sciendo

J Vet Res 63, 41-49, 2019
DOI:10.2478/jvetres-2019-0010

Prevalence and phylogenetic analysis of *Mycoplasma synoviae* strains isolated from Polish chicken layer flocks

Olimpia Kursa, Grzegorz Tomczyk, Anna Sawicka

Department of Poultry Diseases,
National Veterinary Research Institute, 24-100 Puławy, Poland
olimpia.kursa@piwet.pulawy.pl

Received: October 10, 2018 *Accepted: February 25, 2019*

Abstract

Introduction: *Mycoplasma synoviae* (MS) is a chicken pathogen of major economic importance. **Material and Methods:** Between 2010 and 2016, 906 commercial layer chicken flocks in Poland were examined for MS, and the phylogenetic relationship among the strains was established. Regionally dispersed samples were collected and tested with the use of real-time PCR to detect the 16S–23S intergenic spacer region. Positive samples were also tested with LAMP and conventional PCR to detect the *vlhA* gene. **Results:** MS genetic material was detected in 265 (29%) of the tested flocks by real-time PCR, in 227 by the LAMP method and in 202 (22%) by conventional PCR. The by-year percentage of positive samples began at 34% in 2010, rose to 44% in 2012, and declined to 29% in 2016. A phylogenetic analysis of Polish *M. synoviae* strains using a partial sequence of the *vlhA* gene showed nine genotypes (A–I), the most frequently occurring being F and C. Pathogenic Polish MS field isolates (n = 27) collected from chickens with clinical signs of infection were grouped for their characteristic symptoms: respiratory for genotypes C, E, F, and I (n = 13), EAA and a drop in laying for genotypes F, E, and C (n = 12), and synovitis for genotype A (n = 2). **Conclusion:** These data showed the country's isolate diversity. The high prevalence suggests the need to introduce appropriate control programmes. This is the first report of molecular epidemiological data on *M. synoviae* infection in layer chickens in Poland.

Keywords: chickens, *Mycoplasma synoviae, vlhA* gene, genotype, pathogenicity.

Introduction

Mycoplasma synoviae (MS) is a small bacterium, belonging to the *Mollicutes* class. It gives rise to air sac lesions and synovitis in chickens and turkeys. This organism is capable of being transmitted horizontally from bird to bird or vertically from parent to offspring *via* the egg. Birds are infected for the rest of their lives and remain carriers. It is acknowledged to be a cause of great economic loss in the commercial poultry industry due to poor growth and decreased egg production (10, 18). In addition to strains with respiratory tract tropism and arthropathic strains, there are also strains with oviduct tropism which can induce eggshell apex abnormalities (EAA) without any physical abnormalities (9, 22). Significant economic losses may result also from the loss of eggs after laying through eggshell changes characteristic of EAA, such as an altered shell surface and thinning and increased translucency in different areas, leading to a higher incidence of cracks and breakage of the eggshell (3, 9, 12, 17, 29, 32).

The differences between MS strains are related to their pathogenicity and course of the disease. The molecular characterisation of MS strains may assist in epidemiological studies to determine the source of infections and relationships among strains isolated from neighbouring or related flocks. It has been shown that DNA sequence analysis targeting the haemagglutinin-encoding *vlhA* gene is a useful tool for the detection and initial typing of the strains (1, 4, 14, 16, 23), and their differentiation based on the sequences of this gene has already been described (1, 7, 14). The variable lipoprotein haemagglutinin *vlhA* gene is immunodominant and variably expressed membrane lipoproteins that play an important role in binding to the host cell receptors enable the colonisation of host tissues (2, 31, 27). The conserved 5' end of this gene

includes tandem repeats that encode proline-rich repeats (PRR), which is very useful for typing of MS strains based on insertion and deletion of nucleotides and a region which is highly polymorphic (RIII) (1). Therefore, differentiation of MS strains can be achieved without any culture or isolation method (16).

It is therefore becoming increasingly obvious that expanding the existing sequence data on the genetic diversity of MS is necessary to better understand the biology and molecular epidemiology of this microorganism. In the present study, we aimed at an investigation of the prevalence of MS in Polish layer flocks, which has not been published so far. For phylogenetic analysis, we chose pathogenic strains involved in inducing EAA and egg production losses and strains which cause synovitis or respiratory disease.

Material and Methods

Sample collection. In total, 906 flocks of layer chickens from different regions of Poland were tested for the presence of MS. All samples were obtained from commercial chicken layer flocks of different ages (16–56 weeks) from 16 provinces of Poland between 2010 and 2016. The samples originated from both healthy and sick chickens. Individual chickens (60 per flock) were swabbed at the trachea (881 flocks) using sterilised cotton. Swab samples from synovial fluid were taken from birds showing swollen joints (seven flocks). Tissues samples from trachea (nine flocks) and oviduct (nine flocks) were taken *post mortem* from birds producing eggs with EAA.

