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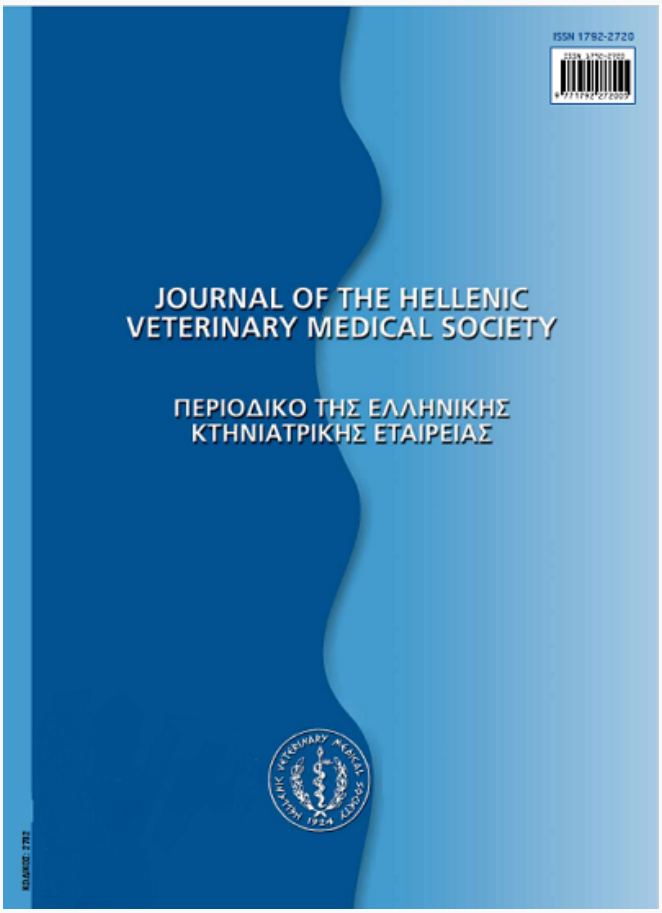

Serotypic diversity and sequence variation of the ompA gene among Mannheimia haemofytica isolates from domestic ruminants

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Ορολογική και γενετική ποικιλότητα του γονιδίου *ompA* στελεχών *Mannheimia haemolytica* που απομονώθηκαν από μηρυκαστικά

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ABSTRACT. Pneumonia caused by *Mannheimia haemolytica* is an important disease of ruminants. Because of its economic significance, several methods have been used to study the pathogenicity and epidemiology of *M. haemolytica*. The objectives of this study were to provide data about the prevalence of the different serotypes of the bacterium and to investigate the genetic diversity of a significant virulence factor, the *ompA* gene. Two methods, DNA sequencing analysis and DGGE, were used to study the polymorphisms of the *ompA* gene. Ninety-four isolates from pneumonic lungs were investigated. Capsular serotyping showed that serotype A2 was the predominant serotype among ovine strains and the only serotype found in caprine strains. This is the first reported analysis of the *ompA* gene of *M. haemolytica* strains isolated from goats. Analysis of the gene revealed five DGGE patterns and nine genotypic groups. The ovine isolates, which belonged to four DGGE patterns, showed a much greater diversity than the caprine strains, which belonged to just two DGGE patterns. Sequence analysis was used to verify the DGGE results and revealed eight genotypic groups among the ovine isolates and three among the caprine ones. Furthermore, the correlation of these results showed a great diversity of the *ompA* gene among serotype A2 isolates.

Keywords: *Mannheimia haemolytica; ompA* gene; genetic diversity

ΠΕΡΙΛΗΨΗ. Η *Mannheimia haemolytica* αποτελεί ένα από τα σημαντικότερα αίτια πνευμονίας στα μηρυκαστικά. Εξαιτίας των σοβαρών οικονομικών επιπτώσεων που προκαλεί, διάφορες μέθοδοι έχουν χρησιμοποιηθεί με στόχο τη επιζωοτιολογική μελέτη και τη μελέτη της παθογένειας του βακτηρίου. Σκοπός της παρούσας έρευνας ήταν η διερεύνηση της συχνότητας απομόνωσης των διαφόρων οροτύπων της *Mannheimia haemolytica* στην Ελλάδα και η γενετική ανάλυση ενός σημαντικού γονιδίου λοιμοτοξικότητας του βακτηρίου, του γονιδίου *ompA*. Για το σκοπό αυτό, μελετήθηκαν ενενήντα τέσσερα στελέχη *M. haemolytica*, που απομονώθηκαν από τεμάχια πνευμονικού ιστού ενός βοοειδούς, προβάτων και αιγών με μακροσκοπικές αλλοιώσεις πνευμονίας. Η ταυτοποίηση των στελεχών έγινε με βασικές βιοχημικές δοκιμές, καθώς και με τη μέθοδο της Πολλαπλής Αλυσιδωτής Αντίδρασης της Πολυμεράσης (Multiplex PCR). Η οροτυποποίηση των στελεχών έγινε με τη μέθοδο της έμμεσης αιμοσυγκόλλησης. Για την μελέτη των πολυμορφισμών του γονιδίου *ompA*, χρησιμοποιήθηκαν δύο μέθοδοι: η μέθοδος της Ηλεκτροφόρησης Πηκτής με Διαβαθμισμένη Αποδιατακτική Σύσταση (DGGE-Denaturing Gradient Gel Electrophoresis) και η ανάλυση της αλληλουχίας του γονιδίου (DNA sequencing).

Ανάμεσα στα ενενήντα τέσσερα στελέχη, βρέθηκαν οι ορότυποι A1, A2, A5, A6, A7, A9 και A12. Ο ορότυπος A2 ήταν ο επικρατέστερος. Το μοναδικό στέλεχος που απομονώθηκε από βοοειδές ήταν A2, όπως και τα στελέχη από αίγες. Τα υπόλοιπα στελέχη A2 προήλθαν από πρόβατα.

