Co-circulation of genetically diverse population of vaccine related and unrelated respiratory mycoplasmas and viruses in UK poultry flocks with health or production problems

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ABSTRACT

Respiratory diseases continue to have a major impact on poultry health, welfare and productivity. However, little information is available on their current status in UK poultry flocks. We investigated the presence of four economically important respiratory pathogens in healthy or problematic flocks; infectious bronchitis virus (IBV), avian metapneumovirus (aMPV), *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms). Samples from 131 UK poultry flocks were received during the 12 month study period. Oropharyngeal (OP) swabs were taken from eight birds per flock and accompanied with flock health information. The study included 118 chicken, 6 pheasant and 5 turkey flocks, and 1 quail and 1 partridge flock. Chicken flocks were of layers (n = 98), broilers (n = 15), breeders (n = 3) and undisclosed (n = 2). Flock ages ranged from 3 to 72 weeks old, and the average flock size was 17,633 birds. PCR detected 65 (49.6%), 59 (45%) and 8 (6.1%) flocks as positive for IBV, Mg/Ms and aMPV respectively. Analysis of the *mgc2* gene of the Mg isolates revealed high similarities to Mg TS-11 and Mg 6/85. Further gene analysis found that the TS-11-like isolates were unrelated to the TS-11 vaccine. Multi-locus sequence typing (MLST) analysis identified the majority of positive Ms as ST21, along with ST2 (MS-H-like), ST6 and ST43. IBV S1 gene sequencing identified strains as 793B (66.7%), Arkansas (23.8%) and Massachusetts (9.5%). All aMPV positive samples belonged to subtype B. Findings indicate that over half of the flocks sampled were positive for at least one of the four vaccine or field strains of mycoplasmas or viruses.

1. Introduction

The UK poultry industry currently produces 1.69 million tonnes of meat and over 10 billion eggs per year (Agriculture and Horticulture Devlopment Board, 2016). In addition, the industry is growing, with a 6.8% year on year increase in the number of poultry slaughtered from January 2016 to January 2017 (Department for Environment, Food and Rural Affairs, 2017). Despite this, respiratory disease still poses an important challenge to the industry, as subclinical and clinical infections cause substantial production losses. In the UK, single or mixed infections with infectious bronchitis virus (IBV), avian metapneumovirus (aMPV), *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms) can cause significant mortality, poor body weight gain and food conversion efficiency, and an increase in carcass condemnation in meat-type birds. In addition, drops in egg production and quality have been reported in layer and breeder birds. As a result, extensive vaccination against these pathogens takes place to protect against disease and production losses. A number of factors, including host migration (Cha et al., 2013) and genetic variations (Woo et al., 2006), can contribute to non-vaccine strains infecting flocks and either causing disease or resulting in a failure to achieve the required production levels. For these reasons, continuous surveillance of the current endemic strains is very important to ensure protection strategies are sufficient (Jones, 2010).

Infections with a number of IBV strains have been previously reported in the UK, including QX (Ganapathy et al., 2012; Irvine et al., 2010), Massachusetts (Hughes et al., 2009), 793B (Worthington et al., 2008), Italy02 (Worthington et al., 2008) and D274 (Worthington et al., 2008). Flock owners regularly vaccinate against IBV, however as the level of cross-protection between serogroups varies (Cavanagh et al., 1997), several different IBV strains are typically included in the vaccination program. Despite vaccine availability, farms are still witnessing infections with IBV, suggesting that further knowledge of circulating strains is required.

To date, four aMPV subtypes (A, B, C and D) are known (Cook and Cavanagh, 2002), but only A and B have been identified in the UK (Jones, 2010), with reports of C and D being infrequent detection in

Europe (Al-Shekaili et al., 2015). Infection causes egg production issues and swollen head syndrome in both chickens and turkeys (Cecchinato et al., 2012; Jones, 2010).

There are over a hundred-mycoplasma species worldwide causing disease in humans and other hosts, however disease in poultry in the UK is typically due to *Mycoplasma synoviae* (Ms) and *Mycoplasma gallisepticum* (Mg). Infection with both species is now controlled through vaccination, but while certain strains induce cross-reactive antibodies to varying degrees, protection is seemingly related to the virulence of the challenge strain rather than antigenic differences between vaccine strains (Lin and Kleven, 1982). Previous report described diseases caused by mycoplasmas in UK poultry flocks (Bradbury et al., 2001), but a nationwide study has not been carried out in recent decades.