Isolation. For each tested flock, four pools of 15 swab samples were inoculated into 15 mL of Frey's broth medium. Tissues were placed in 2 mL of Frey's broth (11). All broth samples were stored at −20°C until processed for DNA analysis of the 16S–23S intergenic spacer region and *vlhA* gene by LAMP and PCR assay.

Detection of *M. synoviae*. The DNA was extracted from a 200 µL sample using a QIAamp DNA Kit (Qiagen, Germany) following the procedure provided by the manufacturer. Purified DNA samples (100 µL) were stored at −20°C for further downstream molecular analysis.

Real-time PCR. For the detection of MS DNA, a real-time PCR was performed using specific primers (30). The reaction was carried out using a QuantiTect Probe PCR Kit (Qiagen, Germany) in a total volume of 25 µl with 1.3 µL of each 10 µM primer (MSF: 5'-CTA AAT ACA ATA GCC CAA GGC AA-3'; MSR: 5'-CCT CCT TTC TTA CGG AGT ACA-3'), 0.5 µL of probe, 7.4 µL of distilled water, and 2 µL of DNA in an ABI 7500 thermal cycler (Applied Biosystems, part of Thermo Fisher Scientific, USA) under the following conditions: 50°C for 2 min, 95°C for 15 min, 40 cycles of 94°C for 30 sec, and 60°C for 1 min.

LAMP method. Positive samples were analysed using a LAMP method with specific primers targeting the *vlhA* gene. Reactions were carried out as described previously by Kursa *et al.* (21).

Polymerase chain reaction. The *vlhA* fragment of MS was amplified from positive samples in real-time PCR and LAMP, using primers as described previously (7, 14). The reaction mixture contained Taq PCR Master Mix (Eurx, Poland) in a volume of 12.5 µL, 1.5 µL of each 10 µM primer (MSvF: 5'-GGC CAT TGC TCC TRC TGT TAT-3'; MSvR: 5'-AGT AAC CGA TCC GCT TAA TGC-3'), and 7.5 µL of distilled water with the addition of 2 µL of DNA to give a total reaction volume of 25 µL. The PCR went through an initial step of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and final elongation at 72°C for 7 min. The PCR products were separated in 2% agarose gel and stained with ethidium bromide (E-gel, Invitrogen, part of Thermo Fisher Scientific, USA).

Sequencing and phylogenetic analysis of MS *vlhA* gene. The amplicons of the selected DNA were sequenced with the forward (MSF) and reverse (MSR) PCR primers (7, 14), sequences were determined in a 3500 Genetic Analyzer (Applied Biosystems). The FinchTV programme in version 1.4.0 was used for aligning all sequences manually. Amino acid sequences were aligned using Clustal W performed in MEGA7 software (20) and the BLAST programme determined, their identities along with those of nucleotides (www.ncbi.nlm.nih.gov/BLAST). The field isolates obtained in the study were compared with the *vlhA* gene sequence of MS reference strain WVU 1853 (AM998371) and other sequences available in GenBank (Table 1). The sequences obtained in this study have been deposited in GenBank, and the accession numbers are listed in Table 2. Phylogenetic trees were constructed using the unweighted pair group method with arithmetic means algorithm. Typing of the MS field isolates was based on the size of the PCR amplicon of the *vlhA* gene, point mutations in the RIII region, and nucleotide grouping using the sequence similarity of the full amplicon (1, 5, 14, 16).

Statistical analysis. The Spearman's rank correlation coefficient test was used to compare the results obtained using the three methods. Statistical analyses of various parameters were performed using Social Science Statistics programme (www.socscistatistics.com). The differences were considered significant at $P < 0.05$.

Results

Detection of MS. The presence of the genetic material of MS was detected in 265 of the tested flocks by real-time PCR, in 227 flocks by LAMP method, and in 202 flocks by PCR. Over the years studied, the

percentage of positive samples began at 34% in 2010, peaked at 44% in 2012, and declined to 29% in 2016 (Tables 3 and 4). Strong correlation was found between the real-time PCR results and PCR results ($r = 0.96$, $P < 0.05$) as well as between real-time PCR results and LAMP results ($r = 1$, $P < 0.05$). The number of flocks tested in each province between 2010 and 2016 and number of positive samples in every year confirmed by PCR are shown in Table 4. Fig. 1 shows the locations of flocks with MS in Poland; they are in regions where a high number of poultry farms are located (Mazowieckie, Kujawsko-Pomorskie, and Wielkopolskie provinces). Most of the positively tested flocks (89.8%) did not show clinical symptoms, however part of the flocks (27 field isolates (10.2%)) showed synovitis (0.75%) or airsacculitis (4.9%). Some also showed a drop in egg production or eggshell changes characteristic of EAA (4.53%).

