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Prevalence of pathogens from *Mollicutes* class in cattle affected by respiratory diseases and molecular characteristics of *Mycoplasma bovis* field strains

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Abstract

Introduction: *Mycoplasma bovis* is one of the main pathogens involved in cattle pneumonia. Other mycoplasmas have also been directly implicated in respiratory diseases in cattle. The prevalence of different *Mycoplasma* spp. in cattle affected by respiratory diseases and molecular characteristics of *M. bovis* field strains were evaluated. **Material and Methods:** In total, 713 nasal swabs from 73 cattle herds were tested. The *uvr*C gene fragment was amplified by PCR and PCR products were sequenced. PCR/DGGE and RAPD were performed. **Results:** It was found that 39 (5.5%) samples were positive for *M. bovis* in the PCR and six field strains had point nucleotide mutations. Additionally, the phylogenetic analysis of 20 *M. bovis* field strains tested with RAPD showed two distinct groups of *M. bovis* strains sharing only 3.8% similarity. PCR/DGGE analysis demonstrated the presence of bacteria belonging to the *Mollicutes* class in 79.1% of DNA isolates. The isolates were identified as: *Mycoplasma bovirhinis*, *M. dispar*, *M. bovis, M. canis*, *M. arginini, M. canadense*, *M. bovoculi*, *M. alkalescens*, and *Ureaplasma diversum*. **Conclusion:** Different *Mycoplasma* spp. strains play a crucial role in inducing respiratory diseases in cattle.

Keywords: cattle, *Mycoplasma*, *Ureaplasma*, pneumonia, molecular characteristics.

Introduction

Mycoplasmas and *Ureaplasma diversum* belong to the *Mollicutes* class. It is well known that *Mycoplasma bovis* is one of the main pathogens involved in cattle pneumonia, especially in bovine respiratory disease complex (BRD). Usually the disease is associated with other pathogenic microorganisms such as viruses and other bacteria (8, 18). Other mycoplasmas including *M. dispar*, *M. canis*, *M. arginini*, and the related species *U. diversum* have been directly implicated in BRD (5, 9, 18). Moreover, other mycoplasma species such as *M. alkalescens*, *M. canadense*, and *M. bovirhinis* are involved in cattle respiratory infection (13, 18, 25). *M. bovis* causes significant economic losses in the cattle industry, due to low growth rate of the animals and increased costs of treatment (21). Due to the difficulty of effective isolation of mycoplasmas under laboratory conditions, molecular methods are considered to be the most suitable and have recently been generally recommended for diagnosis of mycoplasmal infection (28). The survey of *M. bovis* spread and studies on its genetic diversity were performed in different parts of the world because *M. bovis* is well-equipped to generate diversity, and new clones may appear (2, 7, 23, 29).

The aim of the study was to evaluate the prevalence of different *Mycoplasma* spp. and *U. diversum* in cattle affected by respiratory diseases and to study the molecular characteristics of field strains of *M. bovis*. In addition, this study allowed us to create the first report about the prevalence of diversity in the *uvr*C gene, which so far has been considered to be a conservative one in the *M. bovis* genome.

Material and Methods

Samples. The study was performed on 577 Black and White breed calves (ranging in age from two weeks to six months) and 136 Holstein-Friesian cows (ranging in age from two to five years) from 73 herds located in different regions of Poland (Table 1). In total, 713 nasal swabs were collected from the cattle following standard procedures by Veterinary Inspectorate officers and veterinary practitioners during clinical examination in 2014 and 2015. The samples were collected from herds in which the presence of respiratory diseases had been confirmed either by serological examination or the presence of clinical signs of infection. The swabs were collected into transport medium (Copan, USA) in duplicate: the first for DNA extraction and the second for bacterial culture.

Table 1. Number of herds and animals tested considering regions and provinces

Region	Province	Number of herds tested	Number of animals tested
eastern	Lublin	6	119
	Holy Cross	1	11
	Podlasie	14	107
	Podkarpacie	1	6
central	Masovian	14	128
northern	Pomeranian	14	114
north-western	Greater Poland	11	99
south-western	Opole	5	70
southern	Lesser Poland	7	59
total		73	713

Bacterial culture. *M. bovis* strains were isolated from the swabs. For this purpose, culturing in Eaton's agar (19) at 37ºC and in 5% CO_2 for 5–7 d was performed and then culturing was continued in Eaton's broth for 48 h.

