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Characterisation of *Mannheimia* Sp. and *P. multocida* Strains Isolated from Bovine Pneumonic Lungs in Two Slaughterhouses in Mexico

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Abstract: *Mannheimia* sp. and *P. multocida* (Pm) strains were isolated from pneumonic lungs (n = 475) which were obtained in two slaughterhouses in Mexico. Isolation and phenotyping were carried out by means of *in vitro* culture, as well as biochemical and immunological tests. Of the 131 isolates 60.3% was Pm and 39.7% was *Mannheimia* sp. The isolating rate in lungs was 16.6% for Pm, 10.7% *M. haemolytica* (Mh) and 0.2% *M. glucosida* (Mg) in both slaughterhouses. These differences were significant ($p \leq 0.05$). Out of 52 *Mannheimia* sp. isolations, 98% was Mh and 1.9% Mg. Out of 51 Mh isolates 33% was of Serotype 1(S1), 17.6% S6 and 49% Non-Typable (NT). These differences were significant ($p \leq 0.05$). Out of 79 Pm isolations, 98.7% was biotype A and 1.2% biotype D. These differences were significant ($p \leq 0.05$). Pm got the highest rate of isolations in lungs which differs from data of previous results where Mh is considered as the most pathogenic bacteria and the most commonly associated to bovine pasteurelosis pneumonic. The highest frequency of Mh S1 and Pm biotype A confirms that these two bacteria are the most commonly found in pneumonic lungs in Mexico.

Key words: *Mannheimia* serotypes, *P. multocida* biotypes, pneumonic lung, cattle

INTRODUCTION

Among the infectious diseases that affect bovine, the respiratory ones are the main cause of loss worldwide, specially among young animals (Lekeu, 1996). Pneumonia is responsible for approximately 75% of clinical cases and is directly involved between 45-55% of mortality; medical treatment represents 8% of the total cost of production (Zecchinon *et al*., 2005). *Pasteurellaceae* microorganisms are opportunistic and primary pathogens as well as hosts of the upper respiratory tract of domestic and wild animals (Chaslus-Dancla *et al*., 1996). *P. haemolytica* and *P. multocida* may cause primary diseases, such as pneumonias or haemorrhagic septicemia, as well as secondary infections. Furthermore, they are considered as one of the main causes of pneumonia in ruminants all around the world (Blanco-Viera *et al*., 1995; Chaslus-Dancla *et al*., 1996). *P. multocida* subsp. *multocida* is the most widely studied among the *Pasteurella* genus; there are 5 capsular groups (A, B, D, E and F) as well as 16 somatic serotypes (Carter, 1967; Boyce *et al*., 2004; Sleim, 2005). Several diseases are frequently associated with some of these groups, the hemorrhagic septicemia is regularly associated with B and E groups, the respiratory tract infections with group A, fowl cholera with A, F and rarely D groups; pig´s atrophic rhinitis and bovine pneumonia with group D (Boyce *et al*., 2004; García *et al*., 1988).

Pasteurella haemolytica complex negative to trehalose was reclassified as the new *Mannheimia* genus which includes at least 5 species: *M. haemolytica*, *M. granulomatis*, *M. glucosida, M. ruminalis* and *M. varigena*; as well as all serotypes of *P. haemolytica* biotype A (1, 2, 5-9, 12-14, 16 and 17) were reclassified as *M. haemolytica* (Mh). Serotype 11 was reclassified as the new *M. glucosida* species (Mg) (Angen *et al.,* 1999 a,b; Highlander, 2001; Zecchinon *et al*., 2005).

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Mh is found in the nasopharynx and tonsils of apparently healthy animals (Narayanan *et al*., 2002) and it is also the most pathogenic and main microorganism responsible for the bovine pneumonic pasteurellosis or shipping fever (Trigo, 1991; Murphi *et al*., 1993; Pijoan *et al*., 1999; Narayanan *et al*., 2002). The S1 and S2 are the most prevalent in the world and the ones that are most frequently isolated from calves (Wray and Thompson, 1971).

