Ley *et al.*, 1996, Luttrell *et al.*, 1996).

2.5.2. Clinical signs of *M. synoviae* infection

Clinical disease most frequently occurs in 4-16 week-old chickens and 10-24 week-old turkeys. Birds at higher age rarely show symptoms, mainly when their immune systems are attenuated. The *M. synoviae* induced infectious synovitis most frequently manifests in arthritis, tendovaginitis, bursitis, airsacculitis and inflammation of other serous membranes (Ferguson-Noel and Noormohammadi, 2013).

Chicks hatched from infected eggs can usually suffer from airsacculitis. The first sign of the infection in the flock is the difference in the development of the chicks. Infected birds are retarded in growth, show reduced feed intake and utilization, they are lean and weak, their plumage is fluttered and their combs are pale or cyanotic. Diarrhea can also occur with greenish discoloration and contain large amount of uric acid or urate crystals (Ferguson-Noel and Noormohammadi, 2013).

Beside general symptoms, infectious synovitis can be characterized by painful walking, as inflammation usually occurs at the tarsometatarsal area (Figure 2), however, occasionally other joints can be affected as well. Palpating the inflamed joints, they are noticeably warm and painfully swollen by the exudate in the synovial cavity, which is fluent at the beginning, but become thicker as time goes on. The enlargement of the sternal bursa can be conspicuous on the lean animals. Difficulties of moving, lameness can be observed, the birds usually sitting near the food or water source. In case of turkeys, the disease can lead to total immobility, as inflamed joints cannot bear the weight of the birds (Landman and Feberwee, 2012). Acute phase is usually followed by slow recovery, however, disease can become chronic as well and sometimes the joints suffer irreversible damage (Dijkman *et al.*, 2013).

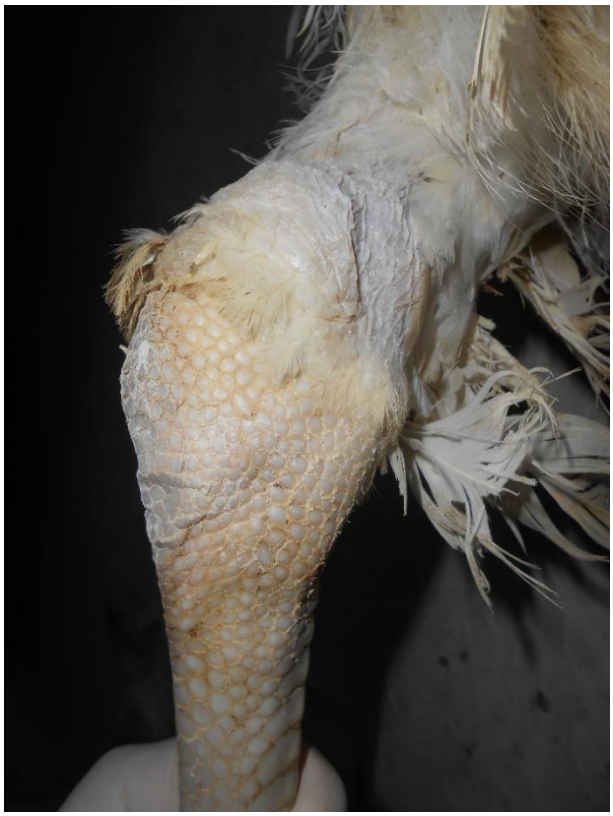


Figure 2. *M. synoviae* induced arthritis and tendovaginitis in turkey
(photo taken by Miklós Gyuranecz)

In respiratory disease caused by *M. synoviae*, the birds are coughing, sneezing and showing nasal and eye discharge. In broilers, the meat gain is remarkably decreased, while downgrading rate is increased (Vardaman *et al.*, 1973). Clinical signs in turkeys are evoked by sinusitis and airsacculitis (Ghazikhanian *et al.*, 1973; Rhoades, 1981), however, respiratory form of the *M. synoviae* induced disease is relatively rare, involvement of joints is more typical (Landman and Feberwee, 2012).

M. synoviae induced eggshell apex abnormalities occur primarily in layer hybrids (Catania *et al.*, 2010; Feberwee and Landman, 2010; Ranck *et al.*, 2010). The sharply demarcated lesion can be observed on the top of the egg expanded about 2 centimeters from the apex (Feberwee *et al.*, 2009). The eggshell is thinned and more transparent, the surface is scraggy and rough, fissures and fractures are typical (Brandão *et al.*, 2014). Broken eggs mean direct losses, but they increase cleaning costs as well, moreover, risk of infections through the thinned eggshell is also extended (Catania *et al.*, 2010; Feberwee and Landman, 2010). Jeon *et al.* (2014) investigated the construction of eggshells in a *M. synoviae* infected Korean layer flock with scanning electron microscope. They found that eggshells had weaker structure even if it cannot be observed macroscopically.

M. synoviae infection can reduce egg production in chickens and turkeys as well, and the quality and hatchability of the eggs are also decreased (Bradbury and Garuti, 1978; Lott *et al.*, 1978; Opitz, 1983; Mohammed *et al.*, 1987; Branton *et al.*, 1999; Gole *et al.*, 2012).

The morbidity of the disease is generally lower in the articular form, it is usually between 5-15 %, but it can reach 75 % occasionally. In respiratory form, the morbidity is higher, it might be even 90-100 %. The mortality varies between 1-10 %, but more often the infection remains completely asymptomatic (Ferguson-Noel and Noormohammadi, 2013).

2.6. Pathological lesions

2.6.1. Pathological lesions of *M. gallisepticum* infection

The most significant pathological lesion is the serous-mucous, serous-fibrinous or purulent inflammation in the mucosa of the upper respiratory tract, bronchial tubes, air sacs, and sometimes conjunctiva. The surface of the tracheal mucosa is red, swollen, covered with serous-fibrinous exudate. Infraorbital sinuses are filled with serous, serous-fibrinous or caseous exudate, especially in turkeys. Walls of the air sacs are blurred, thickened, covered with fibrin plaques. Their cavities filled with at first creamy, later thick, at least dry, friable material. Lungs are edematous, occasionally pneumonia can be observed as well (Kerr and Olson, 1967).

In severe cases, serous membranes can be affected as well. Most commonly, fibrinous pericarditis and perihepatitis can be observed. In case of peritonitis, fibrin plaques can be found in the abdominal cavity. Salpingitis can occur as well with fibrinous exudate filling their cavity (Domermuth *et al.*, 1967).

Histopathological lesions are characterized by edema and inflammatory cell infiltration of the mucosal membranes. Cilia are destructed and mucous glands show hyperplasia. The surface of the mucosa is usually covered with inflammatory exudate (Raviv and Ley, 2013).

2.6.2. Pathological lesions of *M. synoviae* infection

Serous exudate or creamy grayish yellow debris can be found in the cavity of the inflamed bursa, joints and tendon sheets, especially in the sternal bursa and joints of the wings, tarsometatarsus and fingers. The inflammation can affect the surrounding muscles and air sacs as well (Sevoian *et al.*, 1958).

Lesions in the upper respiratory tract are relatively uncommon. These can be for example catarrhal tracheitis, sinusitis and air sacculitis. The cavity of the sinuses and air sacs are filled with yellow thick exudate (Bradbury and Garuti, 1978).

In generalized infection the liver is enlarged and mottled, the gallbladder is stretched, the spleen is enlarged and its structure is granular. Kidneys are enlarged as well, they are pale or hyperemic, sometimes spotted. Intestinal mucosa is edematous and reddened, the feces is stinking and green because of the bile (Ferguson-Noel and Noormohammadi, 2013).
Histopathological examination of the tracheal mucosa reveals edema, increased number of the inflammatory and apoptotic cells and decreased number of the cilia (Buim *et al.*, 2011).

2.7. Diagnosis

The epidemiological anamnesis, clinical signs and pathological lesions of mycoplasmosis are usually non-specific, thus diagnosis requires the identification of the pathogen. Most frequent laboratory tests for the diagnosis are the isolation of the bacterium, serological techniques and molecular biological methods. It is worth to mention, that great genetic similarity between *M. gallisepticum* and *M. imitans* is a difficulty in diagnostics, false results can be seen in several serological and molecular biological tests as a result of cross reaction between the two species (Bradbury *et al.*, 1993).

2.7.1. Isolation of the pathogen

Isolation of *M. gallisepticum* and *M. synoviae* can be performed most successfully by collecting choanal or tracheal swab samples. Sometimes cloacal and conjunctival swab samples can be usable as well for the isolation of *M. gallisepticum*. In case of dead animals, collecting tissue samples is also an option, *M. synoviae* can be cultured from the affected joints, bursa, or oviduct as well (Ferguson-Noel and Noormohammadi, 2013). Optimal time of sampling is during the acute phase, because in the subsequent chronic period, number of bacteria in the trachea can decrease significantly. In the acute state, there is usually no immune response yet, thus serological tests still can be negative; however, it is the most appropriate time for sampling to identify the pathogen by isolation or polymerase chain reaction (PCR) (Yagihashi and Tajima, 1986; Levisohn and Dykstra, 1987; Hyman *et al.*, 1989).
Avian mycoplasmas, especially *M. synoviae*, are fastidious, slow growing microorganisms, thus their laborious and time-consuming culturing is usually performed exclusively by special laboratories. Frequently occurring mixed infections make it more complicating, as saprophytic *Mycoplasma* species usually grow faster than pathogens. They are facultative anaerobic, microaerophilic organisms, their culturing is carried out at 37-38 °C in the presence of 5 % CO_2. Their cell-less, nutrient-rich culture media can be liquid and solid as well and contain DNA, yeast extract, cysteine, glucose, pyruvate and serum, which can be for example heat-inactivated swine, horse or bird serum (Frey *et al.*, 1968). Some mycoplasma species, like *M. synoviae*, can grow exclusively in the presence of nicotinamide adenine dinucleotide (NAD);

in contrast, *M. gallisepticum* does not need NAD in the broth. Growth of glucose fermenting *Mycoplasma* species, like *M. gallisepticum* or *M. synoviae* can be followed up by adding pH sensitive color indicator (e.g. phenol red) to the broth. As cell wall-less organisms, mycoplasmas are *ab ovo* resistant to β-lactam antibiotics, thus penicillin derivatives can be used in selective media to inhibit the growth of Gram-positive bacteria, while thallium acetate supplement can be effective against Gram-negative bacteria (Kleven *et al.*, 1996; Kleven, 1998c; 2008). For culturing avian mycoplasmas, special media are commercially available as well.

Isolation of avian mycoplasmas can take a few days or even weeks. Colonies on solid media are small, usually 0.2-0.3 mm. They form regular circle colonies, which can be typical fried egg-shaped or atypically homogenous in structure (Figure 3). The typical fried egg appearance is the result of insinuation of the pleomorphic organisms among the fibers of the agar where growth is initiated. Further division of cells, as the colony develops, results in spread onto the surface (Adler *et al.*, 1974; Razin, 1983).

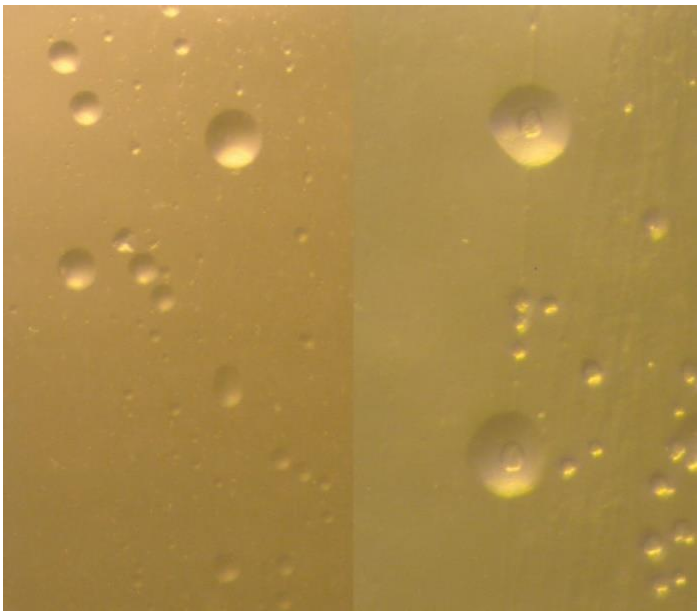


Figure 3. *M. gallisepticum* colonies with homogeneous structure (left side) and typical fried-egg appearance (right side) on solid media

On mycoplasma cultures we can apply growth or metabolic inhibition tests. Identification of *Mycoplasma* species by inhibition of growth with hyperimmune serum originally involved the incorporation of specific antisera into agar. This technique was subsequently refined by allocating drops of antisera or small filter-paper discs saturated with specific antiserum on the surface of the agar plates, so hyperimmune serum can counteract the bacterial growth in the zones of inhibition (Stanbridge and Hayflick, 1967).

In liquid medium, metabolism of certain substrates can be inhibited by adding specific hyperimmune serum, which prevents bacterial metabolism. In case of *M. gallisepticum* and *M. synoviae*, the inhibition can be demonstrated by fail of acid production, but accordingly to certain *Mycoplasma* species, the lack of arginine hydrolysis, urea metabolism, tetrazolium reduction or phosphatase production also can be observable (Hill, 1977).

2.7.2. Serological methods

Serological methods detect antibodies in the serum by using specific antigens. Slide plate agglutination (SPA) is a rapid and simple test, applicable even at the poultry farms. It detects immunoglobulins of the immunoglobulin M (IgM) class, thus it is primarily applicable to explore acute infections. Unfortunately, false positive results can occur, for example due to cross reactions. In laboratory conditions, the easily applicable and cost-effective enzyme-linked immunosorbent assay (ELISA) is considered to be more reliable. Hemagglutination inhibition (HI) tests are based on the detection of IgG antibodies, thus this method is capable to reveal chronic infection as well. This is the so-called „gold standard" method in serology as it is considered to be the most reliable assay, however it is relatively elaborate as well, thus recently these tests are rarely used (Feberwee *et al.*, 2005).

2.7.3. Molecular biological techniques

The most important molecular biological method is PCR, which provides a rapid, simple and cost-effective tool with an appropriate combination of specificity and sensitivity. PCR can detect specific regions of the DNA in the sample examined. In case of conventional PCR, the amplification products can be visualized by agarose gel electrophoresis. Several PCR protocols are available for the detection of mycoplasmas. A PCR test detecting the 16S/23S rRNS intergenic region of all bacteria belonging to the class of Mollicutes has been described by Lauerman *et al.* (1995), while several assays are capable to distinguish on the level of species as well. For example, Garcia *et al.* (2005) designed *M. gallisepticum*-specific PCRs, while a multiplex PCR published by Wang *et al.* (1997) is able to detect the four most important avian pathogen mycoplasmas (*M. synoviae*, *M. gallisepticum*, *M. meleagridis*, *M. iowae*) simultaneously.

Beside conventional PCRs, the use of TaqMan assays in real-time PCR tests are increasingly widespread in the diagnosis of *M. gallisepticum* and *M. synoviae* as well. Taqman probes are labeled with dyes that can be excited by light resulting in fluorescent signal. This signal can be detected on real-time platform. This method is more sensitive than the conventional PCR and suitable for even quantitative measurements, on the other hand it is more expensive and requires special laboratory equipment (Raviv and Kleven, 2009; Sprygin *et al.*, 2010).

For phylogenetic studies and epidemiological investigations, identification of *Mycoplasma* species is not enough, it is necessary to reveal genetic relationships between the strains. Recently, sequence based methods are in use for genotyping *M. gallisepticum* and *M. synoviae* strains.

2.7.3.1. Genotyping *M. gallisepticum* strains

Determining the nucleotide sequence of the *pvpA*, *gapA* and *mgc2* genes (gene-targeted sequencing) is a rapid and cost-effective method to compare different *M. gallisepticum* strains (Ferguson-Noel *et al.*, 2005). The most reliable, but more expensive genotyping method is the multi-locus sequence typing (MLST) assay, which classify the examined strains in different sequence types (STs) due to the nucleotide sequence of certain housekeeping genes (Ghanem and El-Gazzar, 2019). Similarly, core genome MLST (cgMLST) is based on the determination of nucleotide sequences of even hundreds of genes, which make this typing method highly effective (Ghanem *et al.*, 2017). The disadvantage of this test is that it requires whole genome sequencing of the pure cultures, thus it is laborious, time-consuming and expensive as well.

2.7.3.2. Genotyping *M. synoviae* strains

Beside determining the nucleotide sequence of the *vlhA* gene (Benčina *et al.*, 2001; Hong *et al.*, 2004; Jeffery *et al.*, 2007; Hammond *et al.*, 2009; Harada *et al.*, 2009; Wetzel *et al.*, 2010; El-Gazzar *et al.*, 2012; Dijkman *et al.*, 2014a), MLST assays suitable for genotyping *M. synoviae* strains have been developed recently as well (Dijkman *et al.*, 2016; El-Gazzar *et al.*, 2017). The multi-locus variable number tandem repeat analysis (MLVA) examines the numbers of repeat units, which are usually located in non-coding regions of the genome. This method provides a highly discriminative, rapid and cost-effective alternative typing technique for the genetic characterization of *M. synoviae* strains (Kreizinger *et al.*, 2018).

2.8. Treatment and control

The control programs for *M. gallisepticum* and *M. synoviae* are primarily based on eradication of the pathogen and maintaining stocks free of infection. As predisposing factors have great importance in the development and progression of the disease, eliminating these factors is one of the most essential component of the prevention. Providing appropriate housing technology, following epidemiological rules and performing regular tests are all necessary to prevent the infection of the stocks (Ley and Yoder, 1997; Levisohn and Kleven, 2000).

As vertical infection is a significant route of transmission, prevention is primarily based on maintaining commercial breeder stocks free of infection. The immune status of the breeding

pairs should be monitored regularly by serology. Immune response of turkeys is usually slower and weaker, thus PCR tests are recommended instead of serological methods in their case (Landman and Feberwee, 2012). The *Mycoplasma*-free status of the breeders is suggested to be re-investigated after laying, just before the eggs or few days old chicks are utilized in order to exclude the opportunity of vertical transmission (Kleven, 2008).

If a flock is proved to be infected with *M. gallisepticum* or *M. synoviae*, most effective way to eliminate the pathogen is stamping out. After slaughtering the infected birds, hutches should be disinfected and filled with birds originating from *Mycoplasma*-free stocks. This method can be feasible in broiler farms applying all-in-all-out system, while it can be unaccomplishable in multi-age commercial layer flocks (Kleven, 2008).

There are many difficulties in maintaining flocks free of infection. Intensive, continuously growing poultry industry is usually carried out in extended integrations. Moreover, different farms can be located in the same region, close to each other or to a backyard poultry stock. Therefore, aims of elimination programs are almost impossible to fulfil in many poultry farms. In these cases, vaccination provides an effective measure of long-term disease control, while antibiotic therapy can offer a short-term solution (Kleven, 2008).

2.8.1. Antibiotic therapy

Antibiotic treatment can be a good choice in case of *M. gallisepticum* or *M. synoviae* infected backyard and ornamental poultry, as eradication of the whole stock is not reasonable. Besides, antimicrobial therapy can be useful in case of intensive poultry farms affected by outbreaks as well, because the adequate treatment can relieve economic losses through the reduction of clinical signs and vertical transmission (Fiorentin *et al.*, 2003; Hong *et al.*, 2015). However, this strategy can be effective only in the short run, as eradication of these pathogens cannot be achieved by the use of antibiotics. The stock remains infected, the birds are spreading the bacteria and outbreaks can occur from time to time (Levisohn and Kleven, 2000). Moreover, long lasting application of the antimicrobial agents can contribute to the development and spreading of antibiotic resistance (Le Carrou *et al.*, 2006). Therefore, antibiotics can never replace real long-term solutions, like eradication and vaccination.

Medication of avian mycoplasmosis is really challenging. As previously mentioned, epithelial cells of the tracheal mucosa can partially incorporate these pathogens making them difficult to reach by antimicrobial agents (Razin *et al.*, 1998). Moreover, mycoplasmas are naturally resistant against several antibiotics. Because of their lack of cell wall, they are resistant to β-lactams (e.g. penicillin, cephalosporin, monobactam, carbapenem), glycopeptides and fosfomycin (Shelton *et al*., 1958; Taylor-Robinson and Bébéar, 1997; Schultz *et al.*, 2012). Likewise, polymyxins, sulfonamides and trimethoprim are ineffective against mycoplasmas due to their missing lipopolysaccharides and folic acid synthesis (McCormack, 1993; Olaitan

et al., 2014). Natural resistance can be observed against 14-membered lactone macrolides (e.g. erythromycin) (Shelton *et al.*, 1958; Whithear *et al.*, 1983; Bradbury *et al.*, 1994; Gautier-Bouchardon *et al.*, 2002) as well. Besides, a naturally occurring mutation in the *rpoB* gene is responsible for their resistance against rifampicin (Gaurivaud *et al.*, 1996; Bébéar and Bébéar, 2002).

Antibiotics affecting RNA-, DNA- or protein synthesis, or impairing cell membranes can be effective against mycoplasmas, like fluoroquinolones, aminocyclitols, aminoglycosides, tetracyclines, macrolides, lincosamides, pleuromutilins and phenicols (Kleven and Anderson, 1971; Hamdy *et al.*, 1976; Olson and Sahu, 1976; Baughn *et al.*, 1978; Whithear *et al.*, 1983; Jordan *et al.*, 1989; 1999; Bradbury *et al.*, 1994; Hannan *et al.*, 1997; Stanley *et al.*, 2001; Wang *et al.*, 2001; Cerda *et al.*, 2002; Gautier-Bouchardon *et al.*, 2002; Gerchman *et al.*, 2008; Landman *et al.*, 2008; Kreizinger *et al.*, 2017a; Catania *et al.*, 2019), however, susceptibility profiles of the certain *Mycoplasma* strains can be very different (Dufour-Gesbert *et al.*, 2006; Landman *et al.*, 2008).

Due to *in vitro* tests, pleuromutilins seem to be the most effective agents against *M. gallisepticum* and *M. synoviae* isolates, minimal inhibitory concentration (MIC) values of tiamulin and valnemulin are consistently lower than MIC values of any other antibiotics (Jordan *et al.*, 1989; Ammar *et al.*, 2016; Kreizinger *et al.*, 2017a). Most *M. gallisepticum* and *M. synoviae* isolates are susceptible to tetracyclines as well, low MIC values were described especially in case of oxytetracycline and doxycycline (Pakpinyo and Sasipreeyajan, 2007; Gharaibeh and Al-Rashdan, 2011). Spectinomycin in combination with lincosamides is also found to be effective *in vitro* against mycoplasmas (Gautier-Bouchardon *et al.*, 2002). Most strains were found to be sensitive to macrolides; however, several studies reported resistance against tylosin and tilmicosin (Levisohn, 1981; Whithear *et al.*, 1983; Jordan *et al.*, 1989; Tanner and Wu, 1992; Hannan *et al.*, 1997; Gerchman *et al.*, 2011; Gharaibeh and Al-Rashdan, 2011; Ammar *et al.*, 2016; Catania *et al.*, 2019).

Susceptibility of *M. gallisepticum* and *M. synoviae* strains to fluoroquinolones increased over the last few decades, most isolates show high MIC values in case of enrofloxacin and difloxacin (Gerchman *et al.*, 2008; Gharaibeh and Al-Rashdan, 2011). These data are particularly troublesome as importance of fluoroquinolones is critical in the therapy of humans, thus use of fluoroquinolones should be avoided in the treatment of avian mycoplasma infections.

Unfortunately, acquired resistance is a common phenomenon in case of mycoplasmas and can occur against any antibiotic agent threating veterinary and human medicine as well (Gautier-Bouchardon, 2018). The development and spreading of strains bearing mutations associated with decreased susceptibility is frequently a consequence of inadequate antibiotic therapy. Therefore, antibiotics can be applied only in justifiable cases and with special care. Antibiotic susceptibility tests prior to treatment can contribute greatly to prudent antibiotic usage (Kreizinger *et al.*, 2017a; Gautier-Bouchardon, 2018).

2.8.1.1. Determination of antibiotic susceptibility

Most common method of antibiotic susceptibility testing is the determination of MIC values *in vitro* by broth or agar microdilution method. These are very labor-intensive and time-consuming methods, as they require previous isolation and pure culture of the bacterium (Hannan, 2000). Interpretation of the results is difficult as well, because standard breakpoints of susceptible, intermediate and resistant categories to antimicrobial agents concerning *Mycoplasma* species have not been defined yet. In the lack of official breakpoints, the MIC data can be evaluated based on breakpoints of other avian pathogens determined by the Clinical and Laboratory Standards Institute (CLSI, 2018). The MIC values also can be compared to the results of previous publications taking into account that there are no internationally harmonized and accepted testing conditions for avian mycoplasmas. Moreover, the results of *in vitro* antibiotic susceptibility tests can only predict the expected *in vivo* efficacy of the antibiotics (Hildebrand, 1985).

There is an increasing need for rapid antimicrobial susceptibility tests in order to guide therapy more effectively. Mechanisms of resistance, for instance enzymatic inactivation of antibiotics, changes in the binding sites of the agents, or synthesis of efflux pumps and broad substrate transporters decreasing intracellular concentrations of the antibiotics are all mediated by genetic alterations (Sundsfjord *et al.*, 2004). Thus, a plausible method for the exploration of antibiotic resistance is to test the antimicrobial susceptibility at the molecular level.

A rapid and cost-effective method is the detection of resistance-associated mutations by molecular biological assays. These assays are most commonly based on real-time PCR techniques, but conventional PCR also can be applied. For the detection of single nucleotide polymorphisms (SNPs) related to higher MIC values, mismatch amplification mutation assays (MAMAs) can be used, whereas high resolution melt (HRM) analysis is capable of investigating hot-spot regions with several mutations as well (Sulyok et al., 2018).

2.8.2. Vaccination

Currently inactivated and attenuated vaccine types are available to control *M. gallisepticum* or *M. synoviae* infection, while using recombinant vaccines are also an opportunity against *M. gallisepticum* (Ferguson-Noel and Cookson, 2012).

In these recombinant (vector) vaccines, genes encoding protective antigens of a pathogen are implanted into the DNA of another agent (vector). These recombinant strains are able to protect against the infections caused by the recipient and vector as well (Yamanouchi *et al.*, 1998; Robert-Guroff, 2007). The vector of the commercially available recombinant vaccine against *M. gallisepticum* (Vectormune® FP-MG, Ceva Inc.) is the fowl poxvirus (FPV) carrying the *40k* and *mgc* genes of the *M. gallisepticum* (Zhang *et al.*, 2010).

Bacterin vaccines (MG-BAC®, Zoetis; AVIPRO 104 MG BACTERIN®, Elanco; Gallimune MG®, Boehringer Ingelheim) contain inactivated bacteria, thus they are concerned to be safer than live vaccine strains. On the other hand, application of bacterin vaccines is very labor-intensive. As they usually contain oil-based emulsion adjuvant, the vaccine requires subcutan or intramuscular administration, thus animals have to be treated individually. Adjuvant induced local tissue damage is also a common problem leading to decreased quality of meat and increasing the rate of downgrading. The most serious problem with bacterin vaccines is their questionable effectiveness. Certain studies reported that the use of bacterin vaccines reduces significantly the clinical signs and vertical transmission of *Mycoplasma* infection, while other papers suggested that their protective effect is not sufficient (Rimler *et al.*, 1978; Yoder, 1979; 1983; Glisson and Kleven, 1984; 1985; Hildebrand, 1985; Levisohn and Kleven, 2000). Although bacterins induce serological response, the developed immunity is not enough to protect tracheal mucosa from the infection. Hence, bacterins are rarely used, instead, vaccination with live strains are increasingly spreading (Whithear, 1996).

These attenuated avirulent vaccine strains do not cause disease, but - in contrast to the inactivated bacteria in bacterin vaccines - they are able to colonize the mucosa in the upper respiratory tract of the birds, and prevent infection with virulent strains. Moreover, in case of an already existing infection, vaccine strains can occasionally overgrow and eradicate the virulent strains from the habitat (Levisohn and Kleven, 2000). The presence of the vaccine strain, as an antigen in the respiratory system, exposes the host to a continuous challenge, inducing a long lasting effective mucosal immunity (Kleven, 2008). Besides, low-rate bird-to-bird transmission of live vaccine strains can enhance the protective immunity of the flocks (Levisohn and Kleven, 2000).

2.8.2.1. Live *M. gallisepticum* vaccines and methods for differentiating vaccine strains from field isolates

Live *M. gallisepticum* vaccine strains are members of the so-called variant or atypical strains. These strains are characterized by decreased virulence and antigenicity (Levisohn and Kleven, 2000). Variant strains were first described from chickens in the early 1970s (Yoder, 1986). Later, similar strains were isolated from turkeys as well (Avakian *et al.*, 1992). Currently four live vaccine strains are commercially available in different countries: the F (Cevac® MG-F, Ceva Inc.; Poulvac® Myco F, Zoetis; AviPro® MG F, Elanco), 6/85 (Nobilis® MG 6/85, MSD Animal Health), ts-11 (TS-11®, Merial Italia S.p.A.; Vaxsafe® MG, Bioproperties Pty Ltd.) and K vaccine strains (K 5831, Vaxxinova Japan K.K.).

The F strain evolved by natural selection, it was isolated by Yamamoto and Adler in the years of 1950s (Yamamoto and Adler, 1958). Virulence and pathogenicity of the strain is relatively low, nevertheless F strain cannot be used in broiler chickens and turkeys as it can cause respiratory disease in their flocks (Rodriguez and Kleven, 1980a; 1980b; Kleven, 1981; Lin and Kleven, 1982a; 1982b; Ley *et al.*, 1993). On the other hand, F strain was found to be effective in layer flocks. It persists life-long in the upper respiratory tract of the birds, inhibits infection with virulent *M. gallisepticum* strains and prevents the reduction of egg production (Cunningham and Olson, 1978; Carpenter *et al.*, 1981; Kleven, 1981; Cummings and Kleven, 1986; Levisohn and Dykstra, 1987; Mohammed *et al.*, 1987). It can be applied by intraocular or intranasal route or by spray. Optimal time for vaccination is between the age of 8-14 weeks, but if there is high risk for the infection of the chicks, it can be administered earlier as well (Luginbuhl *et al.*, 1967; Fabricant, 1975).

Vaccine strain 6/85 also evolved by natural selection, the vaccine was described and developed by Evans *et al.* (Evans *et al.*, 1992; Evans and Hafez, 1992). It is avirulent in chickens and turkeys as well, nonetheless it is permitted only in layer line chickens to prevent the reduction of egg production. It has low transmissibility and does not induce immune response, though provides sufficient protection against wild strains (Evans and Hafez, 1992; Ley *et al.*, 1997). It is applied by spray, and it can be detected in the upper respiratory tract for 4-8 weeks after the vaccination (Levisohn and Kleven, 2000).

Characterization of the ts-11 strain was performed by Whithear *et al.* (Whithear *et al.*, 1990a; 1990b; 1990c). The strain was developed by chemical mutagenesis of a *M. gallisepticum* isolate from Australia and was selected as a temperature-sensitive mutant. The ts-11 is an avirulent strain characterized by low transmissibility and induces slowly evolving immunity. Vaccination by ts-11 strain exerts long-term protection against wild *M. gallisepticum* strains, as the live vaccine strain persists life-long in the upper respiratory tract of the birds (Whithear, 1996). It can be applied intraocularly (Levisohn and Kleven, 2000).

The K strain is a naturally occurring avirulent strain which originates from the USA and currently available for vaccination only in a few far Eastern countries. The vaccine is proved to be safe and effective (Raviv *et al.*, 2008; Ferguson-Noel and Cookson, 2012), in experimental infections, it reduced virulent *M. gallisepticum* induced clinical signs in broilers and backyard chickens to a similar extent as F and ts-11 vaccine strains (Ferguson-Noel and Williams, 2015). In laboratory conditions, F vaccine strain was proved to be able to overgrow and eradicate the virulent strains from the upper respiratory tract, i.e. the F strain can be effective in an already existing infection as well (Kleven *et al.*, 1990; 1996). For long-term prevention purposes, use of the more avirulent ts-11 or 6/85 strains is recommended. Field experiments showed that proper application of these vaccines provides long-lasting protection against virulent *M. gallisepticum* strains (Levisohn and Kleven, 2000).

