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Varga, Zsuzsanna; Volokhov, Dmitriy V.; Stipkovits, Laszlo; Thuma, Akos; Sellyei, Boglarka; and Magyar, Tibor, "Characterization of *Pasteurella multocida* strains isolated from geese" (2012). *Food and Drug Administration Papers*. 53.

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Characterization of *Pasteurella multocida* strains isolated from geese

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ARTICLE INFO

Article history:

Received 19 April 2011

Received in revised form 20 December 2012

Accepted 22 December 2012

Keywords:

Pasteurella multocida

Goose

Fowl cholera

Pasteurellosis

ABSTRACT

Phenotypic and genotypic diversity of forty-two *Pasteurella multocida* isolates from geese were characterized by analysis of their capsular type, Heddleston serotype, biotype, fimbrial gene allele type, comparative outer membrane protein (OMP) electrophoresis patterns, and were analyzed using PCR for the presence of virulence-associated genes (*toxA*, *tbpA*, *pflA*, *hgbA*, *hgbB*, *nanH*, *nanB*, *fimA*, *hsf-1*, and *pmHAS*). A sequence comparison of the *thdF* and *rpoB* housekeeping genes of twenty representative *P. multocida* strains from three different OMP groups demonstrated that seventeen strains were closely related phylogenetically to previously published strains of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida*, and only three strains from geese were grouped with previously published strains of *P. multocida* subsp. *septica*. This study is the first report regarding the characterization of phenotypic and genotypic features of different *P. multocida* field strains isolated from geese with the different clinical representation of pasteurellosis and provide our overview on the usefulness of these in vitro tests for primary characterization of *P. multocida* strains for the epidemiological studies of fowl cholera.

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1. Introduction

Pasteurella multocida is the causative agent of fowl cholera (FC), a contagious disease that affects over 100 avian species worldwide and leads to significant economic losses in domestic and commercial poultry (Christensen and Bisgaard, 2000; Harper et al., 2006). In the European Union, FC has been eliminated from closed-cycle commercial large scale chicken and turkey flocks; however, disease can still be found in water fowl kept in semi-closed husbandry systems where free access to the outside environment and water is allowed.

P. multocida has been classified by serological methods into five capsular serogroups designated A, B, D, E and F, and 16 serotypes can be distinguished by analysis of heat stable precipitin antigens (Christensen and Bisgaard, 2000; Heddleston et al., 1972). Three subspecies of *P. multocida* have been established (subsp. *multocida*, subsp. *septica* and subsp. *gallicida*) based on results of total genomic DNA hybridization, carbohydrate fermentation, enzymatic activities, and phylogenetic gene sequence comparison studies (Davies, 2004; Hunt Gerardo et al., 2001; Kuhnert and Korczak, 2006; Mutters et al., 1985). Based on differences in fermentation activities, *P. multocida* isolates have been classified into 13 different biotypes (Blackall et al., 1997; Fegan et al., 1995). In addition, more than 60 different outer membrane proteins (OMP), with presumed roles in pathogenesis and immunity, have been identified in *P. multocida* strains isolated from a wide range of animal species (Hatfaludi et al., 2010). Fimbriae of *P. multocida*

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encoded by the *ptfA* gene may play an important role in adhesion of the bacterium to the host epithelial cells, and the *ptfA* gene polymorphism can be a useful tool for epidemiological studies (Sellyei et al., 2010; Tang et al., 2009).

Domestic and wild geese are frequently affected by FC (avian pasteurellosis) (Blanchong et al., 2006; Samuel et al., 2005). The strains of *P. multocida* detected in geese exhibit substantial differences in their virulence and therefore in their ability to cause peracute, acute or chronic forms of FC. At this time, no serological, biochemical or genetic markers are known that permit the differentiation of highly virulent strains from less virulent strains of *P. multocida* isolated from poultry. Experimental animal infection (Wilkie et al., 2000) is still a “gold standard” method for assessing the potential virulence of *P. multocida* isolates. In addition, the phenotypic and genotypic characteristics of *P. multocida* strains and its subspecies associated with the different clinical representation of pasteurellosis in geese are still unknown.

