Molecular characterization and antimicrobial susceptibility of Pasteurella multocida *strains isolated from hosts affected by various diseases in Italy*

Lucilla Cucco[1], Francesca Romana Massacci[1], Carla Sebastiani1, Piermario Mangili[1], Luca Bano[2], Monia Cocchi[3], Andrea Luppi[4], Roberta Ortenzi[1], Giovanni Pezzotti[1] and Chiara Francesca Magistrali[1*]

[1] *Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, via Salvemini 1, 06126 Perugia, Italy.*
[2] *Istituto Zooprofilattico Sperimentale delle Venezie, Treviso, vicolo Mazzini 4, 31020 Villorba (TV), Italy.*
[3] *Istituto Zooprofilattico Sperimentale delle Venezie, Udine, Via della Roggia 92, 33030 Campoformido (UD), Italy.*
[4] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Reggio Emilia, Via Pitagora 2, 42124 Reggio Emilia, Italy.*

[*] *Corresponding author at: Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, via Salvemini 1, 06126 Perugia, Italy. Tel.: +39 075034343045, e-mail: c.magistrali@izsum.it.*

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Antimicrobial susceptibility, Capsular types, *Pasteurella multocida*, Virulence factors.

Summary
Pasteurella multocida is a widespread pathogen associated with major animal diseases of economic significance. Despite this, little is known about the capsular types, virulence gene pattern, and antimicrobial susceptibility of isolates from hosts affected by different diseases, and no data are available in Italy. One hundred eighty six isolates of *P. multocida*, were taken from different species in different states of health in several Italian regions, and were tested for genes encoding for capsular types (cap) and major virulence factors (tbpA, toxA, hgbB and pfhA). Antimicrobial susceptibility was investigated with the agar diffusion test. The majority of isolates was capA+. However, the distribution differed according to species and disease of origin, with a greater heterogeneity in isolates from rabbits; capE was never found, while capB was detected once. Only capA+ and capF+ strains tested positive for pfhA. Conversely, almost all capD+ isolates were hgbB+. In bovine respiratory disease, pfhA+/tbpA+/capA+ isolates predominated, while tbpA+/toxA+/capD+ isolates predominated in sheep. Overall, low levels of resistance were found, with full susceptibility to ceftiofur and florfenicol. Lower susceptibility to older antimicrobials was recorded, since only approximately 1/3 of the isolates showed susceptibility to tylosin and erythromycin, and resistance to tetracycline (7.5%), and trimethoprim - sulphametoxazole (4.8%) was also observed.

Caratterizzazione molecolare e sensibilità agli antimicrobici di ceppi di Pasteurella multocida *isolati da campioni patologici provenienti da diverse specie animali*

Parole chiave
Sensibilità agli antimicrobici, Tipi capsulari, *Pasteurella multocida*, Fattori di virulenza

Riassunto
Pasteurella multocida è un patogeno molto diffuso associato a numerose patologie degli animali domestici, sia come agente primario, sia come batterio di irruzione secondaria. L'impatto economico delle pasteurellosi è quindi rilevante, in particolare in alcune filiere, ad esempio in quella del coniglio, dove è considerata la più importante malattia ad eziologia batterica. Nonostante questo, i dati sui tipi capsulari e i geni codificati per i fattori di virulenza di ceppi di *P. multocida* associati a malattie in diverse specie animali sono scarsi in letteratura, e in particolare in Italia. Scopo di questo lavoro è stato caratterizzare isolati di *P. multocida* provenienti da diverse specie animali e forme morbose in termini di tipo capsulare e fattori di virulenza. Inoltre, la sensibilità agli antimicrobici è stata valutata con un test di diffusione in agar. Complessivamente, sono stati testati 186 ceppi, provenienti da diverse specie e diverse forme cliniche in diverse regioni italiane. La maggior parte degli isolati era capA+, tuttavia la diffusione dei tipi capsulari non si è rilevata omogenea, con una maggiore variabilità riscontrata nel coniglio. Non si sono rilevati isolati capE+, mentre un solo stipite, da un coniglio affetto da setticemia, è stato caratterizzato come capB+. Solo gli isolati positivi per capA e capF erano pfhA+; d'altra parte, tutti i ceppi capD+ si sono mostrati hgbB+. Gli