DNA extraction. DNA extraction from swab samples was performed using the QIAmp DNA Mini Kit (QIAGEN, Germany) following the manufacturer's procedure. In cases where DNA was extracted directly from the swabs, negative results in PCR were obtained. Therefore, the DNA of each strain was extracted from 1 mL of broth culture according to Akwuobu *et al.* (1) and then the QIAmp DNA Mini Kit was applied.

PCR for *uvr*C gene. The specific primers were used (28). PCR was carried out in a T-personal thermocycler (Biometra, Germany) according to Subramaniam *et al.* (28) with modifications of the number of cycles, from 35 to 40; temperature of primer annealing, from 52ºC to 60ºC; and elongation time, from 60 s to 90 s. The DNA from nasopharyngeal swabs or, in cases when DNA concentration was too low, DNA from bacterial culture was used. The positive control (DNA obtained from the reference strain of *M. bovis* PG45: ATCC 25523) and water as negative control were used.

DNA sequencing and phylogenetic analysis. The PCR products were sequenced by Genomed (Poland). Each amplicon was analysed twice (forward and reverse) to ensure the reliability of the sequences. The sequences were compared with nine relevant sequences from GenBank and also with the closely related *M. agalactiae* strain. additionally, the predicted amino acid sequences were aligned. A dendrogram was plotted using a neighbour-joining (NJ) algorithm with 1000-BOOTSTRAP replicate analysis by Mega 6.0 software (The Biodesign Institute, Arizona State University, USA). The sequences were plotted as a similarity matrix with the use of Geneious software v 9.0.5 (Biomatters, New Zealand) based on percentage values.

Random amplification of polymorphic DNA (RAPD). The primers described by Hotzel *et al.* (10) were used. The PCR cycling conditions were reported by Byrne *et al.* (4). DNA from individual *M. bovis* isolates was used. Hybridisation profiles were analysed using BioNumerics software v 7.5 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity between isolates was calculated using the Jaccard coefficient (11) and the dendrogram was constructed by the unweighted pair group method using arithmetic means (UPGMA) (27).

PCR/denaturing gradient gel electrophoresis (PCR/DGGE). PCR/DGGE was performed using the DCode Universal Mutation Detection System (Bio-Rad, USA). The primers and DGGE method were described by McAuliffe *et al.* (16) with modifications (6). DNA from nasopharyngeal swabs was examined. DNA from the following strains was used as control: reference strain of *M. bovis* (ATCC 25523), type strains of *M. bovigenitalium* (ATCC 19852), *M. bovoculi* (ATCC 29104), and *Acholeplasma laidlawii* (ATCC 23206), and NCTC type strains of: *M. canadense*, *M. canis*, *M. arginini*, *M. bovirhinis*, *M. dispar*, *M. alkalescens*, *M. mycoides* subsp. *mycoides* SC, and *U. diversum* obtained from the Animal Plant and Health Agency, Weybridge, U.K.

Results

In total, 329 (46.1%) cattle from all 73 herds showed the clinical signs possibly indicating mycoplasma infection. Apathy and appetite disturbances was observed in all examined herds, nasal discharge in 43 (58.9%) herds, cough and dyspnea in 33 (45.2%), swollen udder and joints in 8 (11%), increased rectal temperature in 6 (8.2%), high somatic cell counts and decreased level of milk production in 5 (6.8%), abortions and infertility in 1 (1.4%), and keratoconjunctivitis in 1 (1.4%) out of all the herds tested.

Thirty-nine (5.5%) samples were positive for *M. bovis* in the PCR. Forty PCR products (*uvr*C gene

fragment), which were received directly from the swab or from culture when DNA concentration was low, were sequenced and submitted to NCBI GenBank (Table 2).

