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Short communication. Isolation of *Aeromonas hydrophila* in piglets

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Abstract

The production of Alentejano breed pig started a recovery two decades ago due to increasing demand for *gourmet* products. These pigs are raised in rotational semi-extensive or extensive outdoor production systems in the "Montado" (green and cork oak forest), grazing and feeding acorns and other associated food resources. Bacteria of the genus *Aeromonas* are considered as emerging pathogens of importance for man and animals, but its involvement in swine is not well documented. In the context of a study made at the University of Évora to assess the specific diseases of Alentejano swine, diseased piglets from two farms were submitted for pathological and bacteriological examinations. Pathological examinations revealed changes characteristic of septicemia, and *Aeromonas hydrophila* was isolated in pure culture from multiple organs of piglets from both farms. Antibiotic sensitivity tests showed that the isolates from one of the farms were susceptible to gentamicin, oxitetracycline, neomycin, enrofloxacin, colistin sulfate, trimethoprim, ceftiofur, and amoxicillin plus clavulanic acid. In contrast, the *A. hydrophila* isolated in the other farm was resistant to all drugs tested but enrofloxacin. This is the first report in the world showing the relationship between septicemia and *A. hydrophila* infection in piglets. The importance of this finding is further reinforced by the fact that these bacteria can be highly resistant to antimicrobial agents.

Additional key words: Alentejano swine breed; Iberian swine; emerging pathogens; resistance to antibiotics.

Resumen

Comunicación corta. Aislamiento de *Aeromonas hydrophila* en lechones

La producción tradicional de cerdo de raza Alentejana se ha recuperado en los últimos años debido a la creciente demanda de productos tipo *gourmet*. Estos cerdos son criados en sistemas extensivos o semi-extensivos en la dehesa arbolada, en los que la ingestión de bellotas (Montanera) se complementa con otras fuentes de alimentación. Las bacterias del género *Aeromonas* son consideradas como patógenos emergentes de importancia para el hombre y los animales, pero su repercusión en ganado porcino es prácticamente desconocida. En un estudio realizado en la Universidad de Évora para determinar las enfermedades de los cerdos de esta raza, se realizó un examen anatomopatológico post-mortem de lechones de dos granjas, se tomaron muestras de diversos órganos durante la necropsia y se examinaron bacteriológicamente. El examen patológico puso en evidencia lesiones típicas de septicemia en las dos granjas y de varios órganos se aisló *Aeromonas hydrophila* en cultivo puro. Las cepas aisladas en una de las granjas se mostraron sensibles, mediante un antibiograma, a gentamicina, oxitetraciclina, neomicina, enrofloxacina, sulfato de colistina, trimetoprim, ceftiofur y amoxicilina más ácido clavulánico. Sin embargo, las cepas aisladas en la otra granja fueron resistentes a todos los antibióticos estudiados con la excepción de la enrofloxacina. Esta constituye la primera cita de aislamiento de *A. hydrophila* en lechones presentando signos clínicos de septicemia.

Palabras clave adicionales: cerdo de raza Alentejana; cerdo Ibérico; patógenos emergentes; resistencia a los antibióticos.

The Alentejano is an Iberian swine breed originated from the Portuguese region of Alentejo, and raised essentially for the production of meat, ham and sausages. The production of Alentejano pig started a recovery two decades ago due to several factors including the increasing demand for these animal *gourmet* products (meat and traditional high grade transformed products). This breed is part of an agro-forestry-pastoral system

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that combines green and cork oak forest ("Montado"), where pigs are fed on acorns, grass and pigswill. Piglets are weaned at 2 months age, and then fattened in semi-extensive or extensive systems in the Montado forest type in Alentejo region (Freitas *et al.*, 2006). This process allows sustainable use of natural resources available in the region even if, occasionally, pig standard commercial feeds are used (Charneca *et al*., 2010).

The pathology of these pigs reared in extensive systems is substantially different from that occurring in intensive farms, since environmental conditions interfere with risk factors for the development of disease. A study was undertaken at the University of Évora to determine the pathological processes associated with these extensively managed pigs (Queiroga *et al.*, 2009).

Bacteria of the genus *Aeromonas* are gram-negative bacilli, facultative anaerobes, usually oxidase and catalase positive, glucose fermenting and capable of reducing nitrate to nitrite (Martin-Carnahan & Joseph, 2005). These bacteria have been associated to aquatic environments and are usually isolated from foods of plant and animal origin (Fontes, 2009), being considered as emerging pathogens for both animals and humans. Infections in humans are usually reported in immunocompromised individuals but can also affect immunocompetent patients (Janda & Abbott, 2010). Although the role of Aeromonads in clinical pathology is a matter of controversy (Ghenghesh *et al.*, 1999; Janda & Abbott, 2010), gastroenteritis and diarrhea are frequent clinical manifestations of this infection in humans (Fontes, 2009). Moreover, these bacteria have been associated with cases of septicemia, cellulitis, myonecrosis, peritonitis, hepatitis, pancreatic abscesses, respiratory, urogenital and eye infections (Talon *et al.*, 1998; Janda & Abbott, 2010), and septic arthritis (Roberts *et al.*, 2006) in humans.