Since live vaccines are used in many parts of the world, differentiation of *M. gallisepticum* vaccine strains from wild, virulent isolates is essential in control programs (Whithear, 1996). It is a difficulty that genetic background of virulence is not completely understood yet. Moreover, avirulent vaccine strains can occasionally retrieve their virulence during circulation in the flock (El-Gazzar *et al.*, 2011).

Previously, DNA fingerprinting methods, such as amplified fragment length polymorphism and random amplified polymorphic DNA were used to discriminate *M. gallisepticum* vaccine strains and field isolates (Fan *et al.*, 1995; Hong *et al.*, 2005). However, these methods had low reproducibility and high labor intensity. Sequence-based methods, such as gene-targeted sequencing, PCR tests, TaqMan probes, HRM analyzes or genotyping assays (MLST, cgMLST) are characterized by higher reproducibility and lower labor intensity (Ferguson-Noel *et al.*, 2005; Evans and Leigh, 2008; Raviv *et al.*, 2008; Ghorashi *et al.*, 2010; 2013; 2015; Ghanem *et al.*, 2017; Ricketts *et al.*, 2017; Ghanem and El-Gazzar, 2019). Disadvantages of these methods are that they are usually expensive and require special laboratory equipment.

Beside the determination of antibiotic susceptibility profile, the aforementioned MAMA method can be suitable for the discrimination of *M. gallisepticum* vaccine strains from field isolates as well. MAMA is a PCR-based technique used for SNP discrimination in many bacteria (Birdsell *et al.*, 2012). In brief, the technique is based on SNP-specific primers, one being marked with an additional 15-20 bp long GC-clamp. The GC-clamp increases the melting temperature and the size of the amplicon as well. The temperature shift can be easily detected in the presence of intercalating fluorescent dye on a real-time PCR platform (melt-MAMA) and the difference in the sizes of the amplicons can be observed in agarose gel electrophoresis (agarose-MAMA), which enable the differentiation of the SNP-specific genotypes.

MAMA provides a comfortable, time- and cost-effective molecular tool for the discrimination of vaccine strains from wild virulent isolates to control vaccination programs. This method is highly specific and sensitive enough to be applicable on DNAs extracted directly from clinical samples avoiding technical problems associated with isolation, which is particularly complicated in the case of mycoplasmas. Further advantage of the assay is that it can be performed on basic real-time PCR platforms and on conventional PCR equipment coupled with agarose gel electrophoresis too.

2.8.2.2. Live *M. synoviae* vaccines and methods for differentiating vaccine strains from field isolates

Temperature-sensitive (ts+) MS-H vaccine strain (Vaxsafe® MS, Bioproperties Pty Ltd.) has been developed from an Australian isolate (86079/7NS) by chemical mutagenesis (Markham *et al.*, 1998a; 1998b; 1998c; Morrow *et al.*, 1998). It is applied as eye drops between the 3rd and 6th week of age and induces even life-long protection against *M. synoviae* infection (Jones *et al.*, 2006a; 2006b; 2006c). It can be horizontally transmitted between the animals of the flock (Nicholas *et al.*, 2009). Due to the *in vivo* passages in the stock, MS-H strain can lose its temperature-sensitivity (ts-) relatively often, but it does not definitely associated with restoring its virulence as well. This means that beside temperature-sensitive phenotype, other factors can also contribute to the attenuation of the MS-H vaccine strain (Noormohammadi *et al.*, 2003).

The NAD-independent MS1 vaccine strain (Nobilis® MS Live, MSD Animal Health Inc.) was developed by spontaneous mutagenesis with *in vitro* passages of the *M. synoviae* type strain NCTC 10124 (WVU 1853, ATCC 25204) (Dijkman *et al.*, 2014b). It is applied by spray and similarly to MS-H, it is able to spread by horizontal transmission within the flock.

Previously, differentiation of MS-H vaccine strains from wild virulent *M. synoviae* isolates was based on the temperature-sensitive phenotype of the MS-H strain, but this method became unreliable with the appearance of the ts- variants (Morrow *et al.*, 1998). Recently, differentiation by molecular biological methods is more preferable.

Initially, the *vlhA* gene sequencing was used for the genotype-based differentiation of the MS-H vaccine strain from wild isolates (Noormohammadi *et al.*, 2000; Jeffery *et al.*, 2007). Later, however, it turned out that the allele sequence of the MS-H strain is not unique, homology could be observed between the vaccine and several wild-type strains (Dijkman *et al.*, 2014a).

Recently, differentiation is based on specific mutations of the thermosensitivity-associated *obg* gene. The MS-H-specific SNPs are detectable by HRM or MAMA tests (Birdsell *et al.*, 2012; Shahid *et al.*, 2014a; Kreizinger *et al.*, 2015). Besides, MLVA assays also proved to be able to identify the MS-H vaccine strain (Kreizinger *et al.*, 2018) independently or in combination with the MAMA tests. To distinguish the *M. synoviae* MS1 vaccine strain from field isolates and the MS-H vaccine strain, a specific SNP in the gene encoding the histidine triad (HIT) protein family has to be detected (Dijkman *et al.*, 2014b; Kreizinger *et al.*, 2017b).

3. Aims of the study

The aims of the study were:

1. to develop an MLST assay applicable for genotyping different *M. gallisepticum* strains.

2. to investigate the genetic relatedness of *M. gallisepticum* strains with the developed MLST assay in order to evaluate the discriminatory power of the method and better understand the epidemiology of *M. gallisepticum*.

3. to develop rapid molecular biological assays (MAMA tests) capable of differentiating *M. gallisepticum* vaccine strains from field isolates.

4. to compare the results of the developed MLST assay with data obtained from the designed MAMA tests in order to confirm their reliability and investigate the congruency of these methods.

5. to investigate the genetic background of decreased susceptibility to fourteen antibiotics of eight antimicrobial groups in *M. synoviae* and to identify potentially resistance-related mutations suitable to be targeted with rapid molecular biological assays.

4. Materials and methods

4.1. *Mycoplasma gallisepticum* samples

4.1.1. Sample collection

For the assay developments, 19 *M. gallisepticum* whole genome sequences (WGSs) available online in GenBank (strain S6, GenBank accession number: NC_023030; strain R_{low}, GenBank accession number: AE015450; strain R_{high}, GenBank accession number: NC_017502; house finch isolates, GenBank accession numbers: NC_018412, NC_018409, NC_018406, NC_018407, NC_018408, NC_018410, NC_018411, NC_018413; and ts-11 re-isolates, GenBank accession numbers: MAFU00000000, MAFV00000000, MAFW00000000, MADW00000000, MATM00000000, MATN00000000, MAGQ00000000, MAGR00000000) were used *in silico*.

DNA of the *M. gallisepticum* type strain ATCC 19610 and the live 6/85 (Nobilis® MG 6/85, MSD Animal Health Hungary, Budapest, Hungary), ts-11 (TS-11®, Merial Italia S.p.A., Assago, Italy), F (Cevac® MG-F, Ceva Inc., Budapest, Hungary) and K (K 5831, Vaxxinova Japan K.K., Tokyo, Japan) vaccine strains were also included in the study.

Besides, DNA of 266 *M. gallisepticum* isolates including pure *M. gallisepticum* cultures (n=212) and clinical samples (n=54) were used in this study. These samples were collected between 1993 and 2019 and originated from 28 countries (Italy, n=82; Spain, n=58; UK, n=25; Israel, n=24; Hungary, n=10; Malaysia, n=7; USA, n=7; Australia, n=6; India, n=6; Thailand, n=4; the Netherlands, n=4; Fiji, n=3; Germany, n=3; Indonesia, n=3; Iraq, n=3; Romania, n=3; China, n=2; Jordan, n=2; Philippines, n=2; Portugal, n=2; Ukraine, n=2; Albania, n=1; Austria, n=1; Czech Republic, n=1; Egypt, n=1; France, n=1; Russia, n=1; Slovenia, n=1; unknown, n=1) and seven avian species (chicken, turkey, partridge, pheasant, goose, guinea fowl, quail) including different production categories of poultry (broiler, layer or breeder chickens, backyard chickens, meat turkeys or turkey breeders).

Out of these 290 *M. gallisepticum* strains including whole genome sequences and DNAs, 131 samples were used for the MLST studies, 250 for the development of MAMAs differentiating the F, 6/85 and ts-11 vaccine strains from field isolates, and 281 to test the MAMA designed for the identification of the K vaccine strain.

Background information of the tested *M. gallisepticum* samples are provided in Table S1.

4.1.2. Sample processing

The DNAs were extracted directly from clinical samples (e.g. tissue samples, swab samples or FTA cards) or from 200 µl of pure *M. gallisepticum* logarithmic-phase broth culture. Pure cultures of *M. gallisepticum* strains were isolated and cultured according to the following protocol: Swab samples taken from the choana or trachea of live birds were pooled, four swabs were placed into 2 ml sterile Frey broth (Frey *et al.*, 1968) (Sigma-Aldrich Inc., St. Louis, USA) immediately. In case of post mortem sampling, tracheal or lung tissue were collected. 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. The broth containing the samples was shaken well, filtered through a 0.45 µm cellulose acetate membrane filter (Sartorius Stedim Biotech GmbH., Göttingen, Germany) and incubated at 37 °C in an atmosphere of 5 % CO_2.

Following color change (red to yellow shift) of the phenol red indicator due to the metabolic activity of mycoplasmas, the culture was inoculated onto solid Frey's media (Frey *et al.*, 1968) (Sigma-Aldrich Inc.) and incubated at 37 °C in an atmosphere of 5 % CO_2 until visible colonies appeared. Filter cloning was performed to gain pure cultures from the isolates. When it was possible, cultures were filter cloned only once to minimize *in vitro* mutations of the isolates.

DNA extraction from 200 µl pure *M. gallisepticum* logarithmic-phase broth culture was performed using the ReliaPrep™ gDNA Tissue Miniprep System (Promega Inc., Madison, USA) according to the manufacturers' instructions for Gram-negative bacteria. *M. gallisepticum* positivity of the used samples was confirmed by conventional or real-time Taqman PCR, both targeting the second cytoadhesin-like protein-encoding gene (*mgc2*) of *M. gallisepticum*. The DNA of the *M. gallisepticum* type strain ATCC 19610 served as positive control, while nuclease-free water was used as negative control.

The conventional PCR amplified a 236-302 bp long part of the *mgc2* gene by using the following primer pair: mgc2-2F: 5'-CGCAATTTGGTCCTAATCCCCAACA-3'; mgc2-2R: 5'-TAAACCCACCTCCAGCTTTATTTCC-3' (Garcia *et al.*, 2005). PCR was carried out in 25 µl total volume, containing nuclease-free water, 5 µl of 5X Green GoTaq Flexi Buffer (Promega Inc.), 2 µl of $MgCl_2$ (25 mM; Promega Inc.), 0.8 µl of deoxynucleoside triphosphates (dNTP; 10 mM; Fermentas, Waltham, USA), 1 µl of each primer (10 pmol/µl), 0.2 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.) and 2 µl of target DNA solution. The conventional PCRs were performed using Biometra T3000 Personal (Biometra Inc., Göttingen, Germany) thermal cycler or Bio-Rad T100 or C1000 Touch (Bio-Rad Laboratories Inc., Hercules, USA) thermal cyclers throughout the study. The PCR consisted of initial denaturation for 3 minutes at 94 °C followed by 35 amplification cycles of denaturation for 30 seconds at 94 °C, primer annealing at 58 °C for 30 seconds and extension at 72 °C for 1 minute. The final extension step was performed for 5 minutes at 72 °C. The amplified gene products were detected by standard

electrophoresis (8 V/cm) loading 5 µl of each sample in 1% agarose gel (SeaKem LE Agarose, Lonza Group AG, Basel, Switzerland). As molecular weight marker, a 100-bp DNA ladder (O'RangeRuler 100 bp, Thermo Fisher Scientific Inc., Waltham, USA) was used. PCR products were visualized with ECO Safe nucleic acid gel stain (Pacific Image Electronics Inc., Torrance, USA) under UV light and documented photographically (Eastman Kodak Company, Rochester, USA) throughout the study.

The real-time PCR amplified a 95 bp long part of the *mgc2* gene by using the following primers: MG-forward: 5'-TTGGGTTTAGGGATTGGGATT-3'; MG-reverse: 5'-CCAAGGGATTCAACCATCTT-3'; MG-probe: 5'-TGATGATCCAAGAACGTGAAGAACACC-3' with 6-FAM fluorophore and BHQ quencher (Raviv and Kleven, 2009). PCR was carried out in 12 µl total volume, containing nuclease-free water, 1.5 µl of GeneAmp 10X Gold Buffer (Thermo Fisher Scientific Inc.), 1.5 µl of $MgCl_2$ (25 mM; Thermo Fisher Scientific Inc.), 0.5 µl of dNTP (10 mM; Fermentas), 0.5 µl of each primer (10 pmol/µl), 0.25 µl of Taqman probe (10 pmol/µl), 0.1 µl of AmpliTaq Gold DNA polymerase (5 U/µl; Thermo Fisher Scientific Inc.) and 2 µl of target DNA solution. The real-time PCRs were performed using Applied Biosystems Step-One Plus real-time PCR system with StepOne Software version 2.3 (Thermo Fisher Scientific Inc.) throughout the study. The PCR consisted of initial denaturation for 10 minutes at 95 °C followed by 40 amplification cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

The presence of other, contaminant mycoplasmas (i.e. *M. synoviae*) was excluded by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region of the Mollicutes by using the following primer pair: F1: 5'-ACACCATGGGAGCTGGTAAT-3'; R1: 5'-CTTCATCGACTTTCAGACCCAAGGCAT-3' (Lauerman *et al.*, 1995). PCR was carried out in 25 µl total volume, containing nuclease-free water, 5 µl of 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl $MgCl_2$ (25 mM; Promega Inc.), 0.5 µl dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl of target DNA solution. The PCRs consisted of initial denaturation for 5 minutes at 95 °C followed by 35 amplification cycles of denaturation for 30 seconds at 95 °C, primer annealing at 54 °C for 30 seconds, and extension at 72 °C for 1 minute. The final extension step was performed for 5 minutes at 72 °C. The amplified gene products were detected by agarose gel electrophoresis, visualized and documented photographically as described above (Figure 4). The PCR products (888-938 bp long in case of *M. gallisepticum*) were submitted to Sanger sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, USA). The reading errors of the chromatograms were trimmed by Lasergene package (DNASTAR Inc., Madison, USA) and corrected sequences were submitted to Standard Nucleotide BLAST search in order to identify the *Mycoplasma* species (http://www.ncbi.nlm.nih.gov/BLAST).

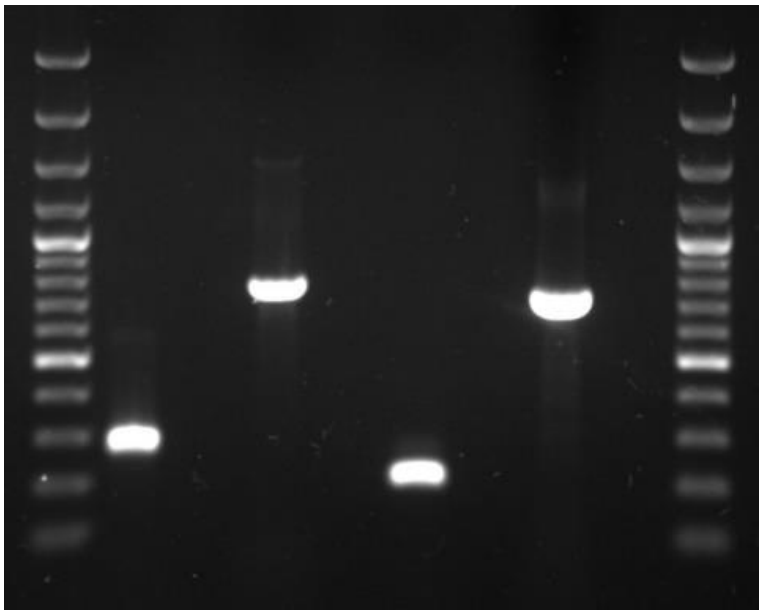


Figure 4. Identification of avian pathogen *Mycoplasma* species by specific PCR (Lauerman *et al.*, 1995) followed by agarose gel electrophoresis

100-bp DNA ladder was used as molecular weight marker (Line 1 and 10). Line 2: *M. iowae*; Line 4: *M. meleagridis*; Line 6: *M. synoviae*; Line 8: *M. gallisepticum*. Line 3, 5, 7, 9: negative control

4.2. Development of multi-locus sequence typing (MLST) assay for genotyping *M. gallisepticum* strains

4.2.1. Target selection and primer design

Based on literature data concerning genotyping different *Mycoplasma* species, several housekeeping genes were examined during the *M. gallisepticum* MLST target selection (data not shown). These corresponding genes of the 19 published *M. gallisepticum* whole genomes were aligned by Geneious software version 10.2.3. (Biomatters Ltd., Auckland, New Zealand) (Kearse *et al.*, 2012) and analyzed manually.

Targeted alleles had to meet the following criteria: 1) have to be single-copy genes which can be found in all published *M. gallisepticum* genomes; 2) possess highly diverse internal fragments 3) contain conserved regions suitable for designing primer pairs which do not form hairpin, self- or cross-dimers, have similar melting temperatures allowing their simultaneous application and eventuate an amplicon size of 300-800 bp making it suitable for Sanger sequencing.

Primer design was performed by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). The melting temperature (Tm) and general suitability of the designed primer sets were calculated by using NetPrimer software (Premier Biosoft International, Palo Alto, USA) (http://www.premierbiosoft.com/netprimer). The specificity of the primers was analyzed *in silico* using Standard Nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

4.2.2. Test of the designed primers, assay development

The designed primer pairs 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 carried out in 25 µl total volume containing nuclease-free water, 5 µl of 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl of $MgCl_2$ (25 mM; Promega Inc.), 0.5 µl of dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), 0.25 µl of GoTaq FlexiDNA polymerase (5 U/µl; Promega Inc.) and 1 µl of target DNA solution. The initial denaturation/enzyme activation for 2 minutes at 95 ºC was followed by 40 cycles consisting of denaturation step at 95 ºC for 30 seconds, primer annealing at 56 ºC for 30 seconds and extension at 72 ºC for 1 minute. A final extension step at 72 ºC for 5 minutes was also included. The amplified gene products were detected by standard electrophoresis (8 V/cm) loading 5 µl of each sample in 1% agarose gel (SeaKem LE Agarose, Lonza Group AG). As molecular weight marker, a 100-bp DNA ladder (O'RangeRuler 100 bp, Thermo Fisher Scientific Inc.) was used. The PCR products were submitted to Sanger sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). For each locus, all sequences were trimmed and aligned using the Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). An allelic number was assigned to each unique allele variant. The discriminatory power for each locus was determined by calculating Simpson's index of diversity (SI) (Hunter and Gaston, 1988).

The results of the preliminary study performed with the ten *M. gallisepticum* samples listed above were evaluated and genes showing the highest discriminatory power (SI) were selected for the MLST scheme.

4.2.3. Genotyping 131 *M. gallisepticum* strains by the developed MLST assay

The developed MLST assay based on the selected housekeeping genes was performed on 131 *M. gallisepticum* samples. The PCR, agarose gel electrophoresis, visualization, photographical documentation and Sanger sequencing were carried out as described above.

Trimmed sequences were concatenated in alphabetical order of the selected genes and aligned with all published corresponding sequences using the Geneious software version 10.2.3. (Biomatters Ltd.) (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 loci. The discriminatory power of the method and for each locus was determined by calculating Simpson's index of diversity (Hunter and Gaston, 1988).

Molecular phylogenetic analysis of the concatenated sequences containing the selected loci of the 131 *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.*, 2016).

Molecular phylogenetic analysis with the inclusion of an outgroup was also prepared. 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 (Bradbury *et al.*, 1993; Kempf, 1998; Volokhov *et al.*, 2012). Thus, for this purpose corresponding sequences of *M. imitans* type strain ATCC 51306 (GenBank accession numbers: NZ_JADI00000000 - NZ_JADI01000029; NZ_KI912416 - NZ_KI912419) were used. 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 in MEGA7.0.26 software (Nei and Saitou, 1987; Tamura, 1992; Kumar *et al.*, 2016).

4.2.4. Specificity and sensitivity tests

The specificity of the assays was tested *in vitro* with the following avian *Mycoplasma* species: *M. anatis* (ATCC 25524), *M. anseris* (ATCC 49234), *M. anserisalpingitidis* (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 the pure culture of the *M. gallisepticum* type strain ATCC 19610 were used in the range of 10^5-10^0 template copy number/µl. Template copy number was calculated with the help of an online tool (Staroscik, 2004) (http://cels.uri.edu/gsc/cndna.html) based on the DNA concentration measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). 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.

4.2.5. Statistical analyzes

The discriminatory power was determined by calculating Simpson's index of diversity (SI) (Hunter and Gaston, 1988) with 95% confidence intervals (CI) using the online tool Comparing Partitions (http://www.comparingpartitions.info/?link=Tool).

4.3. Development of mismatch amplification mutation assays (MAMAs) for differentiating *M. gallisepticum* vaccine strains from field isolates

4.3.1. Whole genome sequencing, target selection and primer design

Genomic DNAs of *M. gallisepticum* 6/85, ts-11 and K vaccine strains were extracted from 10 ml of logarithmic-phase broth cultures using a QIAamp DNA Mini kit (Qiagen Inc., Hilden, Germany). The DNAs were quantified fluorometrically on Qubit 2.0 equipment using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc.).

Next-generation sequencing of the *M. gallisepticum* 6/85 and ts-11 vaccine strains was performed on Ion Torrent platform (New England BioLabs, Hitchin, UK). DNA was subjected to enzymatic fragmentation using the reagents supplied in the NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent Kit (New England BioLabs). The library DNA was clonally amplified by Ion PGM Template Kit on Ion OneTouch 2 system (Thermo Fisher Scientific Inc.). The *M. gallisepticum* K vaccine strain was submitted to the next-generation sequencing on Illumina NextSeq 500 platform (Illumina Inc., San Diego, USA) with NextSeq 500/550 High Output Kit v2.5 (Illumina Inc.). DNA libraries were prepared with the Nextera Mate Pair Library Preparation Kit (Illumina Inc.).

The quality of the short reads were checked with FastQC software version 0.11.8. (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Babraham Bioinformatics, The Babraham Institute, Babraham, UK).

Reads were mapped to *M. gallisepticum* strain R_{low} (GenBank accession number: AE015450) as reference genome and annotated by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). The draft whole genomes of the *M. gallisepticum* vaccine strains and 19 published *M. gallisepticum* genomes were aligned. The SNPs specific for each vaccine strains were explored by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). Target mutations were selected based on the following criteria: 1) SNP is present in a single-copy gene which can be found in the genomes of all examined *M. gallisepticum* strains; 2) SNP results in amino acid change in the protein encoded by the gene 3) SNP is surrounded by conserved regions suitable for primer design. Numbering of nucleotide positions was according to the individual genes of *M. gallisepticum* strain R_{low} (GenBank accession number:

AE015450). The selected SNPs were used as targets for mismatch amplification mutation assays.

Primer design was performed by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). All primer sets consisted of two competing forward primers and a consensus reverse primer. At the 3′ end, competing primers were allele-specific and a single antepenultimate destabilizing mismatch was inserted in each allele-specific primer to enhance the discriminative capacity of the assays. Vaccine-specific primers were marked with an additional 14 bp long GC-clamp at the 5’ end to increase the size and melting temperature of the amplicon. The primers were constructed to limit amplicon lengths of ≤110 bp. The melting temperature (Tm) and general suitability of the designed primer sets were calculated by using NetPrimer software (Premier Biosoft International) (http://www.premierbiosoft.com/netprimer). The specificity of the primers was analyzed *in silico* using Standard Nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

4.3.2. Test of the designed primers, assay development

After *in silico* analysis on the available sequences, preliminary examinations were performed to test the designed primers using the DNA of the targeted vaccine strain and as wild-type controls, the *M. gallisepticum* ATCC 19610 type strain and the other non-targeted vaccine strains. Nuclease-free water was used as negative control in all PCR assays.

Melt-MAMA PCR mixture consisted of nuclease-free water, 2 µl 5X Colour-less GoTaq Flexi Buffer (Promega Inc.), 1 µl $MgCl_2$ (25 mM; Promega Inc.), 0.3 µl dNTP (10 mM; Fermentas), 0.5 µl EvaGreen (20X, Biotium Inc., Hayward, USA), 0.15 µl / 0.6 µl of each primer (10 pmol/µl), 0.08 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl target DNA solution with a final volume of 10 µl. Thermocycling parameters were 95 °C for 10 minutes, followed by 30 cycles / 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. PCR products were subjected to melt analysis using a dissociation protocol comprising 95 °C for 15 seconds, followed by 0.3 °C incremental temperature ramping from 60 °C to 95 °C. EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature.

Agarose-MAMA PCR reactions were carried out in 25 µl total volume containing nuclease-free water, 5 µl 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl $MgCl_2$ (25 mM, Promega Inc.), 0.5 µl dNTP (10 mM, Fermentas), 1 µl / 4 µl of each primer (10 pmol/µl), 0.2 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl target DNA solution under the following PCR conditions: 95 °C for 5 minutes followed by 35 cycles / 40 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The final elongation step was performed at 72 °C for 5 minutes. The amplified gene products were detected by standard electrophoresis (8 V/cm) loading 2.5 µl of each sample in 3% agarose gel (MetaPhor Agarose, Lonza Group AG).

As molecular weight marker, a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific Inc.) was used.

Discriminating potential of the assays was examined based on the following criteria: 1) peaks of the melting curves and band sizes of the two genotypes (vaccine-type and wild-type) were distinguishable; 2) negative control did not amplify or overlap the peaks of the vaccine or the wild-type strains; 3) the mutation was specific to the vaccine strain when all available samples with known vaccination status were tested.

The results of the preliminary examinations were evaluated and the assays showing the highest discriminating potential were selected for further tests. The DNAs of additional 250 (in case of the F, 6/85 and ts-11 differentiating assays) or 281 (in case of the assay developed for differentiation of the K vaccine strain) *M. gallisepticum* isolates including pure cultures and clinical samples were investigated to test the reliability of the developed assays.

Samples which appeared to be ts-11 re-isolates by the developed assays and/or originating from Australia were further tested according to Ricketts *et al.* (2017). In brief, the presence of three additional genes (*vlhA3.04a*, *vlhA3.05* and *mg0359*) was investigated for the discrimination of field isolates from ts-11 vaccine strains. Primer sequences are indicated in Table 1.

Table 1. Examined alleles and primer sequences used for the detection of *M. gallisepticum* ts-11 vaccine strain by Ricketts et al. (2017)

Genes	Primers	Primer sequences (5'-3')
vlhA3.04a	F	TACTGAAAACGCTGATGGAC
	R	GCCACTAGTTCCTGCTGCAT
vlhA3.05	F	CATCCGATAATGTAGGGCTTG
	R	TGCAGAGCTAGATTGATTTCCA
mg0359	F	GGGAGACAGAGCAAGAAATATCA
	R	AGGGAACAATTTATCTCAATCTGAA

PCR was carried out in 25 µl total volume, containing nuclease-free water, 2.5 µl of GeneAmp 10X Gold Buffer (Thermo Fisher Scientific Inc.), 2.5 µl of $MgCl_2$ (25 mM; Thermo Fisher Scientific Inc.), 1 µl of dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), 0.2 µl of AmpliTaq Gold DNA polymerase (5 U/µl; Thermo Fisher Scientific Inc.) and 1.5 µl target DNA solution. PCRs consisted of initial denaturation for 4 minutes at 94 °C followed by 35 amplification cycles of denaturation for 30 seconds at 94 °C, primer annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds. The final extension step was performed for 10 minutes at 72 °C.

The amplified gene products were detected by standard electrophoresis (8 V/cm) loading 5 µl of each sample mixed with 1 µl of loading dye (6X DNA Loading Dye; Thermo Fisher Scientific Inc.) in 1% agarose gel (SeaKem LE Agarose, Lonza Group AG). As molecular weight marker, a 100-bp DNA ladder (O'RangeRuler 100 bp, Thermo Fisher Scientific Inc.) was used.

4.3.3. Stability, specificity and sensitivity tests

In the stability tests of the mutations targeted by the designed assays, the vaccine strains and the *M. gallisepticum* ATCC 19610 type strain were subjected to serial passage: 100 µl broth culture was inoculated into 900 µl Frey's medium (Frey *et al.*, 1968) (Sigma-Aldrich Inc.) 10 times. Each passage resulted in ~3.322 population doubling ($PD=\log(N_f/N_i)/\log 2$ where N_f is the final number of cells, N_i is the initial number of cells), thus cumulative population doubling was ~33.22 to the end of the 10-step serial passage (cPD=10PD) (Choi *et al.*, 2017). After the 10^{th} passage, DNAs were extracted, the designed assays were performed and comparisons were made between genotypes of the parent and derivative strains.

The specificity of the assays was tested using the following avian *Mycoplasma* species: *M. anatis* (ATCC 25524), *M. anseris* (ATCC 49234), *M. anserisalpingitidis* (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 dilutions of each genotype were used in the range of 10^6-10^0 template copy number/µl. Template copy number was calculated with the help of an online tool (Staroscik, 2004) (http://cels.uri.edu/gsc/cndna.html) based on the DNA concentration measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). This method was verified by the qPCR system targeting the *mgc2* gene (Raviv and Kleven, 2009) as described above (Subsection 4.1.2.). Tenfold dilution series of a synthetic sequence (500ng; gBlock, Integrated DNA Technologies Inc., Coralville, USA) containing a 95 bp long fragment of the *mgc2* gene of *M. gallisepticum* (between nucleotides 220622-220716, according to nucleotide numbering of the *M. gallisepticum* type strain NCTC 10115, GenBank accession number: NZ_LS991952) was used as control for the template copy number determinations. The lowest template copy numbers yielding melting temperature (Tm) specific to the genotypes were considered as the detection limit of the assays.

In order to assess the capability of the assays to identify mixed population of the two genotypes in a single specimen, different template copy number combinations of the *M. gallisepticum* ATCC 19610 type strain and the vaccine strains were tested in separate PCRs. The mixtures contained the type strain and the vaccine strain in the following combinations: constant template copy numbers (10^6 copies/µl) of one strain was paired with a member of a series of 10-fold DNA dilutions (10^6-10^3 copies/µl) of the other strain and vice versa.

4.3.4. Statistical analyzes

Adjusted Rand co-efficient with 95 % confidence intervals (CI) was used to determine the congruency of the assays in the comparisons (Pinto *et al.*, 2007). Rand co-efficient was indicated when adjusted Rand co-efficient was not able to detect congruency (adjusted Rand co-efficient: 0.000). Values were calculated with the help of the online tool Comparing Partitions (http://www.comparingpartitions.info/?link=Tool). Samples which showed false negative results in any of the compared assays were excluded from the statistical analyzes.

4.4. Detecting mutations potentially associated with decreased susceptibility to certain antibiotics in *M. synoviae* strains

4.4.1. Sample collection

In total, 96 *M. synoviae* strains, including the *M. synoviae* type strain NCTC 10124 (GenBank accession number: CP011096), the MS-H (Vaxsafe® MS, Bioproperties Pty Ltd., Ringwood, Australia; GenBank accession number: KP704286) and MS1 (Nobilis® MS Live, MSD Animal Health Hungary, Budapest, Hungary) vaccine strains and 93 field isolates were investigated in the present study. Samples were selected to provide a diverse *M. synoviae* strain collection for the analysis concerning the geographical location and date of isolation, however, antibiotic susceptibility profile was the primary consideration when samples were chosen for further investigations. The isolation year of the 93 field strains were between 1982 and 2019, but the majority of these samples (n=84) was collected during the past decade (2010-2019). The samples originated from chickens (n=65) and turkeys (n=28) and from 18 different countries (Hungary, n=25; Italy, n=22; the Netherlands, n=9; Israel, n=4; Spain, n=4; Austria, n=3; Czech Republic, n=3; Russia, n=3; Slovenia, n=3; Ukraine, n=3; USA, n=3; Jordan, n=2; Korea, n=2; Lebanon, n=2; Tunisia, n=2; China, n=1; Serbia, n=1; Taiwan, n=1). The whole genome sequence of the *M. synoviae* strain MS53 (GenBank accession number: AE017245) was also used for this study, as reference genome.

