



BRIEF REPORT

Ureaplasma diversum–*Ureaplasma* sp. alone or concomitantly with *Mycoplasma hyopneumoniae* in pig lungs with and without pneumonia: A descriptive exploratory study in abattoirs

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Abstract Occurrence of *Ureaplasma diversum* (*U. diversum*) has been associated with reproductive failures in cattle and detected in pigs with and without pneumonia. However, its role in the porcine respiratory disease complex (PRDC) is unclear. A cross-sectional study was conducted in abattoirs, inspecting 280 pig lungs from eight herds. All the lungs were inspected, processed and classified according to the histopathological analysis. Moreover, bronchoalveolar lavage (BAL) specimens were collected and processed by PCR for detection of *U. diversum* and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*). *Ureaplasma* sp.–*U. diversum* and *M. hyopneumoniae* were detected in 17.1% and 29.3% of the analyzed BAL specimens, respectively. The concomitant presence of both microorganisms was detected in 12.5% of the inspected lungs. Both agents were found in lungs with and without pneumonia. *M. hyopneumoniae* was detected in 31.8% of pig lungs with enzootic pneumonia-like lesions, while *Ureaplasma* sp.–*U. diversum* was detected in 27.5% of lungs with these lesions. This descriptive exploratory study provides information for future experimental and field-based studies to better define the pathogenic role of this organism within the PRDC.

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PALABRAS CLAVE

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Complejo de
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Lesión compatible
con neumonía
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Ureaplasma sp.;
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Mycoplasma
hyopneumoniae

Presencia de *Ureaplasma diversum*-*Ureaplasma* sp. solo o junto con *Mycoplasma hyopneumoniae* en pulmones de cerdos con y sin neumonía: un estudio exploratorio descriptivo en frigorífico

Resumen La presencia de *Ureaplasma diversum* se ha asociado a fallas reproductivas en el ganado bovino y se ha detectado en cerdos con y sin neumonía. Sin embargo, su participación en el complejo de enfermedades respiratorias porcinas (CERP) no es clara. Se llevó a cabo un estudio transversal en matadero, inspeccionando 280 pulmones de cerdo provenientes de ocho piaras. Todos los pulmones fueron inspeccionados, procesados y clasificados según el análisis histopatológico. También se colectaron muestras de lavado broncoalveolar (LBA) y se procesaron mediante PCR para la detección de *U. diversum* y *Mycoplasma hyopneumoniae*. *Ureaplasma* sp.-*U. diversum* y *M. hyopneumoniae* se detectaron en el 17,1% y en el 29,3% de los LBA analizados, respectivamente. La presencia concomitante de ambos microorganismos se detectó en el 12,5% de los pulmones inspeccionados. Ambos agentes se encontraron en pulmones con y sin neumonía. *M. hyopneumoniae* se detectó en el 31,8% de los pulmones con lesiones compatibles con neumonía enzoótica, mientras que *Ureaplasma* sp.-*U. diversum* se detectó en el 27,5% de los pulmones con estas lesiones. Este estudio exploratorio descriptivo proporciona información para futuros estudios experimentales y de campo tendientes a definir mejor el papel patógeno de este organismo dentro del CERP.

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Ureaplasma diversum (*U. diversum*) is considered one of the most important members of the family *Mycoplasmataceae* causing reproductive failure in cattle. *U. diversum* has also been detected in pneumonic cattle¹⁴. In pigs, it was first detected in animals with and without pneumonia^{1,2} and recently in nasal swabs of live pigs¹¹. However, there is a lack of information on this microorganism with regard to its distribution and participation in the multifactorial porcine respiratory disease complex (PRDC). While *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is the primary agent of the PRDC, co-infections with other pathogens such as viruses and bacteria are frequent¹⁰. Although the concomitant presence of *M. hyopneumoniae* with other *Mycoplasma* species, including *Mycoplasma hyorhinis* (*M. hyorhinis*) and *Mycoplasma flocculare* has been reported^{5,7}, there has been scant confirmatory evidence of the important role of these *Mollicutes* in *M. hyopneumoniae* infections. Furthermore, recent swine lung microbiome studies have demonstrated that members of the family *Mycoplasmataceae* are one of the most common microbial families in pig lungs with and without pneumonia¹³. Although other *Ureaplasma* species, such as *Ureaplasma parvum* and *Ureaplasma urealyticum*, have been detected in pig lungs¹³, there are only limited reports of the presence of *U. diversum* with other *Mollicutes* in pneumonic pig lungs^{1,2}. The objectives of this study were: (i) to detect *U. diversum* in abattoir-collected pig lungs at the individual and herd level and (ii) to determine the concomitant presence of *U. diversum* and *Mycoplasma hyopneumoniae*.