These microorganisms have been recognized also as a source of infections in poikilothermic animals, including amphibians, reptiles and fish (Fontes, 2009). Infections in parrots (Akkoç *et al*., 2008) and other wild birds (Glünder & Siegmann, 1989) have been also reported. *Aeromonas* species have been also considered as pathogens for marine mammals (Krovacek *et al.*, 1998; Pereira *et al*., 2008) and rabbits (Abdel-Gwad & Abdel-Rahman, 2004). Involvement of Aeromonads in musculoskeletal infections, abortion and reproductive problems, diarrhea and septic arthritis has been also reported in horses (Schneider, 1998; Staples, 2000). Septic arthritis, mastitis, seminal vesiculitis and abortions due to *Aeromonas* sp have been reported also in ruminants (Moro *et al*., 1999; Staples, 2000; Ílhan *et al*., 2006; Janda & Abbott, 2010). *Aeromonas hydrophila* has been identified as the responsible agent of septicemia in dogs (Staples, 2000; Janda & Abbott, 2010). Finally, *A. hydrophila* has been reported as a cause of diarrhea in piglets (Staples 2000), but never associated to other pathological processes in pigs. In this work we report the direct relationship between *A. hydrophila* and septicemia in piglets.

Two different disease outbreaks from two different farms were studied. Samples from farm 1 were taken from three piglets that presented as sudden death cases at 2 to 3 days of age. A total of fifty piglets had died in similar conditions in that farm. Samples from farm 2 were taken from two piglets, aged 2 months, which died after a prostration phase. Twenty other piglets had died similarly in farm 2.

The five piglets received were subjected to routine post-mortem examination, and selected organs and tissue fragments of lung, liver, spleen, kidney, small intestine and mesenteric lymph nodes were collected for both pathological and bacteriological examinations.

Samples for pathology were fixed for 24-h in 10% neutral buffered formalin, embedded in paraffin and then processed according to routine histological techniques.

From each farm, samples of lung, liver, spleen, kidney and small intestine of each piglet were aseptically collected and pooled by organ. Each organ pool was cultured on blood agar (Oxoid, CM271 supplemented with 5% defibrinated sheep blood) and MacConkey (Oxoid, CM115) agar plates. Blood agar plates seeded with lung samples were added with nicotinamide adenine dinucleotide (NAD) growth factor (factor V DD4 Oxoid, X3486A) to attempt isolating *Haemophilus parasuis*. Lung samples were also cultured onto tryptose broth (Tryptose Phosphate Broth, Oxoid, CM0283), and intestinal samples were also plated in brilliant green agar (Oxoid, CM0263) and also cultured in tetrathionate broth (Oxoid, CM29 supplemented with iodine as indicated by the supplier) for *Salmonella* spp enrichment. All cultures were incubated at 37°C for 24-h in aerobiosis and, if negative, reincubated in the same conditions for an additional 24-h period. Pooled liver samples were also plated on Wilkins agar (Oxoid, CM619) supplemented with 5% defibrinated sheep blood and incubated at 37°C in an anaerobic jar (Anaerocult A, Merck, 1.13829.0001) for 48-h.

Bacterial isolates were subjected to macro- and microscopical examination, and to oxidase and catalase tests. Preliminary identification of the isolates was made

using the Vitek 2 Compac system (BioMérieux), and the *Aeromonas* species isolated confirmed by sequencing the *gyr*B gene (Molecular Diagnostics Center, Biomolecular Technologies SL, Alicante, Spain) as described elsewhere (Yáñez *et al*., 2003; Fontes, 2009).

The results obtained are summarized in Table 1. All three pigs examined in farm 1 were showing characteristic signs of septicemia, namely intense lung and liver congestion. Pure *Aeromonas* spp cultures were isolated from the lungs, livers and kidneys in that farm. *Aeromonas sobria* was presumptively identified according to biochemical tests, but further molecular sequencing identified *A. hydrophila*. Both piglets from farm 2 were also showing histopathological lesions of intense lung and liver congestion and *Aeromonas* spp. was isolated from the lungs, spleens and kidneys (in pure cultures), and also isolated in mixed cultures from the small intestines from the two piglets examined. The isolates from farm 2 were identified presumptively as *A. hydrophila* or *Aeromonas caviae*, since the methods used (Vitek 2 Compac system, BioMérieux) do not discriminate between the two species. However, further molecular analyses identified *A. hydrophila*. A hemolytic strain of *Escherichia coli* was simultaneously isolated from the liver and small intestine in the piglets of farm 2 (Table 1).