Η ανάπτυξη και εφαρμογή της μεθόδου DGGE αποκάλυψε πέντε διαφορετικά πρότυπα μεταξύ των στελεχών. Τα στελέχη πρόβειας προέλευσης, εμφάνισαν μεγαλύτερη παραλλακτικότητα (τέσσερα διαφορετικά πρότυπα), από αυτά που προέρχονταν από αίγες (δύο διαφορετικά πρότυπα). Το πρότυπο 2 ήταν το πιο διαδεδομένο και ακολουθούσαν τα πρότυπα 1 και 3. Το σύνολο των στελεχών που απομονώθηκαν από αίγες ανήκε σε δύο από αυτά τα πρότυπα. Η μέθοδος της ανάλυσης των αλληλουχιών του DNA εφαρμόστηκε με στόχο την επιβεβαίωση των αποτελεσμάτων της DGGE και την εύρεση των συγκεκριμένων πολυμορφισμών. Η μελέτη του γονιδίου *ompA* ανέδειξε 11 διαφορετικούς γονότυπους μεταξύ των στελεχών της *M. haemolytica*, που χωρίστηκαν σε εννέα συνολικά ομάδες. Τα στελέχη των προβάτων έδειξαν μεγαλύτερη ετερογένεια και κατατάχθηκαν σε οκτώ ομάδες, δύο από τις οποίες ήταν οι πολυπληθέστερες. Η πλειοψηφία των στελεχών που απομονώθηκαν από αίγες ανήκε στην ομάδα *IX*, η οποία συνέπιπτε με το πρότυπο 1 της DGGE. Η μελέτη της δευτεροταγούς δομής της πρωτεΐνης OmpA εντόπισε τις αντικαταστάσεις αμινοξέων στους επιφανειακούς βρόχους της πρωτεΐνης και, πιο συγκεκριμένα, στις υπερμεταβλητές περιοχές. Καθώς, για πρώτη φορά διεθνώς, αναφέρονται πολυμορφισμοί του γονιδίου *ompA* στελεχών της *M. haemolytica* τα οποία έχουν απομονωθεί από αίγες, τα στοιχεία της έρευνας συμβάλλουν στη μελέτη της εξελικτικής πορείας του γονιδίου, και στην αποσαφήνιση της ύπαρξης ή μη ειδικότητας των διαφόρων υπο-ομάδων ως προς τον ξενιστή.

INTRODUCTION

Mannheimia haemolytica is an important pathogen involved in the respiratory disease complex of cattle, sheep and goats (Zecchinon et al. 2005). The bacterium is an apparent commensal on the upper respiratory tract of healthy ruminants but it is also responsible for pneumonic pasteurellosis, a disease that causes considerable economic losses to the cattle and sheep industries (Frank 1989, Gilmour and Gilmour 1989). The bacterium, in conjunction with certain viral infections and stress factors, is capable of evading the host immune defenses and colonizing the lower respiratory tract causing a fibrinous lobar pneumonia (Brogden et al. 1998).

Capsular serotyping is a traditional method still used for the classification of *M. haemolytica*, as well as for epidemiological studies. However, there has been an increasing interest in the genetic nature of its various

virulence factors since it can aid in understanding its pathogenicity and epidemiology at a molecular level.

Seventeen capsular serotypes have been described within the former *Pasteurella haemolytica* complex (Adlam 1989, Younan and Fodar 1995); serotypes 3, 4, 10 and 15 are now classified as *Bibersteinia [Pasteurella] trehalosi* (Blackall et al. 2007), while serotype 11 has been reclassified as *Mannheimia glucosida* (Angen et al. 1999). *M. haemolytica* comprises representatives of the 12 remaining serotypes (A1, A2, A5–A9, A12–A14, A16 and A17) (Angen et al. 1999); among them, serotypes A1 and A2 are the most prevalent worldwide. Whereas A1 is the predominant serotype associated with bovine pneumonic pasteurellosis, other serotypes are recovered infrequently from diseased cattle, although serotype A2 is often associated with healthy animals (Frank 1989). In contrast, A2 is the most commonly isolated serotype from cases of ovine pneumonic pasteurellosis, followed by serotypes A7 and A9. Low numbers of serotype A6 isolates are recovered from cases of both bovine and ovine pneumonic pasteurellosis (Frank 1989, Gilmour and Gilmour 1989). The association of different serotypes with infections in cattle and sheep suggests differences in host specificity and virulence among the different serotypes. It has also been shown that bovine and ovine isolates of the same serotype can be distinguished by differences in chromosomal genotype (Davies et al. 1997) or outer membrane protein profiles (Davies and Donachie 1996).

M. haemolytica possesses several virulence factors including capsular polysaccharide, leukotoxin (LktA), lipopolysaccharide (LPS), adhesins, outer membrane proteins, glycoproteases and neuraminidases (Highlander 2001, Zecchinon et al. 2005). In common with other gram-negative bacteria, *M. haemolytica* possesses outer membrane protein A (OmpA), a heat-modifiable protein which is an integral component of the outer membrane (Beher et al. 1980), with immunogenic activity (Ayalew et al. 2011, Puohiniemi et al. 1990, Zeng et al. 1999). OmpA is also involved in adherence to host tissues in related pathogens such as *Pasteurella multocida* (Dabo et al. 2003). The adherence of respiratory pathogens to the mucosal epithelium is critical in host colonization and infection (Beachey 1981). Based on sequence homology, Davies and Lee (2004) suggested that the OmpA of *M. haemolytica* is involved in binding to bovine and ovine receptors, thus playing an important role in the colonization of the respiratory tracts of cattle and sheep. It has also been demonstrated that OmpA shows a specific interaction with the extracellular matrix (EMC) protein fibronectin (Lo and Sorensen 2007) and that it contributes to adherence of *M. haemolytica* to bovine bronchial epithelial cells (BBEC) *in vitro* (Kisiela and Czuprynski 2009). Moreover, a recent study has shown that two different OmpA subclasses, OmpA1 and OmpA2, could potentially bind to different receptors in cattle and sheep (Hounsome et al. 2011).