A cross-sectional study was conducted in five abattoirs involving eight Argentinian herds (1–8) from three provinces (Table 1). The main characteristics of the herds, such as type of system, size (number of sows) and main aspects of health management are shown in Table 1. Lung samples were col-

lected using systematic random selection procedures where the first unit is randomly selected followed by selection at equal intervals. The herd sample size was set in 20, 40 and 60 lungs for herds with less than 500 sows, between 500 and 1000 sows, and more than 1000 sows, respectively. Each lung was grossly inspected for enzootic pneumonia (EP)-like lesions. Briefly, looking for purple to grey areas of pulmonary consolidation, located in the apical, intermediate, accessory and cranial parts of the diaphragmatic lobes¹⁰. Then, the type of pneumonia was determined by histopathological examination. For each inspected lung two specimens were collected using the following criteria: if the lung showed any macroscopic lesion compatible with EP-like pneumonia, a BAL sample (for PCR) and a piece of lung (for histopathological examination, in 10% buffered formalin) were collected for the affected lobe. Each BAL specimen was collected by a cut made in the affected lobe and a sterile 3 ml plastic pipette with sterile saline was introduced into the secondary or tertiary bronchi adjacent to the lesion. In case no gross lesions were detected, the specimens were collected from the right middle lobe. For the histopathological analysis, lung tissues were formalin-fixed, paraffin wax embedded and haematoxylin and eosin (H-E) stained. To determine EP-compatible lesions, each sample was graded from 0 to 4, according to the criteria described by Calsamiglia et al.³. Lesions scoring 1–2 were considered nonspecific pneumonias and 3–4 were considered to be compatible with EP. DNA from 280 BAL samples was extracted using the commercial Puri-Prep S kit (Inbio-Highway, Argentina) according to the manufacturer's instructions. For both, *U. diversum* and *M. hyopneumoniae* detection, species-specific nested PCRs were performed under the conditions reported by Cardoso et al.⁴ and Calsamiglia et al.³, respectively. Considering that Cardoso et al.⁴ reported a specificity of 73.1% using this

Table 1 Description of study herds, showing production type, number of sows, location and main health and management aspects.