In the UK and other countries, there is a lack of data outlining poultry respiratory infections. In the current study, we investigated the presence of important poultry mycoplasmas (Mg and Ms) and viruses (IBV and aMPV) in samples that were collected from UK poultry flocks presenting with respiratory signs or failing to achieve expected production levels. In addition, we collected farm and flock information at the point of sampling. Molecular techniques that would allow differentiation of field and vaccinal strains were used to detect potential co-circulation.

2. Methods

2.1. Flock sampling

Poultry veterinarians investigating flocks with respiratory signs or drops in production (e.g. number or quality of eggs) utilised a pre-prepared sampling pack (comprised of eight plastic shaft cotton swabs and eight plastic shaft cotton swabs with charcoal transport media) to collect oropharyngeal (OP) swabs from 8 birds per flock. Samples were transported to our laboratory overnight on ice.

2.2. Flock demographic data

Owners completed a questionnaire asking for details about both the farm and the flocks. Questions covered farm size, flock size, vaccination and treatment history, amongst others. The first section of the farm postcode was also obtained to allow results to be stratified by country. All work was granted ethical approval by the University of Liverpool ethics board.

2.3. Extraction of DNA and RNA

Dry swabs from the same flock were pooled and dipped into working solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) (Chomczynski and Sacchi, 2006) and stored at $-20\,°C$ until required. Extractions were carried out using the DNEasy and RNEasy kits (Qiagen, UK), according to manufacturer's instructions for DNA and RNA, respectively.

2.4. Molecular detection of mycoplasmas, IBV and aMPV

Extracted DNA was subjected to PCR assays targeting both the *mgc2* gene of *M. gallisepticum* and the *vlhA* gene of *M. synoviae* (Moscoso et al., 2004). Extracted RNA was subjected to nested RT-PCR assays to detect the S1 gene of IBV and the G gene of aMPV (subtypes A and B) (Cavanagh et al., 1999). Both the IBV and aMPV assays were previously used in epidemiological studies to detect the respective viruses from field samples (Al-Shekaili et al., 2015; Ball et al., 2016b; Ganapathy et al., 2015).

2.5. Differentiation of vaccine and field strains of Mg

Analysis of *mgc*2 gene sequences revealed six isolates as TS-11-like

(> 99% similarity to the vaccine strain). In accordance with Ricketts et al., (2017), three additional genes (*vlhA 3.04a*, *vlhA 3.05* and *mg03659*) were tested to confirm if the isolates were of field or TS-11 Mg vaccines. These three genes are present in the vaccinal strains but absent in the field strains (Ricketts et al., 2017). Twelve isolates had greater than 99% nucleotide relatedness to the 6/85 vaccine strain. Any isolates not identified as either TS-11 or 6/85-like were considered field isolates.

2.6. Sequence analysis

Mycoplasma and IBV amplicons were submitted for commercial sequencing (Source BioScience, UK). Chromatograms were analysed in ChromasPro, and samples with poor read data were re-sequenced. Following sequence clean up, samples were aligned using ClustalW and subjected to a BLAST search (NCBI database) to determine strain identity. The average rate of base substitution was measured and compared between swab and cultured mycoplasma sequences. Phylogenetic analysis was carried out using maximum-likelihood with 1000 bootstrap replicates. Reference mycoplasma strains used for analysis included *M. gallisepticum* TS-11 (JQ770175), *M. gallisepticum* TLS-2 (JN113387), *M. gallisepticum* 6/85 (KP318741), *M. gallisepticum* K6216D (KU577606), *M. gallisepticum* PG31, *M. synoviae* MS-H (KX168666) and *M. synoviae* WVU. Reference IBV strains used for analysis included 793B (AF093794), CR88 (KM067900), 1/96, QX (KF297571), D388, Q1 (AF286302), D274 (X15832), IS/1494/06 (EU780077), IS/885/00 (AY279533), H120 (GU393335), M41 (AY561711) and Arkansas (GQ504721).

2.7. Multi-locus sequence typing (MLST) of Ms

Samples found positive by PCR were further examined using a recently published MLST scheme (El-Gazzar et al., 2017). All seven loci were amplified and sequenced as previously described, then subsequently analysed and compared to the online database (http://pubmlst.org/msynoviae/) to determine allele numbers. The complete allelic profiles were then cross-referenced to identify the sequence type (ST) to which each strain belonged, including the MS-H vaccine sequence type.

2.8. Quantitative PCR analysis of Mg and Ms

A commercial kit (BioChek, The Netherlands) was used to quantify the amount of genomic material extracted from oropharyngeal swab samples. All PCR positive samples were tested using the assay. To generate a log relative expression unit (log REU) value, sample cycle threshold (Ct) values were compared to Mg or Ms reference curves gathered from diluted Mg or Ms reference strains (Ball et al., 2016a; Londt et al., 2013).

