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Antimicrobial resistance in bacteria from breeding dogs housed in kennels with differing neonatal mortality and use of antibiotics

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**Abstract**

This work examines the antimicrobial resistance of potentially pathogenic bacteria (*Staphylococcus pseudintermedius, Streptococcus canis*, *Escherichia coli*) found in the vaginal tract in prepartum mammary secretions and postpartum milk of bitcheshoused in breeding kennels (N \_ 20; 92 bitches). The kennels were divided into three categories: no routine antimicrobial administration around parturition (category 1); routine administration of one antibiotic around parturition (category 2); routine administration of multiple antimicrobials around parturition (category 3). Bacteriological cultures and antibiotic susceptibility tests were performed on vaginal specimens, prepartum mammary secretions, and postpartum milk. Stillbirths and neonatal deaths were recorded for each whelping and analyzed as “within-litter stillbirths” and “within-litter neonatal deaths” according to kennel category, by Pearson \_2 test and the Kruskal-Wallis nonparametric test, respectively. The frequency of isolation and antimicrobial resistance of bacteria were analyzed according to kennel category by Pearson \_2 test. Kennel category was not significantly associated with differing numbers of stillbirths or neonatal death events, nor was the frequency of isolation of potentially pathogenic bacteria in the three kennel categories significantly different. Kennel category 3 had a significantly higher frequency of isolation of multiresistant gram-positive bacterial strains. Our results show that intense administration of antibiotics to breeding bitches does not effectively reduce neonatal mortality; on the contrary, it induces multiresistance in potentially pathogenic prepartum bitches.

*Keywords:* Antimicrobial resistance; Stillbirth; Neonatal death; Bitch

**1. Introduction**

Puppy loss during the first weeks of life ranges between 15% and 40% [1]. Death is usually sudden,

without noticeable signs. Although multifactorial etiology is generally recognized, the dams and the environment are a probable source of infection [2,3]; bacterial infections caused by *Streptococcus canis, Staphylococcus aureus, Staphylococcus pseudintermedius* (new classification [4]), *Escherichia coli, Klebsiella* spp. and *Pseudomonas* spp. are one of the major causes of disease in newborn puppies [1,3,5]. Puerperal mastitis and metritis are known factors leading to neonatal bacteremia and septicemia [6–8]. Subclinical mastitis has also been recognized as a predisposing factor to septicemia in puppies [7] and nursing from bitches with mastitis may be the cause of fatal gastroenteritis [9]. The kennel environment increases the risk of neonatal illness because of high animal concentrations, and many other factors, such as hygiene, the capability and experience of staff, and whelping facilities, may play a predisposing role in neonatal disease and mortality [10]. Many bacterial species colonize the vagina of healthy bitches, among which *Escherichia coli, Streptococcus canis, Staphylococcus pseudintermedius, Pasteurella* spp. and *Klebsiella* spp. are the most frequently isolated [11–16]. Cultures from the milk of healthy bitches often show bacterial growth, and coagulasepositive and coagulase-negative staphylococci, \_*-*hemolytic streptococci, *E. coli* hemolytic and nonhemolytic strains and *Bacillus* spp. are isolated in 70% of cultures [9]. In the case of mastitis, streptococci, staphylococci and *E. coli* are mainly isolated. Ververidis et al. [17] showed the clear-cut role played by *S. pseudintermedius* (*S. intermedius* in the old classification) in the pathogenesis of mastitis, even when cultures are negative. Bacteriologic cultures of the vaginal tract and of the mammary secretions of bitches around parturition have been suggested as suitable for assessing the origin of the pathogenic bacteria responsible for neonatal infections [2,7]. Many dog breeders commonly treat bitches before and after parturition with antimicrobials—and ask veterinarians to prescribe them—with the aim of eliminating bacterial flora and reducing neonatal mortality, but the only consequence of this excessive use is to predispose the vagina to colonization by opportunistic pathogens and to select resistant bacteria [18,19]. Growing resistance of bacteria toward antimicrobials is becoming a concern in veterinary medicine, both for animal health and because of the potential risk that animals may become reservoirs of zoonotic agents or that bacterial resistance may be transferred to pathogens affecting humans [20]. This study was carried out in breeding kennels with high neonatal mortality but differing in antimicrobial use: our aim was to assess the antimicrobial resistance of potentially pathogenic bacteria in the vaginal tract in prepartum mammary secretions and the milk of periparturient bitches and to correlate findings with neonatal mortality rates.