Herd	Size and characteristics	Location (province)	Main health and management aspects
1	Indoor, two-site, 2900-sow, commercial herd	San Luis	Sow vaccination: Parvovirus-leptospirosis- <i>Erysipelothrix rhusopathiae</i> , <i>Mycoplasma hyopneumoniae</i> , atrophic rhinitis and Glässer's disease. Pig vaccination: PCV2 at 3 wks old. Free of Aujeszky's disease, brucellosis, influenza and <i>A. pleuropneumoniae</i> . <i>Pasteurella multocida</i> and <i>Streptococcus suis</i> endemic. In growers, strategic in-feed medication with 100 ppm tiamulin (Denagard® 80% premix, Elanco, Argentina) and 300 ppm chlortetracycline (Diclortet® 15% premix, Elanco, Argentina) for two weeks at 6 wks old
2	Indoor, three-site, 1100-sow, commercial farrow-to-finish herd	Córdoba	Sow vaccination: Parvovirus-leptospirosis- <i>Erysipelothrix rhusopathiae</i> , atrophic rhinitis, <i>A. pleuropneumoniae</i> and Glässer's disease. Pig vaccination: PCV2 and <i>Mycoplasma hyopneumoniae</i> at 3 wks old; <i>A. pleuropneumoniae</i> at 6 and 9 wks old. Free of Aujeszky's disease and brucellosis. <i>Pasteurella multocida</i> and <i>Streptococcus suis</i> endemic. In growers, strategic in-feed medication with generic antibiotics at 12–16 wks old (100 ppm tiamulin and 300 ppm chlortetracycline) and at 16–20 wks old (50 ppm tylosin)
3	Indoor, one site, 550 sow, commercial farrow-to-finish herd	Córdoba	Sow vaccination: Parvovirus-leptospirosis- <i>Erysipelothrix rhusopathiae</i> Pig vaccination: <i>Mycoplasma hyopneumoniae</i> at 3 and 6 wks old. Free of Aujeszky's disease and brucellosis <i>Pasteurella multocida</i> , <i>Streptococcus suis</i> and PCV2 diagnosed In growers, strategic in-feed medication at 3–5, 11–13, and 14–16 wks old (100 ppm tiamulin, Denagard® 80% premix, Elanco, Argentina and 50 ppm tylvalosin, Aivlosin®, 62.5% premix ECO Animal Health)
4	Indoor, one-site, 500-sow, commercial farrow-to-finish herd	Córdoba	Sow vaccination: Parvovirus-leptospirosis- <i>Erysipelothrix rhusopathiae</i> Pig vaccination: <i>Mycoplasma hyopneumoniae</i> at 3 wks old Free of Aujeszky's disease and brucellosis <i>Pasteurella multocida</i> , <i>Streptococcus suis</i> and PCV2 diagnosed In growers, strategic in-feed medication at 3–5, 11–13 and 14–16 wks old (100 ppm tiamulin, Denagard® 80% premix, Elanco, Argentina and 50 ppm tylvalosin – Aivlosin®, 62.5% premix ECO Animal Health)
5	Indoor, one-site, 120-sow, commercial farrow-to-finish herd	Mendoza	Sow vaccination: <i>Mycoplasma hyopneumoniae</i> Pig vaccination: <i>Mycoplasma hyopneumoniae</i> at 3 wks old. Free of Aujeszky's disease and brucellosis <i>Pasteurella multocida</i> and <i>Streptococcus suis</i> endemic. In growers, strategic in-feed medication with generic antibiotics at 7–9 wks old (300 ppm chlortetracycline)
6	Indoor, one-site, 110-sow, commercial farrow-to-finish herd	Mendoza	Sow vaccination: <i>Mycoplasma hyopneumoniae</i> Pig vaccination: <i>Mycoplasma hyopneumoniae</i> at 3 wks old. Free of Aujeszky's disease and brucellosis <i>Pasteurella multocida</i> and <i>Streptococcus suis</i> endemic. In growers, strategic in-feed medication with generic antibiotics at 11–13 wks old (300 ppm chlortetracycline)
7	Indoor, one-site, 100-sow, commercial farrow-to-finish herd	Mendoza	Free of Aujeszky's disease and brucellosis <i>Pasteurella multocida</i> diagnosed. No vaccination In growers, strategic in-feed medication with generic antibiotic at 6–8 wks old (100 ppm of tiamulin)
8	Indoor, one-site, 300-sow, commercial farrow-to-finish herd	Mendoza	Pig vaccination: <i>Mycoplasma hyopneumoniae</i> at 3 wks and 6 wks old Free of Aujeszky's disease, brucellosis No antibiotic treatment

Vaccination schedules and antibiotic treatments of piglets are described in greater detail. For sows, only the vaccines used are described. PCV2: Porcine Circovirus type 2; wks old: weeks old.

Table 2 Proportion (percentage and 95% confidence interval) of BAL PCR-positive specimens for *Ureaplasma* sp.–*U. diversum* and/or *Mycoplasma hyopneumoniae* and lungs with macroscopic and microscopic lung lesions, according to the herds.