The protein has a similar structure to the OmpA protein of *Escherichia coli*, which comprises a C-terminal periplasmic domain and a N-terminal transmembrane domain consisting of eight membrane-traversing antiparallel β-strands and four surface-exposed loops. It has been shown that the majority of polymorphic nucleotide sites in the *ompA* gene of *M. haemolytica* occurs in four hypervariable domains within the surface-exposed loops (Davies and Lee 2004).

In pathogenic bacteria, the detection of single nucleotide polymorphisms (SNPs) is very important, since those present in virulence factors may aid in defining the host specificity at molecular level (Lawrence et al. 2010). Denaturing gradient gel electrophoresis (DGGE) can detect single base mutations in DNA (Fisher and Lerman 1983). In DGGE, DNA fragments of the same length but with different sequence composition can be separated; separation is based on the decreased electrophoretic mobility of a partially melted doublestranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (Muyzer and Smalla 1998). This method can detect nearly 100% of the sequence variants by the attachment of a GC-clamp, a sequence rich in guanines and cytosines, to one side of the DNA fragment (Muyzer and Smalla 1998, Sheffield et al. 1989). DGGE has been used extensively for diversity analysis in microbial ecology (Muyzer and Smalla, 1998). It has also been used for the molecular typing of *Staphylococcus aureus* (Gürtler et al. 2001) and *Campylobacter jejuni* (Nielsen et al. 2000) and for the differentiation of *Mycoplasma* species (McAuliffe et al. 2003).

The aims of this study were to examine the prevalence of *M. haemolytica* serotypes among ovine, caprine and bovine isolates collected from pneumonic lungs in Greece, to study the diversity of the *ompA* gene of these isolates by DNA sequence analysis and DGGE, thus evaluating the efficiency of DGGE for this

purpose, and to correlate variation in capsular serotypes with the *ompA* gene diversity.

Materials and methods

Bacterial strains

Ninety-four isolates of *M. haemolytica* were analysed in this study. The isolates originated from widespread geographical locations within Greece over the period 2006-2007. They were recovered from 395 pneumonic lung tissue samples obtained from 21 slaughterhouses and from eight cases of pneumonia (kindly provided by Regional State Laboratories). Eighty-one strains were recovered from 250 ovine samples, 12 from 49 caprine samples and only one strain was recovered from the 104 bovine lung tissue samples examined.

Bacterial isolation and identification

Lung tissue samples were plated onto blood agar plates [Columbia blood agar base (Oxoid) supplemented with 5% (v/v) defibrinated sheep blood] and incubated overnight at 37 °C. After incubation, the plates were examined for *Mannheimia*-like colonies (greyish, odourless, showing a narrow zone of β-haemolysis). A single haemolytic colony was subcultured and tested by Gram-staining, catalase, oxidase and indole. All Gram-negative, catalase positive, cytochrome-oxidase positive and indole negative isolates having the typical colony morphology were stored at -80 °C in 25% (v/v) glycerol-supplemented (Sigma-Aldrich) Brain Heart Infusion broth (BHIB-Oxoid).

DNA extraction

After centrifuging 1 ml of bacterial culture (BHIB) for 5 min at 8,000 x g, DNA was extracted using a DNA purification kit (NucleoSpin® Tissue/Macherey-Nagel) according to the manufacturer's instructions.

Bacterial identification by multiplex PCR

A multiplex PCR protocol was carried out for the final identification of the isolates (Alexander et al. 2008); this specific PCR assay, containing four primers sets, is able to identify *M. haemolytica, M. glucosida* and *M. ruminalis*, the three most closely related species in the genus *Mannheimia*, without further phenotypical testing.

Reactions were carried out in a 25 μl volume containing (final concentrations): 1× AmpliTaq Gold® 360 Buffer (Invitrogen), 2 mM Magnesium Chloride, 2.5 U AmpliTaq Gold® 360 DNA Polymerase (Invitrogen), 200 μM of each deoxynucleoside triphosphate and 100 ng of sample DNA. Primers were added at a final concentration of 0.8 μM, with the exception of primer set HP which was added at a final concentration of 0.4 μM.

After an initial denaturation of 10 min at 95 °C, reaction mixtures were cycled 45 times in a PTC-200 Peltier Thermal Cycler (MJ Research) under the following conditions: 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The final extension was 10 min at 72 °C.

Serotyping

All serotyping was performed at Moredun Research Institute (Edinburgh, Scotland) using specific antisera. Serotyping was performed by indirect hemagglutination (IHA) using the rapid modification of the test as described by Fraser et al. (1983). Antiserum against serotype 17 was not included in the test and antiserum against serotype 13 was inactive.

Primer design, PCR amplification of the *ompA* gene

The Entrez Nucleotide database (NCBI) was used to retrieve the available *ompA* gene sequence of different *M. haemolytica* strains (accession numbers: AF133259, AY244653, AY244654, AY244655, AY244656, AY244657, AY244658, AY244659, AY244660, AY244661, AY244662, AY244663). The oligonucleotide primers were designed using the online analysis tool Primer3Plus (http://www.bioinformatics.nl/primer3plus) (Untergasser et al. 2007) and were analysed by Melt94 (http://web.mit.edu/osp/www/melt.html) to assist their selection and to determine DGGE conditions. A GC-clamp of 30 nucleotides (GCCCGCCGTCCCGGCCCGACCCCCGCGCGT) was attached to the 5'-end of the forward primer. The oligonucleotide primers were designed to amplify an approximately 768-mer segment of the *ompA* gene comprising three of the four hypervariable domains; primers were the forward primer was 5'-TTTGGTCGTGTTCGTGGTAA-3', and the reverse primer was 5'-TGCATCACATGTGTGACCAG-3'.