Since the 80´s, it has been demonstrated in Mexico that the most significant *M. haemolytica* serotypes in ruminants are the 1 and 2 in bovine and 1, 2, 5 and 9 in ovine according to their pulmonary frequency (Pijoan *et al*., 1999; Blanco-Viera *et al*., 1995; Colin *et al*., 1987; Blanco *et al*., 1993) and within *P. multocida* only the A and D capsular serotypes in ovine and caprine have been isolated (Blanco *et al*., 1993; Blanco-Viera *et al*., 1995) and the A in bovine (Blanco-Viera *et al*., 1995; Pijoan *et al*., 1999; Jaramillo *et al*., 1987). Most of the research work carried out in the country on ruminants´ pneumonia has been on ovine and the available data on bovine is limited.

The aim of this work was to isolate and characterize the strains of *Mannheimia* sp. and *P. multocida* (Pm) genus of pneumonic lungs in sacrificed bovine in two slaughterhouses in 2 different geographical areas in Mexico to determine the differences among the frequencies of the different isolated serotypes and biotypes.

MATERIALS AND METHODS

Characterization of the studied area and sampling design: We carried out a descriptive, transversal and prospective study by means of nonprobability sampling in 2 slaughterhouses, one located in Tlalnepantla (STL) in the state of Mexico and the other in Gómez Palacio in the state of Durango (SGP). We examined a total of 475 pneumonic lungs over a 6 month-period (STL, n = 362; SGP, n = 113).

Sample collecting: Out of the total number of examined lungs, we collected samples of the tissue infected by pneumonia, which is clearly identified by the presence of consolidation areas, blood-red aspect and, occasionally, the presence of adherences and fibrin thrombus. Samples were packed in sterile bags and kept in the refrigerator or the freezer up to their processing time in the bacteriology lab at the Centro Nacional de Investigación Disciplinaria en Microbiología (CENID-Microbiología), of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP).

Strain isolation and identification: The lung tissue samples were grown on blood agar by contact with the addition of 5% of sheep blood (BBL, Becton Dickinson); they were incubated at 37°C for 24 h. Phenotype identification of colonies with the typical *Mannheimia* sp. or Pm shape was carried out by conventional means: Gram staining and biochemical tests (oxidase, carbohydrate fermentation and sulfhydric acid production (TSI), citrate utilization, motility, indole production, urease production, trehalose and aesculine fermentation). A single representative colony was chosen for evaluation. Colonies were grown again in blood agar (5% sheep blood) (37°C 24 h^{-1}) and pure cultures of the strain were obtained.

Final identification was performed with the API 20E bacterial identification system (bioMerieux, Inc.). Briefly, cultures were resuspended in 5 mL of sterile suspension medium at a density of 4.0 in the McFarland scale. The suspension was transferred to strips for biochemical tests and the reading of the reactions was carried out after incubation at 37°C for 24 h, in agreement with the system´s instructions. All biochemical tests were also applied to reference strains of Mh (1, 2, 5-9, 11 and 12), Pm (A and D) and *P. trehalosi* (S3, S4 and S10).

The profile results of each strain were compared to those of the taxons of a data base in the APIWEB Program (http://www.biomerieux.com). Moreover, we considered the phenotypical properties of *Mannheimia* sp. mentioned by other authors (Angen *et al*., 1999a, Angen *et al*., 2002).

Serologic typification of *Mannheimia* sp strains: We performed it by the IHA described by Biberstein (1978); we used monospecific antisera against capsular antigens (1-17) of *Mannheimia* sp. Agglutinations with titres over 1:64 were positive. In each plate we included a positive and a negative control.

Typification of Pm strains: We performed it by means of hyaluronidase decapsulation test (Carter and Rundell, 1975) for biotype A and by acriflavine test (Carter and Subronto, 1973) for biotype D; we used reference biotypes A and D strains.

All reference strains of Mh, Pm and *P. trehalosi* as well as the monospecific antisera of *Mannheimia* sp. were kindly donated by Dr. G.H. Frank and Dr. B. Briggs, NADC, USDA.