Sequence analysis of the *vlhA* gene. Among the isolates sequenced, 27 field strains isolated from chickens showing clinical signs and 31 of the field isolates from birds without clinical symptoms were selected as material with which a phylogenetic tree was constructed and which were compared to sequences available in the GenBank. Using this comparison, the Polish sequences were grouped into nine genotypes: A–I (Fig. 2). Most strains were assigned to genotype F (n = 25) or genotype C (n = 11). The remaining Polish sequences were grouped into genotypes E (n = 8), I (n = 6), B (n = 3), A (n = 2), D, G, and H (n = 1 for each group) (Fig. 2). The variability in nucleotide sequences was reflected in the large number of differences in the amino acid sequences.

A total of 13 Polish MS isolates from chickens showing respiratory signs and collected from different flocks were an assortment of the genotypes C (n = 5), E (n = 1), F (n = 3), and I (n = 4) (Table 2). A decline in egg production and eggshell changes characteristic of EAA were caused by 12 isolates from chickens isolated in 2015 and 2016 and these isolates were in genotypes C (n = 1), F (n = 10), and E (n = 1) (Table 2). Two Polish MS isolates from chickens showing joint swelling were in genotype A. Most of the farms were identified by only one genotype. During the study, only one flock had a mixed infection with two different genotypes, which were E and F.

Table 1. *M. synoviae vlhA* sequences selected from GenBank included in the phylogenetic analysis

	Isolate	Year of isolation	Country	Genotype*	GenBank accession number
1	K1968	1968	USA	B	KJ 606929.1
2	K1	2016	UK	C	AJ580993
3	WT-60	2003	Japan	B	AB501281
4	M2008.13	2014	France	F	KJ606947
5	2002.02a	2002	Netherlands	C	KJ606932
6	AMS-8	2010	Austria	-	KC832807
7	B94-91	2003	UK	E	AJ580992
8	EB-11	2000	Israel	-	KC832809
9	K27	2016	UK	E	AJ581658
10	IZSVE/4383/11	2011	Italy	-	KC832815.1
11	B38-96-170	2003	UK	D	AJ580985
12	AMS-9	2010	Austria	-	KC832808
13	CHN-FJCXZ2-2-2013	2013	China	-	KU572366.1
14	IZSVE/4564	2010	Italy	-	KC832814.1
15	K1938	2016	Australia	D	DQ661613
16	V6	2016	Australia	C	AF464961
17	K3009/37	2015	Slovakia	-	KP055185
18	B48/05	2005	Spain	F	FM164344
19	CBU0866 (EAA)	2014	South Korea	-	KM985992.1
20	B27-00	2000	UK	C	AJ580988
21	EGY.Ras.joint.4	2014	Egypt	-	KT957968.1
22	M2000.05_	2000	Netherlands	E	KJ606931
23	AHRU2015CG0202.1	2015	Thailand	E	KX168668
24	EAA	2005	Netherlands	F	FJ495803
25	WVU 1853 USA	1955	USA	A	AM998371

* Genotyping by Dijkman *et al.* (5)

Table 2. Polish isolates of *M. synoviae* from laying hen flocks used for phylogenetic analysis of the *vlhA* gene