Amplification reaction mixtures were prepared at a final volume of 25 μl containing 1× PCR Buffer

(Invitrogen), 2 mM Magnesium Chloride, 1.25 U Taq DNA Polymerase (Invitrogen), 200 μM of each dNTP, 0.8 μM each primer and 60-80 ng of sample DNA. All PCRs were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research) as follows: initial denaturation at 95 °C for 10 min and 35 cycles of denaturation at 95 °C for 30 sec, annealing at 58.5 °C for 30 sec and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min.

DGGE and DNA sequence analysis

All DGGE assays were carried out using the IngenyPhorU-2×2 System (Ingeny). PCR products (9μl and 3μl loading buffer) were applied directly onto, 21 x 28cm, 6% (w/v) polyacrylamide (37.5 : 1 acrylamide/bisacrylamide) gels in 1× TAE buffer (BioRad), containing a linear gradient of 20% to 80% (w`/v) urea and formamide as denaturants. Electrophoresis was performed at a constant voltage of 80V for 21 h at 60 °C in 0.5× Tris-acetate-EDTA (TAE) buffer (Bio-Rad). Gels were stained with ethidium bromide (1.5μg/ml) and photographed under UV light by a Kodak gel Doc XR system.

In order to have comparable results, PCR reactions were repeated (using the primers without the GC-clamp) and the same 768-mer DNA fragments were sequenced (Lark Technologies Inc.), using the 3730xl DNA Analyzer (Applied Biosystems).

Data analysis

All nucleotide sequences were analysed using Sequence Scanner version 1.0 (Applied Biosystems) and EditSeq, MegAlign modules of the Lasergene Ver.7.1 software (DNASTAR Inc., Madison, WI, USA). Complementary alignments were also performed using ClustalW2 (Thompson et al. 1994) multiple sequence alignment software (http://www.ebi.ac.uk/Tools/msa/clustalw2). The Neighbor-Joining method (Saitou and Nei 1987) was used to construct a dendrogram showing the relationships among the genotypes revealed by DNA sequencing of the *ompA* gene. The dendrogram was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. Evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 707 positions in the final dataset. Genetic diversity analyses were conducted using MEGA version 4 (Tamura et al. 2007).

Comparison of the sequences obtained in the current survey was also performed against the three dimensional structure of the *E. coli* OmpA proteins (MMDB 16249 & PDB 1G90; MMDB 9208 & PDB 1BXW) using the Cn3D software (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). Secondary structure prediction software Psipred was also used (http://bioinf.cs.ucl.ac.uk/psipred).

Nucleotide sequence accession numbers

The GenBank accession numbers for the *ompA* sequences obtained in this study are JN054708, JN054709, JN954710, JN054711, JN054712, JN054713, JN054714, JN054715, JN054716, JN054717 and JN054718 (Table 1).

Results

Multiplex PCR

All isolates were positively identified as *M. haemolytica* by PCR.

Serotyping

The serotyping results revealed clear-cut differences in the isolation rates of the different capsular serotypes between sheep and goats. Among the 83 isolates that were tested (11 isolates were not viable after transportation), seven different serotypes were found. Serotype A2 was the most common one, comprising 36 (43.4%) isolates in total (Table 1). Twenty-three of the A2 isolates originated from sheep (32.8% of the ovine isolates), whereas all the caprine isolates belonged to serotype A2. The one bovine isolate also belonged to serotype A2. From the remaining 47 isolates of sheep origin, 11 (13.2% of the total) belonged to serotype A1 and 10 (12%) to serotype A7. Serotypes A9 and A12 comprised seven (8.4%) isolates each, whereas serotypes A6 and A5 appeared at very low rates (3.6% and 2.4% respectively). Serotypes A8, A14 and A16 were not recovered at all, whereas seven (8.4%) of the isolates remained untypeable.

Table 1. Details of the *M.haemolytica* isolates examined in the study [correlation of the *ompA* genotype with the patterns revealed by DGGE, the capsular serotype and the origin (animal species) of the isolates].

ompA allele	DGGE pattern	Capsular serotype	Host species	No of isolates	GenBank accession no*
I.1	3	A2	calf	1	JN054708
I.2	3	A2	sheep	1	
		A6	sheep	2	
		NT	sheep	4	JN054709
II	2	A1	sheep	10	
		A2	sheep	2	
		A5	sheep	2	
		A7	sheep	8	JN054710
		A9	sheep	7	
		A12	sheep	7	
		UT	sheep	2	
		NT	sheep	2	
		A2	goat	1	
III	2	A1	sheep	1	
		A6	sheep	1	
		A7	sheep	2	JN054711
IV	4	A2	sheep	1	JN054712
		UT	sheep	1	
		NT	sheep	1	
V.1	4	A2	sheep	1	JN054713
V.2	4	A2	sheep	1	JN054714
VI	2	A2	sheep	12	
		UT	sheep	4	
		NT	sheep	3	JN054715
		A2	goat	3	
VII	2	A2	sheep	4	JN054716
VIII	5	A2	sheep	1	
		NT	sheep	1	JN054717
IX	1	A2	goat	8	JN054718

*UT: untypeable, NT: not tested, DGGE: Denaturing Gradient Gel Electrophoresis. *GenBank accession numbers of representative isolates for each ompA allele.*

DGGE

DGGE revealed five distinct patterns (designated 1 – 5) among the isolates, each represented by multiple bands (Fig. 1 and 2). Pattern 2 was the most prevalent comprising 71 isolates (75.5% of the total), followed by pattern 1 which comprised 8 (8.5%) isolates. The one bovine isolate, as well as seven ovine ones belonged to pattern 3 (8.5%). Patterns 4 and 5 were recovered only from sheep, comprising five (5.3%) and two (2.1%) isolates respectively (Table 1).