Table 2. Accession numbers of Polish *M. bovis* field strains and their territorial origin

Province/region	Name of isolate	NCBI GenBank number
Lublin/eastern (herd no. 1)	1_Mbovis_uvrC_PL	KP691391
	2_Mbovis_uvrC_PL	KP691392
	3_Mbovis_uvrC_PL	KP691393
	4_Mbovis_uvrC_PL	KP691394
	5_Mbovis_uvrC_PL	KP691395
	6_Mbovis_uvrC_PL	KP691396
	7_Mbovis_uvrC_PL	KP691397
	8_Mbovis_uvrC_PL	KP691398
Lublin/eastern (herd no. 2)	9_Mbovis_uvrC_PL	KP691399
	10_Mbovis_uvrC_PL	KP691400
	11_Mbovis_uvrC_PL	KP691401
	12_Mbovis_uvrC_PL	KP691402
	13_Mbovis_uvrC_PL	KP691403
	14_Mbovis_uvrC_PL	KP691404
Holy Cross/eastern	30_Mbovis_uvrC_PL	KU168357
	31_Mbovis_uvrC_PL	KU168358
	32_Mbovis_uvrC_PL	KU168359
	33_Mbovis_uvrC_PL	KU168360
	34_Mbovis_uvrC_PL	KU168361
	35_Mbovis_uvrC_PL	KU168362
	36_Mbovis_uvrC_PL	KU168363
	37_Mbovis_uvrC_PL	KU168367
	38_Mbovis_uvrC_PL	KU168364
	39_Mbovis_uvrC_PL	KU168365
	40_Mbovis_uvrC_PL	KU168366
Podlasie/eastern	18_Mbovis_uvrC_PL	KU168351
Masovian/central	15_Mbovis_uvrC_PL	KU168342
	16_Mbovis_uvrC_PL	KU168349
Pomeranian/northern (herd no. 1)	29_Mbovis_uvrC_PL	KU168348
Pomeranian/northern (herd no. 2)	17_Mbovis_uvrC_PL	KU168350
Greater Poland / north-western	19_Mbovis_uvrC_PL	KU168343
	20_Mbovis_uvrC_PL	KU168344
Opole/south-western	21_Mbovis_uvrC_PL	KU168352
	22_Mbovis_uvrC_PL	KU168353
	23_Mbovis_uvrC_PL	KU168354
	24_Mbovis_uvrC_PL	KU168345
	25_Mbovis_uvrC_PL	KU168346
	26_Mbovis_uvrC_PL	KU168355
	27_Mbovis_uvrC_PL	KU168356
	28_Mbovis_uvrC_PL	KU168347

The nucleotide sequences were aligned with relevant sequences in GenBank and a dendrogram was plotted (Fig. 1). Six DNA isolates had point nucleotide mutations, such as transitions and transversions and the mutations resulted in amino acid substitutions (Table 3) as compared with the reference *M. bovis* PG45 (AF003959). The *uvr*C nucleotide sequence of three isolates (KP691391, KP691395, and KP691396) showed 98.5% – 98.7% identity with the reference *M. bovis* PG45 sequence (AF003959). The other three sequences (KP691393, KP691397, and KP691401) showed from 99.7% to 99.9% similarity, while the remaining 34 sequences – 100% similarity.

PCR/DGGE confirmed the presence of bacteria belonging to the *Mollicutes* class in 564 (79.1%) DNA isolates from nasal swabs originating from 713 animals. The particular species identified were as follows: *M. bovirhinis* in 234/713 (32.8%), *M. dispar* in 101/713 (14.2%), *M. bovis* in 66/713 (9.3%), *U. diversum* in 55/713 (7.7%), *M. canis* in 37/713 (5.2%), *M. arginini* in 27/713 (3.8%), *M. canadense* in 21/713 (2.9%), *M. bovoculi* in 13/713 (1.8%), and *M. alkalescens* in 10/713 (1.4%) animals. *M. bovis* was the only species to be detected in 17/713 (2.4%) animals, while in 49/713 (6.9%) animals it co-occurred with another mycoplasma species and/or *U. diversum* (Fig. 2). *M. bovirhinis, M. dispar, U. diversum, M. arginini, M. bovoculi*, and *M. alkalescens* were detected as co-infections with *M. bovis* and in other kinds of co-infections. In DNA isolates from 498/713 (69.8%) animals *U. diversum* and *Mycoplasma* spp. other than *M. bovis* were found. Interestingly, *M. canis* and *M. canadense* were not identified in co-infection with *M. bovis*. Moreover, *M. bovigenitalium*, *M. mycoides* subsp. *mycoides* SC or *A. laidlawii* were not detected, and no mycoplasmas were identified in 149/713 (20.9%) DNA isolates.