Field isolates	Year of isolation	Clinic signs	Genotype	GenBank accession number
OST 14PL	2014	Respiratory signs	F	MF737484
K5/15PL	2015	Respiratory signs	I	MF737510
kur2k_14-4.2PL	2014	No clinical signs	I	MF737513
kur2k_14-4PL	2014	No clinical signs	F	MF737459
kur2k_14-3PL	2014	Respiratory signs	I	MF737512
kur2.14_15PL	2014	Respiratory signs	I	MF737511
kur.K1.12.1PL	2012	No clinical signs	C	MF737471
GK1.15-Z-KPL	2015	Respiratory signs	C	MF737474
568_16PL	2016	No clinical signs	F	MF737502
JAJ/1/14PL	2014	No clinical signs	I	MF737515
FM54_12PL	2012	Respiratory signs	C	MF737470
FM20_13PL	2013	No clinical signs	E	MF737462
FM15_12PL	2012	No clinical signs	E	MF737463
FM6_11PL	2011	No clinical signs	E	MF737460
F573_15PL	2015	No clinical signs	F	MF737488
F572_15PL	2015	No clinical signs	F	MF737489
F529_15	2015	Reproductive signs	C	MF737473
F517_15PL	2015	No clinical signs	F	MF737490
F370_15PL	2015	No clinical signs	E	MF737466
F317_16PL	2016	Reproductive signs	E	MF737461
F240_15PL	2015	No clinical signs	G	MF737492
F225_16PL	2016	No clinical signs	F	MF737504
F221_15PL	2015	No clinical signs	F	MF737491
649_10PL	2010	No clinical signs	B	MF737516
F199_16PL	2016	Respiratory signs	C	MF737476
F197_16PL	2016	Respiratory signs	C	MF737475
F150_16PL	2016	Reproductive signs	F	MF737486
F131_16PL	2016	Reproductive signs	F	MF737495
533_10PL	2010	No clinical signs	F	MF737487
F130_16PL	2016	Reproductive signs	F	MF737496
F123_14PL	2014	No clinical signs	C	MF737472
F51_16PL	2016	Reproductive signs	F	MF737493
F47_16PL	2016	No clinical signs	C	MF737478
F44_16PL	2016	Reproductive signs	F	MF737494
156/14PL	2014	No clinical signs	F	MF737482
W9/14PL	2014	Respiratory signs	I	MF737514
7LpT/16PL	2016	Respiratory signs	F	MF737485
F660_12PL	2012	Joint swelling	A	MF737518
F659_12PL	2012	Joint swelling	A	MF737479
533_12PL	2012	No clinical signs	E	MF737464
F428_12PL	2012	No clinical signs	E	MF737465
F419T_13PL	2013	Respiratory signs	F	MF737480
F156_12PL	2012	Respiratory signs	C	MF737469
1/146/15PLuca-Z	2015	Respiratory signs	E	MF737467
146-2M/15PL	2015	Reproductive signs	F	MF737501
146-3J/15PL	2015	Reproductive signs	F	MF737500
(1.2)146_12PL	2012	No clinical signs	H	MF737509
129_12	2012	No clinical signs	C	MF737468
F474_16PL	2016	No clinical signs	C	MF737477
556-63_16PL	2016	Reproductive signs	F	MF737499
F550_16PL	2016	No clinical signs	B	MF737507
F234_16PL	2016	No clinical signs	D	MF737505
FM7_14PL	2014	No clinical signs	F	MF737483
F92/16PL	2016	Reproductive signs	F	MF737498
206_16PL	2016	Reproductive signs	F	MF737506
566_16PL	2016	No clinical signs	F	MF737497
567_16PL	2016	No clinical signs	F	MF737503
421_16PL	2016	No clinical signs	B	MF737508

Table 3. Detection of *M. synoviae* by real-time PCR, LAMP, and PCR in clinical samples between 2010 and 2016

Year	Number of tested flocks	Number of positive flocks in real-time PCR (%)	Number of positive flocks in LAMP (%)	Number of positive flocks in PCR (%)
2010	65	22 (34)	19 (29)	15 (23)
2011	70	16 (23)	13 (19)	11 (16)
2012	133	59 (44)	50 (38)	45 (34)
2013	128	38(30)	35 (27)	35 (27)
2014	110	34 (31)	31 (28)	29 (26)
2015	213	41 (19)	38 (18)	31 (15)
2016	187	55 (29)	41 (22)	36 (19)
Total	906	265 (29)	227 (25)	202 (22)

Table 4. Prevalence of *M. synoviae* in chicken layer farms between 2010 and 2016

Provinces	2010		2011		2012		2013		2014		2015		2016	
	Total flocks	Positive samples	Total flocks	Positive samples	Total flocks	Positive samples	Total flocks	Positive samples	Total flocks	Positive samples	Total flocks	Positive samples	Total flocks	Positive samples
Dolnośląskie	0	0	0	0	0	0	0	0	1	0	1	0	1	0
Kujawsko-Pomorskie	9	1	4	1	17	5	15	7	11	4	18	3	15	6
Lubelskie	0	0	0	0	0	0	0	0	2	0	3	0	2	0
Lubuskie	4	2	0	0	1	1	5	1	4	0	5	0	4	0
Łódzkie	0	0	0	0	4	1	2	0	2	1	4	1	3	0
Mazowieckie	14	4	44	7	70	27	65	15	51	13	109	16	102	18
Małopolskie	8	0	0	0	1	1	1	0	2	0	5	0	3	0
Opolskie	0	0	0	0	0	0	3	3	2	0	3	0	2	0
Podkarpackie	0	0	0	0	1	1	0	0	1	0	1	0	1	0
Podlaskie	0	0	0	0	1	0	0	0	2	0	5	1	4	0
Pomorskie	1	1	1	0	9	3	5	2	3	1	8	2	7	3
Śląskie	15	3	4	1	4	2	5	1	5	0	11	2	8	1
Świętokrzyskie	3	0	0	0	0	0	3	1	2	0	3	1	2	1
Warmińsko-Mazurskie	2	0	5	0	7	0	5	1	7	3	10	0	8	2
Wielkopolskie	9	4	11	2	13	3	15	3	10	4	19	3	18	2
Zachodnio-Pomorskie	0	0	1	0	5	1	4	1	5	3	8	2	7	3
Total	65	15	70	11	133	45	128	35	110	29	213	31	187	36