Sequence analysis

The *ompA* gene of the 94 isolates was partially (748bp) sequenced and 11 unique *ompA* sequences, representing distinct alleles, were identified. However, they were assigned to one of nine genotypic groups of allelic variants designated *I* to *IX*. Allelic variants within a genotypic group showed only synonymous nucleotide substitutions. Based on the partial sequences, there were 19 (2.54%) polymorphic nucleotide sites and 12 (4.82%) variable inferred amino acid positions among the 11 sequences. The variation degree between alleles representing each group was very low (Table 2). Group *I* was represented by alleles *I.1* and *I.2*, which differed at a single synonymous site, nucleotide C897T (numbering based on AY244653 nucleotide sequence), while group *V* was represented by alleles *V.1* and *V.2* which also differed at one synonymous (C795T) site. There was also an insertion of a GTA triplet in some groups (*IV, V, VI, VII* and *VIII*), between the 306b and 307b positions (AY244653 numbering) which added a putative extra valine in the corresponding amino acid site. In addition, nucleic acid positions 625 to 633 varied greatly among the different groups (Table 2).

Genotypic group *II* was the most prevalent one, consisting of 41 (43.6%) isolates, followed by genotypic group *VI*, consisting of 22 (23.4%) isolates. The remaining genotypic groups were by far less common (Table 1). The genetic relationships of the *ompA* gene sequences are shown in Fig. 3.

The positions of the nucleotide insertions and mutations are shown in the two-dimensional representation of the OmpA structure in Fig. 4.

Discussion

One of the primary aims of the present study was to investigate the prevalence of the different *M. haemolytica* serotypes in Greece as no previous studies have been published describing the distribution pattern of the serotypes of this bacterium in south-east Europe. Since the choice of antigens for vaccines is still based on serotype schemes, conventional serotyping method remains a very important tool in epidemiological studies. Although a substantial number of bovine pneumonic lungs were examined, the number of isolates was inadequate for any conclusions to be drawn. The low recovery rate of *M. haemolytica* from bovine lungs (0.96% as opposed to 24.5% and 32.4% in goats and sheep respectively) may be due to a number of reasons: other bacteria, such as *P. multocida*, were occasionally implicated (the isolation rate for *P. multocida* was 18.4%), and, in many cases, the extensive use of antibiotics may have rendered impossible the isolation of any bacteria at all. It should be mentioned, however, that the only bovine isolate belonged to serotype A2 and not serotype A1, which is usually associated with cattle.

In sheep, our results showed that serotype A2 is the predominant one (32.8%), followed by serotypes A1 (15.7%), A7 (14.3%), A9 and A12 (10% each). Serotypes A5 and A6 were recovered at very low rates (2.9% and 4.3% respectively) and serotypes A8, A14 and A16 were not found at all. Interestingly enough, serotype A2 was the only one recovered from goats, suggesting that a much wider range of serotypes is

Table 2. Depiction of the variable inferred nucleic/amino acid sites and insertions/deletions of the 11 groups of sequences in contrast to strain AY244653 (NCBI), which represents the best BLAST result.

AY244653 nucl. acid numbering (bp)	306-ins-307	310-312	313-315	319-321	325-327	328-330	358-360	460-462	466-468	487-489	595-597	613-615	622-624	625-627	628-630	631-633	637-639	793-795	895-897
AY244653	-	TCT	GGT	GAT	GAA	AAA	GCG	CAT	GAT	GTT	TTA	ACA	GGT	TAT	CTA	ATA	AGA	AGC	CGC
I.1	-	TCT	GGT	GAT	GAA	AAA	GCG	CAT	GAT	GTT	TTA	ACA	GGT	TAT	CTA	ATA	AGA	AGC	CGT
I.2	-	TCT	GGT	GAT	GAA	AAA	GCG	CAT	GAT	GTT	TTA	ACA	GGT	TAT	CTA	ATA	AGA	AGC	CGC
II	-	GCT	-	GAT	GAA	AGA	GCG	CAT	GAT	GCT	GTA	AAA	AGT	-	-	ATT	ACA	AGC	CGT
III	-	GCT	-	GAT	GAA	AGA	GGG	CAT	GAT	GCT	GTA	AAA	AGT	-	-	ATT	ACA	AGC	CGT
IV	GTA	GCT	-	GAT	GAA	AGA	GCG	CAT	GAT	GCT	GCA	AAA	AGT	-	-	ATT	TCA	AGC	CGT
V.1	GTA	GCT	-	GAG	GAA	AGA	GCG	CAT	GAT	GCT	GCA	AAA	AGT	-	-	ATT	TCA	AGC	CGT
V.2	GTA	GCT	-	GAG	GAA	AGA	GCG	CAT	GAT	GCT	GCA	AAA	AGT	-	-	ATT	TCA	AGT	CGT
VI	GTA	TCT	-	AAT	GGA	AGA	GCG	CAT	GAT	GCT	GTA	AAA	AGT	-	-	ATT	ACA	AGC	CGT
VII	GTA	TCT	-	AAT	GGA	AGA	GCG	CGT	GAT	GCT	GTA	AAA	AGT	-	-	ATT	ACA	AGC	CGT
VIII	GTA	GCT	-	AAT	GGA	AGA	GCG	CGT	GAT	GCT	GCA	AAA	AGT	-	-	ATT	TCA	AGC	CGT
IX	-	GCT	GGT	GAT	GAA	AAA	GCG	CGT	GCT	GCT	TTA	AAA	AGT	-	CTT	-	ACA	AGC	CGT