Statistical analysis: Statistical analysis was carried out with the Epi Info® program, Version 3.3.2 for Windows (Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, 2004). We figured out the isolation

frequencies with the data gathered of the different serotypes and biotypes in each of the studied slaughterhouses. We used the square Chi or Fisher test, according to the characteristics of the data in order to evaluate the differences among the isolation frequencies of the serotypes of the *Mannheimia* sp. genus and that of the Pm biotypes.

RESULTS

Strains phenotyping

***Mannheimia* sp.:** All isolates of the STL (n = 37) and the SGP (n = 15) were Gram-negative, haemolytic, non-mobile coccobacilli, positive to cytocrome-oxidase and β-galactosidase production, to nitrate and nitrite reduction, to D-mannitol, D-glucose and D-sacarose fermentation and negative for the following tests: Voges Proskauer, gelatinase, arginine-dehydrolase, lysine decarboxylase, ornitine decarboxylase, citrate utilization, sulfhydric acid, urease and indole production, trehalose, D-sorbitol, L-arabinose, amygdaline and aesculine fermentation and McConkey agar growth.

One isolate of SGP (6.6%) was positive for amygdaline and aesculine and corresponded to *M. glucosida* (Mg) (Table 1).

In accordance with the API 20 E system and the phenotyping properties of the *Mannheimia* sp. (Angen *et al*., 1999a; Angen *et al*., 2002), 97.3% (36/37) of the STL strains and 86.6% (13/15) of the SGP corresponded to Mh and, 2.7% (1/37) of STL and 13.3% (2/15) of SGP to the *Mannheimia* sp.

***P. multocida*:** All STL (n = 62) and SGP (n = 17) isolates were Gram-negative, haemolytic, non-mobile coccobacilli, positive to cytocrome-oxidase and β-galactosidase production, to nitrate and nitrite reduction, to D-mannitol, D-glucose, D-sacarose and trehalose fermentation, to indole production and ornitine decarboxylase test and negative to the following tests: Voges Proskauer, gelatinase, arginine-dehydrolase, lisine decarboxylase, citrate utilization, sulfhydric acid and urease production, D-sorbitol, L-arabinose, amygdaline and aesculine fermentation and McConkey agar growth (Table 1).

Out of the 131 *Mannhemia* sp. and Pm isolates in both slaughterhouses, 60.3% (79/131) were Pm, 39.6% (52/131) Mh and 0.76% (1/131) Mg. In the STL 99 isolates were obtained, of which 62.6% were Pm and 37.3% were *Mannheimia* sp. In the SGP 32 isolates were obtained, of which 53.1% were Pm, 43.7% were Mh and 3.1% were Mg. All these differences were significant (p = 0.05), but not when compared between the differences of both slaughterhouses (Table 2).

Table 1: Phenotypic characteristics of *Mannheimia* spp. (Mspp) and *P. multocida* (Pm) strains isolated in bovine pneumonic lungs in 2 slaughterhouses in Mexico

	Msp.	Pm
Phenotypic characteristics	Positive %	
Haemolysis	100	0
Cytochrome oxidase	100	100
Nitrate reduction	100	100
D-Glucose	100	100
D-Sucrose	100	100
D-Mannitol	100	100
ß-Galactosidase (ONPG)	97.9	100
Ornithine decarboxylase (ODC)	0	100
Indole	0	100
Trehalose	0	100
Amygdalin[a]	6.6	0
Aesculin[a]	6.6	0
D-sorbitol	15	0
Mc Conkey medium growth	0	0
Gelatinase	0	0
Voges Proskauer	0	0
Arginine dehydrolase	0	0
Inositol	0	0
Citrate	0	0
Motility	0	0
H_2S	0	0
Urease	0	0
Tryptophane deaminase	0	0
Lysine decarboxilase	0	0
L-Rhammnose	0	0
D-Melobiose	0	0
L-Arabinose	0	0

[a]One of the SGP´s isolates was positive to these characteristics and corresponded to S11 (*M. glucosida*)

Isolate rate in lungs: Out of 475 Pneumonic Lungs (PL) examined in the STL and the SGP, 16.6% (79/475) of the isolates were Pm, 10.7% (51/475) were Mh and 0.2% (1/475) was Mg. In 362 PL of the STL, 17% (62/362) of the isolates were Pm and 10.2% (37/362) was Mh; these differences were significant (p = 0.05). In 113 PL of the SGP, 15% (17/113) of the isolates were Pm; 12.4% (14/113) was Mh and 0.8% (1/113) was Mg. These differences were only significant (p = 0.05) for the Mg rate in regards to the Mh and Pm rates (Table 3).