Table 3. The type of mutations in the *uvr*C gene fragment and changes in amino acid sequences of Polish *M. bovis* field strains

NCBI GenBank accession number	Position in nucleotide sequence	Change in nucleotide	Type of mutation
KP691391 KP691395 KP691396	99	C → T	transition
	237	C → T	transition
	550	C → T	transition
	441	A → G	transition
	525	A → G	transition
	519	A → T	transversion
	555	C → T	transition
	690	T → C	transition
	810	T → C	transition
	1001	C → T	transition
	1050	C → T	transition
	1056	C → T	transition
	1092	T → C	transition
	1096	A → T	transversion
	1097	A → C	transversion
KP691391	864	G → C	transversion
	1022	T → G	transversion
KP691393	917	G → C	transversion
	920	A → C	transversion
KP691396 KP691397	928	A → C	transversion
KP691401	1079	T → G	transversion
NCBI GenBank accession number		Position in *uvr*C	Change in amino acid
KP691391 KP691395 KP691396		173	L → F
		334	T → M
		366	N → S
KP691391		228	E → D
		341	V → G
KP691393		307	N → T
		306	S → T
KP691396 KP691397		310	T → P
KP691401		360	M → R

Twenty pure field strains of *M. bovis* were isolated, and the RAPD profiles of the isolates analysed with Hum1 contained few bands, so the RAPD profiles were obtained using Hum4 primer. All the isolated *M. bovis* strains showed RAPD patterns of high diversity (Fig. 3). It was demonstrated that the produced profiles formed two distinct groups (I and II) with only 3.8% similarity.

Thirty percent of field strains were classified to group I and were divided into four subgroups (A–D). Seventy percent of field strains fell into group II and were further divided into two subgroups (F and G) with 50% similarity. Nine strains which fell into subgroup F were homologous with five strains of subgroup G.