AY244653 am. acid numbering (aa)	102-ins-103	104	105	107	109	110	120	154	156	163	199	205	208	209	210	211	213	265	299
AY244653	-	S	G	D	E	K	A	H	D	V	L	T	G	Y	L	I	R	S	R
I.1	-	S	G	D	E	K	A	H	D	V	L	T	G	Y	L	I	R	S	R
I.2	-	S	G	D	E	K	A	H	D	V	L	T	G	Y	L	I	R	S	R
II	-	A	-	D	E	R	A	H	D	A	V	K	S	-	-	I	T	S	R
III	-	A	-	D	E	R	G	H	D	A	V	K	S	-	-	I	T	S	R
IV	V	A	-	D	E	R	A	H	D	A	A	K	S	-	-	I	S	S	R
V.1	V	A	-	E	E	R	A	H	D	A	A	K	S	-	-	I	S	S	R
V.2	V	A	-	E	E	R	A	H	D	A	A	K	S	-	-	I	S	S	R
VI	V	S	-	N	G	R	A	H	D	A	V	K	S	-	-	I	T	S	R
VII	V	S	-	N	G	R	A	R	D	A	V	K	S	-	-	I	T	S	R
VIII	V	A	-	N	G	R	A	R	D	A	A	K	S	-	-	I	S	S	R
IX	-	A	G	D	E	K	A	R	A	A	L	K	S	-	L	-	T	S	R

associated with sheep rather than goats. Similar results have been previously reported by other authors: serotype A2 was the most common both in ovine and caprine isolates in a survey conducted in France (Villard et al. 2006) and in ovine isolates from the UK, Germany and the USA (Davies and Donachie 1996). However the isolation rate of the remaining serotypes clearly varies among European countries. Villard et al. (2006) found serotype A6 to be the second most prevalent in sheep (recovered at a rate of 26%), whereas Angen et al. (2002) reported that three out of six ovine isolates belonged to serotype A7. Finally, in the present study, the number of isolates that could not be typed (8.4%) was lower than the numbers reported by the aforementioned writers [14% and 24% of untypeable strains among *M. haemolytica* isolates from various hosts were reported by Villard et al. (2006) and Angen et al. (2002) respectively].

It has been shown that serotyping alone is not a reliable tool for the identification of *M. haemolytica*, as certain serotypes have been found among other bacterial species (Angen et al. 2002). The multiplex PCR (Alexander et al. 2008) is able to differentiate between closely related species without the extensive phenotypic tests that have been previously required to differentiate these species within the family of

Pasteurellaceae (Blackall et al. 2002). 16S rRNA sequencing has revealed that *M. ruminalis*, although it has been isolated solely from the rumen of sheep or cattle, is closely related with *M. haemolytica* and *M. glucosida*. *M. haemolytica* and *M. glucosida* are even more closely related, comprising a single cluster by 16S rRNA sequence homology (Angen et al. 1999). In the present study, all the isolates examined were identified using the multiplex PCR method.

DGGE produced five different patterns. The ovine isolates produced four different patterns (Table 1), exhibiting a greater diversity than the caprine ones which produced only two patterns (1 and 2). However the majority of the ovine strains (82.7%) belonged to pattern 2. Patterns 3, 4 and 5 presented at relatively low rates (8.6%, 6.2% and 2.5% respectively) in sheep. None of the ovine strains belonged to pattern 1. In goats, in contrast to sheep, pattern 1 was the most prevalent, comprising 66.7% of the caprine isolates. So, although a variety of DGGE patterns was present in sheep, there seemed to be a predominant one which differed from its caprine counterpart.

Sequence analysis of the *ompA* gene revealed nine genotypic groups, seven of which were related to the *ompA*2 alleles described by Davies and Lee (2004) (Fig.3). The most common genotypic group recovered from sheep was group *II*, comprising almost half (49.4%) of the ovine isolates and only one (8.3%) of the caprine isolates. The sequence of this group was identical to the sequence of the ovine *M. haemolytica* strain PH346 (accession number AY244658), which represents the previously described allele *ompA*2.1, an allele considered to be exclusively associated with ovine isolates (Davies and Lee 2004). Genotypic group VI was the next most common, consisting of 19 (23.5%) ovine isolates and three (25%) caprine isolates. This group, although related to the *ompA*2 alleles (Fig.3), represented a new group of *ompA* variants. Further in Silico analysis and observation of the 2D and 3D OmpA structure revealed that group *VI*, compared to the *ompA*2 alleles, showed amino acid variability only in the second hypervariable domain

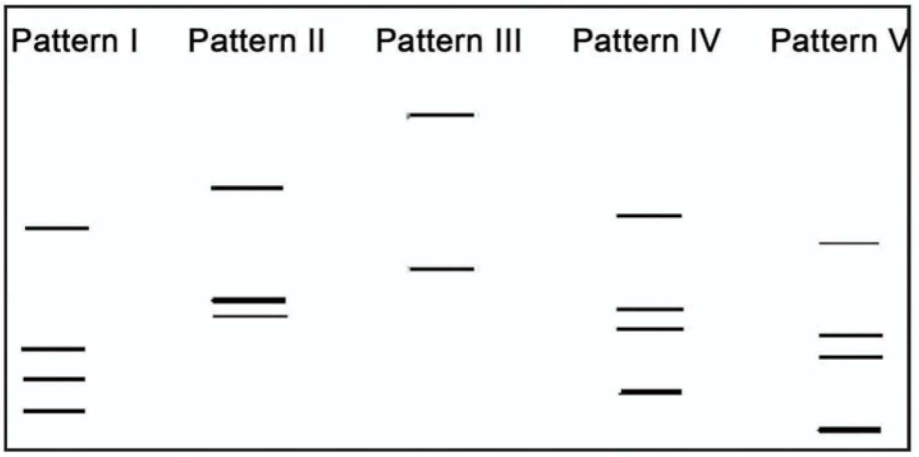


Figure 1: Patterns revealed by DGGE.