Thus, the main goal of this study was to analyze the phenotypic and genotypic characteristics of *P. multocida* field strains isolated from geese afflicted with different forms of pasteurellosis. This study summarizes the characterization of *P. multocida* strains isolated from geese in Hungary and provides our overview on the diversity of *P. multocida* field strains based on in vitro testing results that may reflect to the potential virulence of *P. multocida* isolates.

2. Materials and methods

2.1. *P. multocida* strains studied and cultural conditions

Phenotypic and genotypic characteristics of 42 *P. multocida* isolates from geese were studied. Of these 42 *P. multocida* strains, 34 strains were isolated from geese with confirmed diagnoses of FC at the Central Veterinary Diagnostic Institute of Hungary in 2005–2009. To choose strains representative of a diverse epidemiological setting, strains were selected taking into account the locations and time of their isolation in Hungary as well as the available data on disease cases. Based on the disease profile associated with these strains, these FC isolates were separated into three groups: (i) strains from cases of peracute fowl cholera (pFC) in geese, which were characterized by fulminating acute fatal septicaemia with high mortality in the geese flocks, (ii) strains from cases of typical acute FC (tFC) where the infections developed more slowly and with less fatalities, and (iii) strains from chronic atypical (so-called “pasteurellosis”) cases which were characterized by local, non-systemic inflammations in the respiratory tract, joints, peritoneal cavity or oviducts and caused low mortality in the geese flocks. Eight additional *P. multocida* strains, which were isolated from geese with not reported diagnoses (Table S1) in an earlier period (from 1990 to 2005), were used to compare characteristics among the strains of the focus group (2005–2009). All these *P. multocida* strains were directly isolated from liver, septic blood obtained from heart, or

tissue lesions using sheep blood agar. In the cases diagnosed as pasteurellosis in geese (non-pFC/non-tFC cases), *P. multocida* strains were isolated from local non-systemic infections; phallus inflammation (Strains P747, P898), ovarian inflammation (Strain P770/1), salpingitis (Strain P463), cloacitis (Strain P899) and a site of subcutaneous tissue inflammation (Strain P597). In addition, seven non-geese *P. multocida* strains isolated in Hungary from other species were studied as comparators to the strains isolated from geese (Table S1).

Fifty microliters of pure strains grown in brain–heart infusion broth were centrifuged at $16,000 \times g$ for 10 min. Total DNA from the cell pellet was extracted using a DNeasy blood and tissue kit (Qiagen, Chatsworth, CA) according to the manufacturer’s protocol.

2.2. Biochemical characterization, PCR assays, and phylogenetic analysis of the strains

Biochemical characterization, determination of subspecies of *P. multocida*, somatic serotype and biotype, *P. multocida*-specific PCR, capsule-specific multiplex PCR, and *toxA* gene-specific PCR were carried out as described elsewhere (Kuhnert and Korczak, 2006; Lichtensteiger et al., 1996; Mutters et al., 1985; Townsend et al., 1998; Varga et al., 2007). The typing of the *ptfA* gene for the strains was performed using PCR (Sellyei et al., 2010). The detection of the presence of virulence genes (*toxA*, *tbpA*, *pflA*, *hgbA*, *hgbB*, *nanH*, *nanB*, *fimA*, *hsf-1*, and *pmHAS*) in all strains was carried out as described earlier (Ewers et al., 2006; Tang et al., 2009). In addition, amplification of the *nanB* and *nanH* genes of *P. multocida* strains used in this study was carried out using the next in-house primers designed based on homologous nucleotide sequence regions in these full-length genes available at GenBank (CP003022, CP003313, CP001409, AF274868, and AF274869): *nanB*-Forw (TGGGGCGATATTGATACCGCAATCC), *nanB*-Rev (GGTCATTGACATCTGAAAGCGAGGC), *nanH*-Forw (TACGGTGGGAAGACAGCAAGTGACC) and *nanH*-Rev (TCGGTCCAATACTCAGCTGATATCCG). The sizes of *nanB* and *nanH*-specific amplicons were 2888 bp and 1907 bp, respectively.