Strain serotypification

***Mannheimia* sp.:** Out of the 52 *Mannheimia* sp. isolates in both slaughterhouses, 98% (51/52) corresponded to Mh and, 1.9% (1/52) to Mg. Out of the Mh isolates, 33% (17/51) was serotype 1 (S1), 17.6% (9/51) S6 and, 49% (25/51) NT. These differences were significant (p = 0.05) (Table 2).

All STL isolates (37) were Mh, out of which 35% (13/37) were S1, 24.3% (9/37) S6 and 40.5% (15/37) NT.

Out of the 15 *Mannheimia* sp. isolates of SGP, 93% (14/15) were Mh and 6.7% (1/15) were Mg. Out of the Mh isolates, 28.5% (4/14) were S1 and 71.4% (10/14) NT. The differences among the isolate frequencies of the different Mh serotypes were significant (p≤0.5) for SGP, but not for STL (Table 3, Fig. 1).

Table 2: *Mannheimia* sp. serotypes and P. *multocida* biotypes isolated in pneumonic lungs in 2 slaughterhouses in Mexico

	Isolates																	
	Mannheimia sp.												*P. multocida*					
		Mh																
		S1		S6		NT		Mg				A		D				
Slaughterhouse	Total	N°	(%)	N°	(%)	N°	(%)	N°	(%)	N°	(%)	N°	(%)	N°	(%)	N°	(%)	N°	(%)
STL	99	37	37	37	100	13	35	9	24	15	41	0	0	62	63	62	100	0	0
SGP	32	15	46.8	14	93	4	28.5	0	0	10	71	1	3.1	17	53	16	94	1	5.9
Total	131	52	39.6	51	98	17	33	9	18	25	49	1	0.8	79	60	78	99	1	1.2

STL: Slaughterhouse in Tlalnepantla; SGP: Slaughterhouse in Gomez Palacio

Table 3: Isolating rates of *M. haemolytica* (Mh), *M. glucosida* (Mg) and *P. multocida* (Pm) in Pneumonic Lungs (PL) in 2 slaughterhouses in Mexico

		Mannheimia sp.				*P. multocida*	
		Mh.		Mg.		Pm.	
Slaughterhouse	Total PL	N°	(%)	N°	(%)	N°	(%)
STL	362	37	10.2	0	0	62	17
SGP	113	14	12.4	1	0.8	17	15
Total	475	51	10.7	1	0.2	79	16.6

STL: Slaughterhouse in Tlalnepantla; SGP: Slaughterhouse in Gomez Palacio

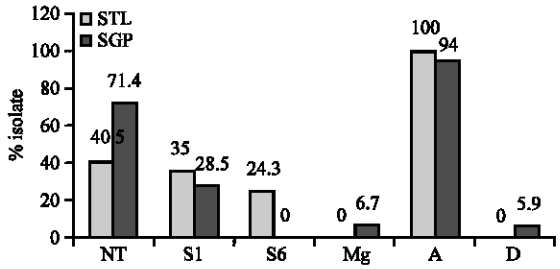

Fig. 1: Serotypes of *Mannheimia* sp. strains and biotypes of *P. multocida* strains isolated of bovine pneumonic lungs in 2 slaughterhouses in Mexico

***P. multocida*:** Out of 79 Pm isolates, 98.7% (78/79) corresponded to A biotype and 1.2% (1/79) to D biotype in both slaughterhouses. These differences were significant ($p \leq 0.5$) (Table 2).

