

# A survey on bacterial involvement in neonatal mortality in dogs

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## Keywords

Antimicrobial susceptibility testing, Bacteriological examination, Dog, Histology, Mortality, Newborn puppies.

## Summary

Bacterial infections represent the second cause of neonatal morbidity and mortality in dogs, so the present study aimed to investigate the bacterial involvement in canine neonatal mortality and to evaluate the antibiotic susceptibility of the isolated bacteria. Fifty-one newborn purebred puppies, born dead or dead within 28 days of age, belonging to 36 different litters, were enrolled and the following procedures were performed on their fresh dead bodies: necropsy, collection of swabs by liver, kidney, lung, small bowel, and possible thoracic and/or abdominal effusion, for both bacteriological examination and antimicrobial susceptibility testing, and collection of samples by the same organs for histology. About 47% of total swabs were positive at bacteriology (pure bacterial culture or bacterial association). In 65% of the newborn puppies the mortality could be attributed to a bacterial infection. Although the high multidrug resistance, the most effective antimicrobials were third generation cephalosporins and fluorquinolones. In case of neonatal mortality, bacterial culture and antimicrobial susceptibility testing become essential for a targeted therapy in surviving littermates and for the management of following pregnancies in bitches with recurrent neonatal loss.

## Studio del ruolo delle infezioni batteriche nella mortalità neonatale dei cani

### Parole chiave

Antibiogramma, Esami batteriologici, Cane, Istologia, Mortalità, Cucciolo.

### Riassunto

Le infezioni batteriche sono la seconda causa di morbilità e mortalità neonatale nel cane. Lo studio si è proposto di indagare il reale coinvolgimento batterico nella mortalità neonatale canina e di valutare la sensibilità delle specie batteriche isolate agli antibiotici. Sono stati studiati 51 cuccioli neonati di razza pura, nati morti o deceduti entro i 28 giorni di età, appartenenti a 36 differenti cucciolate. I cadaveri freschi sono stati soggetti alle seguenti procedure: a) necropsia; b) tamponi di fegato, rene, polmone, piccolo intestino ed eventuale versamento toracico e/o addominale per l'esame batteriologico e l'antibiogramma; c) raccolta di campioni dagli stessi organi per l'esame istologico. Circa il 47% dei tamponi totali è risultato positivo all'esame batteriologico (coltura batterica pura o associazione batterica). Nel 65% dei cuccioli neonati la mortalità è sembrata attribuibile a un'infezione batterica. Nonostante l'elevata multiresistenza agli antibiotici, le cefalosporine di terza generazione e i fluorochinoloni si sono rivelati quelli maggiormente efficaci. In caso di mortalità neonatale, l'esame batteriologico e l'antibiogramma risultano fondamentali sia per instaurare una terapia mirata nei cuccioli superstiti sia per gestire le successive gravidanze in cagne con perdite neonatali ricorrenti.

## Introduction

Neonatal mortality rate in canine species ranges from 9 to 34% (Davidson 2003, Johnson 2006, Mosier 1981, Veronesi 2013), with a greatest risk during the first week of age (Davidson 2003, Mosier 1981, Münnich 2008, Peterson 2011). Beyond dystocia (Moon *et al.* 2000, Moon-Massat and Erb 2002, Münnich *et al.* 1996), bacterial infection was identified as the second main cause of neonatal death in dogs. Indeed, the consequent septicaemia is thought to be the most common cause responsible for puppies mortality within the first 21 days of age (Daniels and Spencer 2011, Münnich *et al.* 1995, Van der Beek *et al.* 1999, Veronesi 2013). Bacterial infection often spreads from mother to foetus during pregnancy, delivery or, after whelping, through infected maternal secretions, as vaginal and oronasal discharges, faeces, and milk (Münnich and Lübke-Becker 2004, Schäfer-Somi *et al.* 2003). Bacterial translocation is also recognized as a cause of neonatal systemic disease (Dahlinger *et al.* 1997, Go *et al.* 1994).