Briefly, the standard PCR mixture (50 μ l) contained 1.5 U of HotStarTaq-Plus DNA polymerase, $1 \times$ reaction buffer supplemented with 2.5 mM MgCl₂ (Qiagen, Chatsworth, CA), 500 nM of each forward and reverse primer, a 200 μ M concentration of each deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), and 7 μ l of DNA template (ca. 0.1–0.2 μ g of total DNA). The PCR was performed using a GeneAmp PCR system model 9600 thermocycler (PE Applied Biosystems, Foster City, CA), with the following cycle conditions: initial activation at 95 °C for 5 min, 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C (extension) for 1.5 min; and a final extension at 72 °C for 7 min. The synthesis of PCR products with the expected molecular weights was confirmed by electrophoresis using a 1% SeaKem Gold TAE-agarose gel with ethidium bromide (Lonza, Allendale, NJ), followed by UV visualization.

Phylogenetic analysis of *thdF* and *rpoB* gene sequences of selected strains was performed as described elsewhere (Kuhnert and Korczak, 2006; Petersen et al., 2001; Stahel

et al., 2009). DNA sequences from this study were deposited in GenBank under accession numbers JF523357–JF523413, KC512818–KC512829.

2.3. Outer membrane protein (OMP) profiling of the strains

The OMP fractions were prepared as described previously (Davies et al., 1992, 2003). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the OMP fractions was carried out in 1.0 mm thick gel (4% stacking and 15% separating) using Bio-Rad Protean II xi Cell equipment and run according to the Bio-Rad protocol. Before the loading step, the samples were mixed (1/4; v/v) with 2× sample buffer contained dithiothreitol and boiled for 5 min, 20 µg protein of each sample was loaded into each well. Bio-Rad Precision Plus Protein Standard (10–250 kDa) was used as the molecular weight marker. The gel was run at constant current (30 mA) as the samples moved through the stacking gel; the current was increase to 40 mA for the continuous run in the separating gel. The electrophoresed proteins were stained with Coomassie Brilliant Blue R-250.

Gels were scanned and analyzed using Fingerprinting II software (Bio-Rad). Based on the comparative OMP profiling analysis of the *P. multocida* strains, the dendrogram representing the similarity among the protein patterns was constructed according to the Dice co-efficient.

3. Results

3.1. Presumptive identification and general characteristics of the strains

All the tested isolates were positive in the *P. multocida*-specific PCR and negative in the *P. multocida* toxin (*tox*A) gene-specific PCR. Table S1 summarizes the clinical diagnoses in geese from which the strains were isolated and the observed characteristics of these strains, i.e. the capsule types, Heddlestone somatic serotypes, biotypes, allele type of fimbrial *ptfA* gene, and the biochemical test results. Of the 42 *P. multocida* strains studied, 40 strains were found to express capsule type A and two strains (P898, P899) expressed capsule type F. Serotyping of the Heddlestone somatic antigen of the strains revealed the presence of serotypes 1, 3, 3,4,(5) and 4,5. In the biotype studies, biotype 3 and an arabinose fermenting unclassified biotype were found among the 21/42 and 15/42 of the geese strains, respectively; only a few strains expressed biotype 2 (3/42), 1 (1/42), and 7 (1/42). One *P. multocida* strain (P770/1, isolated from a goose with ovarian inflammation) fermented maltose and therefore this strain was considered an unclassified maltose-positive biotype. The maltose-positive strains of both *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* have been isolated previously from different hosts including avian species (Fegan et al., 1995; Korbel et al., 1992; Petersen et al., 1998).

Thus, based on the results of the species-specific PCR assay and phenotypic testing, all these strains were recognized to be *P. multocida* species; however, their subspecies affiliation within the species was uncertain based on the biochemical data.

3.2. Outer membrane protein (OMP) profiling

The OMP-based profiling of *P. multocida* geese strains (Fig. 1) revealed a variety of protein bands ranging from approximately 20 to 150 kDa. Based on the similarity of the OMP protein patterns, these strains can be clustered in three main groups (Fig. 1). In addition, the third group can be subdivided in three subgroups (IIIa, IIIb, and IIIc).