isolati da forme respiratorie della specie bovina presentavano prevalentemente il profilo pfhA+/tbpA+/capA+, mentre ceppi tbpA+/toxA+/capD+ sono stati identificati in forme respiratorie nell'ovino. Complessivamente nel corso dello studio è stata descritta una buona sensibilità agli antimicrobici da parte dei ceppi di *P. multocida* esaminati in questo studio, e in particolare non si sono osservate resistenze verso ceftiofur e florfenicolo. Tuttavia, livelli più bassi di sensibilità si sono registrati nei confronti di antibiotici utilizzati più frequentemente e da più tempo in alcune filiere produttive, poiché solo un terzo circa degli isolati si è mostrato completamente sensibile a tilosina e eritromicina e si è osservata la presenza di resistenze nei confronti di tetracicline (7,5%) e thrimethoprim - sulfametossazolo (4,8%).

Introduction

Pasteurella multocida is the causative agent of a wide range of diseases of economic significance worldwide, like haemorrhagic septicaemia in cattle (Katsuda 2013), atrophic rhinitis in swine (Davies 2003), snuffles in rabbits (Stahel 2009), and fowl cholera (Ewers 2006). Moreover, *P. multocida* can be a primary as well as a secondary pathogen in respiratory disease in many domestic species (Ewers 2006). This infection is characterized by a variety of forms, from slow or latent to rapidly developing septicaemia (Harper 2006). Human infections are, in most cases, of animal origin and predominantly occur as a consequence of bites or scratches by carnivores like cats or dogs (Son Millan 2009).

Although this bacterium is frequently isolated during routine diagnostic tests, few data on its epidemiology are available, probably because of the complexity of this species. *Pasteurella multocida* is in fact quite heterogeneous, as it has been divided into 5 capsular serogroups (serogroups A, B, D, E, and F), which are further classified into 16 serotypes (1-16), based on lipopolysaccharide antigens (Dziva 2008). In general, strains possessing a capsule are more virulent than their acapsular variants (Boyce and Adler 2000) and an association between serogroups and specific hosts and diseases has been shown. For example, serogroups A and F are involved in respiratory diseases and fowl cholera; serogroups B and F in haemorrhagic septicaemia in cattle and serogroup D is usually isolated in atrophic rhinitis in swine (Ewers 2006). Capsular typing assays based on polymerase chain reaction (PCR) have been developed to overcome the problems of phenotypic characterization, making the capsular typing affordable in routine diagnostics (Townsend 2001).

Along with the capsular type, the pathogenicity of *P. multocida* is also associated with various virulence factors (VFs), including lipopolysaccharides, fimbriae, and adhesins, toxins, iron regulated and iron acquisition proteins and outer membrane proteins (Harper 2006). Some of these VFs are thought to be important markers for defining the pathogenic potential of *P. multocida* strains. For this reason, the haemoglobin binding protein (hgbB), the transferrin binding protein (tbpA), the filamentous haemagglutinin (pfhA) and the dermonecrotic toxin (toxA) are used to characterize the virulence of *P. multocida* strains (Ewers 2006). A correlation between VFs and capsular types is reported in literature (Ewers 2006). The gene encoding the pfhA is usually associated with serogroups A, B, E, and F. The gene encoding the tbpA is commonly found with serogroups A and B, while the gene encoding the toxA is associated with serogroup D. No distinct association, however, was found between the gene encoding the hgbB and a specific capsular type (Ewers 2006).

In the field, the control of pasteurellosis is still mainly based on antimicrobial therapy (Sellyei 2009). Isolates of *P. multocida* are usually susceptible to antibiotics, but recently, the emergence of resistant strains has been reported in swine, poultry, and cattle (San Millan 2009, Tang 2009, Michael 2012). This finding has raised concerns, since antibiotic resistance in pathogenic bacteria from food-producing animals is recognized as an emerging issue.

Despite the importance of this infection, there is a lack of information on the characteristics of *P. multocida* isolates from outbreaks of pasteurellosis in animals, in particular in the rabbit, and, to the best of our knowledge, no data are available in Italy so far.

The aims of this study was to describe the distribution of genes encoding for virulence factors and capsular types of *P. multocida* isolates from different animal species and diseases in Italy, and to evaluate the antimicrobial susceptibility of these strains.