Herd	Lung pathology (%; 95% CI)		PCR positive (%; 95% CI)		
	Macroscopic EP-like lesions	Microscopic lesions (scored 1–4)	<i>M. hyopneumoniae</i>	<i>U. diversum</i> – <i>Ureaplasma</i> spp.	<i>U. diversum</i> – <i>Ureaplasma</i> spp. and <i>M. hyopneumoniae</i>
1	38/60 (63.3; 50.3–76.3)	41/60 (68.3; 55.7–80.9)	19/60 (31.7; 19.9–43.4)	1/60 (1.7; –1.6–4.9)	9/60 (15; 6–24)
2	21/60 (35; 22–47.9)	23/60 (38.3; 25.2–51.5)	13/60 (21.7; 11.2–32.1)	6/60 (10; 2.4–17.6)	5/60 (8.3; 1.3–15.3)
3	17/40 (42.5; 25.9–59)	19/40 (47.5; 30.7–64.2)	1/40 (2.5; –2.3–7.3)	20/40 (50; 34.5–65.5)	15/40 (37.5; 22.5–52.5)
4	28/40 (70; 54.5–85.4)	28/40 (70; 54.5–85.4)	15/40 (37.5; 22.5–52.5)	0/40 (0; 0–0)	1/40 (2.5; –2.3–7.3)
5	13/20 (65; 40.7–84.6)	15/20 (75; 50.9–91.3)	4/20 (20; 2.5–37.5)	6/20 (30; 9.9–50.1)	3/20 (15; –0.6–30.6)
6	10/20 (50; 25.6–74.4)	13/20 (65; 40.7–84.6)	18/20 (90; 76.9–103.1)	0/20 (0; 0–0)	0/20 (0; 0–0)
7	13/20 (65; 40.7–84.6)	13/20 (65; 40.7–84.6)	0/20 (0; 0–0)	14/20 (70; 49.9–90.1)	1/20 (5; –4.6–14.6)
8	15/20 (75; 50.9–91.3)	18/20 (90; 68.3–98.8)	12/20 (60; 38.5–81.5)	1/20 (5; –4.6–14.6)	1/20 (5; –4.6–14.6)
Total	155/280 (55.3; 49.3–61.4)	170/280 (60.7; 54.8–66.6)	82/280 (29.3; 23.7–34.8)	48/280 (17.1; 12.5–21.7)	35/280 (12.5; 8.4–16.5)

PCR in bovine samples and in order to verify the specificity of this reaction within pig lung samples, two *U. diversum* PCR positive samples from each herd were randomly selected for sequencing. The 18 PCR products obtained were purified (Puriprep-GP Kit, Inbio Highway), quantified and sequenced (ABI 3130xl; Applied Biosystems) using the inner primers described by the authors⁴. The sequences were curated using the BioEdit software⁶ and aligned using Clustal Omega software to obtain a consensus sequence (along with other sequences obtained from nasal swabs specimens from pigs)¹¹. Then, the consensus sequence was aligned against the database using nucleotide BLAST (<http://www.ncbi.nlm.nih.gov/blast>) excluding environmental sample sequences. The proportion of positive pigs was based on the number of PCR-positive specimens for *M. hyopneumoniae*, *Ureaplasma* sp.–*U. diversum* or both, among the number of analyzed samples. Herds were classified as positive if at least one specimen rendered a positive result, and negative if all specimens were negative. The proportion of positive herds was based on the number of positive results among the total number of analyzed herds. The positive pig distribution among the type of microscopic lung was described. A contingency table and a test of independence based on a chi-square distribution was used with the level of significance set at 0.05.