Background information of the used *M. synoviae* strains are provided in Table S2.

4.4.2. Sample processing

Isolation of *M. synoviae* strains was performed similarly to the isolation of *M. gallisepticum* described above (Subsection 4.1.2.).

DNA extraction from 200 µl pure *M. synoviae* logarithmic-phase broth culture was performed using the ReliaPrep™ gDNA Tissue Miniprep System (Promega Inc.) according to the manufacturers' instructions for Gram-negative bacteria. *M. synoviae* positivity of the used samples was confirmed by conventional or real-time Taqman PCR. The DNA of the *M. synoviae* type strain NCTC 10124 served as positive control, while nuclease-free water was used as negative control.

The conventional PCR amplified a 207 bp long part of the 16S rRNA coding sequence of the *M. synoviae* by using the following primer pair: MS-1: 5'-GAAGCAAAATAGTGATATCA-3'; MS-2: 5'-GTCGTCTCCGAAGTTAACAA-3' (Wang *et al.*, 1997). PCR was carried out in 25 µl total volume, containing nuclease-free water, 5 µl of 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl of $MgCl_2$ (25 mM; Promega Inc.), 0.8 µl of dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), 0.2 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.) and 2.5 µl of target DNA solution. The PCRs consisted of initial denaturation for 5 minutes at 94 °C followed by 35 amplification cycles of denaturation for 1 minute at 94 °C, primer annealing at 50 °C for 1 minute and extension at 72 °C for 2 minutes. The final extension step was performed for 10 minutes at 72 °C. The amplified gene products were detected by standard electrophoresis (8 V/cm) loading 5 µl of each sample in 1% agarose gel (SeaKem LE Agarose, Lonza Group AG). As molecular weight marker, a 100-bp DNA ladder (O'RangeRuler 100 bp, Thermo Fisher Scientific Inc.) was used.

The real-time PCR amplified a 119 bp long part of the 16S/23S rRNA intergenic spacer region of the *M. synoviae* by using the following primers: MS-forward: 5'-CTAAATACAATAGCCCAAGGCAA-3'; MS-reverse: 5'-CCTCCTTTCTTACGGAGTACA-3'; MS-probe: 5'-AGCGATACACAACCGCTTTTAGAAT-3' with 6-FAM fluorophore and BHQ quencher (Raviv and Kleven, 2009). The polymerase chain reaction was performed as it has been described above in case of *M. gallisepticum* (Subsection 4.1.2.).

The presence of other, contaminant mycoplasmas (i.e. *M. gallisepticum*) was excluded by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region of the Mollicutes, resulting in a 473-494 bp long PCR product in case of *M. synoviae* (Lauerman *et al.*, 1995). The PCR, agarose gel electrophoresis, visualization, photographical documentation, Sanger sequencing and sequence BLAST were carried out as it has been described above in case of *M. gallisepticum* (Subsection 4.1.2.).

4.4.3. Broth microdilution method

Antibiotic susceptibility profiles of *M. synoviae* strains were determined by broth microdilution method according to the standard guidelines and recommendations of Hannan (2000). The number of microorganisms used for the MIC determination was standardized in order to obtain comparable results. Accepted numbers of microorganisms for the MIC tests were 10^4-10^5 color changing unit (CCU/ml). Determination of this number was performed with a 10-fold dilution series of *Mycoplasma* suspension in liquid Frey's media (Frey *et al.*, 1968) (Sigma-Aldrich Inc.) containing phenol red. The highest dilution, which still resulted in a color change was regarded to contain 10^0 bacterium.

The following antimicrobial agents were examined during the broth microdilution tests: two fluoroquinolones: enrofloxacin and difloxacin; three tetracyclines: doxycycline, oxytetracycline and chlortetracycline; one aminocyclitol: spectinomycin; one aminoglycoside: neomycin; three macrolides: tylosin, tilmicosin and tylvalosin; one lincosamide: lincomycin; one phenicol: florfenicol; and two pleuromutilins: tiamulin and valnemulin; all products originated from VETRANAL (Sigma-Aldrich Inc.) except for tylvalosin (Aivlosin), which was purchased from ECO Animal Health Ltd. (London, UK). The antibiotics were diluted and stored according to the recommendations of Hannan (2000). Stock solutions of 1 mg/ml fluoroquinolones were prepared in 0.1 M NaOH; stock solution of 1 mg/ml florfenicol was prepared in 96 % ethanol and in sterile distilled water; and the rest of the stock solutions of 1 mg/ml were prepared in sterile distilled water and stored at -70 °C. Freshly prepared two-fold dilutions were used in each microtest after checking the thawed antibiotic solutions for any visible changes in their consistency.

Although official MIC breakpoints for *M. synoviae* have not been determined yet, the examined concentration range of the antibiotics was selected to represent previously suggested high and low MIC values as well (Gautier-Bouchardon *et al.*, 2002; Gerchman *et al.*, 2008; Landman *et al.*, 2008; Kreizinger *et al.*, 2017a; Gautier-Bouchardon, 2018) (Table 2 and Table S4).

The 96-well microtiter plates were designed to contain the twofold dilution series of the antibiotic, a growth control (Frey broth medium without antibiotic), a sterility control (Frey broth medium without antibiotic and *Mycoplasma* inoculum) and a pH control (Frey broth medium adjusted to pH 6.8). The duplicates of maximum three clinical isolates and the duplicate of the *M. synoviae* type strain NCTC 10124 were tested on each plate (Figure 5). The reference strain was included in the test to confirm the validity of the results. The microtiter plates were sealed with adhesive film and incubated at a temperature of 37 °C. The MIC value against each isolate was defined as the lowest concentration of the antibiotic that completely inhibited the growth in the broth, i.e. no color change has been observed. The MIC values were read daily and recorded as soon as the growth controls changed color.

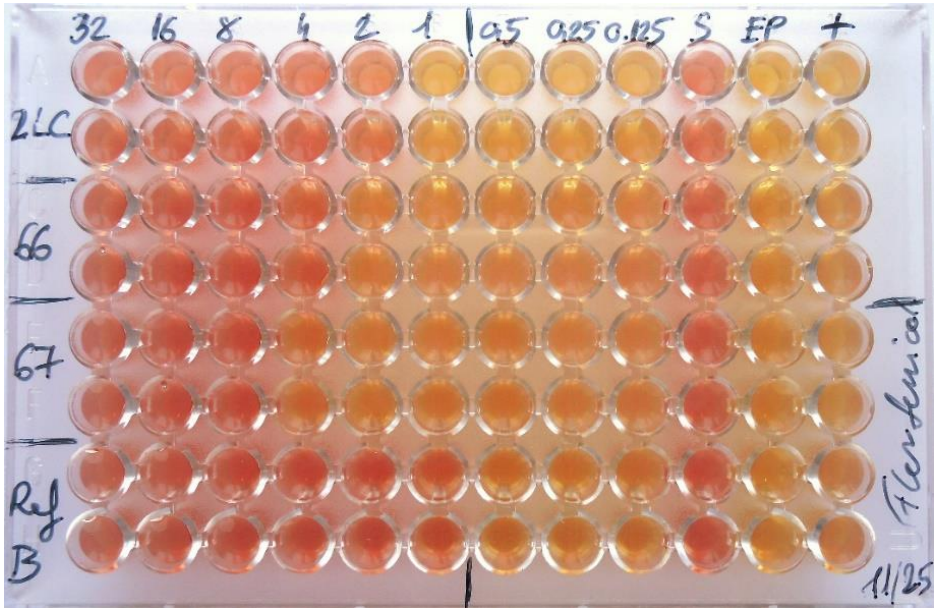


Figure 5. Minimal inhibitory concentration determination of florfenicol with broth microdilution method against four *M. synoviae* isolates

MIC values (expressed as µg/ml) to florfenicol were 2 µg/ml, 4 µg/ml, 8 µg/ml and 1 µg/ml for three isolates and the *M. synoviae* type strain NCTC 10124, respectively. S: sterility control; EP: end point control; +: growth control

The MIC values were interpreted based on the paper of Kempf *et al.* (1989), Gautier-Bouchardon *et al.* (2002), Behbahan *et al.* (2008), Gerchman *et al.* (2008), Landman *et al.* (2008), and van Duijkeren *et al.* (2014). MIC values belonged to susceptible strains according to these publications were considered as low MIC values in this study, while MIC values of resistant strains reported in these papers were considered as high (elevated) MIC values. Intermediate MICs were classified as elevated MIC values, except in the case of fluoroquinolones, in order to divide the examined population into comparable groups based on susceptibility for each antibiotic (Table 2).

Table 2. Details of MIC determination by broth microdilution method

Antimicrobial agents	Tested concentration range (µg/ml)*	Strains tested	Elevated MIC above (µg/ml)
enrofloxacin	0.031 - 64	n=95	1.25
difloxacin	0.039 - 10	n=74	1.25
oxytetracycline	0.25 - 64	n=84	4
chlortetracycline	0.25 - 64	n=84	4
doxycycline	0.039 - 16	n=83	4
spectinomycin	0.25 - 64	n=73	2
neomycin	0.25 - 64	n=78	2
tilmicosin	0.016 - 64	n=87	8
tylosin	0.008 - 64	n=87	1
tylvalosin	0.005 - 64	n=82	0.5
lincomycin	0.031 - 64	n=84	2
florfenicol	0.125 - 32	n=92	2
tiamulin	0.008 - 32	n=91	8
valnemulin	0.039 - 10	n=70	0.125

*Data indicate the minimum and maximum concentrations of the antibiotics used in this study, exact range of the tested concentrations can differ in each strain (Table S4).

4.4.4. Whole genome sequencing and sequence analysis

Genomic DNAs of the pure *M. synoviae* cultures were extracted from 10 ml of logarithmic-phase broth cultures using QIAamp DNA Mini Kit (Qiagen Inc.). The DNA was quantified fluorometrically on Qubit 2.0 equipment using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc.).

Next-generation sequencing of 77 *M. synoviae* field isolates and the vaccine strain MS1 was performed on Ion Torrent platform (New England BioLabs), while an additional 16 *M. synoviae* field isolates were submitted to whole genome sequencing on Illumina NextSeq 500 next-generation sequencing platform (Illumina Inc.) as it has been described above (4.3.1.).

The quality of the short reads were checked with FastQC software version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Babraham Bioinformatics, The Babraham Institute, Babraham, UK).

Reads were mapped to *M. synoviae* strain MS53 as reference genome and annotated by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). Genomic regions which have been previously linked to antibiotic resistance in several *Mycoplasma* species were investigated: *gyrA* and *gyrB* genes encoding the two subunits (GyrA and GyrB) of the DNA gyrase (topoisomerase II) enzyme and *parC* and *parE* genes encoding the two subunits (ParC and ParE) of the topoisomerase IV enzymes for fluoroquinolones (Le Carrou *et al.*, 2006; Ben Shabat *et al.*, 2010; Lysnyansky *et al.*, 2013; Khalil *et al.*, 2016; Sulyok *et al.*, 2017); 16S rRNA coding genes (*rrsA* and *rrsB*) for aminoglycosides and tetracyclines (Idowu et al., 2003; Amram *et al.*, 2015); 23S rRNA coding genes (*rrlA* and *rrlB*) and 50S ribosomal proteins L3, L4 and

L22 for macrolides, pleuromutilins, lincosamides and phenicols (Kobayashi *et al.*, 2005; Li *et al.*, 2010; Tagg *et al.*, 2013; Lerner *et al.*, 2014; Lysnyansky *et al.*, 2015; Ammar *et al.*, 2016; Le Roy *et al.*, 2016; Li *et al.*, 2016).

These genes of *M. synoviae* strains were aligned to detect SNPs by Geneious software (Biomatters Ltd.) (Kearse *et al.*, 2012). In case of protein coding genes (*gyrA*, *gyrB*, *parC*, *parE*, *rplC*, *rplD*, *rplV*) only non-synonymous mutations were included in the study, while all mutations found in the 16S and 23S rRNA coding genes (*rrsA*, *rrsB*, *rrlA*, *rrlB*) were investigated. Numbering of nucleotide and amino acid positions according to *Escherichia coli* strain K-12 substrain MG1655 (GenBank accession number: U00096) was determined to enable the comparison of our results with literature data and indicated where it was necessary. However, nucleotide and amino acid positions referred throughout the text were numbered based on the individual genes and proteins of *M. synoviae* strain MS53 in order to avoid misunderstandings due to gaps generated in the alignment of the corresponding genes of *M. synoviae* and *E. coli*. For the identification of potentially resistance-related SNPs, the correlation between the MIC values and the occurrence of several mutations were analyzed. To this end, the examined *M. synoviae* strains were sorted by their MIC values for each antibiotic and mutations detected in the strains more frequently as MIC values increased or occurred exclusively in isolates with high MIC values were investigated individually. Neighboring or closely located mutations have been evaluated together. Mutations, which have been detected in a large number of strains with high MIC values (at least 33.33 %) or identified at positions previously linked to antibiotic resistance in several *Mycoplasma* species were considered as potentially resistance-related mutations and presented in the study.

4.4.5. Molecular phylogenetic analysis

Molecular phylogenetic analysis of the examined 96 *M. synoviae* strains was performed in order to investigate their genetic diversity and present their phylogenetic relationships and the distribution of strains with elevated and low MIC values for the tested antibiotics in terms of their location on a phylogenetic tree. Concatenated sequences of seven loci were analyzed with multi-locus sequence typing (El-Gazzar *et al.*, 2017) by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al.*, 1985; Kumar *et al.*, 2016) with standard error estimated through 1000 bootstrap replicates in MEGA-X software.

4.4.6. Differentiation of the *rrlA* and *rrlB* genes and determination of the nucleotide at position 2054.

As whole genome sequencing was not able to distinguish between reads belonging to *rrlA* and *rrlB* genes, relevant positions (nucleotide position 2054) with different nucleotides (adenine or guanine) in the two *rrl* genes (IZSVE/2013/2094/D13/10-f, IZSVE/2014/589/D14/1-f-E, IZSVE/2015/2519/D15/1-f, IZSVE/2016/1695/MAV16/2-f, IZSVE/2016/2958/MAV16/11-f, IZSVE/2016/3274/MAV16/3-f) had to be investigated with additional PCR systems. To this end, a PCR was developed for the specific amplification of the partial sequences of 23S rRNA genes *rrlA* and *rrlB*. A common reverse primer was designed targeting the internal sequence of the 23S rRNA genes. Forward primers were developed to bind to the conserved regions of predicted genes encoding hypothetical proteins located close to the *rrlA* and *rrlB* genes. Primer design was performed by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). The melting temperature (Tm) and general suitability of the designed primer sets were calculated by using NetPrimer software (Premier Biosoft International, Palo Alto, USA) (http://www.premierbiosoft.com/netprimer). The specificity of the primers was analyzed *in silico* using Standard Nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

The PCR was carried out in 25 µl total volume, containing nuclease-free water, 5 µl of 5X Color-less GoTaq Flexi Buffer (Promega Inc.), 2.5 µl MgCl2 (25 mM; Promega Inc.), 0.5 µl dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl target DNA solution. The PCRs consisted of initial denaturation for 5 minutes at 95 °C followed by 35 amplification cycles of denaturation for 30 seconds at 95 °C, primer annealing at 54 °C for 30 seconds, and extension at 72 °C for 1 minute. The final extension step was performed for 5 minutes at 72 °C.

The amplified PCR products were submitted to a MAMA test (Birdsell *et al.*, 2012). Primer design was performed by Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012). The primer set consisted of a consensus reverse primer and two competing forward primers designed to specifically target the questionable nucleotide (adenine or guanine) at position 2054. At the allele-specific 3′ end of the competing primers, a single antepenultimate destabilizing mismatch was inserted to enhance the discriminative capacity of the assay. The primer specific for guanine at the 3’ end was marked with an additional 14 bp long GC-clamp at the 5’ end to increase the size and melting temperature of the amplicon. The primers were constructed to limit amplicon lengths of ≤100 bp. The general suitability of the designed primer set was calculated by using NetPrimer software (Premier Biosoft International) (http://www.premierbiosoft.com/netprimer). The specificity of the primers was analyzed *in silico* using Standard Nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). The melt-MAMA PCR mixture consisted of nuclease-free water, 2 µl 5X Color-less GoTaq Flexi Buffer (Promega Inc.), 1 µl MgCl2 (25 mM; Promega Inc.), 0.3 µl dNTP (10 mM; Fermentas), 0.5 µl

EvaGreen (20X, Biotium Inc.), 0.15 µl of each primer (10 pmol/µl), 0.08 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl target DNA solution with a final volume of 10 µl. Thermocycling parameters were 95 °C for 10 minutes, followed by 30 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. PCR products were subjected to melt analysis using a dissociation protocol comprising 95 °C for 15 seconds, followed by 0.3 °C incremental temperature ramping from 60 °C to 95 °C. EvaGreen fluorescence intensity was measured at 525 nm at each ramp interval and plotted against temperature. *M. synoviae* strains with analogous gene duplicates concerning nucleotide position 2054 (adenine in both genes or guanine in both genes) were used as positive controls, while nuclease-free water was utilized as negative control.

Sequences of primers used for the conventional PCRs and the subsequent melt-MAMA tests are indicated in Table 3.

Table 3. Data of amplicons and primers used for the differentiation of the two *rrl* genes and the determination of the nucleotide at position 2054

PCR systems	Position in the genome (bp)[a]	Primers	Primer sequences (5'-3')	Tm (°C)	Product size
conventional PCR	661693-661720	rrlA-F	TTGGTTCTTGATCTAATTTAACTTCTTT		1138 bp
	774826-774851	rrlB-F	GCCAAAGAATTTAGTTTCATTATTTG		1118 bp
	662808-662830; 775921-775943	rrl-R	CGCAATGATCTCTCAACTGTCTC		
melt-MAMA	662749-662768; 775862-775881	rrl-2054-G	ggggcggggcggggGGTACCCGCATCAAGACCAG	82.2 ± 0.3	88 bp
		rrl-2054-A	GGTACCCGCATCAAGACAAA	76.9 ± 0.6	74 bp
	775805-775828; 662692-662715	rrl-2054-con	CACATGTTAGGCCAAATTTCAATA		

Two primer sets (the forward primer rrlA-F or rrlB-F with the common reverse primer rrl-R) were used for the conventional PCR, while one primer set (competing rrl-2054-G and rrl-2054-A primers with the consensus rrl-2054-con primer) was used for the melt-MAMA test. [a]according to *M. synoviae* strain MS53; Tm: melting temperature of the amplicons

4.4.7. Investigating the presence of different *tet* genes

In order to investigate *in silico* the possible presence of *tet* genes in *M. synoviae* strains with high MIC values to tetracyclines, reads of these strains were submitted to analysis by SPAdes software version 3.11.1. (Center for Algorithmic Biotechnology, Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, Russia) (Bankevich *et al.*, 2012). Sequences of primers designed for the detection of *tet*(L), *tet*(M), *tet*(O), *tet*(R) and *tet*(S) genes in different *Mycoplasma* species (Table 4) and sequences of publicly available *tetM* genes of *Mycoplasma* and *Ureaplasma* species (*Mycoplasma gallisepticum* strain GDMT09 *tet*(M) gene, 398 bp, GenBank accession number: GQ424446; *Mycoplasma hominis* strain

Sprott complete genome *tet*(M) gene, 1,920 bp, GenBank accession number: CP011538; *Ureaplasma parvum* strain Ply157 *tet*(M) gene, 1,563 bp, GenBank accession number: KT267561; *Ureaplasma urealyticum tet*(M) gene, 4,793 bp, GenBank accession number: U08812) were mapped to the draft genomes of these strains using Geneious software version 10.2.3. (Biomatters Ltd.) (Kearse *et al.*, 2012).

Beside these *in silico* examinations, *tet*(M) positivity of the *M. synoviae* strains with high MIC values to tetracyclines were investigated with a conventional PCR by using the following primer pair: tetM-F1: 5'-GCAGTTATGGAAGGGATACG-3'; tetM-R1: 5'-TTCTTGAATACACCGAGCAG-3' (Shahid *et al.*, 2014b). PCR was carried out in 25 µl total volume, containing nuclease-free water, 5 µl of 5X Green GoTaq Flexi Buffer (Promega Inc.), 2.5 µl of $MgCl_2$ (25 mM; Promega Inc.), 0.5 µl of dNTP (10 mM; Fermentas), 1 µl of each primer (10 pmol/µl), 0.25 µl of GoTaq Flexi DNA polymerase (5 U/µl; Promega Inc.) and 1 µl target DNA solution. The PCRs consisted of initial denaturation for 2 minutes at 95 °C followed by 40 amplification cycles of denaturation for 30 seconds at 95 °C, primer annealing at 50 °C for 30 seconds and extension at 72 °C for 1 minute. The final extension step was performed for 5 minutes at 72 °C. The amplified gene products were detected by standard electrophoresis (8 V/cm) loading 5 µl of each sample in 1% agarose gel (SeaKem LE Agarose, Lonza Group AG). As molecular weight marker, a 100-bp DNA ladder (O'RangeRuler 100 bp, Thermo Fisher Scientific Inc.) was used. The DNA of a transformed *M. synoviae* strain possessing the *tet*(M) gene served as positive control, while nuclease-free water was used as negative control.

Table 4. Data of primers mapped to the draft genomes of *M. synoviae* strains with high MIC values for tetracyclines

Reference	*Mycoplasma* species	Genes	Primers	Primer sequences (5'-3')
Amram *et al.*, 2015	*M. bovis*	*tet*(L)	tetL-1	CCACCTGCGAGTACAAACTGG
			tetL-3	TCGGCAGTACTTAGCTGGTGA
			tetL-up	ATAAATTGTTTCGGGTCGGTAAT
			tetL-rev	AACCAGCCAACTAATGACAATGAT
		tet(M)	tetM-1	GACAAAGGTACAACGAGGACGGA
			tetM-2	TCCCTCTATTACCGTATCCCAT
			tetM-up	AGTTTTAGCTCATGTTGATG
			tetM-rev	TCCGACTATTTAGACGACGG
			tetM-Fw	ACAGAAAGCTTATTATATAAC
			tetM-Rv	TGGCGTGTCTATGATGTTCAC
		tet(O)	tetO-Fw	ACGGARAGTTTATTGTATACC
			tetO-Rv	TGGCGTATCTATAATGTTGAC
			tetO-1	GGTGCAATTGCAGAACCAGG
			tetO-2	CCAGTTCTGACATTTTAAACGG
			tetO-3	GCTATTGGAGTTATTTACCC
			tetO-4	ACAGACAATGACGATATGGA
			tetO-5	GCTTCCAATAGGGAGCGGCT
			tetO-6	AGACCGCTTAGAAAAGCAGA
			tetO-7	CAAACAGGACACAATATCCA
			tetO-up	AGCGTCAAAGGGGAATCACTATCC
			tetO-rev	CGGCGGGGTTGGCAAATA
Filioussis *et al.*, 2013; 2014	*M. agalactiae*	*tet*(M)	tetM-Fw2	GTRAYGAACTTTACCGAATC
			tetM-Rv1	ATCGYAGAAGCGGRTCAC
		tet(O)	M6	GTTATCACGGAAGYGCWA
			M4	GAAGCCCAGAAAGGATTYGGT
		tet(S)	tetS-Fw1	ATTGCAGAACTTGAAAAGGA
			tetS-Rv1	CATTTGGACCTCACCTAAAA
Breton *et al.*, 2010	*M. agalactiae*	*tet*(R)	tetR-BglII-F	CAACGAAGATCTCATATGTCTAGATTAGATAA
			tetR-BamHI-R	GGCAGGATCCTTAAGACCCACTTTCACAT
Dégrange *et al.*, 2008	*M. hominis*	*tet*(M)	tetM-1	GAACTGTATCCTAATGTGTG
			tetM-2	ATACTCTAACCGAATCTCG
Blanchard *et al.*, 1992	*M. hominis*		sense	TTATCAACGGTTTATCAGG
			antisense	CGTATATATGCAAGACG

5. Results

5.1. Development of multi-locus sequence typing (MLST) assay for genotyping *M. gallisepticum* strains

Among the examined housekeeping genes, 15 loci (*adk*, *atpG*, *dnaA*, *dnaN*, *fusA*, *gltX*, *lepA*, *leuS*, *pta*, *rpoB*, *ruvB*, *tpiA*, *tuf*, *ugpA*, *uvrA*) met the requirements described above (Subsection 4.2.1.). After performing a preliminary 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 scheme based on their genomic location and high diversity.

The chromosomal locations of these six genes, as shown in Table 5, suggests that it is unlikely for any of them to be co-inherited 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. anserisalpingitidis* 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 template copy number/µl for all selected loci. This sensitivity was enough to investigate the 12 tested field samples as well.

Novel sequence data of the six individual loci of the MLST scheme for the 107 tested *M. gallisepticum* isolates and field samples, and the 6/85, ts-11 and K vaccine strains have been deposited in Genbank (*atpG* sequences correspond to accession numbers MK288880-MK288986, MK289522, MH544230-MH544231; *dnaA* to MK288987-MK289093, MK289523, MH544232-MH544233; *fusA* to MK289094-MK289200, MK289524, MH544234-MH544235; *rpoB* to MK289201-MK289307, MK289525, MH544236-MH544237; *ruvB* to MK289308-MK289414, MK289526, MH544238-MH544239; and *uvrA* to MK289415-MK289521, MK289527, MH544240-MH544241).

Based on concatenated sequences of the six loci, the 131 *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 131 *M. gallisepticum* samples into 57 STs resulted in a Simpson's index of diversity of 0.958.

Table 5. Consecutive steps of target gene selection

Gene	Position in the genome of R_{low} (bp)	Primers	Primer sequences (5'-3')	Target selection	Nr. of SNPs/length of the examined locus (bp)	Simpson's index of diversity
adk	77499-78143	adk-F	TCTATTTCGCTTTTATGCTAAAGAAGA	excluded due to insufficient amplification		
		adk-R	TGCTTACCACACTAGGATCATCATC			
gltX	995377-996873	gltX-F	ACCACCAATTTATTCAAGAAAGTGTT			
		gltX-R	AAATAACCTTTTTTTCTAAACCCTTCA			
leuS	614706-617123	leuS-F	GATCATCCCTGTGATTAAGGCTG			
		leuS-R	TTCCCTTTTGATTTTGACATCTTG			
tpiA	808902-809660	tpiA-F	TTGAAAAACATATAAAACTACTCAAGAAGTTA			
		tpiA-R	GCCCAAACTGGTTCATAAGCA			
tuf	329567-330751	tuf-F	CTGCTCAAATGGACGGTGGTA	excluded due to multiple indel events in the sequences		
		tuf-R	CGAAGTAAGATCCCAGCGTTG			
ugpA	46568-47557	ugpA-F	AAGTGGCTAAAGGGTTTTGACAA			
		ugpA-R	CGTATAAAGTTCCAAGAACAAATAGTATGA			
lepA	772880-774679	lepA-F	CCTTAAAGTGGCAATGGAAAAGA	excluded due to lower Simpson's index of diversity	9/378 (2.38%)	0,83
		lepA-R	GATAATTTCAGCTAAAGGAATCTCATAAA			
pta	865219-866196	pta-F	AAAGAAGGGAATGACACTAGAAGTAGC		17/497 (3.42%)	0.87
		pta-R	ATATTAGGGAAGATGAATACATTAGCAC			
dnaN	1011604-1012779	dnaN-F	CTGTTATTACAAAGTAAATGATACTGAAGATG		18/496 (3.63%)	0.9
		dnaN-R	TTCAAGATACAACCAGATATTGCTGAT			
atpG	**427048-427917**	**atpG-F**	**TGGAACTAAACTAAATTCGTTTTTAAGA**	**included in the MLST assay**	**13/341 (3.81%)**	**0.93**
		atpG-R	**TAGCATACTCACACACTTTGGATTCA**			
dnaA	**3163-4548**	**dnaA-F**	**GAGCGTCAAAAATTATTCCCAGA**		**13/415 (3.13%)**	**0.92**
		dnaA-R	**TTACGAATATCGCCTTCATCAAA**			
fusA	**740849-742930**	**fusA-F**	**CAGTAGCAGTATTAGATGCCCAAATG**		**12/544 (2.21%)**	**0.92**
		fusA-R	**TAGTAGGGATCTGTACTTCTTCACCAA**			
rpoB	**303943-308115**	**rpoB-F**	**GTTAATGCTTAAAGAACAACTTGATTTATT**		**21/508 (4.13%)**	**0.94**
		rpoB-R	**GGTTAATTGGTGCGTGTTAAAGAA**			
ruvB	**846984-847904**	**ruvB-F**	**CAACGACAATGTATGGCAGGAT**		**15/338 (4.44%)**	**0.93**
		ruvB-R	**GGTTAATTGGTGCGTGTTAAAGAA**			
uvrA	**102406-105264**	**uvrA-F**	**TTTACCAATCTTAATGTGAATAAAGCC**		**19/490 (3.88%)**	**0.96**
		uvrA-R	**CCGTTCCCTGGGTGGAGTT**			

Number of single nucleotide polymorphisms (Nr. of SNPs) and the Simpson's index of diversity for each locus based on the sequence analysis of 10 *M. gallisepticum* samples. Proportions of the variable nucleotides/genes are in brackets.

Numerous SNPs were detected in the examined loci, with *dnaA* possessing the highest number of variable nucleotide positions (36/415; 8.67%). Beside nucleotide diversity, a 9 bp nucleotide deletion was 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) (Table 6).

Table 6. Number of single nucleotide polymorphisms (Nr. of SNPs) and the Simpson's index of diversity for each locus based on the sequence analysis of 131 *M. gallisepticum* samples

Loci	Length of amplicon (bp)	Nr. of SNPs/length of the examined locus (bp)	Simpson's index of diversity
atpG	395	19/341 (5.57%)	0.833
dnaA	461	36/415 (8.67%)	0.874
fusA	597	24/544 (4.41%)	0.891
rpoB	562	34/508 (6.69%)	0.913
ruvB	388	28/338 (8.28%)	0.884
uvrA	536	37/490 (7.55%)	0.856

Proportions of the variable nucleotides/genes are in brackets.

A phylogenetic tree created by the analysis of the concatenated sequences containing the six loci of the 131 *M. gallisepticum* samples revealed two major clades (Figure 6). 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 (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.

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.