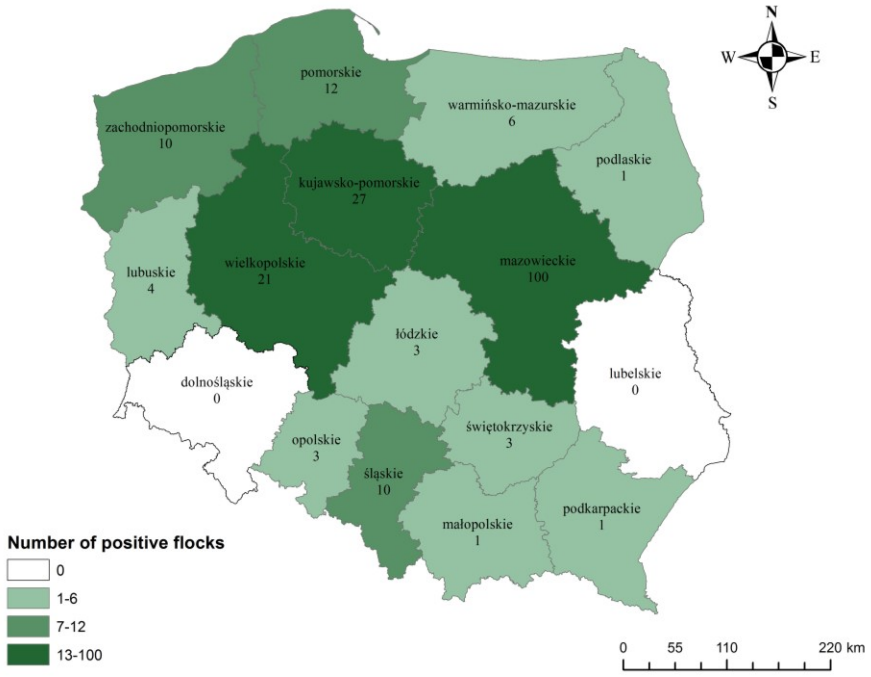

Fig. 1. Map of Poland showing number of all positive cases of *M. synoviae* per province in chicken layer farms between 2010 and 2016

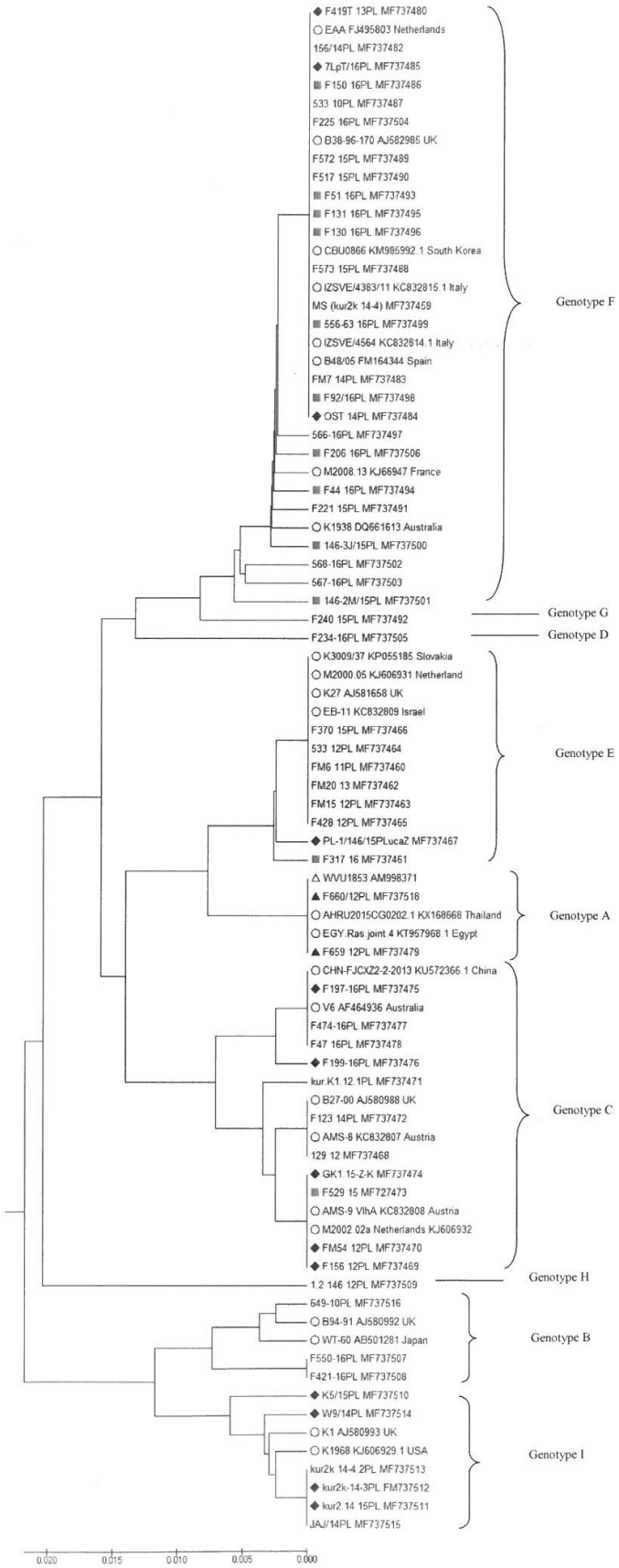

Fig. 2. Phylogenetic tree derived from unweighted pair group method with arithmetic means. It includes 58 Polish MS *vlhA* sequences corresponding to *M. synoviae* reference strain WVU1853 and sequences from other countries. The empty triangle is the reference sequence, and empty circles represent sequences from other countries. Filled squares are sequences from chickens showing reproductive disorders, filled diamonds are those from chickens showing respiratory signs, and filled triangles are those from chickens with swollen joints