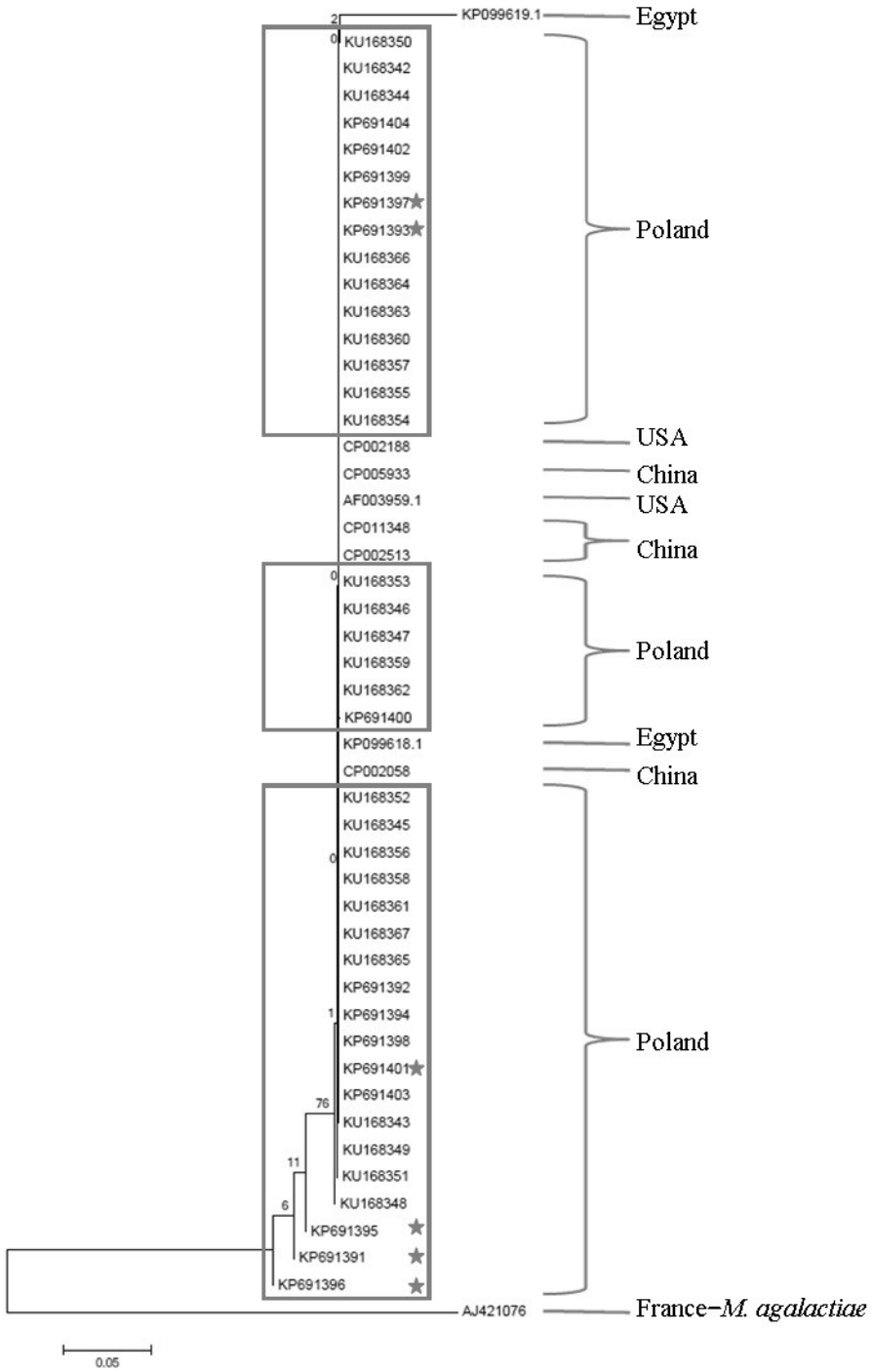

Fig. 1. Dendrogram of the phylogenetic relationship between the *uvr*C gene fragments of Polish field strains, strains from different countries and related *M. agalactiae* strain. The asterisk marks the occurrence of mutation (1000-BOOTSTRAP)

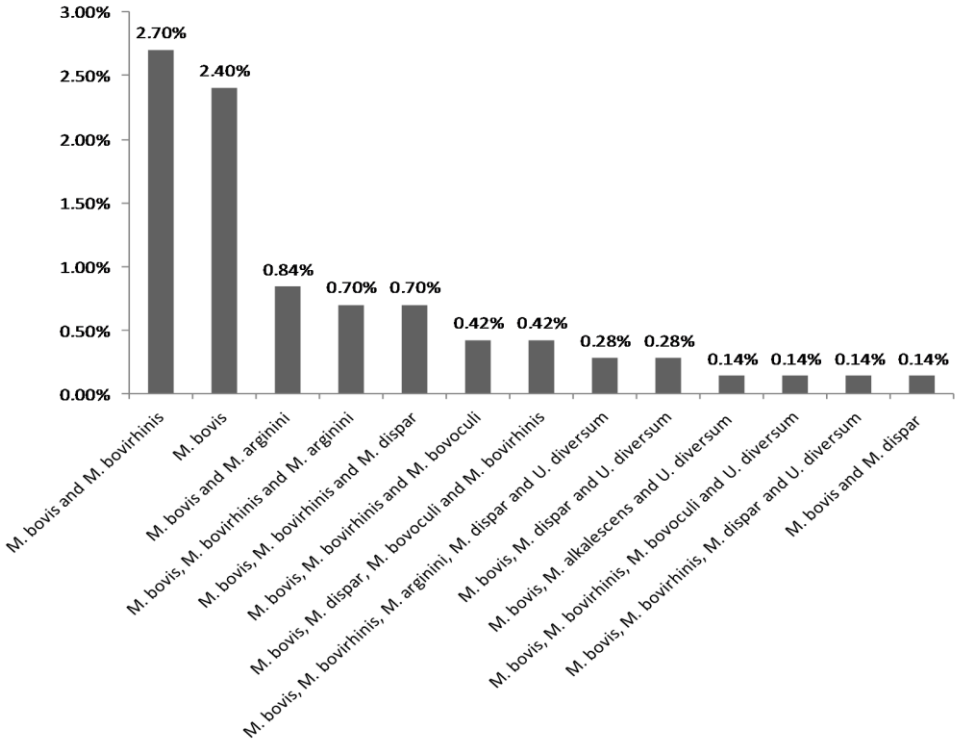

Fig. 2. *M. bovis* in co-infection with other *Mycoplasma* spp. and *U. diversum*

