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BRIEF REPORT

Ureaplasma diversum and *Ureaplasma* sp. in nasal cavity of pigs: Distribution among herds and individual frequency based on pooled samples

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KEYWORDS
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Abstract The role of *Ureaplasma diversum* in the porcine respiratory disease complex (PRDC), its circulation among herds and prevalence in live pigs is unknown; thus, the objectives of this study were: to determine the presence of *U. diversum* in indoor intensive pig herds and to determine the individual frequency of pigs with *U. diversum* from pooled samples. A cross sectional study was carried out in 16 indoor intensive herds from Córdoba and La Pampa provinces, collecting eight nasal swabs specimens that were further processed by two pools of four specimens each by a PCR targeting a fragment of 16S ribosomal RNA gene. Four PCR products were sequenced and aligned against data bases. *U. diversum – Ureaplasma* sp. were detected in 56.3% of the analyzed herds, with 16.5% (95% CI 8.2–30.8) of positive pigs. It was concluded that *U. diversum – Ureaplasma* sp. are present in the nasal cavity of live pigs, being widely distributed among herds.

PALABRAS CLAVE
Cerdos;
Ureaplasma diversum;
Ureaplasma sp.;
Hisopado nasal;

Ureaplasma diversum y _Ureaplasma_ sp. en cavidad nasal de los cerdos: Distribución entre granjas y frecuencia individual basada en muestras agrupadas

Resumen La participación de *Ureaplasma diversum* (*U. diversum*) en el complejo de las enfermedades respiratorias porcinas (CERP), su circulación entre las piaras y la prevalencia en cerdos vivos se desconoce. Este estudio tuvo por objetivos determinar la presencia de *U. diversum* en criaderos intensivos de cerdos y la frecuencia individual de cerdos portadores de dicho agente, a partir de muestras agrupadas. Se realizó un estudio transversal en 16 granjas confinadas de

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PCR;
Sistemas confinados

las provincias de Córdoba y La Pampa, en el que se recogieron ocho muestras de hisopos nasales que se procesaron posteriormente en dos grupos de cuatro muestras cada uno, mediante una PCR dirigida a un fragmento del gen del 16S ARN ribosomal. Se secuenciaron cuatro productos de la PCR y se les alineó contra las bases de datos. Se detectó *U. diversum-Ureaplasma* sp. en el 56,3% de las granjas, con 16,5% (IC del 95%: 8,2-30,8) de cerdos positivos. Se concluyó que *U. diversum-Ureaplasma* sp. están presentes en la cavidad nasal de cerdos vivos, con amplia distribución en las granjas.

Ureaplasma diversum is a bovine-origin Ureaplasma that has been associated with a wide range of reproductive failures in cattle[6-8,10]. The agent has also been detected in pigs from different clinical specimens such as lung swabs, bronchial mucus and tracheobronchial lavages in pigs with and without pneumonia[2,3]. However, the role of *U. diversum* in the porcine respiratory disease complex (PRDC) is unknown. The agent has been detected at pig-level, but there is lack of information about its circulation at herd-level and its prevalence in live pigs. When the prevalence of an agent is unknown, pooled testing offers a cost-effective alternative to estimate the individual prevalence; thus, considering the aforementioned, the objectives of this study were: 1) to determine the presence of *U. diversum* in indoor intensive pig herds and 2) to determine the individual frequency of pigs with *U. diversum*.

The study was approved by the Research Ethics Committee of the National University of Río Cuarto, according to the international guidelines of the Council for International Organizations of Medical Sciences (CIOMS). Production systems and herds participating in this study followed their own management practices.