M. hyopneumoniae and *Ureaplasma* sp.–*U. diversum* were detected in 29.3% (95% CI 24.0–34.6) and 17.1% (95% CI 12.7–21.6) of the analyzed BAL specimens, respectively. The concomitant presence of both microorganisms was detected in 12.5% (95% CI 8.6–16.4) of pig lungs. All herds were positive for at least one of the evaluated specimens;

in all herds *M. hyopneumoniae* was detected alone or in combination with *Ureaplasma* sp.–*U. diversum*, while *U. diversum* was detected in 7 out of the 8 herds. The proportion of positive pigs in each herd ranged between 40% and 90%. Table 2 shows the proportions of PCR positives for *M. hyopneumoniae* alone, *Ureaplasma* sp.–*U. diversum* alone or mixed detection and the proportion of macroscopic and microscopic lung lesions. The 16S ribosomal RNA consensus sequence obtained – 173 pb – (GenBank accession number ON059750) showed 100% similarity with the same region of *U. diversum* strains ATCC 49782 (CP009770.1: GU227397.1), T95 (JN935894.1), ATCC 49783 (GU227398.1) and A417 (NR_025878.1), and *Ureaplasma* sp. strains USP45 (GU227392.1), USP4 (GU227390.1), USP3012 (GU227389.1) and USP47 (GU227388.1). Among the 280 lungs examined, 125 (44.6%) and 110 (39.3%) had no macroscopic and microscopic lesions of pneumonia respectively. Of the 170 lungs with microscopic lesions, 66 (38.8%; 95% CI 31.2–46.4) were diagnosed as EP-like lesions (scored as 3–4) and 104 (61.2%; 95% CI 53.6–68.8) as nonspecific pneumonias, scored as 1–2 (Table 2). Table 3 shows the proportion of each type of pneumonia diagnosed by histopathological examination for all PCR results from the BAL specimens; from the data, there is sufficient evidence to conclude that the histopathological diagnosis and PCR results are dependent on each other. Among the lungs diagnosed as EP-like lesions, an overlap of the confidence intervals was observed, indicating that there were no differences between the proportion of diagnoses by PCR, while among the lungs diagnosed as nonspecific lesions the proportion of PCR positives for *M. hyopneumoniae* alone was higher

Table 3 Proportion (percentage and confidence interval 95%) of BAL PCR-positive or negative specimens for *Ureaplasma* sp.–*U. diversum* and/or *M. hyopneumoniae* according to the type of lesion diagnosed by histopathological examination in 280 lungs.

PCR results	Lung pathology (%; 95% CI)			Total
	No lesion	Nonspecific lesions (scored as 1–2)	EP-like lesions (scored as 3–4)	
<i>M. hyopneumoniae</i>	18/110 (16.4; 9.5, 23.3)	43/104 (41.3; 31.9, 50.8)	21/66 (31.8; 20.6, 43.1)	82/280 (29.2%; 23.7–34.8)
<i>Ureaplasma</i> sp.– <i>U. diversum</i>	16/100 (14.5; 8, 21.1)	14/104 (13.5; 6.9, 20)	18/66 (27.3; 16.5, 38)	48/280 (17.1%; 12.5–21.7)
<i>Ureaplasma</i> sp.– <i>U. diversum</i> and <i>M. hyopneumoniae</i>	17/110 (15.5; 8.7, 22.2)	8/104 (7.7; 2.6, 12.8)	10/66 (15.2; 6.5, 23.8)	35/280 (12.5%; 8.4–16.5)
Negative	59/110 (53.6; 44.3, 63)	39/104 (37.5; 28.2, 46.8)	17/66 (25.8; 15.2, 36.3)	115/280 (41%; 35.1–47)

than for *Ureaplasma* sp.–*U. diversum* alone or mixed detection.