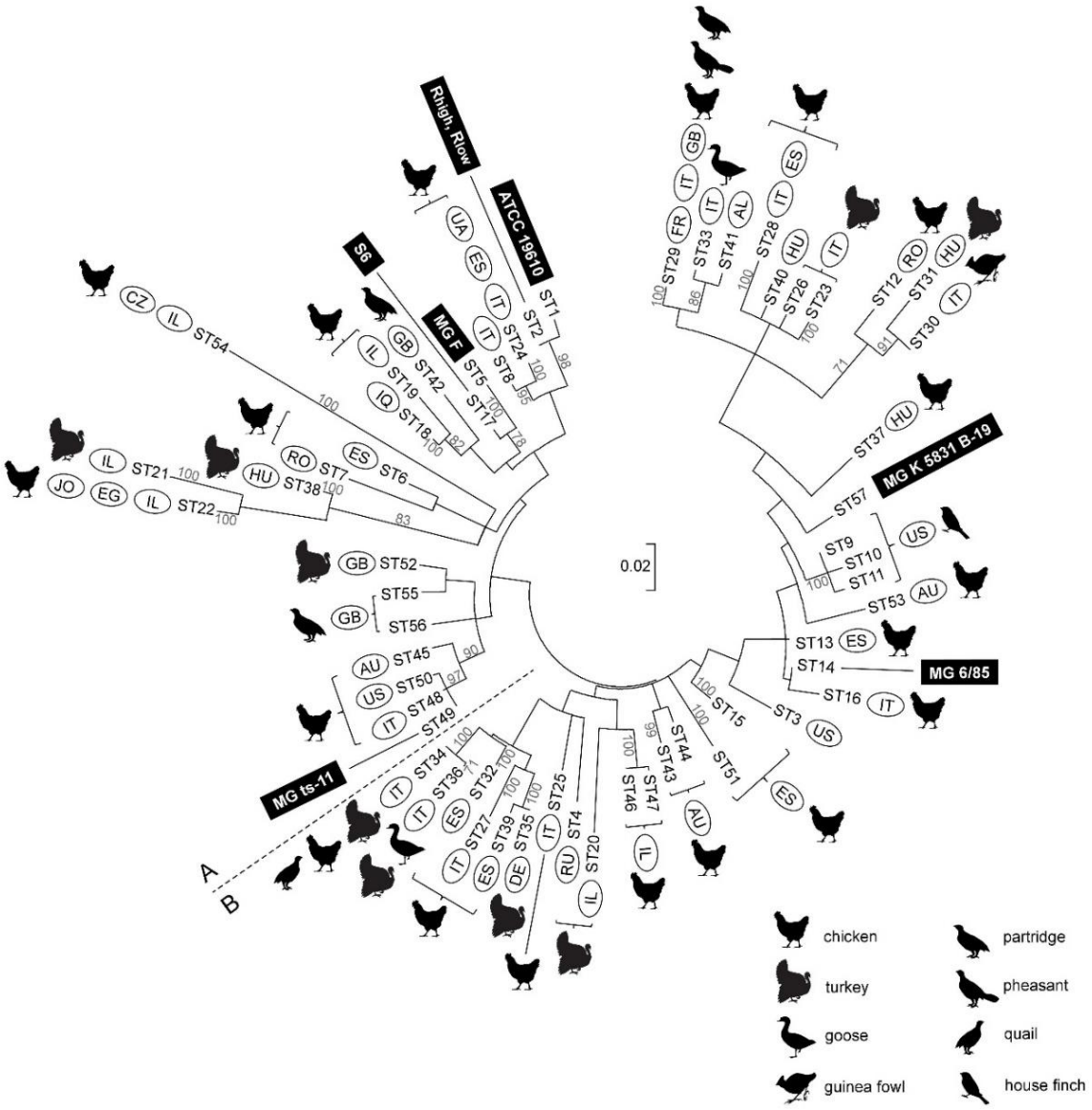


Figure 6. Maximum likelihood phylogenetic tree showing relationships between the sequence types (STs) derived from the examined 131 *M. gallisepticum* strains based on multi-locus sequence typing

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.

ST34 isolates were exclusively Italian samples from 2013 and 2014, but from different host species (turkey, chicken, quail).

ST22 contained samples from chickens originating from three different neighboring 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).

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.

Beside 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 K vaccine strain. 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 (Figure 7).

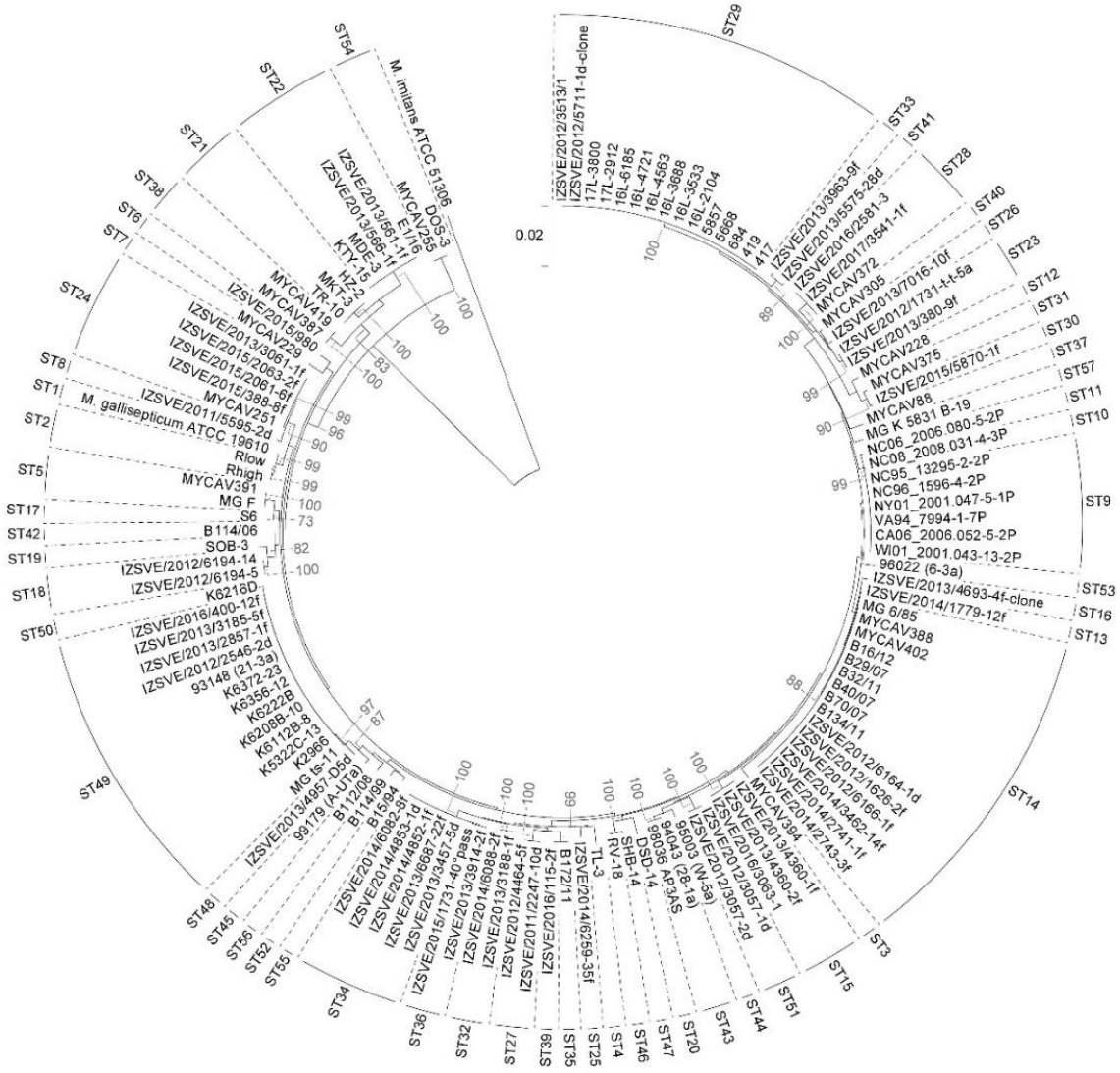


Figure 7. Neighbor-joining phylogenetic tree showing relationships between the examined 131 *M. gallisepticum* strains and the *M. imitans* type strain ATCC 51306 based on multi-locus sequence typing

Bootstrap values of neighbor-joining (1000 replicates) of ≥70 are shown. The scale bar represents the average number of substitutions per site. Sequence types (STs) are indicated around the samples.

5.2. Development of mismatch amplification mutation assays (MAMAs) for differentiating *M. gallisepticum* vaccine strains from field isolates

Whole genome sequencing of the 6/85 and ts-11 vaccine strains on Ion Torrent platform resulted on average 215,429 reads with 167.7 bp length. The mean coverage was 45.7 and 31.3 for the whole genome of 6/85 and ts-11 strains, respectively. Whole genome sequencing of the K vaccine strain on Illumina NextSeq 500 platform generated a total of 463,946

sequence reads with average lengths of 76 bp, the mean coverage was 33.1 for the whole genome. The average Phred score of the short reads were found to be over 23, or 99.5 % base call accuracy.

For the differentiation of *M. gallisepticum* vaccine strains F, 6/85, ts-11 and K, a total of 9, 7, 15 and 7 non-synonymous mutations met the aforementioned criteria and were targeted with MAMA assays, respectively (data not shown). After performing preliminary studies using the DNA of the targeted vaccine strain and as wild-type controls, the *M. gallisepticum* ATCC 19610 type strain and the other non-targeted vaccine strains, the number of assays was narrowed to two MAMAs to strain 6/85, three MAMAs to strain ts-11, two MAMAs to strain F and one MAMA to strain K (Table 7).

The targeted mutations are located in virulence-associated genes (*crmA*, *gapA*, *hlp2*, *lpd*, *plpA, glpK*), a gene (*potC*) coding an ABC transporter protein and a gene (*fruA*) encoding fructose-specific enzyme (EIIABC component) of the phosphotransferase system (PTS).

The assays resulted in wild-type-specific amplicon when the *M. gallisepticum* ATCC 19610 type strain or any of the other three vaccine strains were tested. Negative controls or templates of other avian *Mycoplasma* species were either not amplified or generated non-specific products with melt-profiles differing from the profiles of the expected two allelic states (Figure 8). The non-specific melting temperatures or band sizes were omitted from further analyzes.

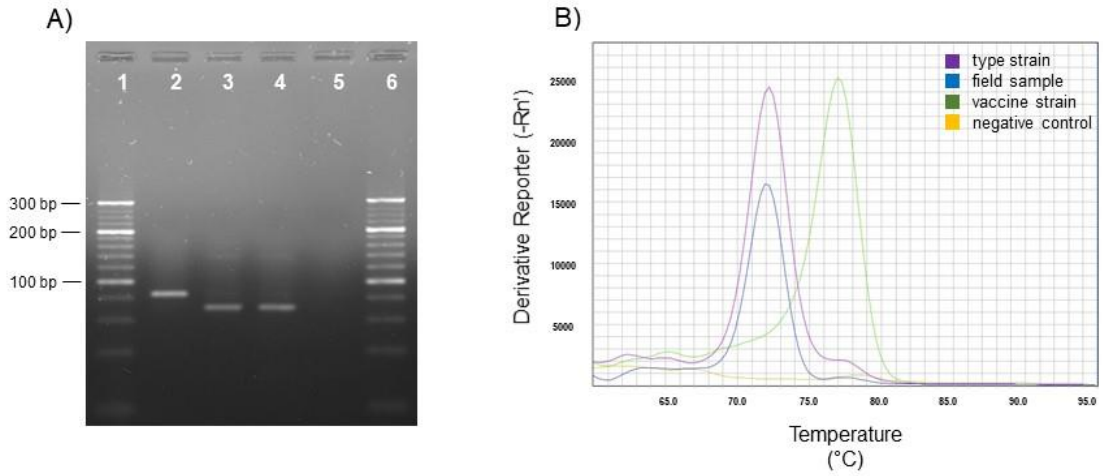


Figure 8. Detection of K vaccine strain-specific SNP in the *fruA* gene

Figure 8A. Discrimination of K vaccine strain with agarose-MAMA. Line 1 and 6: 20-bp DNA ladder was used as molecular weight marker, Line 2: K vaccine strain yielded 85 bp fragments, Line 3: 71 bp fragments of the *M. gallisepticum* ATCC 19610 type strain, Line 4: 71 bp fragments of the *M. gallisepticum* field sample 19135-M1c, Line 5: negative control. **Figure 8B.** Discrimination of K vaccine strain with melt-MAMA. Melting curves of the *M. gallisepticum* ATCC 19610 type strain (purple line; Tm 72.05 °C), the *M. gallisepticum* field sample 19135-M1c (blue line; Tm 72.06 °C) and the K vaccine strain (green line; Tm 77.25 °C). Negative control (yellow line) did not amplify. y-axis: derivative reporter, the negative first-derivative of the normalized fluorescence generated by the reporter during PCR amplification; x-axis: temperature melt curve.

The quantity of *M. gallisepticum* DNA in the samples varied largely and showed wide range of cycle treshold (Ct) values with the *mgc2* gene based qPCR (Raviv and Kleven, 2009). In samples with higher Ct values (usually Ct values above 20 in the *mgc2* gene based qPCR), non-specific PCR product of the negative control was often visible beside the genotype-specific amplicon in the developed assays, detected by real-time PCR as a bimodal peak or by agarose gel-electrophoresis as multiple bands. In 12 cases DNA samples (Ct values above 20 in the *mgc2* gene based qPCR (Raviv and Kleven, 2009)) showed false negative results in at least one of the developed assays: MAMA-F-hlp2 (n=2); MAMA-F-crmA (n=6); MAMA-6/85-lpd (n=5), MAMA-6/85-gapA (n=2); MAMA-ts11-plpA (n=10), MAMA-ts11-glpK (n=4), MAMA-ts11-potC (n=8) and MAMA-K-fruA (n=1).

Detection limit of the assays changed between 10^2 and 10^4 template copy number/µl in melt-MAMA tests and 10^3 to 10^5 in agarose-MAMA tests depending on the assay and the genotype. The assays were able to identify both genotypes in mixed samples at least in one of the following combinations: vaccine-type:wild-type 10^6:10^6, 10^6:10^5, 10^5:10^6 template copy number/µl. Bimodal melting peaks at the specific melting temperatures or two amplicons with the specific band sizes indicated the presence of both genotypes (Figure 9).

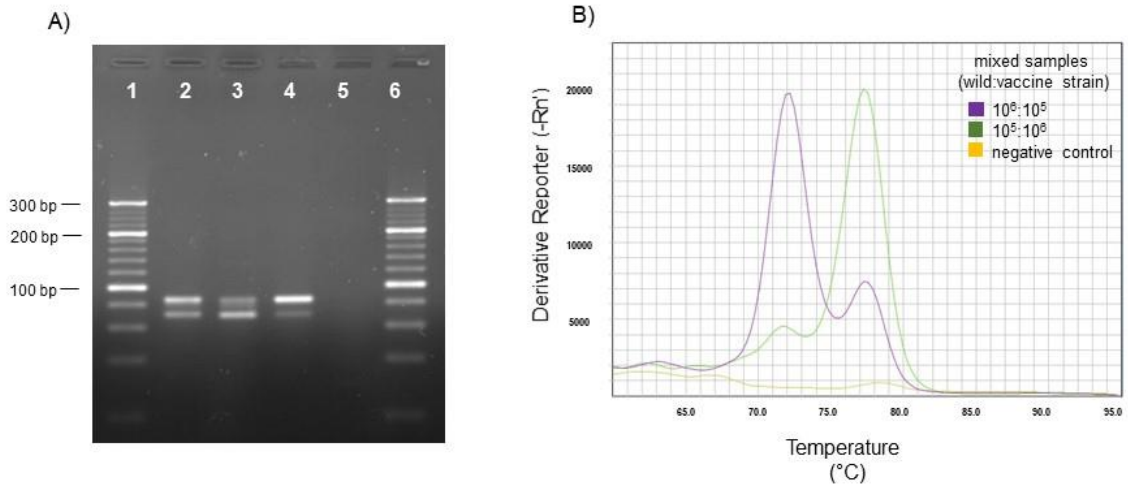


Figure 9. Agarose- and melt-MAMA tests of mixed DNA of *M. gallisepticum* ATCC 19610 type strain and K vaccine strain

Figure 9A. Samples containing wild-type:vaccine strain DNA mix in 10^6:10^6 (Line 2), 10^6:10^5 (Line 3) and 10^5:10^6 (Line 4) template copy number/µl resulted two amplicons at specific band sizes by agarose-MAMA test. Negative control (Line 5) did not amplify. 20-bp DNA ladder was used as molecular weight marker (Line 1 and 6).

Figure 9B. Samples containing the wild-type:vaccine strain DNA mix in 10^6:10^5 (purple line) and 10^5:10^6 (green line) template copy number/µl resulted bimodal peaks in the genotype-specific melting temperatures by melt-MAMA test. Negative control (yellow line) did not amplify. y-axis: derivative reporter, the negative first-derivative of the normalized fluorescence generated by the reporter during PCR amplification; x-axis: temperature melt curve.

In general, the tests showed similar sensitivity to the wild-type and vaccine-type *M. gallisepticum* DNA, however, two assays specific for the ts-11 vaccine strain (MAMA-ts11-glpK and -potC) and one assay specific for the F vaccine strain (MAMA-F-crmA) showed higher sensitivity to the wild-type DNA when mixtures of the wild- and vaccine-type DNA were tested (Table 8).

After the 10-step serial passage (resulting in ~33.22 population doubling), vaccine strains and the *M. gallisepticum* ATCC 19610 type strain showed identical genotypes as their parent strains in the *in vitro* stability test.

In the case of assays differentiating strain F from *M. gallisepticum* field isolates, only one sample, the MYCAV391 was characterized as vaccine type. The two tests (MAMA-F-hlp2 and MAMA-F-crmA) showed maximum congruency (adjusted Rand co-efficient: 1.000).

Assays designed for the differentiation of 6/85 vaccine strain showed high congruency (adjusted Rand co-efficient: 0.959). The results of two samples from Italy, namely IZSVE/2013/4693-4f and IZSVE/2014/6259-35f, showed discrepancy when tested with the developed assays. Both samples were characterized as wild-type *M. gallisepticum* by assay MAMA-6/85-gapA and 6/85 vaccine strain with the assay MAMA-6/85-lpd.

MAMAs developed to differentiate ts-11 vaccine strains also showed high congruency (range of adjusted Rand co-efficient: 0.920-0.961). Contradictory results were found in two cases. The Australian sample 99179 was characterized as wild strain by MAMA-ts11-plpA and vaccine strain by the remaining two assays. The Italian sample IZSVE/2013/4693-4f showed the SNP specific for ts-11 vaccine strain with assay MAMA-ts11-glpK, but was characterized as field strain with the rest of the assays.

According to the method of Ricketts *et al.* (2017), beside the ts-11 vaccine strain and its re-isolates, six Australian samples and one Italian isolate (IZSVE/2013/3185-5f) was characterized as ts-11 vaccine strain out of the 21 examined *M. gallisepticum* samples. These results reveal poor agreement with the assays MAMA-ts11-plpA, MAMA-ts11-glpK and MAMA-ts11-potC (range of Rand co-efficient: 0.495-0.605). Similar results were obtained when the method of Ricketts *et al.* (2017) was compared with the MLST assay (range of Rand co-efficient: 0.486-0.505).

Single MAMA assay was designed for the differentiation of K vaccine strain. All of the tested DNA samples were found to be wild-type strain with the developed MAMA-K-fruA test.

Genotypes of each *M. gallisepticum* strains and clinical samples tested in this study are provided in Table S3.

Table 7. Primer sequences of assays for the differentiation of 6/85, ts-11, F and K vaccine strains from field isolates designed in this study

Vaccine	Gene	Mutation[a]	Assay	Primer	Primer sequence (5'-3')	Primer volume[b]		Cycle number	
						melt-MAMA	agarose-MAMA	melt-MAMA	agarose-MAMA
6/85	***lpd***	G1372T (A-S)	MAMA-6/85-lpd	lpd-1372-6/85	ggggcggggcggggGTTTTTTGTTRAAGTGGTTATAAATCGA	0.15	1	40	40
				lpd-1372-wt	GTTTTTTGTTRAAGTGGTTATAAATAGC	0.6	4		
				lpd-1372-con	GAACAAGCAATTCACCCACACC	0.15	1		
	gapA	A1306G (R-G)	MAMA-6/85-gapA	gapA-1315-6/85	ggggcggggcggggGGTGTTTTTAGAACTAAATTTGAAATCG	0.15	1	40	40
				gapA-1315- wt	GGTGTTTTYAGAACTAAATTTGAAAGCA	0.15	1		
				gapA-1315-con	ATAAAATACCGTATGGATAACCAACAG	0.15	1		
ts-11	***plpA***	C953G (T-S)	MAMA-ts11-plpA	plpA-971-ts11	ggggcggggcggggGCTTCTAGATGAGGTGTGATTGTGC	0.15	1	30	40
				plpA-971- wt	GCTTCTAGATGAGGTGTGATTGAGG	0.15	4		
				plpA-971-con	GGATTATTACCTGAACTTGCCACAG	0.15	1		
	glpK	G67A (D-N)	MAMA-ts11-glpK	glpK-67-ts11	ggggcggggcggggACATCTTGTCGTTCAATCGTTTGTA	0.15	1	40	40
				glpK-67- wt	ACATCTTGTCGTTCAATCGTTTCTG	0.15	1		
				glpK-67-con	GGAAAGTATTGCGTAAATTCGTTTTG	0.15	1		
	potC	C526G (Q-E)	MAMA-ts11-potC	potC-526-ts11	ggggcggggcggggATGAACCCAAATCTAATCTTAGCTTTAG	0.15	1	30	40
				potC-526- wt	ATGAACCCAAATCTAATCTTAGCTTAAC	0.6	4		
				potC-526-con	GCGGGTGTTAAATAAGATAGAGTAATCT	0.15	1		
F	***hlp2***	G5542C (E-Q)	MAMA-F-hlp2	hlp2-5542-F	ggggcggggcggggGTCTTAGTGTGGTTTTTTTAATCTTGTG	0.15	1	40	40
				hlp2-5542- wt	GTCTTAGTGTGGTTTTTTTAATCTTCTC	0.15	1		
				hlp2-5542-con	GAAGTGCAAAAGAAATTAACTGATCTG	0.15	1		
	crmA	C2116G (Q-E)	MAMA-F-crmA	crmA-2116-F	ggggcggggcggggACAACCATTCGGAACAACTCTCG	0.15	4	40	40
				crmA-2116- wt	ACAACCATTCGGAACAACTCACC	0.15	1		
				crmA-2116-con	CTAATATTCTTAATTGATGAGAACTGATCAC	0.15	1		
K	***fruA***	G88A (D-N)	MAMA-K-fruA	fruA-88-K	ggggcggggcggggCGTGTAGCTAGTTCTTTTAAGATATCCTT	0.15	1	30	35
				fruA-88-wt	CGTGTAGCTAGTTCTTTTAAGATATCATC	0.15	1		
				fruA-88-con	AAGCCCGAGTTTGTCTTTATTAATC	0.15	1		

Amino acid changes are indicated in brackets. [a]according to *M. gallisepticum* R_{low} (GenBank accession number: AE015450) nucleotide numbering; [b]primer (10 pmol/µl) volume in 10 µl (melt-MAMA) and 25 µl (agarose-MAMA) reaction mixture

Table 8. Data of amplicons and sensitivity of the assays for the differentiation of 6/85, ts-11, F and K vaccine strains from field isolates designed in this study

Assay	Genotype	Tm (°C)	Amplicon length (bp)	Sensitivity (template copy number/reaction)				Mixed samples (vaccine:wild-type) (template copy number/reaction)						
				melt-MAMA		agarose-MAMA								
				v	wt	v	wt	10^6:10^3	10^6:10^4	10^6:10^5	10^6:10^6	10^5:10^6	10^4:10^6	10^3:10^6
MAMA-6/85-lpd	6/85	79.8±0.1	102	10^3	10^3	10^4	10^4	v	v	v	bm/mb	wt	wt	wt
	wt	76.5±0.3	88											
MAMA-6/85-gapA	6/85	80.3±0.1	99	10^2	10^3	10^3	10^4	v	v	v	bm/mb	wt	wt	wt
	wt	76.0±0.6	85											
MAMA-ts11-plpA	ts-11	82.2±0.0	82	10^3	10^3	10^4	10^4	v	v	bm/mb	bm/mb	wt	wt	wt
	wt	77.5±0.1	68											
MAMA-ts11-glpK	ts-11	79.3±0.1	94	10^1	10^1	10^3	10^3	v	v	v	bm/mb	wt	wt	wt
	wt	76.3±0.1	80											
MAMA-ts11-potC	ts-11	77.6±0.1	106	10^4	10^3	10^4	10^4	v	v	bm/mb	bm/mb	wt	wt	wt
	wt	74.4±0.1	92											
MAMA-F-hlp2	F	78.0±0.1	102	10^3	10^2	10^3	10^3	v	v	v	bm/mb	wt	wt	wt
	wt	74.0±0.1	88											
MAMA-F-crmA	F	81.0±0.0	89	10^4	10^3	10^4	10^4	v	v	bm/mb	wt	wt	wt	wt
	wt	75.5±0.2	75											
MAMA-K-fruA	K	77.3±0.2	85	10^2	10^2	10^3	10^3	v	v	bm/mb	bm/mb	bm/mb	wt	wt
	wt	71.8±0.4	71											

bm: bimodal peak; mb: multiple bands; Tm: melting temperature; v: vaccine-type; wt: wild-type

5.3. Comparison of the results of the developed *M. gallisepticum* MLST assay and MAMA tests

The eight developed MAMA tests showed high congruency with the MLST (range of adjusted Rand co-efficient for 6/85 differentiating tests: 0.826-0.911; for ts-11 differentiating tests: 0.854-0.948; adjusted Rand co-efficient for F and K differentiating tests: 1.000).

Out of the three samples which showed incongruent results with the vaccine differentiating assays, IZSVE/2014/6259-35f, which showed the 6/85 vaccine-type with the MAMA-6/85-lpd, was characterized as wild-type strain by MLST. IZSVE/2013/4693-4f showed mutation specific for ts-11 vaccine with assay MAMA-ts11-glpK and for 6/85 vaccine with MAMA-6/85-lpd, but based on MLST analysis it proved to be a field isolate closely related to the vaccine strain 6/85 (7/2636 nucleotide differences from 6/85 in 1/6 examined alleles). Sample 99179 showed the vaccine-type by MAMA-ts11-glpK and MAMA-ts11-potC assays, while based on MLST analysis it also proved to be a field isolate closely related to the vaccine strain ts-11 (10/2636 nucleotide differences from ts-11 in 3/6 examined genes).

For the K6216D strain originating from the USA, ts-11 vaccine-specific genotype was determined based on *in silico* analysis of the MAMA-targeted mutations in the strain's whole genome sequence (GenBank accession number: MATM00000000). However, MLST analysis defined a unique sequence type (ST50) for this strain, differing in only one nucleotide from the ts-11 MLST profile. Similarly, sample IZSVE/2013/4957-D5d originating from Italy, also differed only in one nucleotide from the ts-11 MLST profile (ST48). This strain showed the ts-11 genotype by the MAMA-ts11-glpK and MAMA-ts11-potC assays, but proved to be false negative by the MAMA-ts11-plpA assay. Sample IZSVE/2014/1779-12f originating from Spain, was determined to harbor the 6/85 vaccine-specific mutations. This strain belonged to the MLST sequence type (ST13) which was the most similar ST to the 6/85 MLST profile (ST14), showing only two nucleotide differences on one allele.

5.4. Detecting mutations potentially associated with decreased susceptibility to certain antibiotics in *M. synoviae* strains

The MIC values of the tested antibiotics against each examined *M. synoviae* strain including previously published MIC data (Kreizinger *et al.*, 2017) are presented in Table S4. The MIC ranges obtained for each tested antimicrobial agent are shown in Table 9. The numbers of the isolates with elevated MIC values for each tested antibiotic are also included in Table 9.

As none of the examined *M. synoviae* strains were found to show high MIC values against doxycycline, tiamulin and valnemulin, relatedness of the MIC values for these antibiotic agents with the detected mutations were not evaluated in the study. In case of neomycin, further investigations were omitted due to the lack of sensitive strains.

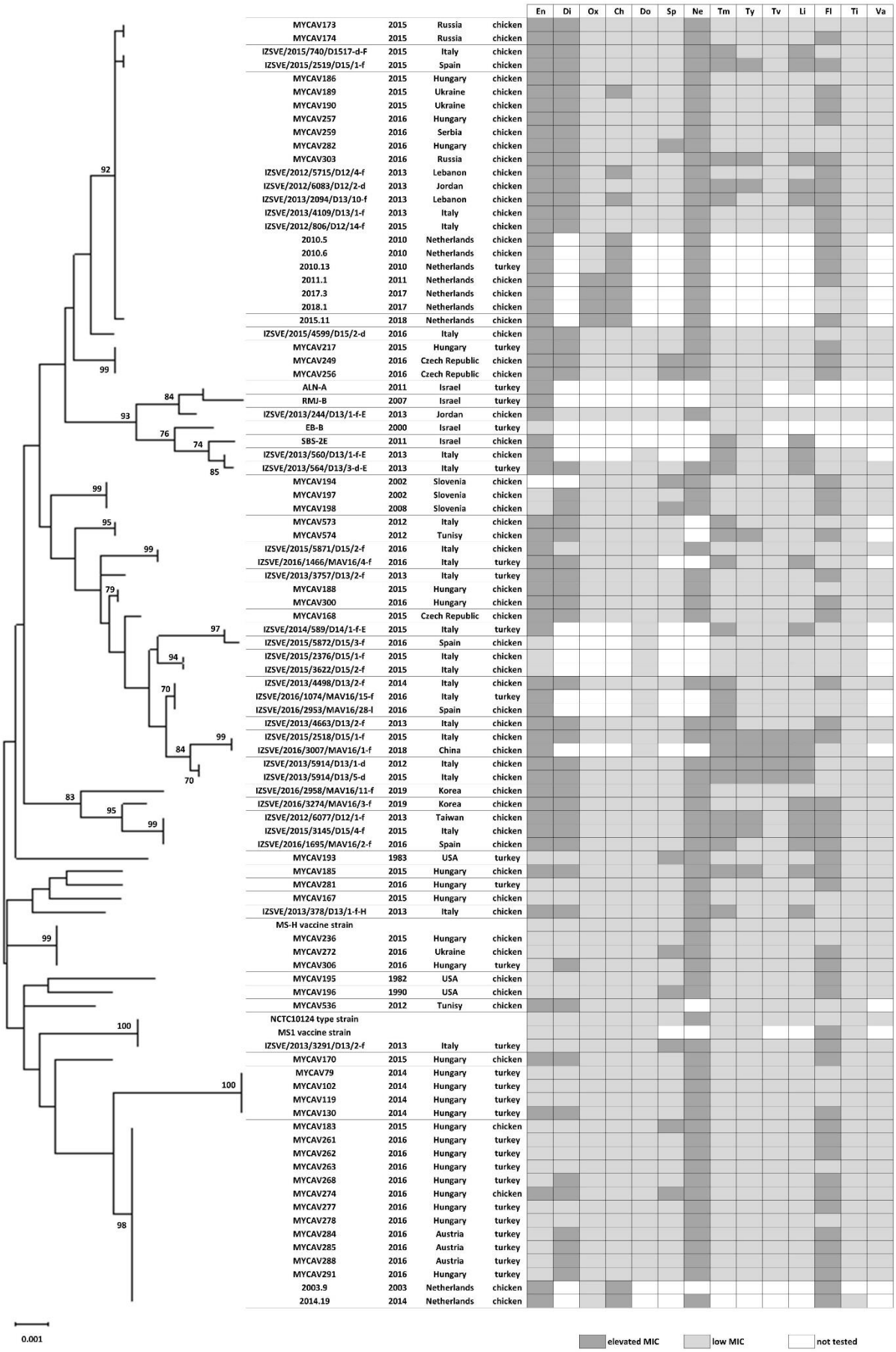
Table 9. Results of the MIC determination by broth microdilution method

Antimicrobial agents	MIC range (µg/ml)	Strains with decreased susceptibility
enrofloxacin	0.156 - >10	n=63 (>1.25)
difloxacin	0.312 - >10	n=52 (>1.25)
oxytetracycline	≤0.25 - 8	n=4 (>4)
chlortetracycline	≤0.25 - >8	n=12 (>4)
doxycycline	≤0.039 - 1.25	n=0 (>4)
spectinomycin	≤0.25 - 8	n=11 (>2)
neomycin	4 - >64	n=78 (>2)
tilmicosin	≤0.25 - >64	n=25 (>8)
tylosin	≤0.25 - 64	n=11 (>1)
tylvalosin	≤0.25 - 4	n=4 (>0.5)
lincomycin	≤0.25 - >64	n=20 (>2)
florfenicol	<0.5 - 8	n=56 (>2)
tiamulin	≤0.039 - 2.5	n=0 (>8)
valnemulin	≤0.039	n=0 (>0.125)

Elevated MIC values of each antibiotic agent are presented in brackets and given in µg/ml.