2.9. Culture and identification of mycoplasmas

Charcoal swabs were plated onto modified Chanock agar medium and then dipped into modified Chanock agar broth. Cultures were checked daily, with colony growth by 24 h post inoculation regarded as contamination. Positive cultures where confirmed as either Mg or Ms using immunofluorescence assays (IFA) (Rosendal and Black, 1972). All positive Mg and Ms isolates were subjected to molecular differentiation, as described above, to determine their relatedness to vaccine strains.

2.10. Statistical analysis

All analysis was carried out using SPSS Statistics 22 (IBM) and an independent t-test. Differences were considered significant at the $p < 0.05$ level.

Table 1
The source of the samples including the regional locations, species of birds, flock age and size derived from the submission forms.

Country	Bird Species				Age Range	Average Flock Size
	Chicken	*Turkey*	*Pheasant*	*Other*		
England	94	5	6	2	3.5–72 weeks	26,377
Wales	4	0	0	0	3–51 weeks	19,950
Scotland	4	0	0	0	41–51 weeks	32,000
Northern Ireland	16	0	0	0	40–74 weeks	8383
Total	118	5	6	2		

3. Results

3.1. Sampled flocks

Over the 12-months period, we obtained swab samples from a total of 131 flocks presenting with respiratory clinical signs. The flocks included 118 chicken (90.1%), 6 pheasant (4.6%), 5 turkey (3.8%) and 2 other (quail and partridge) (1.5%) flocks. Of the chicken flocks, 96 were layers, 15 were broilers and 3 were breeders (Table 1). Ages of all flocks at the time of sampling ranged from 3 to 72 weeks old, and the average flock size was 17,633 birds. Forty five percent and 24.4% of the flocks were reported to be vaccinated against IBV and aMPV, respectively. Based on completed submission forms, for the two mycoplasma species, 33.6% were vaccinated against Mg and 23.7% were vaccinated against Ms, but specific vaccine details (e.g. TS-11, 6/85, Ms1, MS-H) were not disclosed.

Although our sample submission protocol stated that we wanted samples from flocks with respiratory illness or drops in egg production or quality, only 70.2% of the submissions reported these particular signs. Of these, a drop in egg production or egg quality (or both) was described in 44% of flocks, with respiratory signs reported in 26%. Other signs included reduced body weight gain, diarrhoea and lameness (8.4%).

The majority (51.9%) of flocks had reported a previous infection with at least one of the pathogens investigated for this study. Previous infection with either Ms or Mg was the most common (28.2% and 26% respectively), with infection with IBV (22.9%) and aMPV (7.6%) also reported. Mortality rates at the time of sampling differed depending on the bird species. It ranged from 0.1 to 23% in chickens, 5.97 to 8% in turkeys, and 0.3 to 40% in game birds. Veterinarians reported that 79 flocks had not received any treatments prior to sampling.
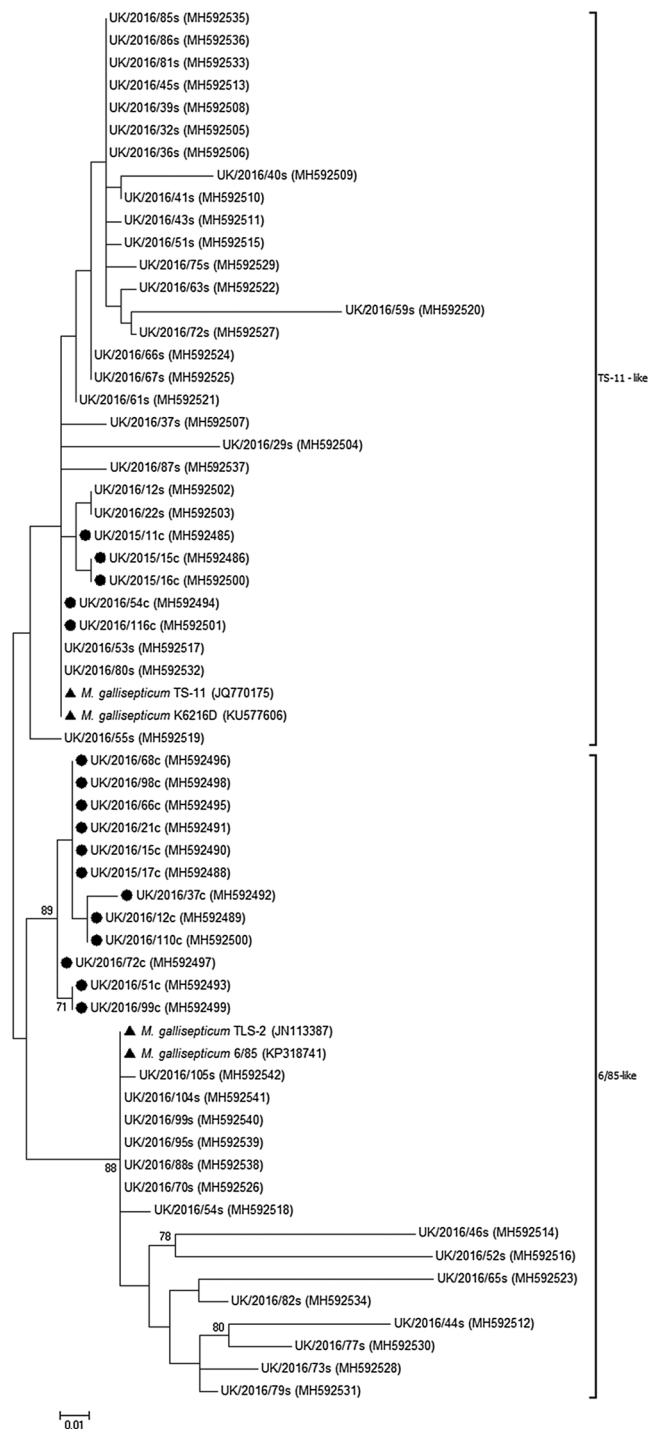
3.2. Molecular detection of Mg, Ms, IBV and aMPV