A hundred percent of STL isolates were A biotype; 94% in the SGP (16/17) were A biotype and 5.9% (1/17) D biotype. These differences were significant ($p \leq 0.5$). (Table 2, Fig. 1).

DISCUSSION

The results of this research show that higher rates of isolates in lungs were of Pm in general level (79%) as well as in each of the traces (STL = 17%; SGP = 15%); we would like to emphasize that these differences were significant. These data is similar to that found in previous studies made on bovine lung samples from necropsies or in slaughterhouses, in which *Pasteurella* sp. isolates have been obtained. Pm has been the main isolate microorganism with rates from 32-62% (Blanco-Viera *et al*., 1995; Pijoan *et al*., 1999) whereas in other studies Mh is reported as the most frequent isolate in lamb pneumonic lungs; Jaramillo *et al*. (1987) found frequencies of 22% for Mh and 19.6% for Pm in Mexico; likewise, in lamb necropsies with pneumonia they reported 55.5% for Mh and 44.5% for Pm in England (Allan *et al*., 1985); 42% for Mh and 8% for Pm in Turkey (Haziroglu *et al*., 199) and 46.3% for Mh and 34.7% for Pm (Welsh *et al*., 2004). Throughout a 9-year-study on isolates found in pnemonic lung samples carried out in a diagnosis lab, they could verify that isolate frequencies varied from 30.6-64.1% for Mh and from 20-47.4% for Pm (Welsh *et al*., 2004).

Even though some authors describe Pm as the most commonly isolate bacteria in lambs (Virtala *et al*., 1996; Welsh *et al*., 2004), it is generally believed that Mh is the most pathogenic bacteria and the most regularly associated with the respiratory diseases complex of bovines, mainly with the mannheimiosis (Trigo, 1991; Murphi *et al*., 1993; Pijoan *et al*., 1999).

Previous studies agree that the reproduction of the phenotyping characterization methods of *Mannheimia* spp is high; however, the serotypification through IHA is not specific enough for a Mh reliable identification as long as it is not supported by a wide batch of biochemical tests, since the genus *Mannheimia* includes phenotypically and genotypically very heterogeneous taxa (Angen *et al*., 1999b; Angen *et al*., 2002).

The highest S1 isolate frequency among the Mh serotypable strains coincides with the findings in other studies that consider this serotype as the most frequent in bovine pneumonic lungs in Mexico (Jaramillo *et al*., 1987; Blanco-Viera *et al*., 1995; Pijoan *et al*., 1999) as well as in North America and Europe (Al-Ghamdi *et al*., 2000; Wray and Thompson, 1971; Quirie *et al*., 1986). These frequencies are fewer that those reported in several studies showing frequencies from 58-88.9% in Mexico (Jaramillo *et al*., 1987; Blanco-Viera *et al*., 1995; Pijoan *et al*., 1999) or 59-63% in the U.S. (Al-Ghamdi *et al*., 2000), or in England (Quirie *et al*., 1986; Wray and Thompson 1971), but higher than 10% reported by Purdy *et al*. (1997) in the United States.

It is widely known that even though other Mh serotypes such as 2, 6, 7, 8, 9 and 11 may be part of the regular flora of the upper respiratory tract of bovine, S1 may experience an explosive development and turn into the dominant isolate in the bovine nasopharynx under an stressful handling, viral infections or environmental changes, in such a way that after colonization of the Mh upper respiratory tract, S1 easily invades the lung through the inhalation of drops or infected tissue residues (Frank-Smith, 1983; Al-Ghamdi *et al*., 2000).

Our findings supports that other serotypes different of S1 together with NT strains may be found in mannheimiosis lesions, as it is shown in other studies (Blanco-Viera *et al*., 1995; Purdy *et al*., 1997; Pijoan *et al*., 1999; Al-Ghamdi *et al*., 2000) and that they may probably be able to produce the pathological changes found in pneumonic lungs (Purdy *et al*., 1997). The S6 isolate frequency in both slaughterhouses coincides with the data reported by Blanco-Viera *et al*. (1995) in Mexico, in spite of STL´s superiority (24%). This serotype has also been isolated in bovine nasal exudate in previous studies (Jaramillo *et al*., 2007a; Jaramillo *et al*., 2007b). Mg isolate is the first achieved in Mexico in bovine pneumonic lungs as it has only been reported in nasal ovine nasal exudates (Wray and Thompson, 1971; Angen *et al*., 1999a; Angen *et al*., 2002; Sisay and Zerihun, 2003). Some strains have also been isolated in bovine (Quirie *et al*., 1986) however, it is rarely isolated or associated with bovine diseases (Angen *et al*., 1999a).