To assess antimicrobial susceptibility of the *A. hydrophila* isolates, the Kirby Bauer method (CLSI, 2006) was used and the following antibacterial agents tested: ampicillin, gentamicin, penicillin G, oxytetracycline, lincomycin, neomycin, streptomycin, enrofloxacin, colistin sulfate, trimethoprim, sulphonamides, tulathromycin, ceftiofur and amoxicillin associated with clavulanic acid (Oxoid).

The *A. hydrophila* isolates from farm 1 were sensitive to gentamicin, oxytetracycline, neomycin, enrofloxacin, colistin sulfate, trimethoprim, ceftiofur and amoxicillin plus clavulanic acid. However, the *A. hydrophila* isolates from farm 2 were only susceptible to enrofloxacin

Aeromonads have been previously associated with diarrhea in swine (Staples, 2000) but never reported as the cause of septicemia and death in this animal species. These bacteria possess several virulence factors (Daily *et al*., 1981; Fujii *et al.,* 2008) and have been reported as responsible of septicemia in both humans (Talon *et al*., 1998; Janda & Abbott, 2010) and dogs (Janda & Abbott, 2010). Besides the association with the above pathological conditions, these bacteria have been isolated from feces of dogs, cats, horses, cattle, sheep and pigs, a fact explaining the ubiquity of these organisms in nature (Ghenghesh *et al*., 1999). They have also been found in carcasses of chickens, sheep and pigs, as well as in slaughterhouses equipment (Gill & Jones, 1995; Mano *et al.*, 2000; Costa & Rossi, 2002; Hinton *et al.*, 2004; Fontes, 2009). The contaminated water and food are important sources of infection (Fontes, 2009). These bacteria persist and multiply in the soil and water, being this a factor of paramount epidemiological importance (Brandi *et al*., 1996).

Table 1. Results of the pathological and bacteriological analyses in the affected piglets

Farm	Gross lesions	Histopathological lesions	Bacteriological isolates
1	Li – Focal necrotic hepatitis. S – Haematoma. K – Surface haematoma. I – Catarrhal enteritis and mild bloat; intense congestion of the mesenteric vessels. ML – Slight hypertrophy.	Lu – interalveolar capillar congestion. Li – Congestion; focal necrotizing hepatitis; interstitial mixed inflammatory cell infiltration. S – Lymphoid hyperplasia; infarction. K – Lymphocytic interstitial nephritis. I – Catarrhal enteritis.	*Aeromonas hydrophyla* (Lu, Li, K).
2	Serious cachexia. Serious atrophy of fat. Lu – Hepatization the apical lobe of the right lung. Li – Hepatic congestion. I – Catarrhal enteritis; congestion of the mesenteric vessels.	Lu – Congestion; exudative pneumonia. Li – Congestion; mild interstitial inflammatory infiltrate. I – Catarrhal/erosive enteritis.	*Aeromonas hydrophyla* (Lu, S, K, I). *Escherichia coli* (haemolytic strain) (Li, I).

I: Small intestine; K: kidney; Li: liver; Lu: lung; ML: mesenteric lymph nodes; S: spleen.

The extensive production systems of Alentejano pigs favor contact with these microorganisms. The oak groves are not confined environments, and there may be muddy areas where animals tend to stay for long periods, especially during hot seasons. The prolonged stay in these flooded areas increase the risk of water contamination by fecal matter, since these microorganisms inhabit frequently the animal's intestines (Ghenghesh *et al*., 1999; Fontes, 2009). It has been reported that *Aeromonas* spp can be excreted by feces and cause abortion in sheep (Ílhan *et al*., 2006). The fact that small ruminants are often raised together with Alentejano pigs (Nunes, 2007) can also contribute to an increased risk of contamination of water and grass, and obviously, also contribute to an increased risk of infection in pigs.

This study reports the first cases in the world of pig deaths associated with *Aeromonas hydrophila* infection, strongly suggesting its direct role as a relevant cause of septicemia in young piglets. The importance of this finding is further strengthened by the fact that some of the isolated strains were resistant to several antimicrobial agents. The resistance pattern in pathogenic bacteria is probably due to a widespread and excessive misuse of antibiotics, and may contribute for the emergence of multi resistant strains with relevant impact on public health (Tollefson & Karp, 2004).

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