The microbial organisms most frequently associated with neonatal death are *Escherichia coli* (Askaa *et al.* 1978, Hoskins 2001), *Staphylococcus aureus* and *Staphylococcus pseudintermedius* (Münnich *et al.* 1995, Sager and Remmers 1990), *Streptococcus canis*, *Streptococcus dysgalactiae* subsp. *equisimilis*, *Streptococcus equi* subsp. *zooepidemicus* (Greene and Prescott 1998, Lamm *et al.* 2010), and *Klebsiella pneumoniae* (Davidson 2003, Münnich 2008). *Proteus mirabilis* and *Pseudomonas aeruginosa* have also been isolated in cases of neonatal loss (Münnich 2008).

Septicaemia may have a hyperacute evolution, with sudden death of the neonates (Askaa *et al.* 1978, Daniels and Spencer 2011, Davidson 2003, Veronesi 2013), or a subacute course (Indrebø *et al.* 2007, Johnston *et al.* 2001, Veronesi 2013). The clinical management of septicaemic newborn puppy is very difficult because of the sudden onset of unspecific symptoms and the fast disease course. Thus, treatment is usually delayed and unsuccessful, and the prognosis is poor.

Post-mortem examination, including necropsy and additional investigations (Johnson 2006, Veronesi 2013), could be helpful to identify the possible cause of neonatal mortality. The detection of involved bacteria allows for performing the subsequent antimicrobial susceptibility testing (AST), essential to choose the most appropriate therapy of surviving littermates and for a better management of following pregnancies in the same bitch.

For all these reasons, the aims of the present study were the investigation of the bacterial involvement in puppies neonatal mortality and the evaluation of the antibiotic susceptibility of the isolated bacteria.

## Materials and methods

### Animals

The study was performed in Northern Italy, between January 2012 and May 2013, on 51 full term newborn puppies, belonging to 36 litters of 17 breeds. All these puppies were stillborn or dead during the neonatal period, considered as the first 28 days of age (Davidson 2003, Veronesi 2013). The 36 bitches, 2-8 year old, 20 primiparous and 16 pluriparous, were healthy before mating, regularly submitted to a vaccination program, and correctly dewormed. Among these, 7 female dogs revealed previous isolated or recurrent, not investigated, neonatal losses. In all the bitches the last gestation showed a normal clinical course.

### Necropsy and sampling

Only fresh dead puppies (stored at 4°C for an elapsing time from death to necropsy of maximum 4 hours) underwent the necropsy, that was mainly focused to a correct bacteriological investigation. For each newborn, body size, maturity, sex, and weight were recorded; gross malformations, when present, were detected. After the careful opening of the abdominal cavity, swabs were collected from liver, kidney, and small bowel, avoiding any possible contamination, and from abdominal effusion, if present. Afterwards, the same organs were also sampled for histology and the specimens were immediately fixed in 10% buffered formalin solution. Finally, the thoracic cavity was opened; lung and possible thoracic effusion were sampled as reported for the abdomen. Only 1 specimen was collected for each organ.

### Bacteriological examination and antimicrobial susceptibility testing

The swabs were immediately plated on Petri plates with first isolation medium (TSA with 5% sheep blood, Oxoid, Milan, Italy), by streaking technique, to obtain the growth of bacterial colonies. Plates were incubated at 37°C for 24 hours under aerobic conditions; swabs collected from lung and effusions were also incubated in modified atmosphere in a candle jar (5% CO<sub>2</sub>). After the first incubation, all the plates that were bacteriological negative underwent a second incubation at 37°C for 24 hours; the plates were considered sterile, when bacterial colonies were not observed after the second incubation.

Isolated bacteria were identified by using different techniques: the macroscopic observation of colonies morphology on blood-agar plates, Gram-stain reaction, cellular morphology, and biochemical tests, particularly catalase and oxidase tests (Oxichrome Reagent, Remel-Oxoid, Milan, Italy).