Discussion

Investigations into the range of prevalence of MS are very important for the poultry industry. To the best of our knowledge, this is the first study describing the prevalence of MS infection among layer flocks in Poland. In the past six years, it seems that MS has taken over the role of *M. gallisepticum* in commercial poultry demonstrating high frequency of detection such that its prevalence clearly as one of the most important avian mycoplasmata, was 29% on average based on the results of the six years of study. Three methods were used to detect MS. The statistical analysis showed strong correlation between the results of real-time PCR and LAMP, which are very sensitive methods. The little difference noted was in the correlation between the results of real-time PCR and PCR methods, which may be related to the lower sensitivity of the latter method (Table 3). Most of the positive flocks were located in regions where the poultry industry is very strongly established, like central Poland (Mazowieckie having 100 flocks, Kujawsko-Pomorskie – 27, and Wielkopolskie – 21) (Fig. 1). The detection rate of MS is in agreement with the rates reported by other researchers from Europe. Epidemiological studies performed in different European countries have shown a high prevalence of MS in commercial flocks (6, 8, 12, 13), and notably infection with MS in commercial flocks was common in the UK (78.6%) and Germany (75%) (13, 19). Comparatively higher prevalences have also been found in Portugal (66.7%) and France (68%) (6, 26). The prevalence of MS varies between different countries. When comparing the prevalence of MS infection in Poland and in other countries, it may be concluded that the level of biosecurity is high, and control of the contact routes to animals on Polish farms is sufficiently stringent. However, MS infection in flocks with no clinical signs had the highest significance for the disease prevalence in this study. Therefore, the use of vaccination may be a good alternative for producing better results in the future.

The genetic analysis of Polish MS field isolates was based on the partial *vlhA* gene sequence. It seems that sequencing the same region of the *vlhA* gene and comparing the found sequence with sequences from different countries in the GenBank database is the most frequently used method (23, 28, 33). A high degree of variability in the virulence of MS strains appears to exist (24, 25) although genotyping in MS relies upon the *vlhA* gene (1, 5, 7, 14, 15, 16). There are differences in pathogenicity between MS strains, but there is no data regarding pathogenicity differences among genotypes (10, 14, 25). This study presents the molecular data of Polish MS isolates causing infection symptoms in chicken joints, respiratory tracts, and reproductive systems, and does it for the first time. Phylogenetic analysis of 58 Polish MS strains showed the presence of nine genotypes (A–I) (Fig 2.). Polish MS isolates from chickens showing respiratory signs were in the four genotypes C, E, G and I, which means that these isolates were associated with respiratory tract infection. Most of them were in genotypes C and I. In our study, only isolates from genotypes C and E could also induce eggshell apex abnormality syndrome, which is consistent with studies carried out in the Netherlands (9). Slightly different results were obtained by researchers from Thailand, where isolates from genotypes C and E were associated only with respiratory tract infection (23).

In this study, we present Polish MS isolates that belong to genotypes C, F, and E causing a drop in egg production and eggshell changes characteristic of EAA. Most of them were from genotype F (10 isolates) obtained from tracheal swabs and oviducts from layer hens (Fig. 2). The prevalence of this genotype in Poland was high in the last two years of the study. Phylogenetic analysis has shown that Polish MS isolates from this genotype were closely related to the sequences reported from the Netherlands (FJ495803), Spain (FM164344), Italy (KC832814.1), and South Korea (KM985992.1), isolated from chickens with reproductive signs. Dutch studies in 2008–2010 also revealed a high prevalence of genotype F among isolates of MS (5).

In Europe, the most frequently found genotypes were A–F: in Slovenia they were B and C; in the UK C, E, and F; and in the Netherlands they were C and F (5, 14, 16). In other countries, the dominant genotypes of MS isolates were F–K in Iran, C in Australia, and A–E in the United States and Canada (1, 23). In Thailand, strains of genotype E often resulted in respiratory signs, and those of genotype L were arthropathic strains (23). In China, native chicken breeds mainly showed synovitis caused by genotype K (33), while in Poland it was genotype A which induced synovitis.

