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

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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 CTC TAC 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 (MSF: 5'-GGG CAT TGC TCC TRC TGT TAT-3'; MSR: 5'-AGT AAC CGA TCC GCT TAA TG-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.

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

| Isolate                  | Year of isolation | Country | Genotype* | GenBank accession number |
|--------------------------|-------------------|---------|-----------|--------------------------|
| K1968                    | 1968              | USA     | B         | KJ606929.1               |
| K1                       | 2016              | UK      | C         | AJ580993                 |
| WT-60                    | 2003              | Japan   | B         | AB501281                 |
| M2008.13                 | 2014              | France  | F         | KJ606947                 |
| 2002.02a                 | 2002              | Netherlands | C         | KJ606932                 |
| AMS-8                    | 2010              | Austria | -         | KC832807                 |
| B94-91                   | 2003              | UK      | E         | AJ580992                 |
| EB-11                    | 2000              | Israel  | -         | KC832809                 |
| K27                      | 2016              | UK      | E         | AJ581658                 |
| IZSVE/4383/11            | 2011              | Italy   | -         | KC832815.1               |
| B38-96-170               | 2003              | UK      | D         | AJ580985                 |
| AMS-9                    | 2010              | Austria | -         | KC832808                 |
| CHN-FIXXZ2-2-2013        | 2013              | China   | -         | KU572366.1               |
| IZSVE/4564               | 2010              | Italy   | -         | KC832814.1               |
| K1938                    | 2016              | Australia | D         | DQ661613                 |
| V6                       | 2016              | Australia | C         | AF464961                 |
| K3009/37                 | 2015              | Slovakia | -         | KP055185                 |
| B48/05                   | 2005              | Spain   | F         | FM164344                 |
| CBU0866 (EAA)            | 2014              | South Korea | -         | KM985992.1               |
| B27-00                   | 2000              | UK      | C         | AJ580988                 |
| EGY.Ras.joint.4          | 2014              | Egypt   | -         | KT957968.1               |
| M2000.05                 | 2000              | Netherlands | E         | KJ606931                 |
| AHRR/2015CG0202.1        | 2015              | Thailand | E         | KX168668                 |
| EAA                      | 2005              | Netherlands | F         | FJ495803                 |
| WVU 1853 USA             | 1955              | USA     | A         | AM998371                 |

* Genotyping by Dijkman et al. (5)
| Field isolates | Year of isolation | Clinic signs | Genotype | GenBank accession number |
|---------------|------------------|-------------|----------|------------------------|
| OST 14PL     | 2014             | Respiratory signs | F        | MF737484               |
| K5/13PL      | 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               |
| FS73 15PL     | 2015             | No clinical signs | F        | MF737488               |
| FS72 15PL     | 2015             | No clinical signs | F        | MF737489               |
| FS29 15       | 2015             | Reproductive signs | C        | MF737473               |
| FS17 15PL     | 2015             | No clinical signs | F        | MF737490               |
| FS30 15PL     | 2015             | No clinical signs | E        | MF737466               |
| FS17 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               |
| I/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 Total flocks | 2010 Positive samples | 2011 Total flocks | 2011 Positive samples | 2012 Total flocks | 2012 Positive samples | 2013 Total flocks | 2013 Positive samples | 2014 Total flocks | 2014 Positive samples | 2015 Total flocks | 2015 Positive samples | 2016 Total flocks | 2016 Positive samples |
|--------------------|-------------------|------------------------|-------------------|------------------------|-------------------|----------------------|-------------------|----------------------|-------------------|----------------------|-------------------|----------------------|-------------------|----------------------|
| Dolnośląskie       | 0                 | 0                      | 0                 | 0                      | 0                 | 0                    | 1                 | 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                    | 2                 | 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                      | 4                 | 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                    | 1                 | 0                    | 1                 | 0                    | 1                 | 0                    | 1                 | 0                    |
| Podlaskie          | 0                 | 0                      | 0                 | 0                      | 0                 | 0                    | 2                 | 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-         | 2                 | 0                      | 5                 | 0                      | 7                 | 0                    | 5                 | 1                    | 7                 | 3                    | 10                | 0                    | 8                 | 2                    |
| Mazurskie          | 9                 | 4                      | 11                | 2                      | 13                | 3                    | 15                | 3                    | 10                | 4                    | 19                | 3                    | 18                | 2                    |
| Zachodnio-         | 0                 | 0                      | 1                 | 0                      | 5                 | 1                    | 4                 | 1                    | 5                 | 3                    | 8                 | 2                    | 7                 | 3                    |
| Pomorskie          | 0                 | 0                      | 0                 | 0                      | 0                 | 0                    | 0                 | 0                    | 0                 | 0                    | 0                 | 0                    | 0                 | 0                    |
| 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 mycoplasma, 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 prevalence 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.

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