The nested PCR used in this study for *U. diversum* detection was developed and originally tested in clinical specimens from bovines with a specificity of about 73%⁴. The obtained sequencing and alignment results showed that their specificity was not enough for the identification of *U. diversum*, since other close-related *Ureaplasma* species could be detected. Other specific molecular techniques should be used in future studies for specific *U. diversum* detection. Despite this fact, there are no antecedents in the literature about the detection of *U. diversum* and other close-related species in pig lungs at herd level. Given the low sensitivity of culture and the availability of PCR in our laboratory, we did not attempt a culture of *Ureaplasma* sp.–*U. diversum* in this study but relied on molecular diagnostics to confirm their presence in the BAL samples. *Ureaplasma* sp.–*U. diversum* were detected in BAL fluid from lungs of approximately 17% of finisher pigs of slaughter age, a proportion greater than that previously reported in pigs from Cuba and Canada, with 6.6% and 3.85%, respectively^{1,2}. It is important to note here that the positive proportion of both, *Ureaplasma* sp.–*U. diversum* and *M. hyopneumoniae*, might be overestimated, considering that the scalding tank has been highlighted as a critical point of cross contamination during the slaughtering process⁸. However, we recently estimated a prevalence of 16.5% of *Ureaplasma* sp.–*U. diversum* in nasal swabs specimens from 6–8 and 15–22 weeks-old pigs¹¹. These results might indicate, that a similar proportion of pigs carry *Ureaplasma* sp.–*U. diversum* at different sites in the respiratory tract and at different ages. Further studies are needed to understand this phenomenon. On the one hand, the evaluation of lung lesions in abattoirs is limited because it only identifies chronic (or recent) lung lesions at the end of the production period and does not provide any information regarding the evolution of respiratory diseases in younger pigs¹². On the other hand, a limitation of gross lung lesion evaluation in abattoirs is that other agents may also cause macroscopic lesions similar to those induced by mycoplasmas^{10,12}. Thus, a histological examination is important in lesion characterization. With regard to microscopic lesions, BALT hyperplasia has so far been associated with *M. hyopneumoniae* and

M. hyorhinis^{7,12} in spite of the fact that the role of *M. hyorhinis* in the PRDC is still controversial^{5,7}. In this case, only *Ureaplasma* sp.–*U. diversum* were detected in 27.3% of EP-like lesions (scored as 3–4). Furthermore, negative PCR results were obtained in 25.8% of EP-like lesions. Not having searched for *M. hyorhinis* is a limitation of this study. However, a putative relationship between the presence of *Ureaplasma* sp.–*U. diversum* with lung lesions cannot be strictly ruled out, especially considering previous findings in which *U. diversum* has been detected in lungs with EP-like lesions². Experimental studies should be conducted to confirm if *Ureaplasma* sp.–*U. diversum* can produce chronic lung inflammation, resulting in BALT hyperplasia. Our second objective, to determine the concomitant presence of *U. diversum* and *M. hyopneumoniae*, resulted in evidence of mixed infection in almost 13% of BAL samples. This is an important finding since there are scarce antecedents about the presence of *Ureaplasma* sp.–*U. diversum* together with other *Mollicutes*². However, this type of mixed infection would not be rare, mainly due to the abundance of *Ureaplasma* and other *Mollicute* species within the pig lungs^{5,7,13}. While pathogenic mechanisms of *U. diversum* in pigs remain unknown, there are already antecedents of the presence of the agent associated with lung lesions in other species. Human *Ureaplasma* sp. closely related to *U. diversum* are capable of causing pneumonia¹⁵, and *U. diversum* has been detected in pneumonic calves¹⁴. In pigs with pneumonia, not only *U. diversum* but also other species of the genus *Ureaplasma* have been detected^{1,13}. The identification of surface molecules and capsular material involved in the interaction of *U. diversum* with the host, and virulence factors including the *Mycoplasma* immunoglobulin binding/*Mycoplasma* immunoglobulin protease (MIB-MIP) system⁹, most of them also present in *M. hyopneumoniae*, suggest that this organism has a pathogenic potential. This descriptive exploratory study provides information that can be used to design experimental and field-based studies to better define the pathogenic role of this organism within the PRDC.

This study represents a comprehensive investigation of *Ureaplasma* sp.–*U. diversum* presence in pig lungs at the individual and herd level. The concomitant presence of *Ureaplasma* sp.–*U. diversum* with *M. hyopneumoniae* was

demonstrated in BAL, being the first study to investigate such an association on a large field-based scale.

Conflict of interest

None reported.

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