The results of this study show that Polish MS isolates more often cause respiratory and reproductive symptoms than arthropathies. Some correlation between phylogenetic analysis and clinical signs of MS infection may be interpreted from this study. The presented results of genotyping of MS isolates do not exclude the existence of such pathotypes, but it seems that the frequency of pathogenic MS strain occurrence is rather low and may depend on the presence of other pathogens, bacteria or viruses. However, this issue requires more in-depth genetic analysis to determine the veracity of these assumptions. Moreover, further studies on the virulence of the MS field isolates should be performed, especially taking into account the high prevalence of MS infection in many countries.

In the course of the study, no geographical relationship was observed with respect to the presence of individual genotypes. There was no clear correlation between the year of isolation and *vlhA* type, although isolates belonging to *vlhA* type F seem to have become predominant in the final years of the study (2015–2016).

In conclusion, the prevalence of MS was found to be quite high in Poland. In most cases, these were subclinical infections. However, both the clinical and subclinical forms of the disease can lead to economic losses resulting from increased mortality, retarded growth, and costs of treatment of secondary infections. The most frequently isolated genotypes in Poland were the genotypes F and C. In these genotypes, association with respiratory tract infection (genotype C) and with reproductive tract infection (genotype F) were most frequent. Many stress factors can lead to a depression of the immune system of hens, predisposing them to infections such as those associated with MS. In Poland, there are no MS eradication programmes yet, and our results show that there may be a threat posed by this pathogen to general poultry health. Fortunately, vaccinations in Poland are available and increasingly widely used. Further studies designed to reveal any form of association between the pathogenicity of MS and the *vlhA*-based genotype are recommended because they may provide key epidemiological information to the poultry industry.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The research was financed from statutory sources of the National Veterinary Research Institute.

Animal Rights Statement: None required.