Materials and methods

Bacterial strains

One hundred and eighty six *P. multocida* field isolates collected in Italy between 2004 and 2011 were investigated. The isolates were from culture

collections of 4 diagnostic laboratories and they originated from different regions of Italy and different animal species affected by pasteurellosis: cattle (39), sheep (19), swine (40), rabbit (88). The strains, stored at - 80°C, were cultured in Brain Heart Infusion (Oxoid Ltd, Basingstoke, United Kingdom) for 24 hours at 37°C; 10 µl of the suspension were then inoculated on blood agar (5% sheep blood red cells) to obtain isolated colonies after incubation for 24 hours at 37°C. All isolates were subsequently tested for capsule typing, presence of specific virulence genes and to determine the susceptibility to antimicrobial agents.

DNA preparation

DNA for PCR analysis was prepared by boiling procedures (Rathnayaka 2011). Bacteria from overnight cultures on Blood Agar (5% sheep blood) were suspended in 200 µl of physiological solution. The suspension was boiled for 10 minutes, immediately chilled on ice, and then centrifuged for 2 minutes at 8000 rpm. The supernatant was used to perform the PCR test.

Capsular typing

The capsular types of the isolate were determined by multiplex capsular PCR typing assay with the capsule-specific primer pairs for genes capA, capB, capD, capE, and capF and with the primers for the detection of the *P. multocida* species specific gene kmt to confirm the identification of the species (Townsend 2001). *Pasteurella multocida* reference strains NTCT 12178 (capD+/toxA+), NTCT 10322 (capA+), NTCT 3195 (capB+), NTCT 10722 (capA+) were used as positive controls for the genes indicated. For all PCR reactions, 1 µl of the boiled supernatant was taken as template DNA and added to the reaction mixture (25 µl) containing: dNTPs 0.2 mm, primers 0.28-4.8 µm (Invitrogen, Carlsbad, CA, USA), 4 µl of 5X PCR Buffer, $MgCl_2$ 1.5 mm and 1 U of GoTaqFlexi DNA Polymerase (Promega, Madison, WI, USA). The following cycling procedure was used: 95°C for 3 minutes; 35 cycles of denaturation at 95°C for 30 seconds; annealing at 55°C for 30 seconds; and extension at 72°C for 5 minutes; with the final extension step at 72°C for 5 minutes. Amplification products were analysed by gel electrophoresis on 2% agarose gel, stained with ethidium bromide, and photographed at UV exposure (Figure 1).

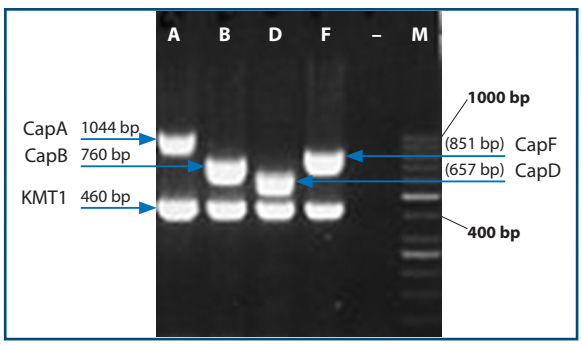

Figure 1. *Agarose gel electrophoresis of* Pasteurella multocida *samples tested using 'Capsular typing' PCR.*
A, B, D, F = capA, capB, capD, capF positive samples; – = amplification negative control; M = DNA marker 50 bp ladder.

Virulence genes

Pasteurella multocida strains were also analyzed by multiplex PCR assay for the presence of the virulence factors using specific primers for the target genes toxA, tbpA, hgbB and pfhA (Atashpaz 2009). Four microliters (4 µl) of the supernatant prepared as previously described, were used as a template for a 50 µl PCR mixture. Briefly, the mixture contained: dNTPs 0.2 mm, primers 0.2-1.6 µm (Invitrogen, Carlsbad, CA, USA), 7 µl of 5X PCR Buffer, $MgCl_2$ 1.5 mm and 1 U of GoTaqFlexi DNA Polymerase (Promega, Madison, WI, USA). PCR conditions were carried out according to the following protocol: 95°C for 5 min; 35 cycles of denaturation at 94°C for 45 s; annealing at 52°C for 50 s; and extension at 72°C for 1 min; with the final extension step at 72°C for 10 min.