Group I consists of only the capsule type F *P. multocida* strains isolated from two independent cases of pasteurellosis in two geese with local infections, i.e. phallus inflammation (P898) and cloacitis (P899). Group II contains only strains of *P. multocida* serotype A:1 isolated from 1990 to 2008. This group represents all eight peracute FC strains studied with only a few strains isolated from geese with typical FC. All of the strains isolated from geese and assigned to group II expressed an unclassified biotype that was characterized by arabinose-positive and dulcitol- and xylose-negative reactions and by the weak ability of these strains to ferment sorbitol (Table S1). In addition to these group II strains from geese, the seven *P. multocida* non-geese strains (Table S1) also expressed the same unclassified biotype characteristics and fell into the OMP group II (data not shown).

Subgroup IIIc consists of only one maltose-positive *P. multocida* strain (770/1) that exhibited a unique OMP electrophoretic pattern. Subgroup IIIb contains *P. multocida* strains that were found to express different biotypes and Heddlestone somatic antigens; these strains were isolated predominantly from geese with “pasteurellosis” (i.e., non-systemic local) infections. Subgroup IIIa consists of strains isolated mainly from tFC cases and contained *P. multocida* strains with biotype 3 and one *P. multocida* strain (P964) that expressed biotype 2.

Thus, we can assume that the OMP pattern-based grouping of these 42 different strains may reflect to their phenotypic and/or genetic similarity, for example, all the strains classified as group II were arabinose-positive and included all the strains associated with peracute FC.

3.3. Detection of virulence-associated genes

The results of PCR analysis for the presence of virulence-associated genes (*tbpA*, *pflA*, *hgbA*, *hgbB*, *nanH*, *nanB*, *fimA*, *hsf-1*, and *pmHAS*) are summarized in Table S2. All the tested isolates were positive for *pmHAS* (hyaluronan synthase), *hgbA* (hemoglobin-binding protein A), *hgbB* (hemoglobin-binding protein B), and *fimA* genes and variable for the presence of *hsf-1*, *nanH* (sialidase NanH), and *nanB* (sialidase NanB) genes. All the OMP group II strains, and maltose-positive strain P770/1 (OMP group IIIc) were found negative for both *nanH* and *nanB* sialidase genes using previously published primers (Tang et al., 2009). Two strains of the OMP group I were also found negative for *nanB* sialidase gene using the same primers. Because the real absence of *nanH* and *nanB* sialidase genes in all OMP group II and IIIc strains was uncertain for us the novel in-house primers for the amplification of *nanH* and *nanB* were designed (see above) and used to clarify the situation. We found that all these strains were positive for *nanB* sialidase gene with the novel primers. The *nanB* gene

Fig. 1. The OMP pattern-based grouping of these studied 42 strains isolated from geese with different disease patterns of FC. Note: ND – no data; UK – unknown; Malt+ – unclassified maltose-positive biotype; P – “pasteurellosis” cases which were characterized by local non-systemic inflammations and low mortality in the geese flocks; pFC – peracute fowl cholera in geese; tFC – typical acute FC in geese.

amplicons sequenced from several strains (Fig. S1) of the OMP group II were phylogenetically closely related to the same gene in *P. multocida* 36950 (GenBank accession number CP003022). Unfortunately, due to the presence of mutations in the binding regions of the published primers within the gene (Tang et al., 2009), the amplification of *nanB* in these strains was failed using the primers. All our attempts to amplify *nanH* sialidase gene in all OMP group II and IIIc strains were unsuccessful with the novel in-house designed primers. However, due to substantial level of nucleotide sequence variability of *nanH* sialidase gene in *P. multocida* found in GenBank (data not shown), we cannot entirely consider that these strains lacking the gene. Thus, biochemical testing of sialidase activity in such strains seems to be most appropriate approach to determine the presence of functional gene (Straus et al., 1996).

Based on our analysis of the presence of virulence-associated genes in the studied *P. multocida* strains we were unable to observe any association between the presence or absence of the tested virulence-associated genes and clinical representation of pasteurellosis in geese.