Mycoplasmas, IBV and/or aMPV were detected in 98 of the 131 flocks (74.8%) (Table 2). The most prevalent pathogen was IBV (n = 64), followed by Mg (n = 43), Ms (n = 13) and aMPV (n = 8). Almost all positive samples were from chicken flocks, with just a single turkey and a single pheasant flock positive for a mycoplasma infection. Infectious bronchitis virus was the only pathogen to be detected within all sampled regions, whereas aMPV was only detected in samples from England.

3.2.1. Mg and Ms

A total of 44 and 15 samples were positive for *M. gallisepticum* and *M. synoviae*, respectively. Nine flocks were PCR positive for both Mg and Ms. Out of 44 samples, interpretable sequence data was obtained from 42 Mg samples (*mgc2* gene), and based on BLAST nucleotide similarity, 27 were identified as TS-11-like and 15 identified as 6/85-like (Fig. 1). From the 15 Ms positive samples, eight returned with interpretable sequence data, of which three were similar to the MS-H strain (UK/2015/3, UK/2015/11 and UK/2016/64) based on the *vlhA* gene (Fig. 2). Sequence analysis determined the average number of base substitutions per site for strains detected from dry swabs as 0.103 for Mg and 0.552 for Ms. Sequence data based on the *mgc2* gene and *vlhA* gene have been submitted to GenBank (NCBI) (accession numbers MH592502-MH592542 and MH588548-MH588553 respectively).

All isolates identified as TS-11-like by *mgc*2 sequencing, were further analysed by PCR for detection of three additional genes (*vlhA* 3.04a, *vlhA* 3.05 and *mg03659*) closely associated with TS-11 (Ricketts

Table 2
PCR and culture results for each bird species tested during the study period, stratified by country.

Bird Species	Pathogen	Total Positive							
		England		Wales		Scotland		Northern Ireland	
		PCR	*Culture*	*PCR*	*Culture*	*PCR*	*Culture*	*PCR*	*Culture*
Chicken	Mg	33	12	0	0	2	2	5	0
	Ms	11	7	0	0	0	0	2	1
	IBV	44	–	3	–	3	–	10	–
	aMPV	8	–	0	–	0	–	0	–
	Negative	22	75	1	4	1	2	2	15
Turkey	Mg	1	3	–	–	–	–	–	–
	Ms	1	0	–	–	–	–	–	–
	IBV	0	–	–	–	–	–	–	–
	aMPV	0	–	–	–	–	–	–	–
	Negative	4	2	–	–	–	–	–	–
Pheasant	Mg	2	1	–	–	–	–	–	–
	Ms	0	0	–	–	–	–	–	–
	IBV	0	–	–	–	–	–	–	–
	aMPV	0	–	–	–	–	–	–	–
	Negative	4	5	–	–	–	–	–	–
Other	Mg	0	0	–	–	–	–	–	–
	Ms	0	0	–	–	–	–	–	–
	IBV	0	–	–	–	–	–	–	–
	aMPV	0	–	–	–	–	–	–	–
	Negative	2	2	–	–	–	–	–	–

(–) denotes no samples tested for that particular bird species and/or country.