Amplification products were analyzed and photographed as above described (Figure 2).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by disk diffusion method by following CLSI 2002 guidelines

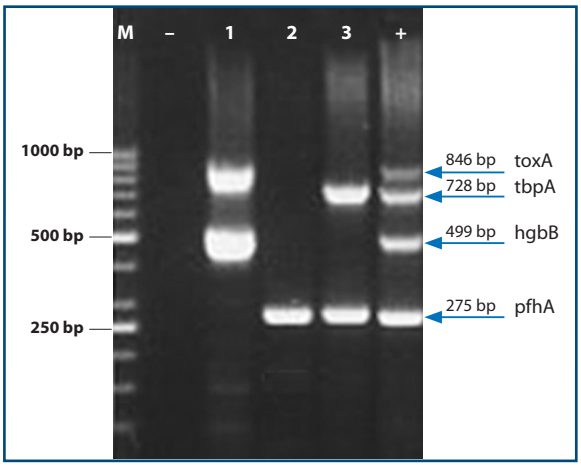

Figure 2. *Agarose gel electrophoresis of* Pasteurella multocida *samples tested using 'Virulence genes' PCR.*
M = DNA marker 50 bp ladder; – = amplification negative control; 1 = Extraction positive control from *P. multocida* NTCT 12178 reference strain; 2 = rabbit isolate; 3 = cattle isolate; + = amplification positive control.

(M31-A2). The antimicrobial agents tested included: ampicillin (Amp 10 µg/ml), clavulanic acid - amoxicillin (Amc 30 µg/ml), enrofloxacin (Eno 5 µg/ml), erythromycin (E 15 µg/ml), gentamicin (Gen 10 µg/ml), tetracycline (Tet 30 µg/ml), sulfamethoxazole - trimethoprim (Sxt 25 µg/ml), tilmicosin (Til 15 µg/ml) (Oxoid Ltd., United Kingdom), ceftiofur (Cef 30 µg/ml), flumequine (Ub 30 µg/ml) (Fatro, Bologna, Italy), florfenicol (Ffc 30 µg/ml) (Merk, Darmstadt, Germany), tylosin (Ty 30 µg/ml) and aminosidine (Am 60 µg/ml) (Mast Group Ltd., Merseyside, United Kingdom). A strain was considered as susceptible, intermediate or resistant according to the CLSI M31-A2 guidelines (CLSI 2002).

Results

All isolates examined were positive for kmt, confirming the species attribution (Adhikary 2013). The occurrence of genes encoding for capsular types and virulence factors, divided into the different species and conditions of origin, is shown in Table I. The distribution of the genes encoding known or putative virulence factors across different capsular types is shown in Table II. Finally, antibiotic susceptibility is described in Table III.

Eleven isolates (5.9%) showed varying resistance, greater than or equal to three, and they were consequently classified as multiresistant. The number of multiresistant strains from each species was as follows: five from cattle (12.8%); four from pigs (10%); two from rabbits (2.3%); no multiresistant isolates were recovered from sheep. Ten (10) out of 11 multiresistant strains were isolated during respiratory disease. All these isolates were resistant to both erythromycin and tetracycline. Resistance to enrofloxacin was observed in isolates from cattle, only. These strains were classified as intermediate to flumequine.

Discussion

In this study, *P. multocida* capsular type A was shown as the most common capsular type associated with various diseases in domestic animals in Italy, while a minor role was played by capsular type D and F. Only one isolate tested positive for cap B, while cap E was never detected. This is not surprising, since these two capsular types had always been associated with the African and the Asian continents, respectively (Dziva 2008).

Table I. *Occurrence of capsular type and virulence factor coding genes among* Pasteurella multocida *isolates.* The data are divided according to species and condition of origin. Prevalences, calculated for each species, are shown in brackets.