3.4. Phylogenetic analysis

Selected strains ($n=20$) from the OMP groups were used to conduct *thdF* and *rpoB*-based phylogenetic

analysis. The strains were selected based on their affiliation to the different OMP groups. The *thdF* and *rpoB*-based analysis revealed that seventeen strains were closely related phylogenetically to previously published strains of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* (Figs. 2 and S1), and only three strains from geese were grouped with previously published strains of *P. multocida* subsp. *septica*. By *thdF* and *rpoB*-based phylogenetic analysis, all these serotype A:1 arabinose-positive strains from the OMP group II were grouped closely to each other and to strains of *P. multocida* subsp. *gallicida* (Figs. 2 and S1). Due to their aberrant phenotype, we cannot say with certainty that these strains were *P. multocida* subsp. *gallicida* strains. However, arabinose-positive isolates among three subspecies of *P. multocida* were previously reported for *P. multocida* subsp. *gallicida* strains only (Kuhnert and Korczak, 2006; Mutters et al., 1985). Thus, we suspect that we have isolated atypical *P. multocida* subsp. *gallicida* strains from these particular geese.

4. Discussion

The results of our study indicate that *P. multocida* strains isolated from geese exhibit substantial phenotypic and genotypic diversity. This diversity could represent

heterogeneity within the species isolated from infected geese. Previously, high levels of diversity have been demonstrated for avian, ovine and porcine isolates, in comparison to cattle respiratory isolates (Hotchkiss et al., 2011).

The OMP-based profiles of *P. multocida* geese strains revealed their clustering in three main groups. Group II was found to contain all the strains of *P. multocida* serotype A:1 that were isolated from peracute cases of FC in geese. All of the strains isolated from geese and assigned to group II expressed an unclassified biotype that was characterized by arabinose-positive and dulcitol- and xylose-negative reactions and by the weak ability of these strains to ferment sorbitol (Table S1). Dulcitol-negative *P. multocida* isolates that demonstrated weak and/or conflicting fermentation reactions in Andrade's sorbitol have been reported previously from infected dog and cat bite wounds (Hunt Gerardo et al., 2001), but similar strains have not been reported yet from poultry.

It has been reported that discrimination between subspecies of *P. multocida*, namely, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida*, can be ambiguous based on sorbitol, trehalose, and dulcitol fermentation reactions (Blackall et al., 1997; Christensen et al., 2004; Fegan et al., 1995; Hunt Gerardo et al., 2001).

Phylogenetically, the 16S rRNA gene sequences of strains of *P. multocida* subsp. *multocida* and strains of *P. multocida* subsp. *gallicida* were found to be almost identical, so use of the 16S rRNA gene was believed to be impracticable for the discrimination between these two subspecies. However, the 16S rRNA gene sequences of strains of *P. multocida* subsp. *septica*, thought previously to form a phylogenetically separate group from the *multocida/gallicida* phylogenetic group, were later found to be a useful marker for the genetic identification of *P. multocida* subsp. *septica* (Petersen et al., 2001; Stahel et al., 2009). In addition, the other housekeeping genes, e.g. *rpoB* and *thdF*, demonstrated higher variability than the 16S rRNA gene and were used as additional markers for phylogenetic studies and identification of *Pasteurella* isolates (Kuhnert and Korczak, 2006; Stahel et al., 2009). Based on previously published data about these genetic markers, we decided to use *rpoB* and *thdF* genes for the assessment of phylogenetic grouping among our *P. multocida* strains as additional information to their observed biochemical characteristics.

The *thdF* and *rpoB*-based phylogenetic analysis of the 20 *P. multocida* strains selected from the three OMP groups revealed that 17 strains were closely related phylogenetically to previously published sequences for strains of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida*; only three strains from geese were grouped with previously published sequences for strains of *P. multocida* subsp. *septica*.

In our study, the use of *thdF* and *rpoB* gene sequencing allowed us to discriminate between the strains belonging to *P. multocida* subsp. *septica* group and the strains belonging to *P. multocida* subsp. *multocida/gallicida* group by comparison of sequence similarity. However, the ability of these markers to discriminate between the strains belonging to *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* was not possible. From our experience, we can conclude that discrimination between *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* strains based on the phylogenetic analysis of these housekeeping genes is problematic due to very limited information about the gene polymorphisms for *P. multocida* subsp. *gallicida* strains, which express the typical biochemical characteristics of this subspecies. Currently, only a few *thdF* and *rpoB* gene sequences of *P. multocida* subsp. *gallicida* are available in GenBank.