For the identification of Gram-negative bacteria (*Enterobacteriaceae*) the growth on selective and differential medium Mc Conkey (Oxoid, Milan, Italy) was evaluated. Moreover, commercially available specific miniaturized methods ('API-20E<sup>®</sup>', 'API-20NE<sup>®</sup>', 'API-20STAPH<sup>®</sup>', Bio-Mérieux, Craponne, France) as well as selective and differential media, such as Mannitol Salt Agar (Oxoid, Milan, Italy) and Brilliant Green Agar (Oxoid, Milan, Italy), were carried out to achieve the biochemical characterization of bacteria (Carter and Wise 2004).

For each cultured bacterial species, susceptibility to the most common antimicrobial drugs was investigated according to CLSI guidelines<sup>1</sup>. Ampicillin, amoxicillin and clavulanic acid, cephalexin, ceftriaxone, enrofloxacin, metronidazole, spiramycin, polymyxins, and trimethoprim-sulfamethoxazole were tested. All the bacteria were classified as being susceptible, intermediate or resistant to antibiotics.

## Histology

Samples were evaluated for possible inflammatory lesions, bacterial emboli within the blood vessels or internal organs, and bacterial growth within the deep tissues.

Four-micrometer-thick serial sections were obtained from each paraffin block and stained with hematoxylin-eosin (H&E).

## Results

### Clinical findings

Out of the 51 newborn purebred puppies (32 born by euthocic delivery and 19 by dystocic delivery), 9 were born dead and 42 died within 28 days after birth. Among the latter ones, 4 newborns died suddenly, 20 displayed noticeable symptoms within 48 hours from birth, whereas the other 18 showed clinical signs after 2 days of age. In 26 of 36 litters, more than 1 newborn puppy was affected by the same symptoms. Clinical signs were always unspecific, sometimes associated, and distributed as follows: lethargy (54.9%), loss of sucking reflex (41.2%), abnormal vocalizations (21.6%), diarrhea (17.6%), failed weight gain (13.7%), hypothermia (5.9%), dermatitis (5.9%), conjunctivitis (5.9%), rigidity (5.9%), dyspnoea (2%), convulsions (2%), jaundice/regurgitation (2%), and heart murmur (2%). Clinical course was fast in 35 puppies, with death within 48 hours since the onset of clinical signs.

<sup>1</sup> Clinical and Laboratory Standards Institute. 2012. M02-A11, Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard, 11<sup>th</sup> Edition.

## Necropsy

Necropsy evidenced that all the newborns were well developed, mature, and of the correct size for the belonging breed. Gross malformations were never detected. Thoracic effusion was found in 6 puppies, whereas abdominal effusion in 4 subjects. In most cases, gross organic lesions were not present, although areas of lung atelectasis were observed in 16 puppies.

A total of 214 swabs were collected. In all the 51 subjects the following organs were sampled for both bacteriology and histology: liver, kidney, lung, and small bowel. Swabs were taken also from thoracic effusion and abdominal effusion in 6 and 4 cases, respectively.

## Bacteriology and antimicrobial susceptibility test

From a total of 214 swabs, 101 (47.2%) were bacteriological positive, whereas 113 (52.8%) were bacteriological negative. For 39 of 51 (76.5%) newborn puppies, at least 1 organ resulted positive at bacteriological examination. In 87 of 101 positive swabs (86.1%) the following bacteria were isolated in pure culture: *E. coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *haemolytic Escherichia coli*, *Proteus mirabilis*,  $\beta$ -*haemolytic streptococci*, *Klebsiella pneumoniae*, *Staphylococcus pseudintermedius*, *Bacillus*, and *Streptococcus faecalis*. Their distribution in the organic swabs is reported in Table I. In the other 14 positive samples (13.9%) different bacterial association were found, as showed in Table II.