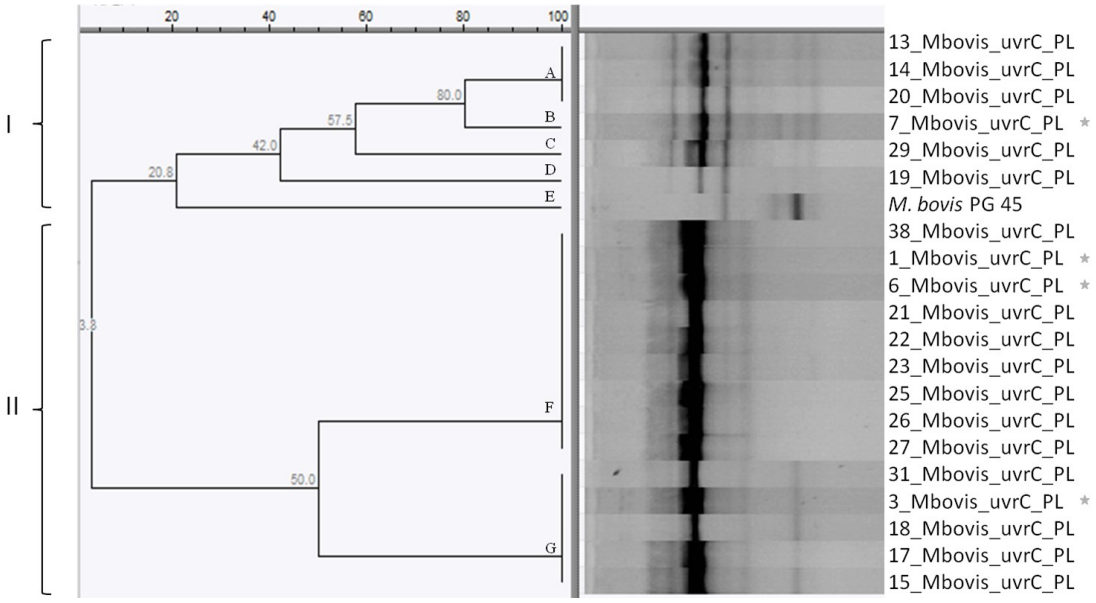

Fig. 3. Dendrogram of the phylogenetic relationship between Polish *M. bovis* isolates and the reference strain. Dendrogram was prepared on the basis of RAPD analysis. The asterisk marks the occurrence of mutation

Discussion

M. bovis infection represents a major disease burden for cattle producers worldwide, but other pathogens from the *Mollicutes* class should also be considered. Obtained results confirmed that the percentage of *Mollicutes* bacteria in nasal swabs from cattle affected with respiratory disease was 79.1. It should be noted that tested isolates contained DNA specific to one or more different *Mycoplasma* spp. and/or *U. diversum*. Co-infection in cattle herds is common in the course of respiratory diseases. Usually it is a co-infection with two or three *Mycoplasma* spp. (12, 13), while in our studies we detected even four or five different mycoplasmas in one animal. The prevalence of *M. bovis,* which is one of the most important bovine mycoplasmas, was 9.3%. It should be noted that other species of bacteria occurred besides *M. bovis*. The highest prevalence was noted for *M. bovirhinis* and *M. dispar*, and lower for other *Mycoplasma* spp. and *U. diversum*. *M. bovis* plays a major role among the mycoplasmas in the infection of