References

1. Benčina D., Drobnič-Valič M., Horvat S., Narat M., Kleven S.H., Dovč P.: Molecular basis of the length variation in the N-terminal part of *Mycoplasma synoviae* hemagglutinin. FEMS Microbiol Lett 2001, 203, 115–123.
2. Berčič R.L., Slavec B., Lavrič M., Narat M., Bidovec A., Dovč P., Benčina D.: Identification of major immunogenic proteins of *Mycoplasma synoviae* isolates. Vet Microbiol 2008, 127, 147–154.
3. Catania S., Bilato D., Gobbo F., Granato A., Terregino C., Iob L., Nicholas R.A.J.: Treatment of eggshell abnormalities and reduced egg production caused by *Mycoplasma synoviae* infection. Avian Dis 2010, 54, 961–964.
4. Catania S., Gobbo F., Bilato D., Gagliazzo L., Moronato M.L., Terregino C., Bradbury J.M., Ramírez A.S.: Two strains of *Mycoplasma synoviae* from chicken flocks on the same layer farm differ in their ability to produce eggshell apex abnormality. Vet Microbiol 2016, 193, 60–66.
5. Dijkman R., Feberwee A., Landman W.J.M.: Variable lipoprotein haemagglutinin (vlhA) gene sequence typing of mainly Dutch *Mycoplasma synoviae* isolates: comparison with vlhA sequences from Genbank and with amplified fragment length polymorphism analysis. Avian Pathol 2014, 43, 465–472.
6. Dufour-Gesbert F., Dheilly A., Marois C., Kempf I.: Epidemiological study on *Mycoplasma synoviae* infection in layers. Vet Microbiol 2006, 114, 148–154.
7. El-Gazzar M.M., Wetzel A.N., Raviv Z.: The genotyping potential of the *Mycoplasma synoviae* vlhA gene. Avian Dis 2012, 56, 711–719.
8. Feberwee A., De Vries T.S., Landman W.J.M.: Seroprevalence of *Mycoplasma synoviae* in Dutch commercial poultry farms. Avian Pathol 2008, 37, 629–633.
9. Feberwee A., De Wit J.J., Landman W.J.M.: Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: field and experimental studies. Avian Pathol 2009, 38, 77–85.
10. Ferguson-Noel N., Noormohammadi A.H.: *Mycoplasma synoviae* infection. In: *Diseases of poultry,* edited by Swayne D.E., Glisson J.R., McDougald L.R., Nolan L.K., Suarez D.L., Nair V.L., Wiley, Ames 2013, pp. 900–906.
11. Frey M.L., Hanson R.P., Anderson D.P.: A medium for the isolation of avian mycoplasmas. Am J Vet Res 1968, 29, 2163–2171.
12. Gole V.C., Chousalkar K.K., Roberts J.R.: Prevalence of antibodies to *Mycoplasma synoviae* in laying hens and possible effects on egg shell quality. Prev Vet Med 2012, 106, 75–78.
13. Hagan J.C., Ashton N.J., Bradbury J.M., Morgan K.L.: Evaluation of an egg yolk enzyme-linked immunosorbent assay antibody test and its use to assess the prevalence of *Mycoplasma synoviae* in UK laying hens. Avian Pathol 2004, 33, 93–97.
14. Hammond P.P., Ramírez A.S., Morrow C.J., Bradbury J.M.: Development and evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing. Vet Microbiol 2009, 136, 1–2.
15. Harada K., Kijima-Tanaka M., Uchiyama M., Yamamoto T., Oishi K., Arao M., Takahashi T.: Molecular typing of Japanese field isolates and live commercial vaccine strain of *Mycoplasma synoviae* using improved pulsed-field gel electrophoresis and vlhA gene sequencing. Avian Dis 2009, 53, 538–543.
16. Hong Y., Garcia M., Leiting,V., Benčina D., Dufour-Zavala L., Zavala G., Kleven S.H.: Specific detection and typing of *Mycoplasma synoviae* strains in poultry with PCR and DNA sequence analysis targeting the hemagglutinin encoding gene vlhA. Avian Dis 2004, 48, 606–616.
17. Jeon E.O., Kim J.N., Lee H.R., Koo B.S., Min K.C., Han M.S., Lee S.B., Bae Y.J., Mo J.S., Cho S.H., Lee C.H., Mo I.P.: Eggshell apex abnormalities associated with *Mycoplasma synoviae* infection in layers. J Vet Sci 2014, 15, 579–582.
18. Kleven S.H.: Mycoplasmas in the etiology of multifactorial respiratory disease. Poultry Sci 1998, 77, 1146–1149.
19. Kohn S., Spergser J., Ahlers C., Voss M., Bartels T., Rosengarten R., Krautwald-Junghanns M.E. Prevalence of *Mycoplasmas* in commercial layer flocks during laying period. Berl Munch Tierarztl Wochenschr 2009, 122, 186–192.
20. Kumar S., Stecher G., Tamura K.: MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Mol Biol Evol 2016, 33, 1870–1874.
21. Kursa O., Woźniakowski G., Tomczyk G., Sawicka A., Minta Z.: Rapid detection of *Mycoplasma synoviae* by loop-mediated isothermal amplification. Arch Microbiol 2015, 197, 319–325.
22. Landman W.J.M.: Is *Mycoplasma synoviae* outrunning *Mycoplasma gallisepticum*? A viewpoint from the Netherlands. Avian Pathol 2014, 43, 2–8.
23. Limpavithayakul K., Sasipreeyajan J., Pakpinyo S.: Characterization of Thai *Mycoplasma synoviae* isolates by sequence analysis of partial vlhA gene. Avian Dis 2016, 60, 810–816.
24. Lockaby S.B., Hoerr F.J., Kleven S.H., Lauerman L.H.: Pathogenicity of *Mycoplasma synoviae* in chicken embryos. Avian Dis 1999, 43, 331–337.
25. Lockaby S.B., Hoerr F.J., Lauerma L.H., Kleven S.H.: Pathogenicity of *Mycoplasma synoviae* in broiler chickens. Vet Pathol 1998, 35, 178–190.
26. Moreira F.A., Cardoso L., Coelho A.C.: Epidemiological survey on *Mycoplasma synoviae* infection in Portuguese broiler breeder flock. Vet Ital 2015, 51, 93–98.
27. Noormohammadi A.H., Markham P.F., Kanci A., Whithear K.G., Browning G.F.: A novel mechanism for control of antigenic

variation in the haemagglutinin gene family of *Mycoplasma synoviae*. Mol Microbiol 2000, 35, 911–923.
28. Ogino S., Munakata Y., Ohashi S., Fukui M., Sakamoto H., Sekiya Y., Noormohammadi A.H., Morrow C.J.: Genotyping of Japanese field isolates of *Mycoplasma synoviae* and rapid molecular differentiation from the MS-H vaccine strain. Avian Dis 2011, 55, 187–194.
29. Ranck M.F., Schmidt V., Philipp H.C., Voss M., Kacza J., Richter A., Fehlhaber K., Krautwald-Junghanns M.E.: *Mycoplasma synoviae*-associated egg-pole shell defects in laying hens. Berl Munch Tierarztl Wochenschr 2010, 123, 111–118.
30. Raviv Z., Kleven S.H.: The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. Avian Dis 2009, 53, 103–107.
31. Razin S., Yogev D., Naot Y.: Molecular biology and pathogenicity of mycoplasmas. Microbiology and molecular biology reviews. MMBR 1998, 62, 1094–1156.
32. Strugnell B.W., McMullin P., Wood A.M., Nicholas R.A.J., Ayling R., Irvine R.M.: Unusual eggshell defects in a free-range layer flock in Great Britain. Vet Rec 2011, 169, 237–238.
33. Sun S., Lin X., Liu J., Tian Z., Chen F., Cao Y., Qin J., Luo T.: Phylogenetic and pathogenic analysis of *Mycoplasma synoviae* isolated from native chicken breeds in China. Poult Sci 2017, 96, 2057–2063.