On the basis of these results, it is reasonable to suppose that 27 of 51 subjects (52.9%) died because of a localized or systemic single bacterial infection. Interestingly, *E. coli* was involved in 15 cases and *haemolytic E. coli* in 4 newborn puppies,  $\beta$ -*haemolytic streptococci* were isolated in 3 subjects, *E. faecalis* was found in 2 cases, whereas in 3 newborn puppies were detected *P. mirabilis*, *K. pneumoniae*, and *S. aureus*, respectively.

In 6 of 51 subjects (11.8%) the death was probably due to a bacterial co-infection. In the first newborn puppy the association between *E. coli* and *K. pneumoniae* was isolated in the kidney, liver, and small bowel, in addition to *Aerococcus viridans* in the lung and thoracic effusion, whereas in the second subject *E. coli* and *K. pneumoniae* were identified in the kidney, liver, and abdominal effusion. In the third neonate, the lethal association was represented by *K. pneumoniae* in the kidney and *E. coli* in the lung, whereas in the fourth subject death might be attributed to *P. mirabilis* in the kidney, liver, and small bowel, *P. aeruginosa* in the small bowel, and *S. aureus* in the lung. In the fifth case the newborn puppy

**Table I.** Organic distribution of bacterial strain isolated in pure culture in 87 samples collected from full term newborn puppies in Northern Italy, between January 2012 and May 2013.

Bacteria	Liver	Kidney	Lung	Small bowel	Abdominal effusion	Thoracic effusion	Total samples
<i>E. coli</i>	12	10	14	13	1	-	50
Haemolytic <i>E. coli</i>	-	2	1	2	-	-	5
<i>S. aureus</i>	1	2	2	1	-	1	7
$\beta$ -haemolytic <i>S.</i>	-	-	3	-	-	-	3
<i>K. pneumoniae</i>	-	1	1	-	-	-	2
<i>P. mirabilis</i>	2	1	1	1	-	-	5
<i>S. pseudintermedius</i>	-	-	1	-	-	-	1
<i>E. faecalis</i>	1	2	1	6	-	-	10
<i>Bacillus</i>	-	-	-	1	-	-	1
<i>S. faecalis</i>	-	-	-	1	-	-	1
<i>A. viridans</i>	-	-	1	-	-	1	2 <sup>a</sup>

<sup>a</sup> *A. viridans* was isolated in lung and thoracic effusion samples belonging to the same subject.

**Table II.** Organic distribution of bacterial association isolated in 14 samples, collected from full term newborn puppies in Northern Italy, between January 2012 and May 2013.

Bacterial association	Liver	Kidney	Lung	Small bowel	Abdominal effusion	Thoracic effusion	Total samples
<i>E. coli</i> + <i>K. pneumoniae</i>	2	2	-	2	1	-	7
<i>E. coli</i> + <i>E. faecalis</i>	1	-	-	1	-	-	2
Haemolytic <i>E. coli</i> + <i>E. faecalis</i>	-	-	1	1	-	-	2
<i>P. mirabilis</i> + <i>E. coli</i> + <i>P. aeruginosa</i>	-	-	-	1	-	-	1
<i>P. mirabilis</i> + <i>E. coli</i>	-	-	-	1	-	-	1
<i>E. coli</i> + haemolytic <i>E. coli</i>	-	-	-	1	-	-	1

probably died because of *E. coli* in all organic samples and abdominal effusion, in addition to *E. faecalis* in the liver, whereas in the sixth newborn the mortality could be due to haemolytic *E. coli* in the kidney, lung, and small bowel, and *E. faecalis* in the lung.

Of the 51 newborn puppies, 6 (11.8%) revealed only physiologic bacteria in the small bowel, and 12 (23.5%) were characterized by having all the organic samples negative at bacteriological examination.

The AST was performed in 33 cases: the most effective drugs were third generation cephalosporins (25 cases, 75.7%) and fluorquinolones (20 cases, 60.6%). In 29 cases (87.9%), a multidrug resistance (resistance to at least 4 antibiotics) of the bacterial strains was noted, whereas in 2 cases (6.1%) the isolated bacteria were resistant to all the tested antibiotics.