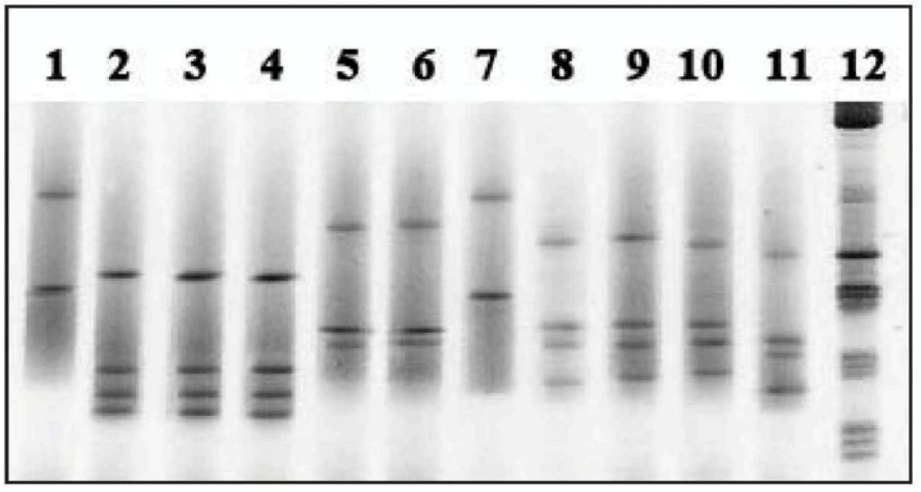


Figure 2: DGGE patterns of PCR amplicons of the *ompA* gene from *M. haemolytica* isolates in negative image of the ethidium bromide-stained DGGE gel. Lanes 1 and 7: pattern 3, lanes 2-4: pattern 1, lanes 5 and 6: pattern 2, lanes 8-10: pattern 4, lane 11: pattern 5, lane 12: 100 bp DNA Ladder

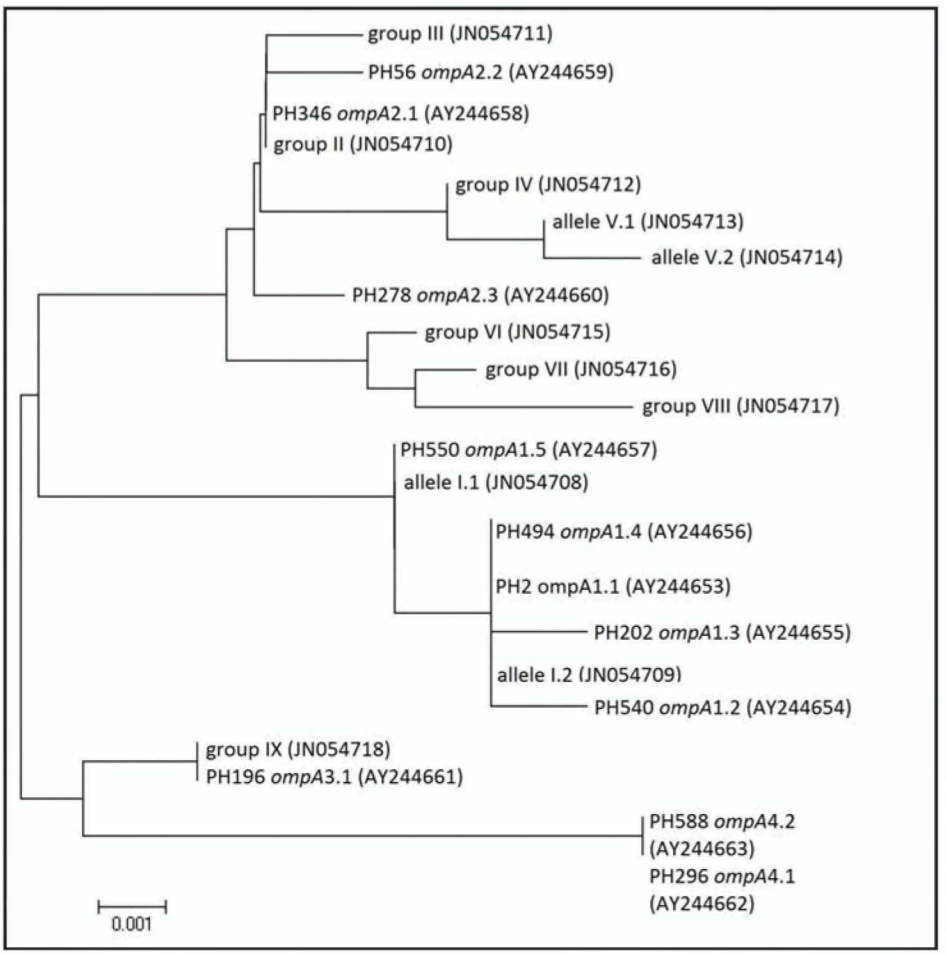
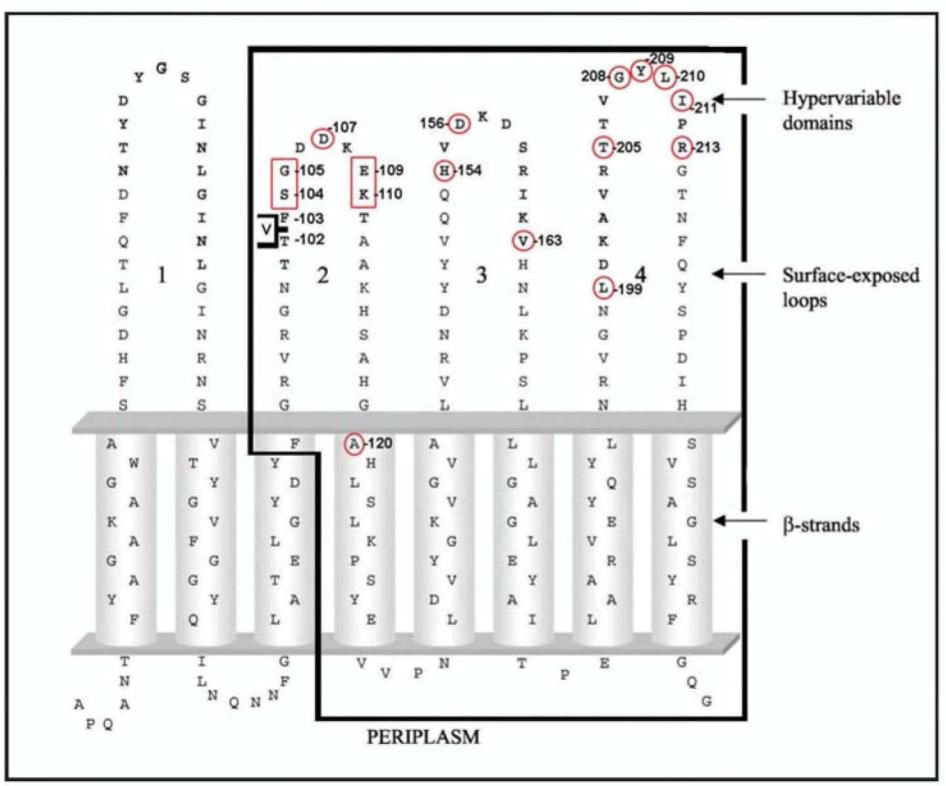