Whole genome sequencing of *M. synoviae* strains resulted on average 144,599 and 2,535,433 reads with 157.36 and 152.08 bp length, and the mean sequencing depth of the whole genomes was 28.41X and 408.42X, generated on Ion Torrent and Illumina NextSeq 500 platform, respectively (Table S5). The average Phred score of the short reads were found to be over 23, or 99.5 % base call accuracy. The raw nucleotide sequence reads of the *M. synoviae* strains were submitted to the Short Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) (BioProject accession numbers: PRJNA634246; PRJNA634252).

Molecular phylogenetic analysis revealed great genetic diversitiy of the 96 examined *M. synoviae* strains as they were classified into 42 sequence types (STs) by multi-locus sequence typing (El-Gazzar *et al.*, 2017). Phylogenetic relationships of the strains are presented by a neighbor-joining tree, demonstrating that the antibiotic susceptibility profile of the strains can differ within the same ST or even in case of identical origin, verifying the inclusion of all these isolates in the study (Figure 10).



				En	Di	Ox	Ch	Do	Sp	Ne	Tm	Ty	Tv	Li	Fl	Ti	Va
MYCAV173	2015	Russia	chicken														
MYCAV174	2015	Russia	chicken														
IZSVE/2015/740/D1517-d-F	2015	Italy	chicken														
IZSVE/2015/2519/D15/1-f	2015	Spain	chicken														
MYCAV186	2015	Hungary	chicken														
MYCAV189	2015	Ukraine	chicken														
MYCAV190	2015	Ukraine	chicken														
MYCAV257	2016	Hungary	chicken														
MYCAV259	2016	Serbia	chicken														
MYCAV282	2016	Hungary	chicken														
MYCAV303	2016	Russia	chicken														
IZSVE/2012/5715/D12/4-f	2013	Lebanon	chicken														
IZSVE/2012/6083/D12/2-d	2013	Jordan	chicken														
IZSVE/2013/2094/D13/10-f	2013	Lebanon	chicken														
IZSVE/2013/4109/D13/1-f	2013	Italy	chicken														
IZSVE/2012/806/D12/14-f	2015	Italy	chicken														
2010.5	2010	Netherlands	chicken														
2010.6	2010	Netherlands	chicken														
2010.13	2010	Netherlands	turkey														
2011.1	2011	Netherlands	chicken														
2017.3	2017	Netherlands	chicken														
2018.1	2017	Netherlands	chicken														
2015.11	2018	Netherlands	chicken														
IZSVE/2015/4599/D15/2-d	2016	Italy	chicken														
MYCAV217	2015	Hungary	turkey														
MYCAV249	2016	Czech Republic	chicken														
MYCAV256	2016	Czech Republic	chicken														
ALN-A	2011	Israel	turkey														
RMJ-B	2007	Israel	turkey														
IZSVE/2013/244/D13/1-f-E	2013	Jordan	chicken														
EB-B	2000	Israel	turkey														
SBS-2E	2011	Israel	chicken														
IZSVE/2013/560/D13/1-f-E	2013	Italy	chicken														
IZSVE/2013/564/D13/3-d-E	2013	Italy	turkey														
MYCAV194	2002	Slovenia	chicken														
MYCAV197	2002	Slovenia	chicken														
MYCAV198	2008	Slovenia	chicken														
MYCAV573	2012	Italy	chicken														
MYCAV574	2012	Tunisy	chicken														
IZSVE/2015/5871/D15/2-f	2016	Italy	chicken														
IZSVE/2016/1466/MAV16/4-f	2016	Italy	turkey														
IZSVE/2013/3757/D13/2-f	2013	Italy	turkey														
MYCAV188	2015	Hungary	chicken														
MYCAV300	2016	Hungary	chicken														
MYCAV168	2015	Czech Republic	chicken														
IZSVE/2014/589/D14/1-f-E	2015	Italy	turkey														
IZSVE/2015/5872/D15/3-f	2016	Spain	chicken														
IZSVE/2015/2376/D15/1-f	2015	Italy	chicken														
IZSVE/2015/3622/D15/2-f	2015	Italy	chicken														
IZSVE/2013/4498/D13/2-f	2014	Italy	chicken														
IZSVE/2016/1074/MAV16/15-f	2016	Italy	turkey														
IZSVE/2016/2953/MAV16/28-I	2016	Spain	chicken														
IZSVE/2013/4663/D13/2-f	2013	Italy	chicken														
IZSVE/2015/2518/D15/1-f	2015	Italy	chicken														
IZSVE/2016/3007/MAV16/1-f	2018	China	chicken														
IZSVE/2013/5914/D13/1-d	2012	Italy	chicken														
IZSVE/2013/5914/D13/5-d	2015	Italy	chicken														
IZSVE/2016/2958/MAV16/11-f	2019	Korea	chicken														
IZSVE/2016/3274/MAV16/3-f	2019	Korea	chicken														
IZSVE/2012/6077/D12/1-f	2013	Taiwan	chicken														
IZSVE/2015/3145/D15/4-f	2015	Italy	chicken														
IZSVE/2016/1695/MAV16/2-f	2016	Spain	chicken														
MYCAV193	1983	USA	turkey														
MYCAV185	2015	Hungary	chicken														
MYCAV281	2016	Hungary	turkey														
MYCAV167	2015	Hungary	chicken														
IZSVE/2013/378/D13/1-f-H	2013	Italy	chicken														
MS-H vaccine strain																	
MYCAV236	2015	Hungary	chicken														
MYCAV272	2016	Ukraine	chicken														
MYCAV306	2016	Hungary	turkey														
MYCAV195	1982	USA	chicken														
MYCAV196	1990	USA	chicken														
MYCAV536	2012	Tunisy	chicken														
NCTC10124 type strain																	
MS1 vaccine strain																	
IZSVE/2013/3291/D13/2-f	2013	Italy	turkey														
MYCAV170	2015	Hungary	chicken														
MYCAV79	2014	Hungary	turkey														
MYCAV102	2014	Hungary	turkey														
MYCAV119	2014	Hungary	turkey														
MYCAV130	2014	Hungary	turkey														
MYCAV183	2015	Hungary	chicken														
MYCAV261	2016	Hungary	turkey														
MYCAV262	2016	Hungary	turkey														
MYCAV263	2016	Hungary	turkey														
MYCAV268	2016	Hungary	turkey														
MYCAV274	2016	Hungary	chicken														
MYCAV277	2016	Hungary	turkey														
MYCAV278	2016	Hungary	turkey														
MYCAV284	2016	Austria	turkey														
MYCAV285	2016	Austria	turkey														
MYCAV288	2016	Austria	turkey														
MYCAV291	2016	Hungary	turkey														
2003.9	2003	Netherlands	chicken														
2014.19	2014	Netherlands	chicken														

Figure 10. Phylogenetic tree generated from seven loci of the examined 96 *M. synoviae* isolates based on multi-locus sequence typing method according to El-Gazzar *et al.* (2017)

The neighbor-joining tree was constructed by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano (HKY) model. Bootstrap values of neighbor-joining (1000 replicates) of ≥70 are shown. The scale bar represents the average number of substitutions per site. The year of isolation, the country of origin, the host species and the susceptibility for each tested antibiotic (indicated by colour codes) are shown next to the ID of the strains. MIC values (μg/ml) were considered to be elevated above: 0.125 for valnemulin (Va); 0.5 for tylvalosin (Tv); 1 for tylosin (Ty); 1.25 for enrofloxacin (En) and difloxacin (Di); 2 for spectinomycin (Sp), neomycin (Ne), lincomycin (Li) and florfenicol (Fl); 4 for oxytetracycline (Ox), chlortetracycline (Ch) and doxycycline (Do); and 8 for tilmicosin (Tm) and tiamulin (Ti).

5.4.1. Fluoroquinolones

Aligning the corresponding genes in the reference genome of the *M. synoviae* strain MS53 and the sequences of the tested 95 *M. synoviae* strains revealed several non-synonymous mutations in the examined genes. Consecutive amino acid substitutions were found in numerous different positions of the GyrA (n=42), GyrB (n=18), ParC (n=56) and ParE (n=35) proteins. Besides, a SNP resulting amino acid deletion in the GyrA protein was also detected (data not shown). Investigating these alterations in context with the MIC values of the *M. synoviae* strains, several mutations were found to be potentially resistance-related (Table 10). These amino acid substitutions are indicated in Table S6 in parallel with the MIC values for the two tested fluoroquinolones of each examined *M. synoviae* strain.

In the *gyrA* gene, the most frequently occurring SNP has been found at position 1651 (G1651A) and resulted in an Asp551Asn amino acid change in 48/71 *M. synoviae* strains with higher MIC values (>1.25 μg/ml) for enrofloxacin and/or difloxacin. A mutation at nucleotide position 1360 (T1360A) of the *gyrA* gene resulted in a Ser454Thr amino acid change in 35 *M. synoviae* strains with higher MIC values, while another SNP in the neighboring nucleotide position (C1361A) resulted in an amino acid substitution at the same position (Ser454Tyr) in one additional *M. synoviae* strain. These two SNPs together affected 36/71 *M. synoviae* strains with decreased susceptibility to fluoroquinolones. A non-synonymous mutation has been detected at nucleotide position 28 of the *gyrA* gene (G28A) resulted in a Glu10Lys substitution in 33/71 *M. synoviae* strains with higher MIC values for enrofloxacin and/or difloxacin. We identified a mutation at nucleotide position 566 (A566G) as well, which resulted in a Glu189Gly amino acid change in the same 33 *M. synoviae* strains and two additional *M. synoviae* isolates with higher MIC values for fluoroquinolones. A SNP in 2/71 *M. synoviae* strains with higher MIC values for enrofloxacin and/or difloxacin has been found as well at position 428 (A428G) of the *gyrA* gene resulting in an Asn143Ser amino acid substitution.

In the *gyrB* gene, non-synonymous mutations at nucleotide positions 446 (C446T) and 1247 (C1247A) resulted in Ala149Val and Ser416Tyr amino acid substitutions, respectively, in the

same 31/71 *M. synoviae* strains with higher MIC values (>1.25 µg/ml) for enrofloxacin and/or difloxacin. In five *M. synoviae* strains with higher MIC values for fluoroquinolones, a mutation at position 1250 (G1250A) of the *gyrB* gene resulted an amino acid substitution (Ser417Asn) at the neighboring position of the Ser416Tyr. These two mutations together affected 36/71 *M. synoviae* strains with decreased susceptibility to fluoroquinolones.

In the *parC* gene, a mutation at nucleotide position 254 (C254T) resulted in a Thr85Ile amino acid change in 56 *M. synoviae* strains with higher MIC values (>1.25 µg/ml) for enrofloxacin and/or difloxacin, while another SNP in the neighboring nucleotide position (A253G) resulted in an amino acid substitution at the same position (Thr85Ala) in two additional *M. synoviae* strains. Besides, a mutation at nucleotide position 256 (T256C) resulted in a Ser86Pro amino acid change (n=1), while at nucleotide position 265, polymorphisms G265C and G265T resulted in amino acid substitutions Asp89His (n=1) and Asp89Tyr (n=1), respectively. Mutations at nucleotide positions coding the region 85-89 amino acids of the ParC together affected 61/71 *M. synoviae* strains with higher MIC values for fluoroquinolones. A mutation at nucleotide position 1798 (G1798A) of the parC gene resulting in a Val600Ile amino acid change was also identified. This mutation was found in 45/71 strains with higher MIC values for enrofloxacin and/or difloxacin. Non-synonymous mutation at nucleotide position 1354 (G1354A) resulted in an amino acid change Glu452Lys in the same 45 *M. synoviae* strains and one additional isolate (MYCAV536) with higher MIC value. Similarly, the mutation at position 2442 (C2442A) resulting in an Asn814Lys amino acid change was detected in the same 45 *M. synoviae* strains and one additional isolate (RMJ-B) with higher MIC value.

In the *parE* gene, a non-synonymous mutation was identified at nucleotide position 260 (C260T) resulting in a Ser87Phe amino acid change in 27/71 *M. synoviae* strains with higher MIC values (>1.25 µg/ml) for enrofloxacin and/or difloxacin.

At least one of the described mutations were carried by 88.73 % of the *M. synoviae* strains with higher MIC values for fluoroquinolones (>1.25 µg/ml for enrofloxacin and/or difloxacin). Interestingly, potentially resistance-related mutations could be detected in six *M. synoviae* strains with lower MIC values (≤1.25 µg/ml) for enrofloxacin and/or difloxacin, however, four of these strains were not examined with both antibiotics. Besides, eight strains were found to have higher MIC values (>1.25 µg/ml) for enrofloxacin and/or difloxacin without any potentially resistance-associated mutations found in the examined genes, however, six of these strains had lower MIC values (≤1.25 µg/ml) or missing MIC data for one of the tested antibiotics.

5.4.2. 30S inhibitors

Aligning the corresponding genes of the *M. synoviae* strain MS53 reference genome and the sequences of the tested *M. synoviae* strains (n=92 for tetracyclines; n=73 for spectinomycin), mutations in 38 different positions of the *rrsA* and/or *rrsB* genes have been detected in this study (data not shown). None of these mutations could be related to the decreased susceptibility to tetracyclines (MIC values of >4 µg/ml for oxytetracycline and/or chlortetracycline, n=12) or spectinomycin (MIC values of >2 µg/ml; n=11). Investigating *in silico* the 12 *M. synoviae* strains with higher MIC values (>4 µg/ml) for tetracyclines, none of the tested *tet* primers or partial gene sequences (*tet*(L), *tet*(M), *tet*(O), *tet*(R), *tet*(S)) could be mapped suitably to the scaffolds of the strains. Likewise, no amplification of specific gene sequences could be detected by PCR (Shahid *et al.*, 2014b) when the DNAs of the 12 *M. synoviae* strains with higher MIC values for tetracyclines were tested.

5.4.3. 50S inhibitors

Aligning the corresponding genes of the *M. synoviae* strain MS53 reference genome and the sequences of the tested *M. synoviae* strains (n=87 for macrolides; n=84 for lincomycin; n=92 for florfenicol), mutations in 79 different positions of the *rrlA* and/or *rrlB* were identified in this study. Besides, non-synonymous mutations of the *rplC*, *rplD* and *rplV* genes resulted amino acid substitutions in 9, 17 and 6 different positions of the 50S ribosomal protein L3, L4 and L22, respectively (data not shown). None of these mutations could be related to the decreased susceptibility to florfenicol (MIC values of >2 µg/ml; n=56) and no resistance-related mutation could be identified in the *rplC* and *rplD* genes. However, potentially resistance-associated mutations have been identified in the *rrl* genes in case of macrolides and lincomycin, and *rplV* gene in case of macrolides (Table 10). These SNPs are indicated in Table S7 in parallel with the MIC values for the three tested macrolides and lincomycin of each examined *M. synoviae* strain.

In 20 *M. synoviae* strains, a mutation has been found at position 2054 (A2054G) in the *rrlA*/*B* genes. Out of these, 14 strains were found to possess this SNP simultaneously in both *rrl* genes, while six strains were found to be heterozygous concerning this position. The PCR designed for the differentiation of the two *rrl* genes followed by the developed melt-MAMA test revealed that IZSVE/2014/589/D14/1-f-E carried this mutation in the *rrlB* gene, while the rest of these strains possessed this SNP in the *rrlA* gene (IZSVE/2013/2094/D13/10-f, IZSVE/2015/2519/D15/1-f, IZSVE/2016/1695/MAV16/2-f, IZSVE/2016/2958/MAV16/11-f, IZSVE/2016/3274/MAV16/3-f). Besides, a SNP at the adjacent position (A2055G) of both *rrl* genes has been observed as well in one *M. synoviae* strain. These two SNPs together affected 19/25 *M. synoviae* strains with high MIC values for macrolides (>8 µg/ml for tilmicosin and/or >1 µg/ml for tylosin and/or >0.5 µg/ml for tylvalosin) and all of the strains (20/20) with MIC

values of >2 µg/ml to lincomycin. In the *rplV* gene, a mutation at nucleotide position 276 (A276C/T) resulting in a Gln92His amino acid change of the L22 protein was also identified in 6/25 *M. synoviae* strains with high MIC values for macrolides. All *M. synoviae* strains with higher MIC values for these 50S inhibitors (>8 µg/ml for tilmicosin and/or >1 µg/ml for tylosin and/or >0.5 µg/ml for tylvalosin and/or >2 µg/ml for lincomycin) have been found to carry exactly one of the described mutations (considering the same position at *rrlA* and *rrlB* as one), while no occurrence of the mentioned SNPs could be observed in *M. synoviae* strains with low MIC values for all of these antibiotics, except in one case: A2054G could be detected in the *rrlA* gene of the IZSVE/2016/2958/MAV16/11-f isolate. In this strain, unique amino acid changes were seen in the 50S ribosomal protein L3 and L22. The mutations in the *rplC* (G752A) and *rplV* (C229T) genes resulted in Arg251Lys and His77Tyr amino acid changes of the L3 and L22 protein, respectively. Besides, a mutation in the *rplV* gene (C124T) resulting in a Pro42Ser amino acid change of the L22 protein which seems to be specific for sensitive strains (≤0.25 µg/ml for macrolides; ≤2 µg/ml for lincomycin; n=8) could be observed in the isolate IZSVE/2016/2958/MAV16/11-f as well (data not shown).

Table 10. Potentially resistance-related mutations identified in *M. synoviae* strains

Antibiotics	Genes	SNP[a]	AA substitution[a]	Strains with lower MIC values possessing the mutation[b]		Strains with higher MIC values possessing the mutation[c]	
FLUOROQUINOLONES	*gyrA*	G28A	Glu10Lys	-	-	n=33	46.48 %
		A428G	Asn143Ser	-	-	n=2	2.82 %
		A566G	Glu189Gly	-	-	n=35	49.3 %
		T1360A	Ser454Thr	-	-	n=36	50.7 %
		C1361A	Ser454Tyr				
		G1651A	Asp551Asn	n=1	5 %	n=48	67.61 %
	gyrB	C446T	Ala149Val	-	-	n=31	43.66 %
		C1247A	Ser416Tyr	-	-	n=36	50.7 %
		G1250A	Ser417Asn				
	parC	A253G	Thr85Ala	-	-	n=61	85.92 %
		C254T	Thr85Ile				
		T256C	Ser86Pro				
		G265C	Asp89His				
		G265T	Asp89Tyr				
		G1354A	Glu452Lys	-	-	n=46	64.79 %
		G1798A	Val600Ile	-	-	n=45	63.38 %
		C2442A	Asn814Lys	-	-	n=46	64.79 %
	parE	C260T	Ser87Phe	n=1	5 %	n=27	38.03 %
	Total number and percentage of strains with high MIC values for fluoroquinolones containing at least one of the listed mutations					**n=63**	**88.73 %**
MACROLIDES	*rrlA* and/or *rrlB*	A2054G	n.a.	n=2	3.45 %	n=19	76 %
		A2055G	n.a.				
	rplV	A276C/T	Gln92His	-	-	n=6	24 %
	Total number and percentage of strains with high MIC values for macrolides containing at least one of the listed mutations					**n=25**	**100 %**
LINCOMYCIN	*rrlA* and/or *rrlB*	A2054G	n.a.	n=1	1.56 %	n=20	100 %
		A2055G	n.a.				
	Total number and percentage of strains with high MIC values for lincomycin containing at least one of the listed mutations					**n=20**	**100 %**

Mutations underlined were previously associated with fluoroquinolone, macrolide or lincosamide resistance in *M. synoviae*. [a]Numbering according to *M. synoviae* strain MS53 (GenBank accession number: AE017245); [b]in case of fluoroquinolones: strains with MIC values of ≤1.25 µg/ml for enrofloxacin and difloxacin (n=20; isolates with missing MIC data (n=4) are excluded from the evaluation); in case of macrolides: strains with MIC values of ≤8 µg/ml for tilmicosin and ≤1 µg/ml for tylosin and ≤0.5 µg/ml for tylvalosin (n=58; isolates with missing MIC data (n=4) are excluded from the evaluation); in case of lincomycin: strains with MIC values of ≤2 µg/ml (n=64); [c]in case of fluoroquinolones: strains with MIC values of >1.25 µg/ml for enrofloxacin and/or difloxacin (n=71); in case of macrolides: strains with MIC values of >8 µg/ml for tilmicosin and/or >1 µg/ml for tylosin and/or >0.5 µg/ml for tylvalosin (n=25); in case of lincomycin: strains with MIC values of >2 µg/ml (n=20); SNP: single nucleotide polymorphism; AA: amino acid; n.a.: not applicable

6. Discussion

6.1. Development of multi-locus sequence typing (MLST) assay for genotyping *M. gallisepticum* strains

M. gallisepticum can cause significant economic losses to the poultry industry by inducing respiratory syndromes 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 131 *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, collected between 1985 and 2017. However, common features of the examined samples did not correlate with identical sequence types 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 genetic instability, samples belonging to a certain sequence type were isolated most commonly in the same year, or within a few consecutive years. However, in some cases strains with identical sequence types were detected in a longer period of time. 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 time period in wild birds and also in semi-wild birds such as game birds. This lesser selection pressure may contribute to the subsistence of certain sequence types 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, detected 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 domestic fowl, 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 sequence types 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 generated maximum likelihood 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 sequence types 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 transmitted 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 a common parent stock, the same strain can spread worldwide. This extensive international trade could explain the occurrence of identical sequence types in distant regions.

Beside the good capability for discriminating different *M. gallisepticum* strains, the developed MLST was shown to be suitable for epidemiological investigations, as the same sequence types 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.

Analyzing 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 whole genome sequences 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 sequence types 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 sequence type 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 reliable tool for this purpose, as analyzing more genes provides more established results.

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. 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 elimination of this avian pathogen from poultry industry due to its multiple applicability.

6.2. Development of mismatch amplification mutation assays (MAMAs) for differentiating *M. gallisepticum* vaccine strains from field isolates

Although maintenance of *M. gallisepticum*-free flocks is the most viable method to control the infection, elimination programs are not feasible in a large number of poultry farms. In these cases, vaccination provides an effective measure of long-term disease control. The use of *M. gallisepticum* live vaccines led to the need for a reliable technique, which can differentiate vaccine strains from wild, virulent isolates. This is crucial in epidemiological investigations, vaccination and eradication programs.

This study revealed mutations in *M. gallisepticum* vaccine strains that are absent in R_{low} and other publicly available *M. gallisepticum* isolates. Real-time (melt-MAMA) and conventional (agarose-MAMA) PCR assays were developed for the detection of these vaccine-specific, candidate mutations. Most of the targeted mutations are located in genes associated with virulence. Cytadhesins, encoded by *gapA* and *crmA* genes play a major role in *M. gallisepticum* host colonization (Indiková *et al.*, 2013). Gene *hlp2,* similar to *hlp3,* encodes cytadherence-associated protein (high molecular weight protein 2), while *plpA* encodes pneumoniae-like protein A (PlpA) which is capable of binding fibronectin (May *et al.*, 2006). The dihydrolipoamide dehydrogenase encoded by *lpd* is a component of the pyruvate dehydrogenase complex, which is also identified as virulence-associated determinant, as it is required for *in vivo* growth and survival in the host (Hudson *et al.*, 2006). The *glpK* has a role in H_2O_2 production thereby affecting host-cell cytotoxicity (Szczepanek *et al.*, 2010; Hames *et al.*, 2009).

The *potC* encoded protein is the permease component of the ABC-type spermidine/putrescine transport system. Direct evidence of its role in virulence is lacking, however, plasticity of the ABC transporter component genes is likely important for survival in the host environment (Szczepanek *et al.*, 2010). The *fruA* gene encoding fructose-specific enzyme (EIIABC component) of the phosphotransferase system (PTS). The bacterial PTS is a multi-protein

system involved in the regulation of a variety of metabolic and transcriptional processes (Västermark and Saier, 2014). This system catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane (Deutscher *et al.*, 2006). Although direct evidence of its role in virulence is lacking, a connection between the PTS and the virulence of certain pathogens was suggested by the observation that some virulence genes underlie a kind of carbon catabolite repression (CCR) (Trappetti *et al.*, 2017). This is a regulatory mechanism enabling bacteria to increase their fitness by optimizing growth rates in natural environments providing different nutrients (Stülke and Hillen, 1999). Although several virulence genes and factors have been already identified, genetic background of virulence is not completely understood (Szczepanek *et al.*, 2010). Role of the PTS in the virulence of *M. gallisepticum* has not been described yet, thus relevance of the revealed SNP in the attenuation of the K vaccine strain merits further investigations.

The targeted SNPs showed firm genetic stability through the passages of the vaccine strains and the *M. gallisepticum* ATCC 19610 type strain, however, it should be noted, that the *in vitro* test may not reflect completely the *in vivo* genetic stability of the strains.

After performing the designed MAMA-K-fruA tests, we found that the targeted mutation occurs exclusively in the K vaccine strain. Although vaccination status of the animals was unknown in many cases, none of the tested samples originated from Japan, where K vaccine strain is in use supporting the results of the MAMA-K-fruA assay. Similarly, in case of assays differentiating the F vaccine strain from *M. gallisepticum* field isolates, only one sample was characterized as vaccine-type. The two tests showed maximum congruency confirming the reliability of these tests. However, a limitation of this study is that no or few field samples originating from flocks vaccinated with commercial K or F vaccine strains were investigated. Evaluation of K and additional F strain re-isolates should further increase the reliability of the presented assays.

Samples harboring at least one SNP specific to ts-11 and/or originating from the same country (Australia) as the parent strain of the ts-11 were checked with PCR systems specific to ts-11 described by Ricketts *et al.* (2017). The disagreement was remarkable between the results of assays developed in the current study and the PCR systems of Ricketts *et al.* (2017), as all five Australian wild-type samples carried the ts-11-specific regions, while five out of six samples containing ts-11-specific SNPs lacked the ts-11-specific sequences. The interpretation of negative results is difficult because beside the presence of the specific regions in the samples, the quality of the DNA and the sensitivity of the PCR systems also influence the results. Although detection limit is not published for the PCR systems of Ricketts *et al.* (2017), according to our results, the detection limit of these assays are similar to that of the currently developed assays for the detection of the ts-11 vaccine strain (10^3 template copy number/reaction). Similarly to the PCRs of Ricketts *et al.* (2017), the developed assays were

unable to discriminate ts-11 strains with reverted virulence, as all non-virulent and virulent ts-11 re-isolates contained the targeted mutations according to the sequences available in the GenBank.

The developed assays aim to support routine diagnostics by determining the successful vaccination of the animals or confirming *M. gallisepticum*-free status of a flock. The combined use of the presented assays provide feasible option for the rapid differentiation of vaccine strains from field isolates with high approximation. These tests are highly specific to be applicable directly on clinical samples avoiding technical problems associated with isolation, which is particularly complicated in case of mycoplasmas. However, due to the moderate sensitivity of certain assays, clinical specimens with lower DNA load may show false negative results, and in these cases strain isolation or enrichment may be required indeed. The new MAMAs are suitable for the detection of simultaneous presence of the vaccine strain and wild, virulent isolates in mixed samples, which is especially useful in monitoring of vaccination programs.

Further advantages of the assays are that they were all designed with the same thermal profile, allowing their simultaneous application. The tests can be performed on basic real-time PCR platforms (without high-resolution melt function) and on conventional PCR equipment coupled with agarose gel electrophoresis too. The designed assays reported here represents a convenient, rapid and cost-efficient tool for differentiating the F, 6/85, ts-11 and K vaccine strains from field isolates and from each other, thereby contribute to the effectiveness of control programs against *M. gallisepticum* infections.

6.3. Comparison of the results of the developed *M. gallisepticum* MLST assay and MAMA tests

Considering the different sensitivity of the assays, congruent results were observed among the developed MAMA and MLST tests, confirming the competence of the designed assays. In line with the results of the MAMA tests, the *M. gallisepticum* K vaccine strain had unique sequence type (ST57) and the MLST system could not identify any closely related strains among the tested samples. Likewise, only one sample (MYCAV391) was classified in the sequence type of the F vaccine strain (ST5) by MLST, which was consistent with the results of the MAMA assays.

However, the MLST system could distinguish other, closely related (differing at 1-10 positions in the examined 2636 bp long concatenated sequences) field strains from vaccines ts-11 and 6/85, which showed the vaccine-type (with 1-2 nucleotide difference by MLST) or incongruent results (with 7-10 nucleotide difference by MLST) with the MAMA assays. It is noteworthy, that a ts-11 re-isolate with reverted virulence (strain K6216D, isolated from a progeny flock of a ts-

11 vaccinated broiler flock which was not distinguishable from ts-11 vaccine strain by previous DNA sequence and RAPD analyzes (El-Gazzar *et al.*, 2011)) showed unique sequence type (ST50) by MLST.

Dissimilar genotype calls of the MAMAs compared with the results of the MLST assay indicate that MAMA-6/85-gapA is the most reliable test to distinguish vaccine strain 6/85, while MAMA-ts11-plpA proved to be the most reliable assay for the discrimination of the vaccine strain ts-11. However, in order to achieve the most definite results, the combined use of all designed assays for each vaccine strain is recommended. In ambiguous cases, MLST can be useful in data interpretation and it can be applied as a confirmatory method to differentiate wild-type and vaccine strains.

6.4. Detecting mutations potentially associated with decreased susceptibility to certain antibiotics in *M. synoviae* strains

Prudent use of antibiotics in the management of *M. synoviae* infection is improved by the determination of antibiotic susceptibility prior to the treatment, however, the most commonly performed broth and agar microdilution tests are very labor-intensive and time-consuming methods (Hannan, 2000). There is an increasing need for exploring resistance-related mutations in the bacterial genomes, which can be targeted by rapid molecular biological tests in order to guide antimicrobial therapy more effectively. The aim of this study was to identify mutations potentially associated with decreased antibiotic susceptibility in *M. synoviae* strains. Interpretation of the results were challenging, as SNPs may have cumulative or opposite effect, and their impact can be modified by unexplored mutations or other unknown factors as well. Moreover, small differences between the elevated MIC values according to this study and the MIC values related to certain strains further complicated the evaluation. In some cases, discrepancies between the MIC values and the presence of the identified mutations may be due to this phenomenon, as there is only one dilution step difference between the concentrations regarded as low or high MIC values. Considering these, all data have been examined individually and in context as well, and evaluated cautiously when SNPs were identified as potentially resistance-associated mutations.

Numerous potentially resistance-related mutations could be detected in the *gyrA*, *gyrB*, *parC* and *parE* genes of *M. synoviae* strains with higher MIC values for fluoroquinolones, however, not all of them were located in the quinolone resistance determining region (QRDR) of these genes. Previous studies suggest that the primary target of fluoroquinolones in *M. synoviae* is the ParC subunit of the topoisomerase IV enzyme (Le Carrou *et al.*, 2006; Lysnyansky *et al.*, 2013). In this study, a hot spot region could be identified in the QRDR of the *parC* gene located at nucleotide positions 253-265 resulted in alterations at the amino acid positions 85-89 (or 80-

84 amino acids according to *E. coli* numbering) of the DNA topoisomerase IV A subunit (ParC). SNPs at the same or adjacent positions have already been mentioned in several studies as resistance-associated mutations. Le Carrou *et al.* (2006) reported a 2-4-fold increase of the enrofloxacin MIC value in *M. synoviae* strains, which had a Ser to Pro substitution at position 81 in ParC. Lysnyansky *et al.* (2013) described full correlation between decreased susceptibility of *M. synoviae* to enrofloxacin and the amino acid substitutions at positions 79-81 and 84 in ParC. This region seems to have a principal role according to our study as well, as mutations located here were possessed by most *M. synoviae* strains (85.92 %) with MIC values of >1.25 µg/ml and all of the isolates with MIC values of ≥5 µg/ml for enrofloxacin and/or difloxacin. The most frequently occurring mutation of this hot spot region was C254T resulting in a Thr85Ile amino acid substitution in the ParC. This mutation could be related to decreased susceptibility to both fluoroquinolones, while the SNP A253G affecting the same amino acid position (Thr85Ala) could be detected for the first time in *M. synoviae* strains resistant against difloxacin but not enrofloxacin. However, additional isolates carrying this mutation should be investigated in order to confirm association between this genotype and the increased difloxacin MIC values. Outside of the hot spot region in the *parC* gene, three potentially resistance-related SNPs were detected for the first time. However, fluoroquinolone resistant strains carrying these mutations were all affected by a mutation of the hot spot region as well. Therefore, impact of these detected novel mutations is difficult to assess.