Fig. 1. Maximum likelihood phylogenetic analysis of sequenced *M. gallisepticum* positive samples based on the *mgc2* gene. Analysis was carried out with the Jukes-Cantor model and gamma distribution. Bootstrap values below 70 are not displayed. Sequences from swab samples have no icon, sequences from cultured isolates are shown with a black circle, and reference strains are indicated with a black triangle along with accession numbers if applicable.

et al., 2017). Our analysis showed that all 27 isolates were unrelated to TS-11, and were considered field strains, as none of the three additional genes were amplified. In contrast, all three gene targets were positive for the TS-11 vaccine, which was used a positive control.

Multi-locus sequence typing (MLST) data demonstrated that 3 of the 15 PCR positive Ms flocks belonged to sequence type 2 (ST2), the same sequence type as the MS-H vaccinal strain. The majority of strains

(n = 9) belonged to ST21, which has been reported previously in the Netherlands. The remaining three samples were identified as ST6, ST7 and ST43. A single strain could not be typed.

Quantitative PCR data showed that, on average, *M. synovaie*-positive flocks carried a significantly greater ($p < 0.005$) bacterial load (4.21 log CFU/ml) when compared to *M. gallisepticum*-positive flocks (2.56 log CFU/ml). For the nine samples from flocks co-infected with Mg and Ms, the concentrations of Ms were also higher than the concentrations of Mg (3.29 compared to 2.68; $p > 0.05$).

3.2.2. Infectious bronchitis virus

IBV was detected in 64 (49.6%) flocks. The majority of the sequences belonged to the 793B genotype (66.7%), with the remaining samples identified as Arkansas (23.8%) and Massachusetts (9.5%). Roughly half of the sequences were vaccine-related (> 99% nucleotide similarity) (Ganapathy et al., 2015; Worthington et al., 2008), with the remaining samples potential field samples (at least 85% similarity) of genotypes previously reported in the UK (Fig. 3) (Worthington et al., 2008). Sequence data has been submitted to GenBank (NCBI) (accession numbers MH589998-MH590043).

3.2.3. Avian metapneumovirus

Only eight of the sampled flocks were RT-PCR positive for aMPV (6.1%). Positive samples were obtained from both layer and breeder chicken flocks, and all were identified as aMPV subtype B. All strains contained the A to G mutation at position 91 in the G gene, which differentiates field isolates from the VC03 vaccinal strain (Listorti et al., 2014). Sequence data has been submitted to GenBank (NCBI) (accession numbers MH562700-MH562707).

3.3. Co-detection of mycoplasmas, IBV or aMPV

A co-infection with at least two of Mg, Ms, IBV and/or aMPV was detected in 42 (32.1%) flocks by PCR (Fig. 4). Of these, a combination of Mg and IBV was the most common (19 flocks), with Ms and aMPV the least common infection (one flock). Three flocks were infected with Mg, Ms and IBV, and a single flock was infected with Mg, Ms and aMPV. We did not detect any flocks infected with Ms, IBV and aMPV, or all four pathogens.

3.4. Culture and identification of mycoplasmas

Mycoplasma cultures were obtained from 26 flocks (19.8%) following either initial plate or broth culture. Broth cultures showing a positive colour change were subsequently plated onto agar to obtain colonies. Following IFA testing, 18 were found to be Mg positive and 9 were Ms positive, with a single chicken flock positive for both. England had the highest culture positive counts (16 Mg, 7 Ms and one co-infection), with Scotland having two Mg infections and Ireland having one Ms infected flock. Flocks from Wales were culture negative for Mg and Ms.