Animal species	Disease	Total	Capsular types					Virulence factors (VF)						
			A	B	D	E	F	hgbB	pfhA	tbpA	pfhA + tbpA	pfhA + hgbB	tbpA + toxA	No VFs
Cattle		39												
	Respiratory disease	36	36 (92.3)	0	0	0	0	2 (5.1)	1 (2.6)	2 (5.1)	31 (79.5)	0	0	0
	Others	3	3 (7.7)	0	0	0	0	2 (5.1)	0	0	1 (2.6)	0	0	0
Pig		40												0
	Respiratory disease	40	32 (80.0)	0	8 (20.0)	0	0	24 (60.0)	15 (37.5)	0	0	1 (2.5)	0	0
Sheep		19												
	Respiratory disease	12	9 (47.4)	0	2 (10.5)	0	1 (5.3)	3 (15.8)	0	0	2 (10.5)	0	7 (36.8)	0
	Mastitis	5	5 (26.3)	0	0	0	0	1 (5.3)	0	0	0	0	4 (21.1)	0
	Septicaemia	2	2 (10.5)	0	0	0	0	2 (10.5)	0	0	0	0	0	0
	Others	0	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit		88												
	Respiratory disease	31	20 (22.7)	0	5 (5.7)	0	6 (6.8)	15 (17.0)	13(14.8)	0	0	3 (3.4)	0	0
	Mastitis	9	8 (9.1)	0	1 (1.1)	0	0	4 (4.5)	1 (1.1)	0	0	2 (2.3)	0	2 (2.3)
	Septicaemia	22	10 (11.4)	1 (1.1)	4 (4.5)	0	7 (7.9)	7 (7.9)	12 (13.6)	0	1 (1.1)	2 (2.3)	0	0
	Reproductive disease	12	8 (9.1)	0	2 (2.3)	0	2 (2.3)	10 (11.4)	2 (2.3)	0	0	0	0	0
	Others	14	5 (5.7)	0	2 (2.3)	0	7 (7.9)	7 (7.9)	6 (6.8)	0	0	0	0	1 (1.1)
Total		186	138 (74.2)	1 (0.5)	24 (12.9)	0	23 (12.4)	77 (41.4)	50 (26.9)	2 (1.1)	35 (18.8)	8 (4.3)	11 (5.9)	3 (1.6)

hgbB = haemoglobin binding protein; pfhA = filamentous haemagglutinin; tbpA = transferrin binding protein; toxA = dermonecrotic toxin.

Table II. *Distribution of virulence factor coding genes among isolates belonging to different capsular types (percentage).*

	cap A	cap B	cap D	cap F	Total
hgbB	52 (37.7)	/	21 (87.5)	4 (17.4)	77
hgbB+pfhA	7 (5.1)	/	1 (4.2)	/	8
pfhA	31 (22.5)	/	/	19 (82.6)	50
pfhA+tbpA	36 (26.1)	1 (100.0)	/	/	35
tbpA	2 (1.4)	/	/	/	2
tbpA+toxA	7 (5.1)	/	2 (8.3)	/	11
No virulence	3 (2.2)	/	/	/	3
Total	138	1	24	23	186

hgbB = haemoglobin binding protein; pfhA = filamentous haemagglutinin; tbpA = transferrin binding protein; toxA = dermonecrotic toxin.

Table III. *Antimicrobial sensitivity related to all isolates (percentage).*

	Susceptible	Intermediate	Resistant
Clavulanic acid - amoxicillin	186 (100.0)	0 (0.0)	0 (0.0)
Ampicillin	177 (95.2)	2 (1.1)	7 (3.8)
Ceftiofur	186 (100.0)	0 (0)	0 (0)
Aminosidine	179 (96.2)	1 (0.5)	6 (3.2)
Gentamicin	179 (96.2)	4 (2.2)	3 (1.6)
Enrofloxacin	182 (97.8)	1 (0.5)	3 (1.6)
Flumequine	180 (96.8)	6 (3.2)	0 (0)
Erythromycin	61 (32.8)	107 (57.5)	18 (9.7)
Tylosin	75 (40.3)	16 (8.6)	95 (51.1)
Tilmicosin	180 (96.8)	3 (1.6)	3 (1.6)
Florfenicol	186 (100.0)	0 (0.0)	0 (0.0)
Thrimethoprim - sulfamethoxazole	171 (91.9)	6 (3.2)	9 (4.8)
Tetracycline	169 (90.9)	3 (1.6)	14 (7.5)

An association between pfhA and capsular types was observed, since only strains belonging to capsular type A or F tested positive for this virulence factor. Moreover, almost all isolates belonging to capsular type D were positive for hgbB. The transferrin binding protein was less frequent, and it was found exclusively in strains from ruminant species, as already reported (Ewers 2006), and usually in association with pfhA and with respiratory disease. In the present study, toxA was rare: it was detected only in isolates from sheep and never alone. However, our study was based on the amplification of target genes using PCR and a false negative result due to a sequence polymorphism of the target genes affecting the primer annealing cannot be ruled out.

Consequenty, the absence of a virulence factor shown in our study should only be considered as putative.