Figure 3: Dendrogram showing the relationships among the nine genotypic groups revealed by DNA sequencing of the *ompA* gene in relation to the alleles described by Davies and Lee (2004).



Figures 4: Schematic representation of N-terminal transmembrane domain of the OmpA proteins of *M. haemolytica* as proposed by Davies and Lee (2004) modified based on the *in silico* analysis of the results obtained with the current survey. Enclosed in the frame is the part of the sequence amplified by the PCR. All of the numbered amino acid positions are involved in a mutation event. Shortly, valine is an inserted amino acid between positions 102aa and 103aa; amino acids 209aa up to 211aa were either present or totally absent between alleles. The rest of the marked sites presented amino acid variability.

(Table 2 and Fig.4). Genotypic groups *IV, V, VII* and *VIII* represented also new *ompA* variants related to the *ompA*2 alleles (Fig.3). These groups showed amino acid variability in all three hypervariable domains that were examined and they were characterised, along with group *VI*, by a putative valine insertion between 102aa and 103aa (Fig.4).

The remaining eight (66.7%) caprine isolates belonged to genotypic group *IX*, which was identical to the bovine strain PH196 (accession number AY244661). This genotypic group was associated strictly with goats, as none of the ovine isolates belonged to it, despite their greater number and diversity. Future investigations of higher numbers of caprine isolates, including isolates from other countries, are required to clarify whether this finding indicates host specificity, as it has been suggested for the subclasses OmpA1 and OmpA2 (Davies and Lee 2004, Hounsome et al. 2011).

Finally, our bovine isolate was identical to the bovine strain PH550 (accession number AY244657) and belonged to allele *I.1*, differing from the ovine allele *I.2* at a single synonymous site. The dendrogram in Fig.3 reveals that genotypic group I (alleles *I.1* and *I.2*) is related to the *ompA*1 alleles described by Davies and Lee (2004), which are considered to be associated only with bovine isolates. The low percentage (8.6%) of the ovine isolates that belonged to this group may suggest that these isolates have been transferred to sheep from cattle.

It is worth mentioning the correlation between genotypic profile and DGGE pattern. First, genotypic groups *II, III, VI* and *VII* – i.e. the majority of the ovine strains - were associated exclusively with DGGE pattern 2. Second, genotypic group *IX* occurred only in caprine strains of DGGE pattern 1. Finally, genotypic group *I* was associated only with DGGE pattern 3, genotypic groups *IV* and *V* with pattern 4 and group *VIII*, consisting of only two strains, was associated with DGGE pattern 5. So, DGGE was able to distinguish genotypic group *IX* (comprising the majority of the caprine strains), but failed to discriminate between the major «ovine» groups (*II* and *VI*).

In Silico analysis and observation of the 2D and 3D OmpA structure revealed that there was sequence variability only in the hypervariable domains as it has been previously described by Davies and Lee (2004). A single exception was alanine in position 120 which, in group *III*, was replaced by glycine (Fig.4). This mutation, even though unexpected within a β-strand, could possibly be tolerated as both amino acids have non-polar side-chains which are neutrally charged, and their only difference lies in the level of hydrophobicity.

Comparison of the serotyping results with the genotypic profiles and the DGGE patterns, revealed a wide range of diversity in the *ompA* gene of serotype A2 isolates, which were distributed among almost all genotypic groups (except group *III*) and produced all five DGGE patterns. In contrast, serotype A5, A9 and A12 isolates were associated with group *II* and with DGGE pattern 2, whereas A1 and A7 isolates were associated with groups *II* and *III* and with DGGE pattern 2. This observation indicates unambiguous differences in the *ompA* gene of serotype A2 isolates. Furthermore, the untypeable strains, which were all ovine, could be differentiated on the basis of their *ompA* profiles; the majority (85.7%) of these strains belonged either to group *II*, or group *VI* and DGGE pattern 2, similarly to the rest of the ovine strains that belonged to a specific serotype.

Conclusions

In conclusion, our study showed that, in Greece, a wide range of serotypes can be isolated from ovine pneumonic lungs while, in contrast, serotype A2 is the only one isolated from goats. Analysis of the *ompA* gene of the different serotypes identified eleven distinct alleles and five DGGE patterns among the different serotypes. Significantly, the majority of the ovine and caprine isolates could be differentiated based on their *ompA* gene profiles. Finally, most of the isolates from goats belonged to one genotypic group that comprised solely caprine isolates, despite the greater number and diversity of the ovine isolates.

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