Of the Mg isolates, twelve were genetically related to the 6/85 genotype (98–100% *mgc2* nucleotide identity) and six were similar to TS-11 (> 99% *mgc*2 sequence identity). When the six TS-11-like isolates were further analysed for additional TS-11 genes, as described above, all six isolates were found to lack the *vlhA 3.04a*, *vlhA 3.05* and *mg03659* genes. For this reason, these isolates were classified as field strains of Mg. BLAST comparisons of the *vlhA* gene revealed that five Ms sequences were similar to the MS-H vaccine strain (96–100% identity), with a further three strains having a lower shared nucleotide identity (84%). MLST analysis showed that four cultured isolates belonged to ST2 (MS-H sequence type), two isolates belonged to ST21 and the remaining two belonged to ST43. Sequence analysis highlighted a lower than average number of base substitutions per site for the cultured isolates (Mg = 0.041; Ms = 0.506) than strains detected on the dry swabs (Mg = 0.103; Ms = 0.552). Sequence data for cultured isolates
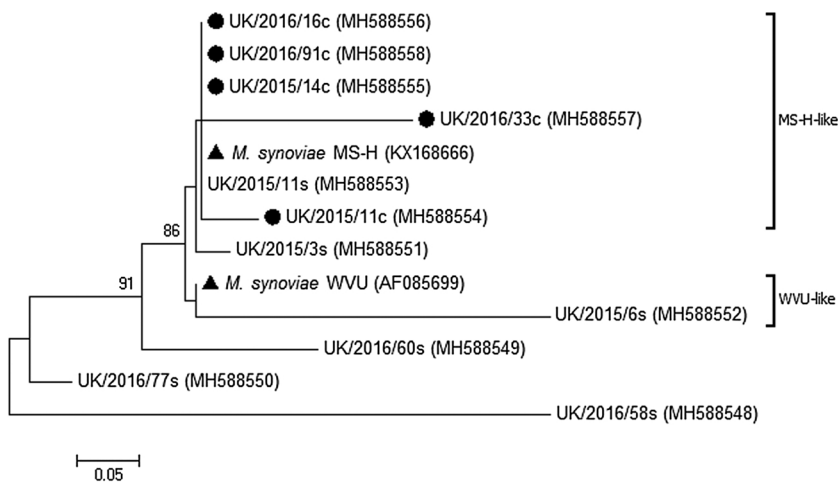
Fig. 2. Maximum likelihood phylogenetic analysis of sequenced *M. synoviae* positive samples based on the *vlhA* gene. Analysis was carried out with the Jukes-Cantor model and gamma distribution. Bootstrap values below 70 are not displayed. Sequences from swab samples have no icon, sequences from cultured isolates are shown with a black circle, and reference strains are indicated with a black triangle along with accession numbers if applicable.

based on the *mgc2* gene and *vlhA* gene have been submitted to GenBank (NCBI) (accession numbers MH592485-MH592501 and MH588554-MH588558 respectively).

4. Discussion

In this study, oropharyngeal swabs were collected from flocks using a non-invasive method for detection of a range of respiratory pathogens (Al-Shekaili et al., 2015; Worthington et al., 2008). The effect of different swab types has previously been investigated for the isolation of mycoplasmas (Zain and Bradbury, 1995) and the use of a cotton swab containing transport medium facilitated successful culture of both Mg and Ms from field cases.

Analysis of the *mgc2* gene sequences from the 44 Mg positive flocks revealed the presence of two Mg genotypes (TS-11 and 6/85). Both genotypes correspond to vaccine strains, but recent work has documented a field case in which an isolate with increased virulence, including vertical transmission, was identified as closely related to TS-11 (El Gazzar et al., 2011). Further PCR analysis showed that despite a high *mgc2* gene similarity with the TS-11 vaccine, none of the TS-11-like strains were positive for the genes (*vlhA3.04a*, *vlhA3.05* and *mg0359)* specific for the TS-11 vaccine. Thus, it appears that despite high similarities to TS-11 seen in the *mgc2* gene sequences, these isolates are likely to have been field strains. Even those Mg detected on the seven farms that had used the TS-11 vaccine were found to be field strains. Our findings show that *mgc2* sequencing alone may not be sufficient to conclude an Mg isolate is TS-11-like, and that instead, as demonstrated by Ricketts et al. (2017), sequencing should be extended to include the *vlhA3.04a*, *vlhA3.05* and *mg0359* genes. The absence of TS-11 on TS-11 vaccinated farms demonstrated that despite vaccination, field Mg strains managed to enter into the flock at some point of rearing. A longitudinal epidemiological study may provide further comprehensive information on the circulation of vaccine and field Mg strains in Mg-vaccinated flocks.

Multi-locus sequence typing (MLST) offers more detailed epidemiological analysis of *M. synoviae* strains than the *vlhA* gene analysis alone (El-Gazzar et al., 2017). Data from MLST analysis further emphasised the high variability between Ms strains. Only three sequences were identified as belong to ST2, which includes the MS-H vaccine, two of which came from MS-H vaccinated flocks. The majority of samples were found to contain ST21, which has previously been detected in the Netherlands. Based on the online *M. synoviae* MLST database (https://pubmlst.org/msynoviae/), the detection of ST6, 7 and 43 in this study appears to be the first detection of these types outside the USA and Australia. Isolates identified as ST6 and ST7 have previously been reported in the USA and this is the first report of all four sequence types in the UK. Analysis using eBurst revealed that ST2 and 43 clustered in the

same clonal complex (CC1), ST6 and ST7 lay in CC4 and ST21 did not lay in a clonal complex. The MLST scheme utilised for *M. synoviae* typing in this study is a recent development and, as such, there is a limited global database of strains reported for each ST.