The range of serotypes found in this work coincides with that reported in several studies in Mexico and other countries (Quirie *et al*., 1986; Jaramillo *et al*., 1987; Wray and Thompson, 1971; Pijoan *et al*., 1999), but it is lower that reported by others (Blanco-Viera *et al*., 1995; Al-Ghamdi *et al*., 2000). It is not clearly stated whether the isolate of other serotypes different from S1 represent genetic changes in the capability of other serotypes to colonize and reproduce in the respiratory tract and therefore induce or not lesions (Al-Ghamdi *et al*., 2000).

In this research, the frequencies of NT strains in both slaughterhouses (49%) and in each of them (STL, 40.5%; SGP, 71.4%) are higher than that found in other studies. These NT strain frequencies are varied and sometimes they may be high (Fraser *et al*., 1982) and they may diverge according to the source of isolation. The frequencies of pneumonic lungs reported in Mexico vary from 8% to not more than 18% (Jaramillo *et al*., 1987; Blanco-Viera *et al*., 1995; Pijoan *et al*., 1999) and in other countries frequencies from 5-24% have been reported (Quirie *et al*., 1986; Wray and Thompson, 1871; Al-Ghamdi *et al*., 2000).

These NT strains generally correspond to the A biotype (Frank, 1980) and have been previously described as Mh mutants; some of which are deficient in the production of soluble antigens. Regarding this matter, we believe that perhaps the serotypification was not sensitive and specific enough to classify all strains. Some of the NT could possible correspond to 1 or 2 serotype or any other Mh serotype. In regards to this, some studies cited by Frank (1980) mention the possibility that strains already classified as NT by IHA, may be, in fact, strains that belong to serotypes typified by IHA, but that the impossibility to react to IHA may be due to a loss of specific serotype antigens in the cell layer. This corroborates the difficulties that arise in the IHA test in Mh serotypification.

The highest Pm biotype A frequency found in this work coincides with the data reported by other national and foreign studies. Most of the studies carried out in pneumonic lungs in Mexico identify A biotype as the only isolate with 100% frequencies (Jaramillo *et al*., 1987; García *et al*., 1988; Pijoan *et al*., 1999) which agrees with the STL isolates. Other studies report lower frequencies regardless of the A biotype dominance; Madsen *et al*. (1983) report 81%, Blanco-Viera *et al*. (1995) 61% and De Rosa *et al*. (2000) 3 out of 4 isolates (75%).

The D biotype finding in the SGP, despite its lower frequency (5.9%), coincides with the findings by Blanco-Viera *et al*. (1995) who report 25%.

These results verify the Pm A biotype dominance, which is considered to be associated with bovine pneumonic problems, while the D biotype is more associated to occasional infections (Jaramillo *et al*., 1987; Pijoan *et al*., 1999). These data lead to the belief that this is an indicator that A biotype is the one that most frequently affects bovines in Mexico.

CONCLUSION

With the data gathered here we can conclude that the highest rate of isolates in lungs, in both and each of the slaughterhouses studied corresponded to Pm. This coincides with some studies but differs from others that consider Mh as the most pathogenic bacteria and the one most frequently associated to bovine pneumonic pasteurellosis. Moreover, the highest Mh frequency of S1 and the Pm A biotype verify that these two bacteria are the most frequently found in pneumonic lungs in Mexico. Furthermore, Mg isolation is the first achieved in bovine pneumonic lungs in Mexico and the Mh isolation of NT strains is higher than that reported in other studies in Mexico or abroad. All isolates are being characterized with techniques of molecular biology to differentiate the genotypes.

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