The vast majority (92%) of isolates from cattle examined in the present study were from respiratory disease. They belonged exclusively to capsular type A and, in most cases (80%), were pfhA+/tbpA+. Moreover, cattle respiratory isolates from different origins have shown a limited diversity when analyzed using multilocus sequence typing (MLST), being predominantly associated with cattle affected by respiratory disease (Hotchiss 2011). Therefore, targeted measures, such as vaccines, can be successful in reducing the effect of this infection in the respiratory disease complex in cattle.

The strains from swine were all from pneumonic pigs, whereas cases of atrophic rhinitis were not included in this work. It has been demonstrated that different subpopulations of *P. multocida* are responsible for pneumonia and progressive atrophic rhinitis (PAR) in pigs (Davies 2003). In our investigation, capsular type A predominated, while a lower proportion of isolates belonging to capsular type D was shown. Again, this finding is in agreement with previous studies, which describes these two capsular types as being the most common among isolates from lungs of pigs affected by pneumonia (Davies 2003, Ewers 2006, Bethe 2009).

In sheep, *P. multocida* is generally associated with pneumonia and less frequently, otitis media and arthritis (Miller 2011, Petridou 2011). In the pathogenesis of ovine pneumonia, *P. multocida* plays a less important role than *Mannheimia haemolytica*; therefore, data on capsular types and virulotypes of *P. multocida* in sheep are scarce in literature. In our study, capsular type A was dominant, but D and F types were also observed. Traditionally, *P. multocida* capsular type A has been linked to pneumonia in small ruminants; more recently, however, capD+/toxA+ strains have been isolated from pneumonic sheep (Ewers 2006). As far as we know, this is the first paper reporting virulotypes and capsular types of *P. multocida* from sheep mastitis; our data showed a predominance of capsular A, tbpA+/toxA+ strains.

In our study, a great variability was shown among isolates from rabbits: even though type A predominated, all capsular types were detected, apart from capsular E. The only isolate belonging to capsular type B during our investigation was from a case of septicaemia in this animal species. This isolate harbored hgbB, pfhA and tbpA. Traditionally, capB+ strains had been reported in cases of haemorragic septicaemia (HS) in cattle and water buffalo in the Asian continent (Petersen 2014) and they have recently been associated with haemorrhagic septicaemia (HS) in pigs in Europe (Cardoso 2013). Therefore, the recovery of a capB+ isolate from a case of septicaemia in rabbits is of particular interest and deserves further investigation. No other association between clinical presentations and specific capsular

types was found in rabbits. Despite its importance in rabbitries, few studies are currently available in literature on pasteurellosis in this animal species; however, a great heterogeneity in *P. multocida* isolates from rabbits, which was shown by our study, has already been reported (Stahel 2009). This variability should be taken into account for the development of appropriate vaccines.

The data on the antibiotic susceptibility of *P. multocida* available in literature show a relatively low frequency of resistance (Sellyei 2009, Watts 2010); recently, isolates collected during a pan European survey confirmed this finding (de Jong 2014). Our study is in agreement with these results, since low levels of resistance were found, with a full susceptibility to ceftiofur and florfenicol. On the other hand, only approximately one third of the isolates showed a full susceptibility to tylosin and erythromycin, and strains resistant to tetracycline (7.5%) and thrimethoprim-sulphametoxazole (4.8%) were observed, as well. A limited number of isolates resistant to gentamycin (n = 3), ampicillin (n = 7) and enrofloxacin (n = 3) was found. All these isolates were from respiratory disease in cattle. The presence of a limited number of *P. multocida* isolates resistant to these families of antimicrobials has already been reported in Europe (de Jong 2014, Hendriksen 2008, Rerat 2012). In our study, multiresistant isolates were found in samples from cattle, pigs and rabbits, but not in sheep. Our results are in agreement with a recent European study, which showed lower susceptibility of *P. multocida* to older antimicrobials (de Jong 2014), probably as a consequence of their extensive use in primary production.

In conclusion, this study showed the distribution of capsular types and toxin genes in isolates from different animal species affected by various diseases in Italy. Even though a great heterogeneity of *P. multocida* was shown, some specialisation in certain host niches and conditions was found. Future vaccine strategies can therefore be developed to finally reduce the use of antimicrobials in the control of diseases caused by *P. multocida* in domestic animals.

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