It has been suggested that Ms is now more economically important than Mg in the Netherlands (Landman, 2014). Interestingly, when tested by both molecular and traditional culture methods, we detected a greater number of Mg than Ms positive flocks. This may have been influenced by the limited number of flocks studied in the current study. Thus, a larger epidemiological study is needed for an accurate assessment. Samples from the positive Ms flocks had a significantly higher concentration of mycoplasmas than those from the Mg positive flocks. Strain variation was also higher for both Mg and Ms samples obtained through swabs with a higher number of base substitutions per site compared to the cultured isolates (7.185 compared to 0.077). The higher level of variation witnessed in the swab samples suggests subpopulation diversity in Mg and Ms infected flocks.

The most common IBV genotype detected during the study was 793B. This is not surprising given that its presence has been widely reported in a number of previous studies (Al-Shekaili et al., 2015; Ball et al., 2016b; Cavanagh et al., 1999; Ganapathy et al., 2015; Worthington et al., 2008). Furthermore, based on the partial S1 sequence, the majority of the 793B-like strains (57.2%) were of field variants, rather than vaccinal strains (Ganapathy et al., 2015; Worthington et al., 2008). Earlier work in the UK has also shown that 793B is the most prominent strain type, but over half of these were identified as 793B vaccines (Worthington et al., 2008). In this study, a number of the 793B sequences were distantly related to common 793B vaccines being used in UK, reflecting possible mutations of the vaccine strains or invasion of field strains despite single or multiple application of live 793B vaccines. There is a potential for vaccine strains to undergo genetic variation should they persist in flocks (Cook et al., 2012; Toro, 2010), which may explain the differences in the 793B sequences. The remaining samples were identified as Arkansas and Massachusetts strains, both of which are routinely used as vaccines in the UK (Worthington et al., 2008). We did not detect any Italy-02 or QX strains in the flocks, despite previous reports of their presence in commercial and backyard flocks in the UK (Valastro et al., 2010; Worthington et al., 2008). The reasons for this could be because the samples in the current study originated mostly from layer flocks, also heterologous vaccination practices in recent years may have led to a substantial reduction in the circulation of IBV QX.

In contrast to the higher levels of IBV detection, we only found aMPV in eight flocks. The low prevalence may be associated with the relatively rapid host response to infection, as there is a low likelihood of a virulent strain being detected ten days after infection (Ganapathy and Jones, 2007). In addition, all strains detected during this study
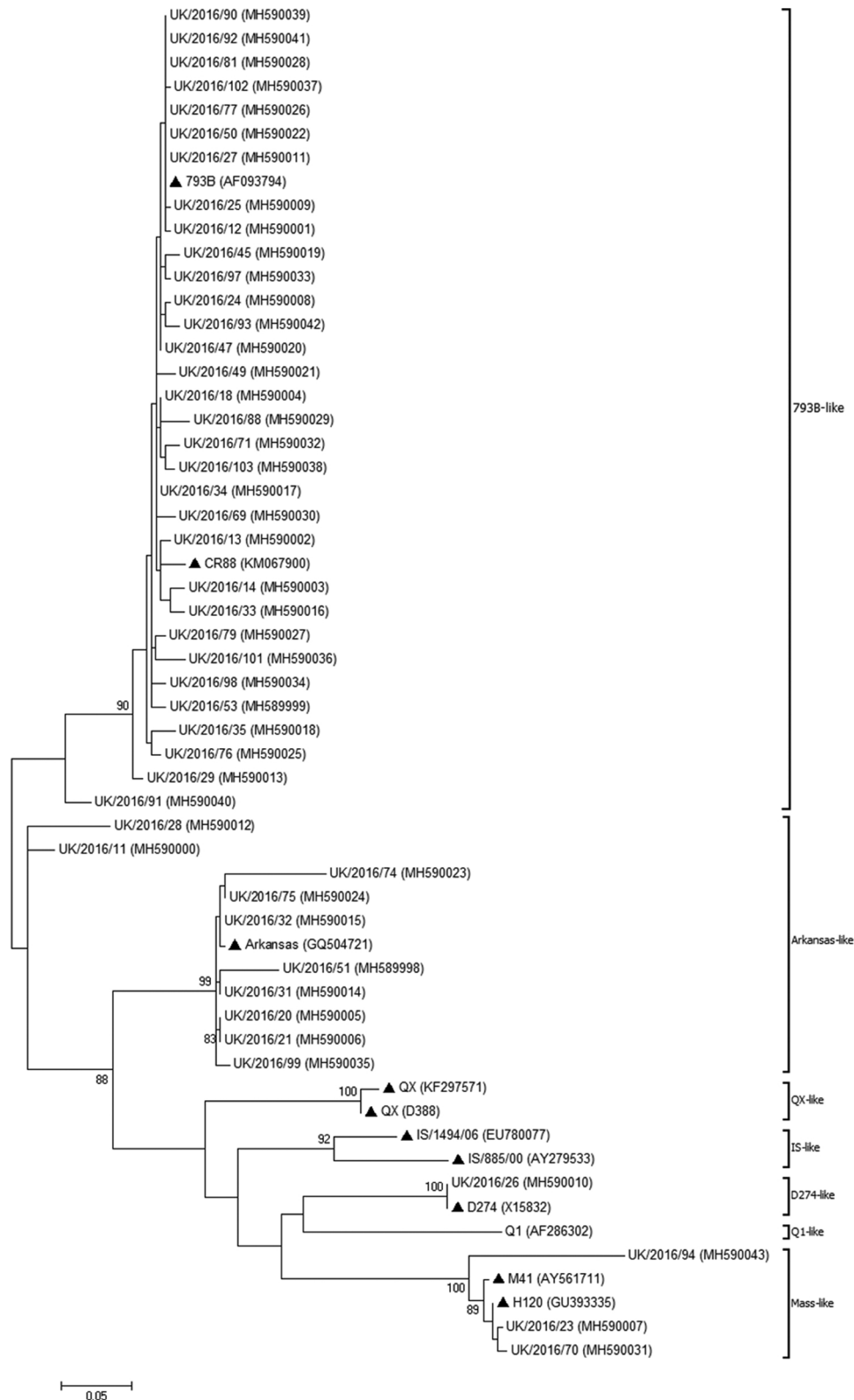
Fig. 3. Maximum likelihood phylogenetic analysis of sequenced IBV strains based on partial S1 gene data. Analysis was carried out with the Jukes-Cantor model and gamma distribution. Bootstrap values below 70 are not displayed. Reference strains are indicated with a black triangle along with accession numbers.