## Histology

Histological examination evidenced a multi-organ morphology characterized by typical findings of an on-going maturation process. Despite gross

lesions were detected during necropsy in only 8 of 51 puppies, noteworthy histological findings were found in 16 cases (31.4%). The most affected organ was the lung (12 cases, 75%) and the lesions included fibrino-purulent, purulent and necrotizing bronchopneumonia or pneumonia (5), necrosis (3), and oedema (6). In particular, 3 newborn puppies showed more than 1 of these pulmonary alterations simultaneously. In 4 subjects (25%) only, or also, the kidney was affected by some lesions, such as cortical or tubular necrosis (3) and acute infarctions (1). Furthermore, in 2 newborn puppies (12.5%) the liver was characterized by multifocal necrosis. However, bacterial aggregates were never detected. Hyperemia was found in all the samples belonging to 9 of the 51 puppies (17.6%).

## Discussion

Despite the high percentage of neonatal death rate in dog, in the last decades few studies investigated the role of bacterial infections in newborn puppies mortality (Daniels and Spencer 2011,

Münnich *et al.* 1995, Poffenbarger *et al.* 1991, Sager and Remmers 1990, Veronesi 2013).

Neonatal mortality is underestimated by both owners and breeders, as proved by the about 20% of bitches which have experienced neonatal loss, whose causes have not been investigated. In over 70% of cases, multiple puppies in the same litter were affected by neonatal diseases, showing that the breeders should consider with great attention also the first sign of sickness even in a single newborn. In agreement with extant literature, also in the present study, clinical signs, when present, were not specific, abrupt, and were followed by fast and fatal clinical course, not allowing for a possible patient treatment (Davidson 2003, Johnston *et al.* 2001, Veronesi 2013).

Post-mortem examination confirmed the limited value of necropsy alone; indeed, in most cases gross organic lesions were not or minimally detectable, as previously reported (Daniels and Spencer 2011, Lamm *et al.* 2010, Schäfer-Somi *et al.* 2003, Veronesi 2013). The usefulness of necropsy is to allow the collection of samples for bacteriology, AST, and, at a less extent, histology.

On a total of 214 collected swabs, more than 47% was bacteriological positive. It is reasonable to believe that in 65% of puppies bacterial infection might have been involved in neonatal death, confirming that bacteria could play an important role in canine neonatal mortality (Lamm *et al.* 2010, Schäfer-Somi *et al.* 2003). In about 23% of newborns all the swabs were bacteriological negative, whereas in about 12% of cases only physiological bacteria were isolated in the small bowel.

Literature reports that the bacterial organisms most frequently responsible for neonatal mortality in dogs are *E. coli*, staphylococci, streptococci, and *Klebsiella* spp. However, *P. mirabilis* and *P. aeruginosa* can be also isolated (Bjurström 1993, Daniels and Spencer 2011, Davidson 2003, Greene and Prescott 1998, Johnston *et al.* 2011, Mosier 1981, Münnich 2008, Sager and Remmers 1990, Schäfer-Somi *et al.* 2003, Veronesi 2013). The results of the present study confirmed that the just mentioned bacteria, alone or in association, are often involved in neonatal death in dogs, above all *E. coli* (19 of 51 cases). The newborn puppy enteric epithelium is more permeable to *E. coli* than the adult one (Young *et al.* 1983) and the most important infection sources are represented by mother (Bjurström 1993, Bjurström and Linde-Forsberg 1992, Münnich and Lübke-Becker 2004), or other subjects living in the kennel, as well as the environment (Münnich 2008). Among staphylococci, the principal pathogenic species are thought to be *S. aureus* and *S. pseudintermedius*. Despite the former usually being described as frequent cause of neonatal septicaemia (Münnich *et al.* 1995, Sager and Remmers 1990), in the