Resistance-related alterations of the GyrA found in our study have not been mentioned in the literature before, except for the mutation in the QRDR of the *gyrA* gene displaying an Asn to Ser amino acid change at position 143 (or 87 according to *E. coli* numbering) of the GyrA (Le Carrou *et al.*, 2006; Lysnyansky *et al.*, 2013). The mutations in the QRDR of the *gyrB* gene resulting in amino acid changes at positions 416 (Ser to Tyr) and 417 (Ser to Asn) in GyrB (or 401 and 402 according to *E. coli* numbering) have been also reported before in the paper of Lysnyansky *et al.* (2013), however their role in decreased fluoroquinolone susceptibility was not clarified in that study. On the other hand, no literature data have been found concerning the mutations detected at positions 149 (Ala to Val) in GyrB and 87 (Ser to Phe) in ParE proteins.

The SNPs identified for the first time as potentially resistance-associated mutations in the *gyrA*, *gyrB* and *parE* genes mainly occurred simultaneously with the mutations of the hot spot region in the fluoroquinolone resistant strains. However, in two strains with increased MIC values, mutations were only detected outside of the hot spot region: IZSVE/2014/589/D14/1-f-E carried all novel SNPs in the *gyrA* and *gyrB* genes, while MYCAV170 had the SNP in the *parE* gene only. These data support the presumption, that beside the importance of the *parC* hot spot region, these mutations may also play a role in the development of fluoroquinolone resistance.

Mutations in the central loop of the domain V (peptidyl transferase region) of 23S rRNA confer resistance to 50S inhibitors in many bacteria (Garza-Ramos *et al.*, 2001). In *M. gallisepticum*, mutations in positions 2057-2059 (according to *E. coli* numbering) can lead to a disruption of the rRNA structure, thus alterations in this area can prevent the attachment of the antimicrobial agents to their binding site (Ammar *et al.*, 2016). Mutations A2058G and A2059G have been previously associated with decreased susceptibility for macrolides and lincomycin in *M. synoviae* as well (Lysnyansky *et al.*, 2015).

In our study, all *M. synoviae* strains with higher MIC values for lincomycin possessed the mutation A2054G (or A2058G according to *E. coli* numbering) in the domain V of the *rrlA* and/or *rrlB* genes, except one strain (IZSVE/2016/1466/MAV16/4-f), which carried the A2055G (or A2059G according to *E. coli* numbering) SNP in the adjacent position of the *rrlA* and *rrlB* gene as well. The same mutations were found to affect the susceptibility of macrolides.

It seems that in *M. synoviae*, resistance to lincomycin and tilmicosin does not require the A2054G mutation in both *rrl* genes, as the presence of this mutation in the *rrlA* gene only was enough to increase the MIC values above 64 µg/ml, while a single isolate carrying the mutation in the *rrlB* gene only (IZSVE/2014/589/D14/1-f-E) showed higher but not extremely high MIC values for lincomycin (4 µg/ml) and tilmicosin (16 µg/ml). *M. synoviae* strains which showed high MIC values for all tested macrolide antibiotics and lincomycin as well carried this mutation in both *rrl* genes. Nevertheless, based on these data, differences between the two heterozygous or between hetero- and homozygous resistant genotypes concerning macrolide and lincomycin susceptibility are difficult to assess.

Beside the SNPs A2054G and A2055G of the *rrlA*/*B* genes, non-synonymous mutations revealed in the *rplV* gene were found to decrease the susceptibility for macrolides, especially in case of tilmicosin. The SNP A276C, as well as A276T resulted in a glutamine-histidine amino acid change at the position 92 (or 90 according to *E. coli* numbering) of the 50S ribosomal protein L22. This amino acid change in the L22 protein has been already described previously in *M. synoviae* strains (Lysnyansky *et al.*, 2015), although its role in macrolide resistance was not suggested in that study. According to our results, a mutation can occur in the closely located position (C274A) resulting in a glutamine-lysine change in the same amino acid position as well, however, it does not seem to affect the susceptibility for macrolides (data not shown). All *M. synoviae* strains with higher MIC values for lincomycin and/or macrolides have been found to possess one of the above mentioned mutations (considering the same position at *rrlA* and *rrlB* as one) indicating their significance. However, other SNPs might play a role as well, for example mutations detected in the *rplC* (G752A) and *rplV* (C124T, C229T) genes of a sensitive strain (IZSVE/2016/2958/MAV16/11-f) may also have an impact on the susceptibility of this isolate modulating the effect of the mutation A2054G in the *rrlA*.

No potentially resistance-related mutations could be identified in case of tetracyclines, spectinomycin and florfenicol based on the data of this study. Moreover, no *tet* genes could be detected in the DNAs of the 12 *M. synoviae* strains with higher MIC values for tetracyclines. In case of these antibiotics, it is plausible that other resistance mechanisms play a role. For example, genes encoding bacterial virulence factors can be associated with resistance of microorganisms (Chernova *et al.*, 2016), or decreased susceptibility of bacterial strains may be induced by increased efflux of the antimicrobial agent leading to lower intracellular concentrations (Piddock, 1999). Active efflux systems might occur in *M. synoviae*, as it has been already described for the human pathogen *M. hominis* (Raherison *et al.*, 2002) and suggested for *M. mycoides* subsp. *capri* as well (Khalil *et al.*, 2016).

The revealed mutations can contribute to the extension of knowledge about the genetic background of antibiotic resistance in *M. synoviae*. Moreover, the explored potentially resistance-related nucleotide positions can be investigated by molecular biological assays (Sulyok *et al.*, 2018). Targeting the most frequently occurring C254T mutation in the *parC* gene by MAMA or analyzing the whole hot spot region by HRM could provide feasible options for the rapid detection of fluoroquinolone resistance. However, simultaneous detection of several mutations in different genes could enhance the reliability of the method. Beside a previously reported SNP of the *gyrB* gene (C1247A), mutations reported here for the first time in the *gyrA* (A566G and T1360A), *gyrB* (C446T) and *parE* (C260T) genes could serve also as appropriate targets for molecular biological assays, based on their frequent occurrence in resistant isolates and low prevalence in sensitive strains. In case of lincomycin, molecular detection of the described A2054G mutation in the *rrlA*/*B* genes might be able to identify almost all resistant strains according to our results. The MAMA test developed in this study for the determination of the nucleotide at this position could be applied for this purpose as well. Beside this mutation, targeting the nucleotide position 276 of the *rplV* gene should be also appropriate to reveal macrolide resistance according to our data. Nevertheless, examination of the antibiotic susceptibility profiles and the listed genetic markers in further isolates should confirm the described correlations.

Supporting the results of conventional *in vitro* sensitivity tests, molecular biological assays could provide excellent guidance for antibiotic therapy, especially when susceptibility data are required quickly or when isolation of *Mycoplasma* fails. Reducing the detection time of antibiotic susceptibility, the use of these methods could contribute to achieve therapeutic success, thereby significantly reduce economic losses. Furthermore, data provided by these assays could support prudent antibiotic usage instead of empirical treatment. This trend could help to reduce the impact of antibiotic resistance and preserve critically important antibiotics for human medicine.

7. Overview of the new scientific results

Ad 1. The designed MLST method is able to differentiate *M. gallisepticum* strains with high discriminatory power and identify closely related strains as well. Relatively high sensitivity of the assay makes it suitable for examining DNAs extracted directly from clinical samples. It can be used in practice for phylogenetic studies and epidemiological investigations as well.

Ad 2. According to the results of the designed MLST assay, the examined *M. gallisepticum* strains can be characterized by high genetic heterogeneity. Our data indicates the high impact of extensive international trade on the worldwide spread of different *M. gallisepticum* strains and also confirms that closely related *M. gallisepticum* strains can infect both domestic fowl and game birds, providing a possible source of infection for chickens and turkeys.

Ad 3. In total, eight MAMA tests were designed and found to be suitable for the differentiation of *M. gallisepticum* vaccine strains from field isolates. The assays reported here represent convenient, rapid and cost-efficient tools for the monitoring of vaccination and eradication programs against *M. gallisepticum* infections.

Ad 4. The developed MLST and MAMA tests showed high congruency confirming the competence of the designed assays. However, the MLST system could distinguish closely related field strains from vaccines ts-11 and 6/85, which showed the vaccine-type or incongruent results with the MAMA tests. Therefore, the highly reliable MLST assay can be applied as a confirmatory test for the MAMAs.

Ad 5. Previously described molecular markers of high MICs for fluoroquinolones, macrolides and lincosamides were detected within the examined *M. synoviae* strain collection. Besides, genetic alterations presumably associated with decreased susceptibility to fluoroquinolones were identified in the *gyrA*, *gyrB*, *parC* and *parE* genes of *M. synoviae* strains for the first time.

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10. Supplements

Table S1. Data of *M. gallisepticum* samples used in this study

ID of *M. gallisepticum* strains	Sample type	Origin	Year	Host
ATCC 19610	type strain	n.a.	n.a.	chicken
MG F	vaccine strain; GenBank Acc. N.: NC_017503	USA	n.a.	n.a.
MG 6/85	vaccine strain	USA	n.a.	n.a.
MG ts-11	vaccine strain	Australia	1985	chicken
MG K 5831	vaccine strain	USA	2005	chicken
S6	GenBank Acc. N.: NC_023030	USA	2015	n.a.
R_{low}	GenBank Acc. N.: AE015450	USA	2015	n.a.
R_{high}	GenBank Acc. N.: NC_017502	USA	2015	n.a.
CA06_2006.052-5-2P	GenBank Acc. N.: NC_018412	California, USA	2006	house finch
NY01_2001.047-5-1P	GenBank Acc. N.: NC_018409	New York, USA	2001	house finch
VA94_7994-1-7P	GenBank Acc. N.: NC_018406	Virginia, USA	1994	house finch
NC95_13295-2-2P	GenBank Acc. N.: NC_018407	North Carolina, USA	1995	house finch
NC96_1596-4-2P	GenBank Acc. N.: NC_018408	North Carolina, USA	1996	house finch
WI01_2001.043-13-2P	GenBank Acc. N.: NC_018410	Wisconsin, USA	2001	house finch
NC06_2006.080-5-2P	GenBank Acc. N.: NC_018411	North Carolina, USA	2006	house finch
NC08_2008.031-4-3P	GenBank Acc. N.: NC_018413	North Carolina, USA	2008	house finch
K2966 (=ts-11)	GenBank Acc. N.: MAFU00000000	Australia	1985	chicken
K5322C-13	GenBank Acc. N.: MAFV00000000	Georgia, USA	2007	chicken
K6112B-8	GenBank Acc. N.: MAFW00000000	Georgia, USA	2007	chicken
K6208B-10	GenBank Acc. N.: MADW00000000	Georgia, USA	2007	chicken
K6216D	GenBank Acc. N.: MATM00000000	Georgia, USA	2008	chicken
K6222B	GenBank Acc. N.: MATN00000000	Georgia, USA	2008	chicken
K6356-12	GenBank Acc. N.: MAGQ00000000	Georgia, USA	2008	chicken
K6372-23	GenBank Acc. N.: MAGR00000000	Georgia, USA	2008	chicken
MYCAV88	*M. gallisepticum* isolate	Hungary	2010	chicken
MYCAV175	*M. gallisepticum* isolate	Hungary	2015	chicken
MYCAV228	*M. gallisepticum* isolate	Romania	2015	chicken
MYCAV229	*M. gallisepticum* isolate	Romania	2015	chicken
MYCAV231	*M. gallisepticum* isolate	Romania	2015	chicken
MYCAV251	*M. gallisepticum* isolate	Ukraine	2016	chicken
MYCAV255	*M. gallisepticum* isolate	Checz Republic	2016	chicken
MYCAV258	*M. gallisepticum* isolate	Ukraine	2016	chicken
MYCAV305	*M. gallisepticum* isolate	Hungary	2016	chicken
MYCAV372	*M. gallisepticum* isolate	Spain	2017	n.a.
MYCAV375	*M. gallisepticum* isolate	Hungary	2017	turkey
MYCAV387	*M. gallisepticum* isolate	Hungary	2017	turkey
MYCAV388	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV389	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV390	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV391	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV392	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV393	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV394	*M. gallisepticum* isolate	USA	n.a.	n.a.
MYCAV395	*M. gallisepticum* isolate	The Netherlands	n.a.	n.a.
MYCAV396	*M. gallisepticum* isolate	Austria	n.a.	n.a.
MYCAV397	*M. gallisepticum* isolate	The Netherlands	n.a.	n.a.
MYCAV398	*M. gallisepticum* isolate	The Netherlands	n.a.	n.a.
MYCAV399	*M. gallisepticum* isolate	The Netherlands	n.a.	n.a.
MYCAV400	*M. gallisepticum* isolate	United Kingdom	2008	chicken
MYCAV401	*M. gallisepticum* isolate	United Kingdom	2008	chicken
MYCAV402	*M. gallisepticum* isolate	Slovenia	n.a.	n.a.
MYCAV403	*M. gallisepticum* isolate	Germany	n.a.	n.a.
MYCAV404	*M. gallisepticum* isolate	Germany	n.a.	n.a.
MYCAV405	*M. gallisepticum* isolate	United Kingdom	2013	chicken

ID of *M. gallisepticum* strains	Sample type	Origin	Year	Host
MYCAV406	*M. gallisepticum* isolate	United Kingdom	2014	chicken
MYCAV407	*M. gallisepticum* isolate	n.a.	n.a.	n.a.
MYCAV419	*M. gallisepticum* isolate	Hungary	2017	turkey
MYCAV420	*M. gallisepticum* isolate	Hungary	2017	turkey
MYCAV604	*M. gallisepticum* isolate	Hungary	2019	turkey
MYCAV605	*M. gallisepticum* isolate	Hungary	2019	turkey
MYCAV612	*M. gallisepticum* isolate	Philippines	2019	chicken
MYCAV617	*M. gallisepticum* isolate	Philippines	2019	chicken
MYCAV623	*M. gallisepticum* isolate	Indonesia	2019	chicken
MYCAV627	*M. gallisepticum* isolate	Thailand	2019	chicken
MYCAV656	*M. gallisepticum* isolate	China	2019	chicken
MYCAV679	*M. gallisepticum* isolate	China	2019	chicken
MYCAV683	*M. gallisepticum* isolate	Russia	2019	n.a.
MYCAV684	*M. gallisepticum* isolate	Hungary	2019	turkey
MYCAV690	*M. gallisepticum* isolate	Malaysia	2019	chicken
MYCAV694	*M. gallisepticum* isolate	Malaysia	2019	chicken
417	*M. gallisepticum* isolate	United Kingdom	2017	pheasant
419	*M. gallisepticum* isolate	United Kingdom	2017	partridge
684	*M. gallisepticum* isolate	United Kingdom	2017	partridge
5668	*M. gallisepticum* isolate	United Kingdom	2017	partridge
5857	*M. gallisepticum* isolate	United Kingdom	2017	pheasant
16L-2104	*M. gallisepticum* isolate	United Kingdom	2016	pheasant
16L-3533	*M. gallisepticum* isolate	United Kingdom	2016	pheasant
16L-4563	*M. gallisepticum* isolate	United Kingdom	2016	partridge
16L-4721	*M. gallisepticum* isolate	United Kingdom	2016	partridge
16L-6185	*M. gallisepticum* isolate	United Kingdom	2016	pheasant
B15/94	*M. gallisepticum* isolate	United Kingdom	1994	partridge
B16/12	*M. gallisepticum* isolate	United Kingdom	2012	chicken
B29/07	*M. gallisepticum* isolate	United Kingdom	2007	chicken
B32/11	*M. gallisepticum* isolate	United Kingdom	2011	chicken
B40/07	*M. gallisepticum* isolate	United Kingdom	2007	chicken
B70/07	*M. gallisepticum* isolate	United Kingdom	2007	chicken
B112/08	*M. gallisepticum* isolate	United Kingdom	2008	partridge
B114/06	*M. gallisepticum* isolate	United Kingdom	2006	partridge
B114/99	*M. gallisepticum* isolate	United Kingdom	1999	turkey
B134/11	*M. gallisepticum* isolate	United Kingdom	2011	chicken
B172/11	*M. gallisepticum* isolate	Germany	2011	turkey
B227/13	*M. gallisepticum* isolate	United Kingdom	2013	partridge
93148 (21-3a)	*M. gallisepticum* isolate	Australia	1993	chicken
99179 (A-UTa)	*M. gallisepticum* isolate	Australia	2000	chicken
95003 (W-5a)	*M. gallisepticum* isolate	Australia	1995	n.a.
94043 (28-1a)	*M. gallisepticum* isolate	Australia	1994	chicken
96022 (6-3a)	*M. gallisepticum* isolate	Australia	1996	chicken
98036 AP3AS	*M. gallisepticum* isolate	Australia	1998	chicken
SHB-14	*M. gallisepticum* isolate	Israel	1997	chicken
HZ-2	*M. gallisepticum* isolate	Israel	1997	turkey
DSD-14	*M. gallisepticum* isolate	Israel	2000	turkey
TR-10	*M. gallisepticum* isolate	Israel	2004	turkey
MKT-3	*M. gallisepticum* isolate	Israel	2005	turkey
TL-3	*M. gallisepticum* isolate	Israel	2005	turkey
MDE-3	*M. gallisepticum* isolate	Israel	2006	chicken
RV-18	*M. gallisepticum* isolate	Israel	2007	chicken
KTY-15	*M. gallisepticum* isolate	Israel	2009	chicken
SOB-3	*M. gallisepticum* isolate	Israel	2017	chicken
DOS-3	*M. gallisepticum* isolate	Israel	2017	chicken
ZR50-1	*M. gallisepticum* isolate	Israel	1995	turkey
SDM-10	*M. gallisepticum* isolate	Israel	1997	turkey
VR-4	*M. gallisepticum* isolate	Israel	2009	chicken
BNC-3	*M. gallisepticum* isolate	Israel	2005	turkey
SLH-1	*M. gallisepticum* isolate	Israel	2013	quail

ID of *M. gallisepticum* strains	Sample type	Origin	Year	Host
BSY-12	*M. gallisepticum* isolate	Israel	1998	chicken
BLF-10	*M. gallisepticum* isolate	Israel	2008	chicken
IZSVE/2014/2741-1f	*M. gallisepticum* isolate	Portugal	2014	chicken
IZSVE/2014/2743-3f	*M. gallisepticum* isolate	Portugal	2014	chicken
IZSVE/2012/6164-1d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/1626-2f	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/6166-1f	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2014/3462-14f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/1779-12f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2012/2546-2d	*M. gallisepticum* isolate	Italy	2012	chicken
IZSVE/2013/2857-1f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/3185-5f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/4957-D5d	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/4693-4f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2014/6259-35f	*M. gallisepticum* isolate	Italy	2014	chicken
IZSVE/2012/5711-1d	*M. gallisepticum* isolate	France	2012	partridge
IZSVE/2011/5595-2d	*M. gallisepticum* isolate	Italy	2011	chicken
IZSVE/2011/2247-10d	*M. gallisepticum* isolate	Italy	2011	chicken
IZSVE/2012/4464-5f	*M. gallisepticum* isolate	Italy	2012	chicken
IZSVE/2012/1731-t-t-5a	*M. gallisepticum* isolate	Italy	2012	turkey
IZSVE/2015/1731-40°	*M. gallisepticum* isolate	Italy	2015	turkey
IZSVE/2013/3061-1f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/3914-2f	*M. gallisepticum* isolate	Italy	2013	goose
IZSVE/2013/380-9f	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2013/3457-5d	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2013/3963-9f	*M. gallisepticum* isolate	Italy	2013	goose
IZSVE/2013/6687-22f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/7016-10f	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2014/4852-1f	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2014/4853-1d	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2014/6082-8f	*M. gallisepticum* isolate	Italy	2014	quail
IZSVE/2015/5870-1f	*M. gallisepticum* isolate	Italy	2015	guinea fowl
IZSVE/2017/3541-1f	*M. gallisepticum* isolate	Italy	2017	chicken
IZSVE/2013/561-1f	*M. gallisepticum* isolate	Jordan	2013	chicken
IZSVE/2012/3057-1d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/3057-2d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2013/4360-1f	*M. gallisepticum* isolate	Spain	2013	chicken
IZSVE/2013/4360-2f	*M. gallisepticum* isolate	Spain	2013	chicken
IZSVE/2013/3188-1f	*M. gallisepticum* isolate	Spain	2013	chicken
IZSVE/2015/388-8f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/2061-6f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/2063-2f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2014/3567-5d	*M. gallisepticum* isolate	Italy	2014	chicken
IZSVE/2014/6716-2f	*M. gallisepticum* isolate	Italy	2014	chicken
IZSVE/2012/6162-2f	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/1627-1f	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/3189-1d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/3189-2f	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2014/1779-8f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/1445-1fwt	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/3462-16f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/5570-1d	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2010/3559-tr.F	*M. gallisepticum* isolate	Italy	2010	turkey
IZSVE/2011/6344-3d	*M. gallisepticum* isolate	Italy	2011	turkey
IZSVE/2011/6488-1f	*M. gallisepticum* isolate	Italy	2011	turkey
IZSVE/2011/3954-15d	*M. gallisepticum* isolate	Italy	2011	turkey
IZSVE/2012/2911-21f	*M. gallisepticum* isolate	Italy	2012	chicken
IZSVE/2012/3616-1f	*M. gallisepticum* isolate	Italy	2012	chicken
IZSVE/2012/3616-2f	*M. gallisepticum* isolate	Italy	2012	chicken
IZSVE/2012/3653-10d	*M. gallisepticum* isolate	Italy	2012	turkey

ID of *M. gallisepticum* strains	Sample type	Origin	Year	Host
IZSVE/2013/3791-1f	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2013/6142-10d	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/1705-2d	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/691-13f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/692-13f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/693-12f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/4268-6d	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/695-12f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/48-2f-p	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2013/3116-17f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/2999-2f	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2013/2093-1f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/5146-1d	*M. gallisepticum* isolate	Italy	2013	turkey
IZSVE/2013/5352-1d	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/5949-8f	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/2013/4816-5d	*M. gallisepticum* isolate	Italy	2013	chicken
IZSVE/723-1d	*M. gallisepticum* isolate	Italy	2014	chicken
IZSVE/2014/722-17d	*M. gallisepticum* isolate	Italy	2014	chicken
IZSVE/2014/6715-1d	*M. gallisepticum* isolate	Italy	2014	chicken
IZSVE/2014/4487-2d	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2014/5452-1d	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2014/5459-1f	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2014/6215-1d	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2014/6435-1f	*M. gallisepticum* isolate	Italy	2014	turkey
IZSVE/2015/3193-23d	*M. gallisepticum* isolate	Italy	2015	chicken
IZSVE/2015/2646-40°	*M. gallisepticum* isolate	Italy	2015	turkey
IZSVE/2015/1811-3f	*M. gallisepticum* isolate	Italy	2015	chicken
IZSVE/2015/4744-85f	*M. gallisepticum* isolate	Italy	2015	chicken
IZSVE/2015/4783-2f	*M. gallisepticum* isolate	Italy	2015	chicken
IZSVE/2015/2541-7f	*M. gallisepticum* isolate	Italy	2015	chicken
IZSVE/2016/852-1f	*M. gallisepticum* isolate	Italy	2016	turkey
IZSVE/2016/121-8d	*M. gallisepticum* isolate	Italy	2016	chicken
IZSVE/2016/149-2f	*M. gallisepticum* isolate	Italy	2016	turkey
IZSVE/2016/231-1f	*M. gallisepticum* isolate	Italy	2016	chicken
IZSVE/2016/2769-7f	*M. gallisepticum* isolate	Italy	2016	chicken
IZSVE/2016/2326-3d	*M. gallisepticum* isolate	Italy	2016	chicken
IZSVE/2016/2947-13d	*M. gallisepticum* isolate	Italy	2016	chicken
IZSVE/2016/3024-2f	*M. gallisepticum* isolate	Italy	2016	chicken
IZSVE/3540-1f	*M. gallisepticum* isolate	Italy	2017	chicken
IZSVE/2017/514-1f	*M. gallisepticum* isolate	Italy	2017	turkey
IZSVE/2017/517-6f	*M. gallisepticum* isolate	Italy	2017	chicken
IZSVE/2012/236-2d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/3058-1d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2012/3058-2d	*M. gallisepticum* isolate	Spain	2012	chicken
IZSVE/2013/6752-3f	*M. gallisepticum* isolate	Spain	2013	chicken
IZSVE/2013/3188-2f	*M. gallisepticum* isolate	Spain	2013	chicken
IZSVE/2013/1772-2f	*M. gallisepticum* isolate	Spain	2013	chicken
IZSVE/2014/3164-2f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/3776-1f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/3188-D13-1f	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2014/4868-4d	*M. gallisepticum* isolate	Spain	2014	chicken
IZSVE/2015/979-1f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/388-4f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/388-3f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/388-7d	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/2061-8d	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/2062-3f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2015/2063-16f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2016/2958-1d	*M. gallisepticum* isolate	Spain	2016	chicken
IZSVE/2017/3571-2d	*M. gallisepticum* isolate	Spain	2017	chicken

ID of *M. gallisepticum* strains	Sample type	Origin	Year	Host
IZSVE/2015/2062-4f	*M. gallisepticum* isolate	Spain	2015	chicken
IZSVE/2016/1934-2f	*M. gallisepticum* isolate	Italy	2016	chicken
LUM-5	clinical sample	Israel	2016	chicken
SLY-12	clinical sample	Israel	2012	quail
BYM-14	clinical sample	Israel	2013	chicken
KFM-12	clinical sample	Israel	2009	chicken
SU-4	clinical sample	Israel	2009	turkey
MSC-14	clinical sample	Israel	2013	chicken
IZSVE/2012/3513/1	clinical sample	Italy	2012	chicken
IZSVE/2016/400-12f	clinical sample	Italy	2016	chicken
IZSVE/2013/5575-28d	clinical sample	Albania	2013	chicken
IZSVE/2012/6194-5	clinical sample	Iraq	2012	chicken
IZSVE/2012/6194-14	clinical sample	Iraq	2012	chicken
IZSVE/2013/566-1f	clinical sample	Jordan	2013	chicken
IZSVE/2014/6088-2f	clinical sample	Spain	2014	chicken
IZSVE/2015/980	clinical sample	Spain	2015	chicken
IZSVE/2016/115-2f	clinical sample	Spain	2016	chicken
IZSVE/2016/2581-3	clinical sample	Spain	2016	chicken
IZSVE/2016/3063-1	clinical sample	Spain	2016	chicken
IZSVE/2016/3274-3f	clinical sample	Spain	2016	chicken
IZSVE/2014/2036-142	clinical sample	Italy	2014	chicken
IZSVE/2014/3712-5f	clinical sample	Italy	2014	chicken
IZSVE/2015/5859-4d	clinical sample	Italy	2015	chicken
IZSVE/2017/10-33	clinical sample	Italy	2017	chicken
IZSVE/2013/3188-2f	clinical sample	Spain	2013	chicken
IZSVE/2014/6089-1d	clinical sample	Spain	2014	chicken
IZSVE/2016/2581-4	clinical sample	Spain	2016	chicken
IZSVE/2016/3274-2f	clinical sample	Spain	2016	chicken
IZSVE/2017/199-3f	clinical sample	Spain	2017	chicken
IZSVE/2012/6194-2	clinical sample	Iraq	2012	chicken
IZSVE/2013/3188-6f	clinical sample	Spain	2013	chicken
IZSVE/2016/115-4f	clinical sample	Spain	2016	chicken
IZSVE/2017/1039-66	clinical sample	Spain	2017	chicken
IZSVE/2012/1183-9f	clinical sample	Spain	2012	chicken
IZSVE/2016/3483-3f	clinical sample	Italy	2016	chicken
IZSVE/2016/2581-2	clinical sample	Spain	2016	chicken
19039-4	clinical sample	Indonesia	2019	chicken
19039-8	clinical sample	Indonesia	2019	chicken
19053-TII-19w	clinical sample	Thailand	2019	chicken
19053-TAL-33w	clinical sample	Thailand	2019	chicken
19053-TB1-9w	clinical sample	Thailand	2019	chicken
19105-5B	clinical sample	Fiji	2019	chicken
19105-5C	clinical sample	Fiji	2019	chicken
19105-5D	clinical sample	Fiji	2019	chicken
19135-M1a	clinical sample	Malaysia	2019	chicken
19135-M1b	clinical sample	Malaysia	2019	chicken
19135-M1c	clinical sample	Malaysia	2019	chicken
19137-IF25	clinical sample	Malaysia	2019	chicken
19141-7CL	clinical sample	Malaysia	2019	chicken
19144-33w	clinical sample	India	2019	chicken
19144-48w	clinical sample	India	2019	chicken
19167-C	clinical sample	India	2019	chicken
19167-G	clinical sample	India	2019	chicken
19171-LI-F1	clinical sample	India	2019	chicken
19171-LM-F1	clinical sample	India	2019	chicken
E1/16	clinical sample	Egypt	2016	chicken

GenBank Acc. N.: Genbank accession number; n.a.: not available

Table S2. Data of *M. synoviae* samples used in this study

ID of *M. synoviae* strains	Sample type	Origin	Year	Host
MS53	GenBank Acc. N.: AE017245	n.a.	n.a.	n.a.
NCTC 10124	type strain; GenBank Acc. N.: CP011096	n.a.	1969	chicken
MS-H	vaccine strain; GenBank Acc. N.: KP704286	Australia	1998	chicken
MS1	vaccine strain	n.a.	n.a.	chicken
MYCAV79	*M. synoviae* isolate	Hungary	2014	turkey
MYCAV102	*M. synoviae* isolate	Hungary	2014	turkey
MYCAV119	*M. synoviae* isolate	Hungary	2014	turkey
MYCAV130	*M. synoviae* isolate	Hungary	2014	turkey
MYCAV167	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV168	*M. synoviae* isolate	Czech Republic	2015	chicken
MYCAV170	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV173	*M. synoviae* isolate	Russia	2015	chicken
MYCAV174	*M. synoviae* isolate	Russia	2015	chicken
MYCAV183	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV185	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV186	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV188	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV189	*M. synoviae* isolate	Ukraine	2015	chicken
MYCAV190	*M. synoviae* isolate	Ukraine	2015	chicken
MYCAV193	*M. synoviae* isolate	USA	1983	turkey
MYCAV194	*M. synoviae* isolate	Slovenia	2002	chicken
MYCAV195	*M. synoviae* isolate	USA	1982	chicken
MYCAV196	*M. synoviae* isolate	USA	1990	chicken
MYCAV197	*M. synoviae* isolate	Slovenia	2002	chicken
MYCAV198	*M. synoviae* isolate	Slovenia	2008	chicken
MYCAV217	*M. synoviae* isolate	Hungary	2015	turkey
MYCAV236	*M. synoviae* isolate	Hungary	2015	chicken
MYCAV249	*M. synoviae* isolate	Czech Republic	2016	chicken
MYCAV256	*M. synoviae* isolate	Czech Republic	2016	chicken
MYCAV257	*M. synoviae* isolate	Hungary	2016	chicken
MYCAV259	*M. synoviae* isolate	Serbia	2016	chicken
MYCAV261	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV262	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV263	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV268	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV272	*M. synoviae* isolate	Ukraine	2016	chicken
MYCAV274	*M. synoviae* isolate	Hungary	2016	chicken
MYCAV277	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV278	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV281	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV282	*M. synoviae* isolate	Hungary	2016	chicken
MYCAV284	*M. synoviae* isolate	Austria	2016	turkey
MYCAV285	*M. synoviae* isolate	Austria	2016	turkey
MYCAV288	*M. synoviae* isolate	Austria	2016	turkey
MYCAV291	*M. synoviae* isolate	Hungary	2016	turkey
MYCAV300	*M. synoviae* isolate	Hungary	2016	chicken
MYCAV303	*M. synoviae* isolate	Russia	2016	chicken
MYCAV306	*M. synoviae* isolate	Hungary	2016	turkey
IZSVE/2012/6077/D12/1-f	*M. synoviae* isolate	Tunisy	2012	chicken
IZSVE/2012/5715/D12/4-f	*M. synoviae* isolate	Italy	2012	chicken
IZSVE/2012/6083/D12/2-d	*M. synoviae* isolate	Tunisy	2012	chicken
IZSVE/2013/378/D13/1-f-H	*M. synoviae* isolate	Taiwan	2013	chicken
IZSVE/2013/244/D13/1-f-E	*M. synoviae* isolate	Lebanon	2013	chicken
IZSVE/2013/560/D13/1-f-E	*M. synoviae* isolate	Jordan	2013	chicken
IZSVE/2013/2094/D13/10-f	*M. synoviae* isolate	Italy	2013	chicken
IZSVE/2013/564/D13/3-d-E	*M. synoviae* isolate	Jordan	2013	chicken
IZSVE/2013/3757/D13/2-f	*M. synoviae* isolate	Italy	2013	chicken
IZSVE/2013/3291/D13/2-f	*M. synoviae* isolate	Lebanon	2013	chicken