belonged to subtype B and, upon further molecular analysis (G gene), were found to be closely related to a subtype B vaccine. Historically, only subtypes A and B have been known to cause infections in the UK (Jones, 2010), and both subtype A and B are currently being used for vaccination of poultry in the UK.

In our study, single swab sampling was carried out only in flocks with respiratory signs or drops in egg production/quality. Almost three quarters of these flocks were positive for one of the vaccine or field mycoplasmas or viruses investigated in this study. Detection of field strains of IBV, Mg and Ms, including in vaccinated flocks, reflects the
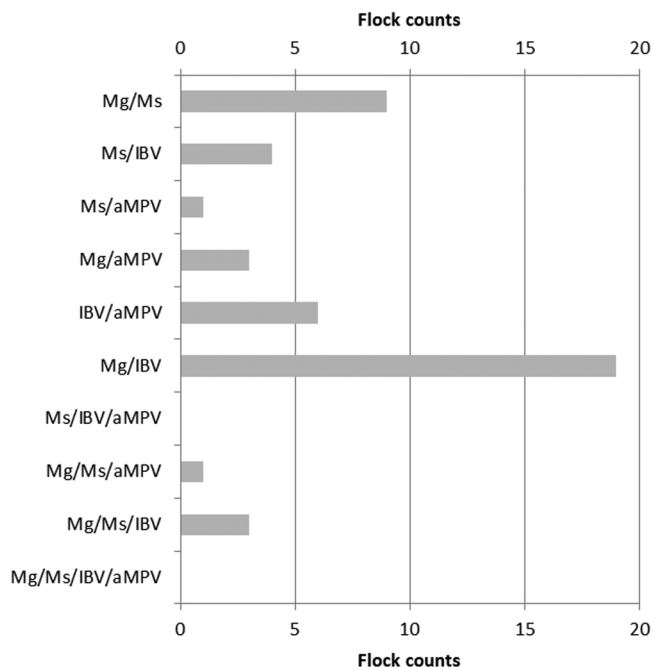
Fig. 4. A cross-comparison on the number of flocks with single or multiple detections of Mg, Ms, IBV or aMPV.

constant challenges posed by these pathogens, either as single or mixed infections, and their contribution to production losses. Further longitudinal studies would improve our understanding of the persistence and evolution of mycoplasmas and viral vaccines in commercial poultry farms, and the complex interactions between these vaccines and pathogens as causes of health and production problems.

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