A cross-sectional study was carried out in 16 herds selected using a convenience sampling strategy with the criterion being that the herds were close to the National University of Río Cuarto (UNRC) and willingness to participate in the investigation. Sixteen intensive indoor herds, ranging between 70 and 560 sows, (media: 211, Standard deviation: 163) from Córdoba and La Pampa provinces were included in the study. In each farm, eight nasal swabs were collected from randomly selected pigs (four 6–8 week-old pigs and four 15–22-week-old pigs). The number and size of the evaluated pools were calculated assuming a prevalence of 5%, considering previous reports[2,3], with a confidence interval (CI) of 95% and 5% accuracy, considering that the sensitivity and specificity of the technique used were 0.73 and 1 respectively[16]. Nasal swabs were collected by the introduction of a sterile dacron swab (DELTALAB®, Spain) into each nostril, rotating clockwise and counterclockwise. All swabs were placed into sterile tubes containing 1 ml sterile Phosphate Buffered Saline (PBS) and were labeled, refrigerated during transport, and stored at −20 °C in the laboratory until DNA extraction was performed. For DNA extraction, nasal swabs specimens from each herd were pooled into groups by age (one pool from pigs of 6–8 weeks old and another pool from pigs of 15–22 weeks old). Briefly, each swab was vigorously vortexed and then removed and 250 μl of the suspension were mixed in a new tube for further processing. In total, DNA from 32 nasal swab pools was extracted as follows: each tube containing the pool was centrifuged at 10 000 rpm for 5 min and the supernatant was discarded. Then, 45 μl of Lysis buffer (10 mM Tris pH 8.3, 50 mM KCL, 0.5% Tween 20) and 1 μl of proteinase K (10 mg/ml) were mixed by pipetting with the pellet and incubated at 37 °C for 40 min and then at 95 °C for 15 min. Detection of *U. diversum* was performed by using a nested-PCR as previously reported[16]. To minimize the potential for false positive results and to prevent carryover contamination of the samples, stringent precautions were taken: DNA extraction, amplification and visualization were performed in a compartmentalized fashion, where three different rooms were used. Negative controls were included every five samples, and filter tips were used in all the processes. As positive control, DNA PCR positive from a previous study was used[13]. Herds were classified as positive if at least one pool rendered a positive result and, negative if all pools were negative. The proportion of positive herds was obtained by dividing the number of positive herds by the total evaluated herds. Pooled prevalence was calculated by using an online pooled prevalence calculator of the Epitools epidemiological calculators[11]. The individual prevalence for fixed pool size and known test sensitivity and specificity with exact confidence limits was estimated[1].

In order to verify the specificity of the utilized nested-PCR in pig respiratory tract specimens, four PCR positive samples from different herds were randomly selected for sequencing. The four obtained PCR products were purified (Puriprep-GP Kit, Inbio Highway), quantified and sequenced (ABI 3130xl; Applied Biosystems) using the inner primers described by the authors[16]. The sequences were curated using the BioEdit software[4] and aligned (together with other sequences obtained from lung pigs – data not shown) using Clustal Omega software. Since all the sequences were identical, a unique consensus sequence was obtained and then aligned against the database using nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast) excluding environmental sample sequences.

Nine out of 16 indoor intensive pig herds rendered positive results (56.3%). Of the positive herds, three were positive in both pools, three in younger (6–8 weeks of

age) and three in older pigs (15–20 weeks of age). Of the total pooled samples ($n = 32$), 12 were positive (six from 6 to 8 week old pigs and six from 15 to 20 week old pigs), resulting in a prevalence of 16.5% (95% CI 8.2–30.8). The 16S ribosomal RNA consensus sequence obtained 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).

The nested PCR used in this study was developed and originally tested in clinical specimens from bovines with a specificity of 73%[16]. 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, in agreement with a previous study, in which it could not be concluded that they were the same species[9]. In any case, there are no antecedents in the literature about de detection of *U. diversum* or *Ureaplasma* sp. from porcine nasal swab specimens. As mentioned above, *U. diversum* has been detected from different types of clinical specimens such as trachea, lung pieces, lung swabs, and bronchial mucus[2,3] and *Ureaplasma* sp. has been detected only in lungs[5,12]. The proportion of *U. diversum* – *Ureaplasma* sp. positives was 16.5% in pigs from more than a half of the analyzed indoor intensive herds from Córdoba and La Pampa provinces, indicating a high circulation of the agents within and among the sampled herds. It is worthy of note that the prevalence at individual level and the detection rate at herd level could be underestimated, because of having worked with pools. However, there are no previous results in the literature to compare our results. Although the pig herds analyzed did not raise cattle on the same farm (data not shown), occurrence of *U. diversum* in dairy cattle has been already reported in Argentina[13]. As there are no antecedents about the detection of the microorganism from porcine nasal swabs, this study is the first to demonstrate that *U. diversum* – *Ureaplasma* sp. are present in the nasal cavity of live pigs. The occurrence of *U. diversum* – *Ureaplasma* sp. in the nasal cavity of pigs does not provide information about the role that the agent plays within the PRDC. However, taking into account that other species of Ureaplasma genus have been detected in pig lungs[5,12] and that *U. diversum* has been detected in pneumonic calves[14,15], further experimental and field studies should be conducted in order to determine the role of the agent within PRDC.

Although the herd sampling was not at random, our results provide important information about the occurrence and circulation of *U. diversum* – *Ureaplasma* sp. in live pigs among herds. Considering that this is the first study conducted for the detection of the agent at herd level, there are no antecedents to compare this detection rate, neither in Argentina nor in other countries. In spite of the limitations of the study, as neither culture of the agent was attempted nor rRNA 16S full length sequence was obtained from clinical specimens, we consider relevant to communicate these results to the veterinary research community. This study provides baseline information for sample size calculations of future studies, in order to determine if *U. diversum* – *Ureaplasma* sp. are opportunistic pathogens or primary agents involved in the PRDC.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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