ID of *M. synoviae* strains	Sample type	Origin	Year	Host
IZSVE/2013/4109/D13/1-f	*M. synoviae* isolate	Italy	2013	turkey
IZSVE/2013/4663/D13/2-f	*M. synoviae* isolate	Italy	2013	turkey
IZSVE/2013/4498/D13/2-f	*M. synoviae* isolate	Italy	2013	turkey
IZSVE/2013/5914/D13/1-d	*M. synoviae* isolate	Italy	2013	chicken
IZSVE/2013/5914/D13/5-d	*M. synoviae* isolate	Italy	2013	chicken
IZSVE/2014/589/D14/1-f-E	*M. synoviae* isolate	Italy	2014	chicken
IZSVE/2012/806/D12/14-f	*M. synoviae* isolate	Italy	2012	chicken
IZSVE/2015/740/D1517-d-F	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2015/2519/D15/1-f	*M. synoviae* isolate	Italy	2015	turkey
IZSVE/2015/2376/D15/1-f	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2015/2518/D15/1-f	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2015/3145/D15/4-f	*M. synoviae* isolate	Spain	2015	chicken
IZSVE/2015/3622/D15/2-f	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2015/4599/D15/2-d	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2015/5871/D15/2-f	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2015/5872/D15/3-f	*M. synoviae* isolate	Italy	2015	chicken
IZSVE/2016/1074/MAV16/15-f	*M. synoviae* isolate	Italy	2016	chicken
IZSVE/2016/1466/MAV16/4-f	*M. synoviae* isolate	Italy	2016	chicken
IZSVE/2016/1695/MAV16/2-f	*M. synoviae* isolate	Spain	2016	chicken
IZSVE/2016/2953/MAV16/28-l	*M. synoviae* isolate	Italy	2016	turkey
IZSVE/2016/3007/MAV16/1-f	*M. synoviae* isolate	Italy	2016	turkey
IZSVE/2016/2958/MAV16/11-f	*M. synoviae* isolate	Spain	2016	chicken
IZSVE/2016/3274/MAV16/3-f	*M. synoviae* isolate	Spain	2016	chicken
MYCAV536	*M. synoviae* isolate	China	2018	chicken
MYCAV573	*M. synoviae* isolate	Korea	2019	chicken
MYCAV574	*M. synoviae* isolate	Korea	2019	chicken
2003.9	*M. synoviae* isolate	The Netherlands	2003	chicken
2010.5	*M. synoviae* isolate	The Netherlands	2010	chicken
2010.6	*M. synoviae* isolate	The Netherlands	2010	chicken
2010.13	*M. synoviae* isolate	The Netherlands	2010	turkey
2011.1	*M. synoviae* isolate	The Netherlands	2011	chicken
2014.19	*M. synoviae* isolate	The Netherlands	2014	chicken
2015.11	*M. synoviae* isolate	The Netherlands	2018	chicken
2017.3	*M. synoviae* isolate	The Netherlands	2017	chicken
2018.1	*M. synoviae* isolate	The Netherlands	2017	chicken
MS-ALN-A	*M. synoviae* isolate	Israel	2011	turkey
MS-EB-B	*M. synoviae* isolate	Israel	2000	turkey
MS-RMJ-B	*M. synoviae* isolate	Israel	2007	turkey
MS-SBS-2E	*M. synoviae* isolate	Israel	2011	chicken

GenBank Acc. N.: GenBank accession number; n.a.: not available

Table S3. Results of the PCRs, MLST assay and MAMA tests performed on *M. gallisepticum* samples

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
ATCC 19610	6.84	wt	wt	wt	wt	wt	wt	wt	wt	wt	1	n.t.	n.t.	n.t.
MG F	7.94	F	F	wt	wt	wt	wt	wt	wt	F	5	n.t.	n.t.	n.t.
MG 6/85	7.23	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
MG ts-11	6.38	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
MG K 5831	6.12	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	K	K	57	n.t.	n.t.	n.t.
S6[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	17	n.t.	n.t.	n.t.
R_{low}[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	2	n.t.	n.t.	n.t.
R_{high}[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	2	n.t.	n.t.	n.t.
CA06_2006.052-5-2P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	9	n.t.	n.t.	n.t.
NY01_2001.047-5-1P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	9	n.t.	n.t.	n.t.
VA94_7994-1-7P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	9	n.t.	n.t.	n.t.
NC95_13295-2-2P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	9	n.t.	n.t.	n.t.
NC96_1596-4-2P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	9	n.t.	n.t.	n.t.
WI01_2001.043-13-2P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	9	n.t.	n.t.	n.t.
NC06_2006.080-5-2P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	10	n.t.	n.t.	n.t.
NC08_2008.031-4-3P[is]	n.t.	wt	wt	wt	wt	wt	wt	wt	wt	wt	11	n.t.	n.t.	n.t.
K2966 (=ts-11)[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
K5322C-13[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
K6112B-8[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
K6208B-10[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
K6216D[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	wt*	50	+	+	+
K6222B[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
K6356-12[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
K6372-23[is]	n.t.	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
MYCAV88	6.29	wt	wt	wt	wt	wt	wt	wt	wt	wt	37	n.t.	n.t.	n.t.
MYCAV175	6.79	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV228	7.11	wt	wt	wt	wt	wt	wt	wt	wt	wt	12	n.t.	n.t.	n.t.
MYCAV229	8.49	wt	wt	wt	wt	wt	wt	wt	wt	wt	7	n.t.	n.t.	n.t.
MYCAV231	6.22	wt	wt	wt	wt	wt	wt	wt	wt	wt	12	n.t.	n.t.	n.t.
MYCAV251	8.68	wt	wt	wt	wt	wt	wt	wt	wt	wt	24	n.t.	n.t.	n.t.
MYCAV255	7.35	wt	wt	wt	wt	wt	wt	wt	wt	wt	54	n.t.	n.t.	n.t.
MYCAV305	7.82	wt	wt	wt	wt	wt	wt	wt	wt	wt	40	n.t.	n.t.	n.t.
MYCAV372	7.46	wt	wt	wt	wt	wt	wt	wt	wt	wt	28	n.t.	n.t.	n.t.
MYCAV375	6.67	wt	wt	wt	wt	wt	wt	wt	wt	wt	31	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
MYCAV387	8.14	wt	wt	wt	wt	wt	wt	wt	wt	wt	38	n.t.	n.t.	n.t.
MYCAV258	6.41	wt	wt	wt	wt	wt	wt	wt	wt	wt	24	n.t.	n.t.	n.t.
MYCAV388	13.61	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
MYCAV389	7.45	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV390	7.03	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV391	11.52	F	F	wt	wt	wt	wt	wt	wt	F	5	n.t.	n.t.	n.t.
MYCAV392	11.22	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV393	8.55	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV394	9.65	wt	wt	wt	wt	wt	wt	wt	wt	wt	3	n.t.	n.t.	n.t.
MYCAV395	8.99	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV396	9.64	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV397	7.49	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV398	11.74	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV399	12.21	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV400	9.08	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV401	9.66	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV402	8.29	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
MYCAV403	11.54	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV404	10.44	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV405	8.95	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV406	6.27	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV407	13.56	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV419	6.99	wt	wt	wt	wt	wt	wt	wt	wt	wt	38	n.t.	n.t.	n.t.
MYCAV420	7.83	wt	wt	wt	wt	wt	wt	wt	wt	wt	38	n.t.	n.t.	n.t.
MYCAV604	12.2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV605	18.79	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV612	17.07	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV617	14.74	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV623	14.07	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV627	18.48	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV656	17.56	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV679	14.3	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV683	12.11	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV684	15.34	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV690	9.98	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MYCAV694	11.5	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
417	10.4	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
419	6.31	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
684	7.69	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
5668	7.21	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
5857	8.57	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
16L-2104	8.64	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
16L-3533	6.94	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
16L-4563	8.56	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
16L-4721	6.29	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
16L-6185	9.51	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
B15/94	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	55	n.t.	n.t.	n.t.
B16/12	9.67	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
B29/07	7.6	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
B32/11	7.48	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
B40/07	8.36	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
B70/07	8.55	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
B112/08	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	56	n.t.	n.t.	n.t.
B114/06	8.19	wt	wt	wt	wt	wt	wt	wt	wt	wt	42	n.t.	n.t.	n.t.
B114/99	7.43	wt	wt	wt	wt	wt	wt	wt	wt	wt	52	n.t.	n.t.	n.t.
B134/11	9.21	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
B172/11	11.39	wt	wt	wt	wt	wt	wt	wt	wt	wt	35	n.t.	n.t.	n.t.
B227/13	10.11	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
93148 (21-3a)	13.7	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
99179 (A-UTa)	7.84	wt	wt	wt	wt	wt	ts-11	ts-11	wt	wt**	45	+	+	+
95003 (W-5a)	9.73	wt	wt	wt	wt	wt	wt	wt	wt	wt	44	(+)	(+)	+
94043 (28-1a)	7.96	wt	wt	wt	wt	wt	wt	wt	wt	wt	43	(+)	+	+
96022 (6-3a)	6.15	wt	wt	wt	wt	wt	wt	wt	wt	wt	53	+	+	(+)
98036 AP3AS	6.38	wt	wt	wt	wt	wt	wt	wt	wt	wt	43	(+)	+	+
SHB-14	12.91	wt	wt	wt	wt	wt	wt	wt	wt	wt	47	n.t.	n.t.	n.t.
HZ-2	11.89	wt	wt	wt	wt	wt	wt	wt	wt	wt	21	n.t.	n.t.	n.t.
DSD-14	13.74	wt	wt	wt	wt	wt	wt	wt	wt	wt	20	n.t.	n.t.	n.t.
TR-10	12.39	wt	wt	wt	wt	wt	wt	wt	wt	wt	21	n.t.	n.t.	n.t.
MKT-3	11.89	wt	wt	wt	wt	wt	wt	wt	wt	wt	21	n.t.	n.t.	n.t.
TL-3	13.47	wt	wt	wt	wt	wt	wt	wt	wt	wt	4	n.t.	n.t.	n.t.
MDE-3	11.98	wt	wt	wt	wt	wt	wt	wt	wt	wt	22	n.t.	n.t.	n.t.
RV-18	10.22	wt	wt	wt	wt	wt	wt	wt	wt	wt	46	n.t.	n.t.	n.t.
KTY-15	12.78	wt	wt	wt	wt	wt	wt	wt	wt	wt	22	n.t.	n.t.	n.t.
SOB-3	13.49	wt	wt	wt	wt	wt	wt	wt	wt	wt	19	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
DOS-3	15.13	wt	wt	wt	wt	wt	wt	wt	wt	wt	54	n.t.	n.t.	n.t.
ZR50-1	10.89	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
SDM-10	11.48	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
VR-4	13.66	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
BNC-3	17.85	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
SLH-1	21.42	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
BSY-12	18.33	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
BLF-10	15.97	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/2741-1f	13.79	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
IZSVE/2014/2743-3f	14.88	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
IZSVE/2012/6164-1d	19.02	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
IZSVE/2012/1626-2f	13.54	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
IZSVE/2012/6166-1f	25.99	wt	wt	-	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
IZSVE/2014/3462-14f	14.39	wt	wt	6/85	6/85	wt	wt	wt	wt	6/85	14	n.t.	n.t.	n.t.
IZSVE/2014/1779-12f	15.23	wt	wt	6/85	6/85	wt	wt	wt	wt	wt[#]	13	n.t.	n.t.	n.t.
IZSVE/2012/2546-2d	13.59	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	(+)	-	-
IZSVE/2013/2857-1f	24.71	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	-	-
IZSVE/2013/3185-5f	11.68	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	+	+	+
IZSVE/2013/4957-D5d	24.2	wt	wt	wt	wt	-	ts-11	ts-11	wt	wt*	48	-	-	-
IZSVE/2013/4693-4f	13.24	wt	wt	6/85	wt	wt	ts-11	wt	wt	wt[##]	16	(+)	-	-
IZSVE/2014/6259-35f	14.22	wt	wt	6/85	wt	wt	wt	wt	wt	wt	25	n.t.	n.t.	n.t.
IZSVE/2012/5711-1d	13.54	wt	wt	wt	wt	wt	wt	wt	wt	wt	29	n.t.	n.t.	n.t.
IZSVE/2011/5595-2d	14.06	wt	wt	wt	wt	wt	wt	wt	wt	wt	8	n.t.	n.t.	n.t.
IZSVE/2011/2247-10d	13.88	wt	wt	wt	wt	wt	wt	wt	wt	wt	27	n.t.	n.t.	n.t.
IZSVE/2012/4464-5f	12.74	wt	wt	wt	wt	wt	wt	wt	wt	wt	27	n.t.	n.t.	n.t.
IZSVE/2012/1731-t-t-5a	13.37	wt	wt	wt	wt	wt	wt	wt	wt	wt	23	n.t.	n.t.	n.t.
IZSVE/2015/1731-40°	16.3	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	wt	36	n.t.	n.t.	n.t.
IZSVE/2013/3061-1f	16.51	wt	wt	wt	wt	wt	wt	wt	wt	wt	24	n.t.	n.t.	n.t.
IZSVE/2013/3914-2f	12.45	wt	wt	wt	wt	wt	wt	wt	wt	wt	36	n.t.	n.t.	n.t.
IZSVE/2013/380-9f	13.93	wt	wt	wt	wt	wt	wt	wt	wt	wt	23	n.t.	n.t.	n.t.
IZSVE/2013/3457-5d	16.66	wt	wt	wt	wt	wt	wt	wt	wt	wt	34	n.t.	n.t.	n.t.
IZSVE/2013/3963-9f	12.75	wt	wt	wt	wt	wt	wt	wt	wt	wt	33	n.t.	n.t.	n.t.
IZSVE/2013/6687-22f	24.6	wt	wt	wt	wt	-	wt	wt	n.t.	wt	34	n.t.	n.t.	n.t.
IZSVE/2013/7016-10f	12.46	wt	wt	wt	wt	wt	wt	wt	wt	wt	26	n.t.	n.t.	n.t.
IZSVE/2014/4852-1f	11.73	wt	wt	wt	wt	wt	wt	wt	wt	wt	34	n.t.	n.t.	n.t.
IZSVE/2014/4853-1d	11.83	wt	wt	wt	wt	wt	wt	wt	wt	wt	34	n.t.	n.t.	n.t.
IZSVE/2014/6082-8f	11.59	wt	wt	wt	wt	wt	wt	wt	wt	wt	34	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
IZSVE/2015/5870-1f	13.97	wt	wt	wt	wt	wt	wt	wt	wt	wt	30	n.t.	n.t.	n.t.
IZSVE/2017/3541-1f	13.71	wt	wt	wt	wt	wt	wt	wt	wt	wt	28	n.t.	n.t.	n.t.
IZSVE/2013/561-1f	28.03	wt	wt	wt	wt	wt	wt	wt	wt	wt	22	n.t.	n.t.	n.t.
IZSVE/2012/3057-1d	26.97	wt	wt	wt	wt	wt	wt	wt	wt	wt	51	n.t.	n.t.	n.t.
IZSVE/2012/3057-2d	27.55	wt	wt	wt	wt	wt	wt	wt	wt	wt	51	n.t.	n.t.	n.t.
IZSVE/2013/4360-1f	13.28	wt	wt	wt	wt	wt	wt	wt	wt	wt	15	n.t.	n.t.	n.t.
IZSVE/2013/4360-2f	26.75	wt	wt	wt	wt	wt	wt	-	wt	wt	15	n.t.	n.t.	n.t.
IZSVE/2013/3188-1f	12.61	wt	wt	wt	wt	wt	wt	wt	wt	wt	32	n.t.	n.t.	n.t.
IZSVE/2015/388-8f	14.71	wt	wt	wt	wt	wt	wt	wt	wt	wt	24	n.t.	n.t.	n.t.
IZSVE/2015/2061-6f	15.01	wt	wt	wt	wt	wt	wt	wt	wt	wt	24	n.t.	n.t.	n.t.
IZSVE/2015/2063-2f	13.24	wt	wt	wt	wt	wt	wt	wt	wt	wt	24	n.t.	n.t.	n.t.
IZSVE/2014/3567-5d	16.67	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/6716-2f	14.74	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/6162-2f	14.12	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/1627-1f	12.75	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3189-1d	13.59	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3189-2f	17.84	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/1779-8f	15.76	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/1445-1fwt	15.19	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/3462-16f	13.87	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/5570-1d	14.61	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2010/3559-tr.F	19.27	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2011/6344-3d	18.78	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2011/6488-1f	11.59	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2011/3954-15d	11.47	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/2911-21f	12.92	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3616-1f	14.63	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3616-2f	11.23	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3653-10d	14.39	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/3791-1f	16.62	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/6142-10d	20.08	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/1705-2d	16.14	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/691-13f	17.47	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/692-13f	23.28	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/693-12f	24.27	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/4268-6d	14.42	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/695-12f	19.31	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
IZSVE/2013/48-2f-p	13.27	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/3116-17f	10.42	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/2999-2f	10.77	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/2093-1f	14.92	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/5146-1d	11.12	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/5352-1d	13.57	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/5949-8f	21.34	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/4816-5d	10.24	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/723-1d	11.69	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/722-17d	15.36	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/6715-1d	27.78	wt	-	wt	wt	wt	wt	-	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/4487-2d	24.58	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/5452-1d	11.37	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/5459-1f	13.44	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/6215-1d	17.86	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/6435-1f	12.33	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/3193-23d	11.61	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/2646-40°	14.11	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/1811-3f	13.55	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/4744-85f	14.21	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/4783-2f	15.19	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/2541-7f	13.86	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/852-1f	15.45	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/121-8d	13.99	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/149-2f	11.46	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/231-1f	10.99	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/2769-7f	16.16	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/2326-3d	12.48	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/2947-13d	9.21	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/3024-2f	12.66	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/3540-1f	11.94	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2017/514-1f	16.31	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2017/517-6f	17.19	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/236-2d	12.72	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3058-1d	18.24	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3058-2d	20.47	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/6752-3f	14.76	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
IZSVE/2013/3188-2f	19.43	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/1772-2f	9.36	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/3164-2f	10.97	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/3776-1f	12.19	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/3188-D13-1f	13.36	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/4868-4d	11.44	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/979-1f	11.02	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/388-4f	7.49	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/388-3f	8.21	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/388-7d	9.75	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/2061-8d	12.46	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/2062-3f	12.35	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/2063-16f	8.25	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/2958-1d	14.23	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2017/3571-2d	17.45	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2015/2062-4f	26.57	wt	-	wt	wt	-	-	-	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/1934-2f	28.66	-	-	-	-	-	-	-	wt	n.t.	n.t.	n.t.	n.t.	n.t.
LUM-5	15.77	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
SLY-12	16.06	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
BYM-14	17.38	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
KFM-12	17.99	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
SU-4	18.32	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
MSC-14	15.54	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/3513/1	18.18	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	wt	29	n.t.	n.t.	n.t.
IZSVE/2016/400-12f	14.53	wt	wt	wt	wt	ts-11	ts-11	ts-11	wt	ts-11	49	-	-	-
IZSVE/2013/5575-28d	17.01	wt	wt	wt	wt	wt	wt	wt	wt	wt	41	n.t.	n.t.	n.t.
IZSVE/2012/6194-5	27.78	wt	wt	wt	wt	-	wt	-	wt	wt	18	n.t.	n.t.	n.t.
IZSVE/2012/6194-14	12.74	wt	wt	wt	wt	wt	wt	wt	wt	wt	18	n.t.	n.t.	n.t.
IZSVE/2013/566-1f	25.65	wt	wt	wt	wt	-	wt	wt	n.t.	wt	22	n.t.	n.t.	n.t.
IZSVE/2014/6088-2f	12.88	wt	wt	wt	wt	wt	wt	wt	wt	wt	32	n.t.	n.t.	n.t.
IZSVE/2015/980	15.97	wt	wt	wt	wt	wt	wt	wt	wt	wt	6	n.t.	n.t.	n.t.
IZSVE/2016/115-2f	13.78	wt	wt	wt	wt	wt	wt	wt	wt	wt	39	n.t.	n.t.	n.t.
IZSVE/2016/2581-3	16.77	wt	wt	wt	wt	wt	wt	wt	wt	wt	28	n.t.	n.t.	n.t.
IZSVE/2016/3063-1	14.96	wt	wt	wt	wt	wt	wt	wt	wt	wt	15	n.t.	n.t.	n.t.
IZSVE/2016/3274-3f	12.74	wt	wt	6/85	6/85	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/2036-142	11.26	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/3712-5f	11.87	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.

ID of *M. gallisepticum* strains	qPCR[a] (Ct)	F		6/85		ts-11			K	MLST		vlhA 3.04A[b]	vlhA 3.05[b]	mg 0359[b]
		hlp2	crmA	lpd	gapA	plpA	glpK	potC	fruA					
IZSVE/2015/5859-4d	12.43	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2017/10-33	11.25	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/3188-2f	12.58	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2014/6089-1d	10.95	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/2581-4	15.14	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/3274-2f	13.78	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2017/199-3f	15.91	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/6194-2	20.11	wt	wt	wt	wt	-	wt	wt	-	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2013/3188-6f	20.96	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/115-4f	23.32	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2017/1039-66	23.14	wt	wt	wt	wt	wt	wt	wt	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2012/1183-9f	23.77	wt	-	-	wt	-	wt	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/3483-3f	28.27	wt	-	-	-	-	-	-	wt	n.t.	n.t.	n.t.	n.t.	n.t.
IZSVE/2016/2581-2	30.09	-	-	-	wt	-	-	-	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19039-4	27.12	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19039-8	26.48	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19053-TII-19w	17.07	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19053-TAL-33w	22	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19053-TB1-9w	14.74	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19105-5B	26.14	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19105-5C	27.43	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19105-5D	27.76	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19135-M1a	25.77	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19135-M1b	25.63	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19135-M1c	26.35	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19137-IF25	27.87	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19141-7CL	24.49	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19144-33w	27.97	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19144-48w	26.64	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19167-C	23.94	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19167-G	23.1	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19171-LI-F1	22.24	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
19171-LM-F1	22.74	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	n.t.	n.t.	n.t.	n.t.	n.t.
E1/16	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	wt	22	n.t.	n.t.	n.t.

[a]according to Raviv and Kleven, 2009; [b]according to Ricketts *et al.*, 2017; [is]analyzed *in silico*; *[#]1-2 nucleotide (0.04-0.08 %) difference from ts-11* or 6/85[#] vaccine strain; 7-10 nucleotide (0.27-0.37 %) difference from ts-11** or 6/85[##] vaccine strain; Ct: cycle threshold; n.t.: not tested; wt: wild-type; -: non-detectable; +: positive; (+): weak positive

Table S4. MIC values (µg/ml) of the examined *M. synoviae* strains for the fourteen tested antimicrobial agents

ID of *M. synoviae* strains	Enroflox.	Diflox.	Oxytet.	Chlortet.	Doxyc.	Spectin.	Neom.	Tilmic.	Tylos.	Tylval.	Lincom.	Florfen.	Tiam.	Valnem.
NCTC 10124[a]	0.312	0.625-1.25	≤0.25	1	≤0.039	1	>64	≤0.25	≤0.25	≤0.25	≤0.25	1-2	0.078	≤0.039
MS-H[a]	0.156	0.312	≤0.25	≤0.25	≤0.039	1	64	≤0.25	≤0.25	≤0.25	≤0.25	0.5	0.078	≤0.039
MS1[a]	0.625	1.25	≤0.25	≤0.25	≤0.039	n.t.	n.t.	≤0.25	≤0.25	n.t.	n.t.	4	0.156	n.t.
MYCAV79[a]	0.312	1.25	0.5	≤0.25	0.078	1	32	≤0.25	≤0.25	≤0.25	1	2	0.078	≤0.039
MYCAV102[a]	1.25	1.25	0.5	0.5	0.078	1	32	≤0.25	≤0.25	≤0.25	1	2	0.078	≤0.039
MYCAV119[a]	0.312	1.25	≤0.25	≤0.25	0.078	2	32	≤0.25	≤0.25	≤0.25	1	2	0.078	≤0.039
MYCAV130[a]	10	5	0.5	0.5	0.078	2	32	≤0.25	≤0.25	≤0.25	1	4	0.156	≤0.039
MYCAV167[a]	0.625	0.625	≤0.25	0.5	≤0.039	1	8	≤0.25	≤0.25	≤0.25	≤0.25	2	≤0.039	≤0.039
MYCAV168[a]	>10	5	1	2	0.312	2	8	0.5	≤0.25	≤0.25	1	8	0.156	≤0.039
MYCAV170[a]	2.5	2.5	0.5	0.5	0.078	2	16	≤0.25	≤0.25	≤0.25	0.5	8	0.156	≤0.039
MYCAV173[a]	10	2.5	0.5	1	0.156	1	4	1	≤0.25	≤0.25	0.5	2	≤0.039	≤0.039
MYCAV174[a]	>10	10	1	4	0.312	2	32	4	≤0.25	≤0.25	1	8	0.078	≤0.039
MYCAV183[a]	1.25	1.25	≤0.25	0.5	0.078	8	>64	≤0.25	≤0.25	≤0.25	0.5	8	0.078	≤0.039
MYCAV185[a]	>10	5	0.5	0.5	0.312	2	16	64	2	0.5	>64	8	0.156	≤0.039
MYCAV186[a]	>10	2.5	0.5	2	0.156	2	64	≤0.25	≤0.25	≤0.25	0.5	2	0.312	≤0.039
MYCAV188[a]	>10	10	1	4	0.312	2	32	≤0.25	≤0.25	≤0.25	0.5	2	≤0.039	≤0.039
MYCAV189[a]	10	5	1	8	0.312	2	32	2	≤0.25	≤0.25	1	8	0.078	≤0.039
MYCAV190[a]	>10	10	0.5	4	0.312	2	32	2	≤0.25	≤0.25	1	8	0.078	≤0.039
MYCAV193[a]	1.25	0.625	≤0.25	≤0.25	0.078	8	32	0.5	≤0.25	≤0.25	2	8	0.625	≤0.039
MYCAV194[a]	n.t.	n.t.	≤0.25	≤0.25	≤0.039	4	32	≤0.25	≤0.25	≤0.25	0.5	4	0.156	≤0.039
MYCAV195[a]	1.25	1.25	0.5	0.5	0.078	2	32	≤0.25	≤0.25	≤0.25	1	8	0.078	≤0.039
MYCAV196[a]	0.625	1.25	0.5	1	0.156	4	16	≤0.25	≤0.25	≤0.25	0.5	8	0.156	≤0.039
MYCAV197[a]	0.625	10	≤0.25	0.5	≤0.039	≤0.25	32	≤0.25	≤0.25	≤0.25	0.5	4	0.078	≤0.039
MYCAV198[a]	0.625	5	0.5	≤0.25	0.156	4	16	≤0.25	≤0.25	≤0.25	0.5	8	0.312	≤0.039
MYCAV217[a]	2.5	10	≤0.25	1	0.078	1	64	0.5	≤0.25	≤0.25	1	8	0.625	≤0.039
MYCAV236[a]	0.312	1.25	≤0.25	1	0.078	1	16	≤0.25	≤0.25	≤0.25	0.5	0.5	0.078	≤0.039
MYCAV249[a]	5	10	0.5	≤0.25	0.156	4	>64	≤0.25	≤0.25	≤0.25	1	4	0.078	≤0.039
MYCAV256[a]	10	5	0.5	1	0.156	4	>64	≤0.25	≤0.25	≤0.25	1	4	0.156	≤0.039
MYCAV257[a]	>10	10	1	2	0.156	2	32	≤0.25	≤0.25	≤0.25	1	4	0.156	≤0.039
MYCAV259[a]	>10	5	≤0.25	0.5	0.078	2	32	≤0.25	≤0.25	≤0.25	≤0.25	2	0.312	≤0.039
MYCAV261[a]	1.25	1.25	≤0.25	0.5	0.078	2	16	≤0.25	≤0.25	≤0.25	≤0.25	4	≤0.039	≤0.039
MYCAV262[a]	0.625	1.25	≤0.25	≤0.25	0.078	1	16	≤0.25	≤0.25	≤0.25	0.5	8	≤0.039	≤0.039
MYCAV263[a]	1.25	1.25	≤0.25	0.5	0.078	1	8	≤0.25	≤0.25	≤0.25	≤0.25	2	≤0.039	≤0.039
MYCAV268[a]	1.25	2.5	≤0.25	0.5	0.078	2	64	≤0.25	≤0.25	≤0.25	0.5	8	0.156	≤0.039