the respiratory tract in cattle, being isolated mainly from sick animals and rarely from healthy ones (30). Similarly to the results obtained in different countries (5, 12), in our study *M. bovirhinis* was the most often isolated mycoplasma. It is ubiquitous and known as a secondary pathogen in cases of calf pneumonia, and was most often detected among cattle with respiratory diseases (18). *M. dispar* and *M. arginini* were the next bacteria frequently encountered in our research. The first pathogen plays the primary role in infection, and the second one is regarded as an opportunist, being isolated rather rarely and mainly in co-infections with *M. bovis* (5, 9). *U. diversum* and *M. canis* are also significant in cattle respiratory diseases. *M. canis* is a canine mycoplasma, but it is also increasingly isolated from cattle pneumonia cases (5, 18). interestingly, *M. bovoculi*, which is responsible mainly for keratoconjunctivitis (18, 26), was found in nasal swabs in our study. *M. canadense* is not able to induce lung infection without other pathogens (25). In our study, *M. canis* and *M. canadense* were not found in co-infection with *M. bovis*, but in co-infection with other mycoplasmas (12, 20).

Following molecular typing with RAPD, the field strains isolated in Poland are known to fall into two groups: I and II. The mutations in the *uvr*C gene were detected among the strains from RAPD group II and the strains originating from different regions of Poland, despite the fact that this group seemed to be more homogeneous. This finding is consistent with the data presented in Europe, where the lack of correlation between geographical origins of strains was also observed (17, 24). It is possible to speculate that the two distinct groups identified by RAPD in this study represent two clonal lines of origin. The isolates from group I were relatively more heterogeneous than isolates of group II, which may indicate slightly different rates of genetic drift between the two putative clonal lines. However, the highest number of mutations in the *uvr*C gene was detected in strains from group II (subgroup F) and the mutations were 16 and 17 Single Nucleotide Polymorphisms (SNPs) for strains KP691391 and KP691396, respectively. It should be highlighted that this is the first report about the prevalence of mutations in the *uvr*C gene, which is considered conservative. In our study, we observed that some samples were negative in PCR testing while positive results were noted in PCR/DGGE. We can assume that it could be the result of mutations in the place of primer annealing in the *uvr*C gene sequence. Due to lack of PCR product, sequencing confirming the presence of mutations in these strains was impossible.

So far, *M. bovis* isolates have been characterised in some countries using genotyping tools such as RAPD, multilocus variable number tandem repeat (VNTR) analysis, and multilocus sequence typing (MLST) (2, 3, 14, 15, 23, 29) and it revealed that *M. bovis* strains are divided into two major clonal complexes. However, MLST results published by Dudek *et al.* (7) revealed that a Polish strain (not included in this study) did not cluster with any previously described clonal complexes. Therefore, in the future, other genotyping methods should be included in order to compare genetic diversity of Polish *M. bovis* strains with isolates from other countries. The nucleotide sequences derived from *uvr*C gene may provide additional information regarding genetic diversity of *M. bovis* field isolates. In our study, Polish *M. bovis* strains are found to be relatively homogenous and cluster closely with the type strain *M. bovis* PG45, which has a distinct geographical and temporal origin. When we compared the sequences of Polish strains with sequences of *M. bovis* from GenBank, all of them appeared to be closely related to the reference strain *M. bovis* PG45. It should be noted that the available sequences were obtained only from the USA, Egypt, and China, while there are no sequences (*uvr*C gene) of *M. bovis* strain from Europe in GenBank. Therefore, future analysis of the range of molecular characterisation of *M. bovis* in European countries, including Poland, is important.

In conclusion, different *Mycoplasma* spp. strains are present in cattle affected with respiratory diseases. *M. bovis* isolated from clinical cases plays an important role in aggravation of cattle pneumonia, but the role of other mycoplasmas cannot be ruled out. The demonstrated genomic heterogeneity among *M. bovis* strains confirms molecular and epidemiological differences and signifies an evolutionary dynamics of the *M. bovis* genome. The nature of the origin of *M. bovis* in Polish cattle is still not established, and it is only known that they are derivative of *M. bovis* PG45 strain. These findings underline the need for continuous surveillance not only in Poland but also in other European countries, to monitor the spread of *M. bovis* infection and mount effective control in order to minimise losses in the cattle industry.

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

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Animal Rights Statement: According to the Local Ethical Committee on Animal Testing at the University of Life Sciences in Lublin (Poland) formal ethical approval is not required for this kind of study (22).

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