present study *S. aureus* was probably responsible for neonatal death in only 1 case. Streptococcal septicaemia is another common cause of miscarriage or neonatal loss in the dog (Greene and Prescott 1998, Kornblatt *et al.* 1982, Kornblatt *et al.* 1983, Münnich 2008), nevertheless in this research only 3 newborn puppies died because of  $\beta$ -haemolytic *Streptococcus* infection. Also streptococci often have a maternal origin (Bjurström 1993, Bjurström and Linde-Forsberg 1992, Vela *et al.* 2006); the consumption of contaminated milk represents another possible, but unusual, source of infection (Schäfer-Somi *et al.* 2003). Regarding *K. pneumoniae*, it was likely responsible for neonatal loss in only 1 case, as well as *P. mirabilis*. The use of antibiotics that reduce resistance to colonization, nutrition with commercial formulas, and iatrogenic transmission seem to predispose the newborn puppies to *K. pneumoniae* infection.

In 4 puppies it was supposed that less frequent, but potentially, pathogenic bacteria, such as *E. faecalis* (2), *A. viridans* (1), and *P. aeruginosa* (1), contributed to the neonatal death. *Enterococcus faecalis* is a bacterium belonging to the normal endogenous flora of humans and animals but its intestinal excess or systemic diffusion represent a real problem, above all in the newborn. *Aerococcus viridans* is a Gram-positive coccus rarely found as human pathogen (Leite *et al.* 2010), but in literature it has been reported that, in vulnerable patients, this organism could have a clinically significant role in systemic infections (Uh *et al.* 2002); this fact might happen also in canine species. *Pseudomonas aeruginosa* is frequently involved in diseases caused primarily by other bacteria; immunosuppressive or too long antibiotic therapies are predisposing factors for the infection.

In 14 of 101 positive samples, bacterial associations were isolated, as already reported by other authors (Schäfer-Somi *et al.* 2003), and in 6 out of 51 newborn puppies death was probably due to the bacterial co-infection.

Obviously, other possible causes of mortality should be considered in those cases in which all the swabs were bacteriological negative (12) or only physiological bacteria were isolated in the small bowel (6).

Beyond bacterial detection, the present study was also aimed to assess antibiotic sensitivity of isolated bacteria. In 33 cases the antimicrobial susceptibility was tested: the most effective drugs were third generation cephalosporins, in agreement with data reported by other authors (Daniels and Spencer 2011, Davidson 2003, Johnston *et al.* 2001, Münnich 2008, Veronesi 2013), and fluoroquinolones. In 88% of cases the bacterial strains showed a multidrug resistance, and in 6% of cases the bacteria were

resistant to all the tested antibiotics. It has been already demonstrated that bacterial antimicrobial resistance and multiresistance represent an emerging problem (Guardabassi *et al.* 2004). The spread use of antibiotics by many breeders to reduce the neonatal mortality might be responsible for the dam vagina colonization by opportunistic pathogens and the selection of resistant bacteria, which may cause septicaemia in newborn puppies (Milani *et al.* 2012). Therefore, the AST should be strongly recommended after bacterial detection, to optimize the efficacy of therapy and to avoid dangerous bacterial resistance (Daniels and Spencer 2011, Davidson 2003, Veronesi 2013).

Histology, reported as a useful tool for septicaemia diagnosis, was of limited value in the present study. Indeed, bacterial emboli within the blood vessels or internal organs, or bacterial growth within the

deep tissues (Daniels and Spencer 2011, Farstad 2003) were never seen, in contrast to what reported by other authors (Lamm *et al.* 2010, Vela *et al.* 2006). A possible explanation for this finding could be the fast course of bacterial infection in newborn puppies, so that the neonates died before the establishment of the typical histological changes (Morris *et al.* 2007).

The present study demonstrates the involvement of bacterial infections in neonatal mortality in canine species and the alarming antibiotic resistance of the isolated bacterial strains.

Neonatal loss should not be underestimated by owners and breeders, and necropsy, coupled to bacteriological examination and AST, should be always suggested in even isolated neonatal mortality occurrence.

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