In our study, two strains (P898 and P899) that belong phylogenetically to the *P. multocida* subsp. *septica* group expressed serotype F:1 and were negative for the fermentation of dulcitol and trehalose but positive for the fermentation of xylose and sorbitol. Also, only these two strains were PCR-positive for the F serotype specific marker glycosyltransferase (*fcgD*) gene (Townsend et al., 2001) and PCR-negative for putative filamentous hemagglutinin (*pfhA*) and transferrin binding protein A (*tbpA*) genes (Table S2). The absence of *pfhA* and *tbpA* genes in these two serotype F:1 strains could be explained by the main association of *pfhA* with serogroups B and E, and *tbpA* with serogroups A and B, respectively. Another strain (P964) that belonged to *P. multocida* subsp. *septica* group based on the phylogenetic analysis of the *thdF* and *rpoB* genes also expressed serotype A:1 and was negative for the fermentation of dulcitol and positive for the fermentation of xylose, trehalose and sorbitol. The P964 strain was also found to be PCR-negative for the *tbpA* gene but positive for the *pfhA* gene. The presence of dulcitol-negative, sorbitol-positive strains of *P. multocida* subsp. *septica* has been previously reported (Hunt Gerardo et al., 2001). We agree with the authors of that study that sorbitol fermentation cannot be a reliable marker for differentiation among subspecies of *P. multocida* and additional phenotypic and/or genotypic tests are required for their unambiguous identification (Hunt Gerardo et al., 2001; Stahel et al., 2009).

In our phylogenetic analysis of *rpoB* gene sequences of our strains compared with the gene sequences that are available in GenBank, we found eight previously published sequences for strains of *P. multocida* subsp. *septica* that were isolated from large-cat bite wounds and reported to be sucrose-, dulcitol- and sorbitol-negative strains (Christensen et al., 2005). These strains were not grouped with all other available sequences of *P. multocida* subsp. *septica*; however, they were grouped (with bootstrap supporting value of 100%) with other

Fig. 2. Dendrogram showing phylogenetic relationships between *P. multocida* isolates from geese and *P. multocida* strains available in GenBank based on nucleotide sequence data for the *rpoB* gene. The trees were constructed by the minimum evolution method in the MEGA 4 package. The bootstrap values presented at corresponding branches were evaluated from 1000 replications. GenBank accession numbers are indicated for each strain used in creating the dendrograms.

P. multocida subsp. *multocida* (Fig. 2). Thus, this case may represent a taxonomic misidentification of sucrose-, dulcitol- and sorbitol-negative strains, which could represent atypical strains of *P. multocida* subsp. *multocida*. Further research is needed to find unambiguous markers for the differentiation between *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* strains. Because the complete genome sequence of a common avian clone of *P. multocida* Pm70 is available now (May et al., 2001), we believe that genomic tools (i.e. microarrays, suppression subtractive hybridization and massive parallel sequencing) can be helpful for differentiation of these two subspecies and in the search for possible genetic markers (pathogenicity islands) of the *P. multocida* pathogenicity (Hert et al., 2008).

Finally, we can conclude that in our study the arabinose fermenting serotype A:1 isolates of the *P. multocida* strains that expressed A type *ptfA* gene were associated with peracute FC in the geese. The *P. multocida* strains of A:1 serotype have been previously reported to be associated with serious cases of fowl cholera (Christensen and Bisgaard, 2000; Leotta et al., 2006), including highly pathogenic “Vietnam type” strains of *P. multocida* that have been detected in Southeast Asia and also found to express serotype A:1 and the A type *ptfA* allele (Doughty et al., 2000; Wilkie et al., 2000). Further studies are required to examine the prevalence of these arabinose fermenting *P. multocida* strains in poultry in the European Union and other countries as well as the analysis of their contribution to causes of acute and peracute FC in geese.

Acknowledgements

We thank Drs. Maureen K. Davidson, Donna K. Chandler and Christine Anderson for their suggestions and critical review of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2012.12.023>.

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