ID of *M. synoviae* strains	Enroflox.	Diflox.	Oxytet.	Chlortet.	Doxyc.	Spectin.	Neom.	Tilmic.	Tylos.	Tylval.	Lincom.	Florfen.	Tiam.	Valnem.
MYCAV272[a]	0.625	1.25	≤0.25	≤0.25	0.078	4	>64	≤0.25	≤0.25	≤0.25	2	4	0.625	≤0.039
MYCAV274[a]	2.5	2.5	≤0.25	≤0.25	0.078	4	64	≤0.25	≤0.25	≤0.25	1	8	0.312	≤0.039
MYCAV277[a]	1.25	1.25	≤0.25	≤0.25	0.078	2	32	≤0.25	≤0.25	≤0.25	0.5	4	0.078	≤0.039
MYCAV278[a]	1.25	1.25	≤0.25	≤0.25	0.156	2	16	≤0.25	≤0.25	≤0.25	0.5	2	≤0.039	≤0.039
MYCAV281[a]	0.312	0.625	≤0.25	≤0.25	0.156	2	32	≤0.25	≤0.25	≤0.25	1	8	0.156	≤0.039
MYCAV282[a]	10	5	0.5	≤0.25	0.156	4	>64	≤0.25	≤0.25	≤0.25	1	0.5	≤0.039	≤0.039
MYCAV284[a]	1.25	2.5	≤0.25	≤0.25	0.078	2	32	≤0.25	≤0.25	≤0.25	0.5	4	0.156	≤0.039
MYCAV285[a]	1.25	2.5	≤0.25	0.5	0.078	2	32	≤0.25	≤0.25	≤0.25	≤0.25	4	0.078	≤0.039
MYCAV288[a]	1.25	2.5	≤0.25	1	0.078	1	>64	≤0.25	≤0.25	≤0.25	0.5	4	0.078	≤0.039
MYCAV291[a]	0.625	2.5	0.5	1	0.156	1	32	≤0.25	≤0.25	≤0.25	≤0.25	4	0.078	≤0.039
MYCAV300[a]	>10	10	1	2	0.312	2	64	1	≤0.25	≤0.25	1	4	0.078	≤0.039
MYCAV303[a]	10	5	0.5	0.5	0.312	2	32	>64	2	0.5	>64	4	0.312	≤0.039
MYCAV306[a]	1.25	2.5	≤0.25	0.5	0.078	1	16	≤0.25	≤0.25	≤0.25	1	4	0.078	≤0.039
IZSVE/2012/6077/D12/1-f[a]	10	5	1	4	0.312	1	4-8	>64	2	≤0.25	>64	8	0.312	≤0.039
IZSVE/2012/5715/D12/4-f[a]	10	10	0.5	8	0.156	1	8	≤0.25	≤0.25	≤0.25	0.5	4	0.312	≤0.039
IZSVE/2012/6083/D12/2-d[a]	5	5	0.5	4	0.156	2	32	>64	2	0.5	>64	8	0.312	≤0.039
IZSVE/2013/378/D13/1-f-H[a]	5	2.5	0.5	1	0.312	2	>64	>64	1	0.5	>64	2	0.156	≤0.039
IZSVE/2013/244/D13/1-f-E[a]	2.5	1.25	0.5	2	0.078	2	32	1	≤0.25	≤0.25	0.5	2	0.156	≤0.039
IZSVE/2013/560/D13/1-f-E[b]	>10	n.t.	n.t.	n.t.	0.156-0.312	n.t.	n.t.	>32	0.5	≤0.25	>32	0.5	0.312-0.625	n.t.
IZSVE/2013/2094/D13/10-f[a]	5	5	0.5-1	8	0.312	0.5-1	32	>64	0.5	0.5	>64	8	0.312	≤0.039
IZSVE/2013/564/D13/3-d-E[a]	5	2.5	0.5	1	0.156	1	32	64	1	≤0.25	>64	2	0.156	≤0.039
IZSVE/2013/3757/D13/2-f[a]	10	10	1	2	0.312	2	16	≤0.25	≤0.25	≤0.25	2	4	0.625	≤0.039
IZSVE/2013/3291/D13/2-f[a]	1.25	1.25	0.5	1	0.078	4	>64	≤0.25	≤0.25	≤0.25	1	4	0.156	≤0.039
IZSVE/2013/4109/D13/1-f[a]	10	10	1	2	0.625	2	32	≤0.25	≤0.25	≤0.25	2	8	0.312	≤0.039
IZSVE/2013/4663/D13/2-f[a]	10	5	0.5	1	0.312	1	16	16	0.5	≤0.25	0.5	4	0.156	≤0.039
IZSVE/2013/4498/D13/2-f[a]	5	2.5	1	2	0.078	2	32	64	0.5	≤0.25	0.5	8	0.156	≤0.039
IZSVE/2013/5914/D13/1-d[a]	5	2.5	0.5	0.5	0.156	0.5	32	>64	16	4	>64	1	0.078	≤0.039
IZSVE/2013/5914/D13/5-d[a]	5	2.5	0.5	1	0.156	0.5	8	>64	32	4	>64	2	0.156	≤0.039
IZSVE/2014/589/D14/1-f-E[b]	2.5-5	n.t.	n.t.	n.t.	0.156-0.312	n.t.	n.t.	16	0.5	≤0.25	4	<0.5	0.312-0.625	n.t.
IZSVE/2012/806/D12/14-f[a]	>10	10	2	1	0.312	2	16	≤0.25	≤0.25	≤0.25	2	4	0.312	≤0.039
IZSVE/2015/740/D1517-d-F[a]	10	5	≤0.25	0.5	0.156	0.5	32	32	0.5	≤0.25	>64	2	0.156	≤0.039
IZSVE/2015/2519/D15/1-f[a]	5	2.5	0.5	0.5	0.078	0.5	16	>64	2	0.5	>64	4	0.078	≤0.039
IZSVE/2015/2376/D15/1-f[b]	0.625-1.25	n.t.	n.t.	n.t.	0.312-0.625	n.t.	n.t.	1	≤0.25	≤0.25	<0.5	1	0.156-0.312	n.t.
IZSVE/2015/2518/D15/1-f[a]	10	5	1	1	0.156	2	16	>64	64	4	>64	4	0.156	≤0.039
IZSVE/2015/3145/D15/4-f[a]	10	5	0.5	1	0.078	2	16	>64	2	0.5	>64	4	0.312	≤0.039
IZSVE/2015/3622/D15/2-f[b]	0.625-1.25	n.t.	n.t.	n.t.	0.312-0.625	n.t.	n.t.	1	≤0.25	≤0.25	<0.5	1	0.156-0.312	n.t.
IZSVE/2015/4599/D15/2-d[a]	10	10	0.5	1	0.078	2	32	≤0.25	≤0.25	≤0.25	1	2	0.078	≤0.039

ID of *M. synoviae* strains	Enroflox.	Diflox.	Oxytet.	Chlortet.	Doxyc.	Spectin.	Neom.	Tilmic.	Tylos.	Tylval.	Lincom.	Florfen.	Tiam.	Valnem.
IZSVE/2015/5871/D15/2-f[a]	2.5	1.25	≤0.25	≤0.25	0.078	0.5	8	≤0.25	≤0.25	≤0.25	0.5	0.5	≤0.039	≤0.039
IZSVE/2015/5872/D15/3-f[b]	0.625-1.25	n.t.	n.t.	n.t.	<0.156	n.t.	n.t.	1	≤0.25	≤0.25	<0.5	<0.5	<0.078	n.t.
IZSVE/2016/1074/MAV16/15-f[b]	>10	n.t.	n.t.	n.t.	0.312-0.625	n.t.	n.t.	>32	0.5	≤0.25	<0.5	1	0.156-0.312	n.t.
IZSVE/2016/1466/MAV16/4-f[a]	10	2.5	0.5	2	0.625	n.t.	n.t.	32	1	0.5	4	2	0.625	n.t.
IZSVE/2016/1695/MAV16/2-f[a]	10	5	0.5	0.5	0.156	1	32	>64	0.5	≤0.25	>64	4	0.312	≤0.039
IZSVE/2016/2953/MAV16/28-l[b]	>10	n.t.	n.t.	n.t.	0.625-1.25	n.t.	n.t.	>32	0.5	≤0.25	<0.5	2	0.156-0.312	n.t.
IZSVE/2016/3007/MAV16/1-f[b]	>10	n.t.	n.t.	n.t.	0.312-0.625	n.t.	n.t.	>32	32	4	>32	1	0.312-0.625	n.t.
IZSVE/2016/2958/MAV16/11-f[a]	5	2.5	1	2	0.312	2	32	≤0.25	≤0.25	≤0.25	2	2	0.078	≤0.039
IZSVE/2016/3274/MAV16/3-f[a]	5	2.5	0.5	0.5	0.078	2	32	8	≤0.25	≤0.25	4	4	0.078	≤0.039
MYCAV536[a]	>10	>10	4	4	1.25	1	n.t.	1	≤0.25	≤0.25	0.5	2	0.078	n.t.
MYCAV573[a]	5	2.5	0.5	0.5	0.156	2	n.t.	16	0.5	≤0.25	1	2	0.078	n.t.
MYCAV574[a]	10	2.5	≤0.25	≤0.25	0.078	1	n.t.	32	2	≤0.25	≤0.25	4	0.156	n.t.
2003.9[c]	>2	n.t.	1	>8	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	4	n.t.	n.t.
2010.5[c]	>2	n.t.	4	>8	n.t.	n.t.	32	n.t.	n.t.	n.t.	n.t.	4	≤0.5	n.t.
2010.6[c]	>2	n.t.	4	>8	n.t.	n.t.	>32	n.t.	n.t.	n.t.	n.t.	4	≤0.5	n.t.
2010.13[c]	>2	n.t.	4	>8	n.t.	n.t.	32	n.t.	n.t.	n.t.	n.t.	4	≤0.5	n.t.
2011.1[c]	>2	n.t.	8	>8	n.t.	n.t.	>32	n.t.	n.t.	n.t.	n.t.	4	1.25-2.5	n.t.
2014.19[c]	1.25-2.5	n.t.	2	>8	n.t.	n.t.	>32	n.t.	n.t.	n.t.	n.t.	4	≤0.5	n.t.
2015.11[c]	>2	n.t.	8	>8	n.t.	n.t.	>32	n.t.	n.t.	n.t.	n.t.	4	≤0.5	n.t.
2017.3[c]	>2	n.t.	8	>8	n.t.	n.t.	>32	n.t.	n.t.	n.t.	n.t.	2	≤0.5	n.t.
2018.1[c]	>2	n.t.	8	>8	n.t.	n.t.	>32	n.t.	n.t.	n.t.	n.t.	2	≤0.5	n.t.
ALN-A[d]	5-10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.5	≤0.25	n.t.	0.5	n.t.	n.t.	n.t.
EB-B[d]	0.156-0.312	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	≤0.25	≤0.25	n.t.	n.t.	n.t.	n.t.	n.t.
RMJ-B[d]	2.5-5	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	≤0.25	≤0.25	n.t.	n.t.	n.t.	n.t.	n.t.
SBS-2E[d]	>10	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	>8	1	n.t.	>8	n.t.	n.t.	n.t.

MIC data of isolates MYCAV79-306 were partly published previously by Kreizinger *et al.* (2017). Exact range of the tested concentrations (µg/ml) in each strain was: enrofloxacin (enroflox.): 0.039-10[a] / 0.125-16[b] / 0.12-2[c] / 0.03125-64[d]; difloxacin (diflox.): 0.039-10[a]; oxytetracycline (oxytet.): 0.25-64[a] / 0.5-8[c]; chlortetracycline (chlortet.): 0.25-64[a] / 0.5-8[c]; doxycycline (doxyc.): 0.039-10[a] / 0.125-16[b]; spectinomycin (spectinom.): 0.25-64[a]; neomycin (neom.): 0.25-64[a] / 4-32[c]; tilmicosin (tilmic.): 0.25-64[a] / 0.015625-32[b] / 0.03125-8[d]; tylosin (tylos.): 0.25-64[a] / 0.0078125-32[b] / 0.015625-4[d]; tylvalosin (tylval.): 0.25-64[a] / 0.005-32[b]; lincomycin (lincom.): 0.25-64[a] / 0.5-32[b] / 0.03125-8[d]; florfenicol (florfen.): 0.125-32[a] / 0.5-16[b] / 0.25-8[c]; tiamulin (tiam.): 0.039-10[a] / 0.0078125-16[b] / 0.5-32[c]; valnemulin (valnem.): 0.039-10[a]; n.t.: not tested

Table S5. Details of whole genome sequences of *M. synoviae* strains

ID of *M. synoviae* strains	Sequencing platform and model	Number of reads	Average length of reads (bp)	Mean sequencing depth (X)*
MS1	IonTorrent PGM	129978	160.1	25.3
MYCAV79	IonTorrent PGM	176483	147.7	31.6
MYCAV102	IonTorrent PGM	116957	163.1	23.3
MYCAV119	IonTorrent PGM	128483	163.0	25.6
MYCAV130	IonTorrent PGM	163292	148.5	29.4
MYCAV167	IonTorrent PGM	85000	156.4	16.0
MYCAV168	IonTorrent PGM	129422	160.6	25.3
MYCAV170	IonTorrent PGM	119322	155.6	22.4
MYCAV173	IonTorrent PGM	144595	145.6	25.4
MYCAV174	IonTorrent PGM	174052	150.8	31.8
MYCAV183	IonTorrent PGM	128244	151.8	23.5
MYCAV185	IonTorrent PGM	126536	163.1	24.8
MYCAV186	IonTorrent PGM	132011	151.7	24.2
MYCAV188	IonTorrent PGM	109692	149.7	20.1
MYCAV189	IonTorrent PGM	109692	149.7	20.1
MYCAV190	IonTorrent PGM	141596	157.8	27.0
MYCAV193	IonTorrent PGM	99659	160.7	19.6
MYCAV194	IonTorrent PGM	106763	152.4	19.7
MYCAV195	IonTorrent PGM	133825	168.9	27.4
MYCAV196	IonTorrent PGM	151005	151.1	27.5
MYCAV197	IonTorrent PGM	99673	158.9	19.1
MYCAV198	IonTorrent PGM	139380	155.3	26.2
MYCAV217	IonTorrent PGM	87339	151.0	15.9
MYCAV236	IonTorrent PGM	135140	151.8	24.7
MYCAV249	IonTorrent PGM	147965	159.4	28.5
MYCAV256	IonTorrent PGM	125774	157.9	23.9
MYCAV257	IonTorrent PGM	173810	152.1	32.0
MYCAV259	IonTorrent PGM	74270	161.7	14.4
MYCAV261	IonTorrent PGM	121632	159.0	23.6
MYCAV262	IonTorrent PGM	157768	154.1	29.6
MYCAV263	IonTorrent PGM	139560	151.5	25.6
MYCAV268	IonTorrent PGM	133099	153.5	24.7
MYCAV272	IonTorrent PGM	145456	163.7	28.7
MYCAV274	IonTorrent PGM	156340	150.9	28.9
MYCAV277	IonTorrent PGM	181044	155.6	34.2
MYCAV278	IonTorrent PGM	146608	145.5	25.9
MYCAV281	IonTorrent PGM	81423	150.4	14.8
MYCAV282	IonTorrent PGM	110113	151.5	20.4
MYCAV284	IonTorrent PGM	139750	156.4	26.4
MYCAV285	IonTorrent PGM	139284	158.0	26.7
MYCAV288	IonTorrent PGM	130121	162.6	25.7
MYCAV291	IonTorrent PGM	268755	166.4	54.9
MYCAV300	IonTorrent PGM	254538	172.0	53.6
MYCAV303	IonTorrent PGM	151104	167.7	32.4
MYCAV306	IonTorrent PGM	206192	180.9	45.7
IZSVE/2012/6077/D12/1-f	IonTorrent PGM	161512	156.2	32.4
IZSVE/2012/5715/D12/4-f	IonTorrent PGM	144592	156.3	29.0
IZSVE/2012/6083/D12/2-d	IonTorrent PGM	185964	169.7	40.3
IZSVE/2013/378/D13/1-f-H	IonTorrent PGM	93285	156.4	18.5
IZSVE/2013/244/D13/1-f-E	IonTorrent PGM	92342	174.3	20.6
IZSVE/2013/560/D13/1-f-E	IonTorrent PGM	163722	160.7	33.9
IZSVE/2013/2094/D13/10-f	IonTorrent PGM	123010	159.2	24.9
IZSVE/2013/564/D13/3-d-E	IonTorrent PGM	168556	156.5	33.7
IZSVE/2013/3757/D13/2-f	IonTorrent PGM	117059	159.3	23.8
IZSVE/2013/3291/D13/2-f	IonTorrent PGM	67538	160.2	13.7
IZSVE/2013/4109/D13/1-f	IonTorrent PGM	218037	161.3	45.0

ID of *M. synoviae* strains	Sequencing platform and model	Number of reads	Average length of reads (bp)	Mean sequencing depth (X)*
IZSVE/2013/4663/D13/2-f	IonTorrent PGM	216330	166.6	45.4
IZSVE/2013/4498/D13/2-f	IonTorrent PGM	145884	154.2	28.9
IZSVE/2013/5914/D13/1-d	IonTorrent PGM	103985	155.4	20.5
IZSVE/2013/5914/D13/5-d	IonTorrent PGM	307844	168.2	65.4
IZSVE/2014/589/D14/1-f-E	IonTorrent PGM	109493	160.2	22.2
IZSVE/2012/806/D12/14-f	IonTorrent PGM	149540	148.2	28.2
IZSVE/2015/740/D1517-d-F	IonTorrent PGM	140179	147.1	26.2
IZSVE/2015/2519/D15/1-f	IonTorrent PGM	191521	158.9	38.3
IZSVE/2015/2376/D15/1-f	IonTorrent PGM	105153	136.3	18.2
IZSVE/2015/2518/D15/1-f	IonTorrent PGM	293210	167.9	61.6
IZSVE/2015/3145/D15/4-f	IonTorrent PGM	234625	145.7	44.0
IZSVE/2015/3622/D15/2-f	IonTorrent PGM	96406	137.3	16.8
IZSVE/2015/4599/D15/2-d	IonTorrent PGM	117861	141.4	21.2
IZSVE/2015/5871/D15/2-f	IonTorrent PGM	116043	159.5	23.7
IZSVE/2015/5872/D15/3-f	IonTorrent PGM	112852	136.9	19.6
IZSVE/2016/1074/MAV16/15-f	IonTorrent PGM	206970	169.3	44.7
IZSVE/2016/1466/MAV16/4-f	IonTorrent PGM	171340	161.5	35.3
IZSVE/2016/1695/MAV16/2-f	IonTorrent PGM	154554	172.1	34.0
IZSVE/2016/2953/MAV16/28-l	IonTorrent PGM	188418	173.3	41.3
IZSVE/2016/3007/MAV16/1-f	IonTorrent PGM	109755	168.1	23.5
IZSVE/2016/2958/MAV16/11-f	IonTorrent PGM	114864	161.2	23.9
IZSVE/2016/3274/MAV16/3-f	IonTorrent PGM	103559	158.8	21.1
MYCAV536	Illumina NextSeq 500	3572077	115.3	516.1
MYCAV573	Illumina NextSeq 500	2814552	118.2	416.5
MYCAV574	Illumina NextSeq 500	3285332	121.3	499.1
2003.9	Illumina NextSeq 500	3677132	117.7	543.9
2010.5	Illumina NextSeq 500	3192206	121.1	486.1
2010.6	Illumina NextSeq 500	3678079	118.2	544.9
2010.13	Illumina NextSeq 500	3275302	118.6	488.3
2011.1	Illumina NextSeq 500	2971371	120.6	449.6
2014.19	Illumina NextSeq 500	2574744	118.8	383.7
2015.11	Illumina NextSeq 500	2967382	122.5	457.0
2017.3	Illumina NextSeq 500	2744503	120.9	417.2
2018.1	Illumina NextSeq 500	3052896	121.5	464.5
ALN-A	Illumina NextSeq 500	667021	249.8	209.9
EB-B	Illumina NextSeq 500	787744	249.5	247.4
RMJ-B	Illumina NextSeq 500	638584	249.6	201.1
SBS-2E	Illumina NextSeq 500	668002	249.6	209.4

*Reads were mapped to the whole genome of *M. synoviae* strain MS53 (GenBank accession number: AE017245); bp: base pair

Table S6. MIC values and potentially resistance-related alterations in GyrA, GyrB, ParC and ParE proteins of *M. synoviae* strains

ID of *M. synoviae* strains	MIC of enroflox. (µg/ml)	MIC of diflox. (µg/ml)	GyrA					GyrB			ParC						ParE	
			7-8	87	133	399	529	142	401	402	80	81	84	441	582	732	83	Ec
			10	143	189	454	551	149	416	417	85	86	89	452	600	814	87	Ms
MS-H	0.156	0.312	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EB-B	0.156-0.312	n.t.	.	.	.	.	D-N	.	.	.	.	.	.	.	.	.	.	
MYCAV281	0.312	0.625	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
NCTC 10124	0.312	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV79	0.312	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV119	0.312	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV236	0.312	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV167	0.625	0.625	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MS1	0.625	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV196	0.625	1.25	.	.	.	.	D-N	.	.	.	.	.	.	.	.	.	.	
MYCAV262	0.625	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV272	0.625	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV291	0.625	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV198	0.625	5	.	.	.	.	.	.	.	.	T-A	.	.	.	.	.	.	
MYCAV197	0.625	10	.	.	.	.	.	.	.	.	T-A	.	.	.	.	.	.	
IZSVE/2015/2376/D15/1-f	0.625-1.25	n.t.	E-K	.	E-G	S-T	D-N	.	.	S-N	.	.	.	E-K	V-I	N-K	.	
IZSVE/2015/3622/D15/2-f	0.625-1.25	n.t.	E-K	.	E-G	S-T	D-N	.	.	S-N	.	.	.	E-K	V-I	N-K	.	
IZSVE/2015/5872/D15/3-f	0.625-1.25	n.t.	E-K	.	E-G	S-T	D-N	.	.	S-N	.	.	.	.	.	.	.	
MYCAV193	1.25	0.625	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S-F	
MYCAV102	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV183	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV195	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV261	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV263	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV277	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV278	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
IZSVE/2013/3291/D13/2-f	1.25	1.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV268	1.25	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV284	1.25	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV285	1.25	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV288	1.25	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV306	1.25	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
2014.19	1.25-2.5	n.t.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
2003.9	>2	n.t.	.	.	.	.	.	.	.	.	T-I	.	.	.	.	.	.	

ID of *M. synoviae* strains	MIC of enroflox. (µg/ml)	MIC of diflox. (µg/ml)	GyrA					GyrB			ParC						ParE	
			7-8	87	133	399	529	142	401	402	80	81	84	441	582	732	83	Ec
			10	143	189	454	551	149	416	417	85	86	89	452	600	814	87	Ms
2010.5	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
2010.6	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
2010.13	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
2011.1	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
2015.11	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
2017.3	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
2018.1	>2	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2013/244/D13/1-f-E	2.5	1.25	.	.	.	.	D-N	.	.	.	.	S-P	.	.	.	.	.	
IZSVE/2015/5871/D15/2-f	2.5	1.25	.	.	E-G	S-T	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
MYCAV170	2.5	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S-F	
MYCAV274	2.5	2.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
MYCAV217	2.5	10	.	.	.	.	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	.	
RMJ-B	2.5-5	n.t.	.	.	.	.	D-N	A-V	S-Y	.	.	.	D-H	.	.	N-K	.	
IZSVE/2014/589/D14/1-f-E	2.5-5	n.t.	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	.	.	.	.	.	.	.	
IZSVE/2013/378/D13/1-f-H	5	2.5	.	.	.	S-Y	.	.	.	.	T-I	.	.	.	.	.	S-F	
IZSVE/2013/564/D13/3-d-E	5	2.5	.	N-S	.	.	.	.	.	.	T-I	.	.	.	.	.	.	
IZSVE/2013/4498/D13/2-f	5	2.5	.	.	.	.	D-N	.	.	.	T-I	.	.	.	.	.	.	
IZSVE/2013/5914/D13/1-d	5	2.5	.	.	.	.	.	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2013/5914/D13/5-d	5	2.5	.	.	.	.	.	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2015/2519/D15/1-f	5	2.5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2016/2958/MAV16/11-f	5	2.5	E-K	.	E-G	S-T	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2016/3274/MAV16/3-f	5	2.5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	.	
MYCAV573	5	2.5	.	.	.	.	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2012/6083/D12/2-d	5	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2013/2094/D13/10-f	5	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV249	5	10	.	.	.	.	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	.	
ALN-A	5-10	n.t.	.	.	.	.	.	.	.	.	T-I	.	.	.	.	.	.	
MYCAV173	10	2.5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2016/1466/MAV16/4-f	10	2.5	.	.	E-G	S-T	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
MYCAV574	10	2.5	.	.	.	.	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
MYCAV130	10	5	.	.	.	.	.	.	.	.	.	.	D-Y	.	.	.	.	
MYCAV189	10	5	E-K	.	E-G	S-T	D-N	.	.	S-N	T-I	.	.	E-K	V-I	N-K	.	
MYCAV256	10	5	.	.	.	.	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	.	
MYCAV282	10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV303	10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2012/6077/D12/1-f	10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	.	

ID of *M. synoviae* strains	MIC of enroflox. (µg/ml)	MIC of diflox. (µg/ml)	GyrA					GyrB			ParC						ParE	
			7-8	87	133	399	529	142	401	402	80	81	84	441	582	732	83	Ec
			10	143	189	454	551	149	416	417	85	86	89	452	600	814	87	Ms
IZSVE/2013/4663/D13/2-f	10	5	.	.	.	.	D-N	.	.	.	T-I	.	.	.	.	.	.	
IZSVE/2015/740/D1517-d-F	10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2015/2518/D15/1-f	10	5	.	.	.	.	.	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2015/3145/D15/4-f	10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2016/1695/MAV16/2-f	10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2012/5715/D12/4-f	10	10	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2013/3757/D13/2-f	10	10	E-K	.	E-G	S-T	D-N	.	.	S-N	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2013/4109/D13/1-f	10	10	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
IZSVE/2015/4599/D15/2-d	10	10	E-K	.	E-G	S-T	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV186	>10	2.5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV168	>10	5	.	.	.	.	.	.	.	S-N	T-I	.	.	E-K	V-I	N-K	.	
MYCAV185	>10	5	.	.	.	.	D-N	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
MYCAV259	>10	5	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV174	>10	10	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV188	>10	10	E-K	.	E-G	S-T	D-N	.	.	S-N	T-I	.	.	E-K	V-I	N-K	.	
MYCAV190	>10	10	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV257	>10	10	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV300	>10	10	E-K	.	E-G	S-T	D-N	.	.	S-N	T-I	.	.	E-K	V-I	N-K	.	
IZSVE/2012/806/D12/14-f	>10	10	E-K	.	E-G	S-T	D-N	A-V	S-Y	.	T-I	.	.	E-K	V-I	N-K	S-F	
MYCAV536	>10	>10	.	.	.	.	.	.	.	.	T-I	.	.	E-K	.	.	.	
IZSVE/2016/1074/MAV16/15-f	>10	n.t.	.	.	.	.	D-N	.	.	.	T-I	.	.	.	.	.	.	
IZSVE/2016/2953/MAV16/28-l	>10	n.t.	.	.	.	.	D-N	.	.	.	T-I	.	.	.	.	.	.	
IZSVE/2016/3007/MAV16/1-f	>10	n.t.	.	.	.	.	.	.	.	.	T-I	.	.	E-K	V-I	N-K	.	
SBS-2E	>10	n.t.	.	.	.	.	D-N	.	.	.	T-I	.	.	.	.	.	.	
IZSVE/2013/560/D13/1-f-E	>10	n.t.	.	N-S	.	.	.	.	.	.	T-I	.	.	.	.	.	.	

Elevated MIC values are highlighted in grey. Ec: amino acid numbering according to *Escherichia coli* strain K12 substrain MG1655 (GenBank accession number: U00096); Ms: amino acid numbering according to *M. synoviae* strain MS53 (GenBank accession number: AE017245); enroflox.: enrofloxacin; diflox.: difloxacin; n.t.: not tested

Table S7. MIC values and potentially resistance-related alterations in *rrlA/B* and *rplV* genes of *M. synoviae* strains

ID of *M. synoviae* strains	MIC of tilmicosin (µg/ml)	MIC of tylosin (µg/ml)	MIC of tylvalosin (µg/ml)	MIC of lincomycin (µg/ml)	*rrlA* 2058 2054	*rrlA* 2059 2055	*rrlB* 2058 2054	*rrlB* 2059 2055	*rplV* 270 276	Ec Ms
EB-B	≤0.25	≤0.25	n.t.	n.t.	.	.	.	.	.	
RMJ-B	≤0.25	≤0.25	n.t.	n.t.	.	.	.	.	.	
MS1	≤0.25	≤0.25	n.t.	n.t.	.	.	.	.	.	
NCTC 10124	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MS-H	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV167	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV259	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV261	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV263	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV285	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV291	≤0.25	≤0.25	≤0.25	≤0.25	.	.	.	.	.	
MYCAV170	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV183	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV186	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV188	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV194	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV196	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV197	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV198	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV236	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV262	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV268	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV277	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV278	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV284	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV288	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
IZSVE/2012/5715/D12/4-f	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
IZSVE/2015/5871/D15/2-f	≤0.25	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV79	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV102	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV119	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV130	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV195	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV249	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV256	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV257	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV274	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV281	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV282	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV306	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
IZSVE/2013/3291/D13/2-f	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
IZSVE/2015/4599/D15/2-d	≤0.25	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV272	≤0.25	≤0.25	≤0.25	2	.	.	.	.	.	
IZSVE/2013/3757/D13/2-f	≤0.25	≤0.25	≤0.25	2	.	.	.	.	.	
IZSVE/2013/4109/D13/1-f	≤0.25	≤0.25	≤0.25	2	.	.	.	.	.	
IZSVE/2012/806/D12/14-f	≤0.25	≤0.25	≤0.25	2	.	.	.	.	.	
IZSVE/2016/2958/MAV16/11-f	≤0.25	≤0.25	≤0.25	2	A-G	.	.	.	.	
ALN-A	0.5	0.25	n.t.	0.5	.	.	.	.	.	
MYCAV168	0.5	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV217	0.5	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV193	0.5	≤0.25	≤0.25	2	.	.	.	.	.	
IZSVE/2015/2376/D15/1-f	1	≤0.25	≤0.25	<0.5	.	.	.	.	.	
IZSVE/2015/3622/D15/2-f	1	≤0.25	≤0.25	<0.5	.	.	.	.	.	
IZSVE/2015/5872/D15/3-f	1	≤0.25	≤0.25	<0.5	.	.	.	.	.	

ID of *M. synoviae* strains	MIC of tilmicosin (µg/ml)	MIC of tylosin (µg/ml)	MIC of tylvalosin (µg/ml)	MIC of lincomycin (µg/ml)	*rrlA*		*rrlB*		*rplV*	
					2058	2059	2058	2059	270	Ec
					2054	2055	2054	2055	276	Ms
MYCAV173	1	≤0.25	≤0.25	0.5	.	.	.	.	.	
IZSVE/2013/244/D13/1-f-E	1	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV536	1	≤0.25	≤0.25	0.5	.	.	.	.	.	
MYCAV300	1	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV189	2	≤0.25	≤0.25	1	.	.	.	.	.	
MYCAV190	2	≤0.25	≤0.25	1	.	.	.	.		
MYCAV174	4	≤0.25	≤0.25	1	.	.	.	.	.	
IZSVE/2016/3274/MAV16/3-f	8	≤0.25	≤0.25	4	A-G	.	.	.	.	
SBS-2E	>8	1	n.t.	>8	A-G	.	A-G	.	.	
IZSVE/2013/4663/D13/2-f	16	0.5	≤0.25	0.5	.	.	.	.	A-T	
MYCAV573	16	0.5	≤0.25	1	.	.	.	.	A-C	
IZSVE/2014/589/D14/1-f-E	16	0.5	≤0.25	4	.	.	A-G	.	.	
IZSVE/2015/740/D1517-d-F	32	0.5	≤0.25	>64	A-G	.	A-G	.	.	
IZSVE/2016/1466/MAV16/4-f	32	1	0.5	4	.	A-G	.	A-G	.	
MYCAV574	32	2	≤0.25	≤0.25	.	.	.	.	A-C	
IZSVE/2016/1074/MAV16/15-f	>32	0.5	≤0.25	<0.5	.	.	.	.	A-T	
IZSVE/2016/2953/MAV16/28-l	>32	0.5	≤0.25	<0.5	.	.	.	.	A-T	
IZSVE/2013/560/D13/1-f-E	>32	0.5	≤0.25	>32	A-G	.	A-G	.	.	
IZSVE/2016/3007/MAV16/1-f	>32	32	4	>32	A-G	.	A-G	.	.	
IZSVE/2013/4498/D13/2-f	64	0.5	≤0.25	0.5	.	.	.	.	A-T	
IZSVE/2013/564/D13/3-d-E	64	1	≤0.25	>64	A-G	.	A-G	.	.	
MYCAV185	64	2	0.5	>64	A-G	.	A-G	.	.	
IZSVE/2016/1695/MAV16/2-f	>64	0.5	≤0.25	>64	A-G	.	.	.	.	
IZSVE/2013/2094/D13/10-f	>64	0.5	0.5	>64	A-G	.	.	.	.	
IZSVE/2013/378/D13/1-f-H	>64	1	0.5	>64	A-G	.	A-G	.	.	
IZSVE/2012/6077/D12/1-f	>64	2	≤0.25	>64	A-G	.	A-G	.	.	
IZSVE/2012/6083/D12/2-d	>64	2	0.5	>64	A-G	.	A-G	.	.	
IZSVE/2015/3145/D15/4-f	>64	2	0.5	>64	A-G	.	A-G	.	.	
MYCAV303	>64	2	0.5	>64	A-G	.	A-G	.	.	
IZSVE/2015/2519/D15/1-f	>64	2	0.5	>64	A-G	.	.	.	.	
IZSVE/2013/5914/D13/1-d	>64	16	4	>64	A-G	.	A-G	.	.	
IZSVE/2013/5914/D13/5-d	>64	32	4	>64	A-G	.	A-G	.	.	
IZSVE/2015/2518/D15/1-f	>64	64	4	>64	A-G	.	A-G	.	.	

Elevated MIC values are highlighted in grey. Ec: nucleotide numbering according to *Escherichia coli* strain K12 substrain MG1655 (GenBank accession number: U00096); Ms: nucleotide numbering according to *M. synoviae* strain MS53 (GenBank accession number: AE017245); n.t.: not tested

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