**2. Materials and methods**

*2.1. Animals and kennels*

The whelping of 92 healthy bitches of various breeds (American Staffordshire Terrier, Dachshund, Boxer, Chihuahua, Cocker Spaniel, Flat-Coated Retriever, Golden Retriever, Labrador Retriever, Lagotto, Norfolk Terrier, German Shepherd, Rottweiler, Schnauzer, Italian Hound, Italian Spinone, Staffordshire Terrier, West Highland White Terrier, and Yorkshire Terrier), of various ages (range 1.5–9 yr) and parity (range 0–7) were included in this study, which was carried out from 2006 to 2009. The bitches were housed in 20 breeding kennels in Northern Italy, all with similar characteristics as regards number of housed animals, type of management, and whelping facilities. The kennels were divided into three categories according to the frequency of antimicrobial use, ranging from no routine administration before and after parturition (category 1 \_ 8 kennels), to almost routine administration of amoxicillin or amoxicillin-clavulanic acid given to nonprimiparous bitches which had had stillbirths or high mortality rates in the previous parturitions, 7 to 10 days before whelping (category 2 \_ 9 kennels), and to routine administration of various antimicrobial agents before and after parturition (second and third generation cephalosporins, macrolids, adopted after amoxicillin/ampicillin, amoxicillin-clavulanic acid, and aminoglycosides had, in the breeders’opinion, become ineffective) (category 3 \_ 3 kennels; Table 1). Thirty-eight bitches belonged to kennels in category 1, 30 to kennels in category 2, and 24 to kennels in category 3. Stillbirths and neonatal deaths occurring within 2 wk of parturition were recorded for each whelping. “Stillbirths” include all deaths which we considered were due to pathologic conditions related to pregnancy/parturition; neonatal deaths were presumed to be due to conditions occurring after birth.

*2.2. Sample collection and bacteriologic identification*

A deep vaginal swab for bacteriologic examination was collected from each bitch, 7 to 10 days before parturition and just before the start of antibiotic treatment. The sterile swab (Copan Innovation, Brescia, Italy) was introduced into the cranial vagina, after thorough disinfection of the vulval labia with a povidoneiodine solution, and placed in the Amies transport medium provided with the swab. On the same occasion, whenever it could be obtained, a drop of secretion was expressed from each of the caudal mammary glands on a sterile swab, after local disinfection. A milk sample was obtained in a similar way, approximately 7 days postpartum, a few days after interruption of antibiotic administration. Bacteriologic cultures were grown following standard methods for isolation and identification of bacteria. Inoculated media (blood agar base [Oxoid, Basingstoke, UK] with 5% of buck blood [DiaTech, Jesi, Italy] and a selective medium for *Enterobacteriaceae* [Mac Conkey agar, Oxoid]) were incubated at 37 °C in both aerobic conditions and a modified atmosphere (5% CO2) for 24 h. Initial identification of bacteria was performed by macroscopic observation of colonies, Gram-stain reaction, cellular morphology, growth on selective medium, catalase and oxidase tests, mobility test, and oxidation-fermentation test. Commercial media incorporating biochemical tests and specific miniaturized methods (API System; BioMérieux, Marcy l’Etoile, France) were also carried out to identify bacteria. Streptococci were identified according to the type of hemolysis on blood agar, evaluation of serologic Lancefield groups, and API 20 strep (BioMérieux). Specific coagulase-positive staphylococci were identified by coagulase reaction on rabbit plasma (Istituto Zooprofilattico delle Venezie, Legnaro, Italy) and API Staph ID 32 (BioMérieux). Molecular analysis were carried out to differentiate *Staphylococcus aureus* and *Staphylococcus pseudintermedius* [19,21]. Gram-negative bacteria were grouped into classes according to their oxidase activity, *Enterobacteriaceae* as oxidase-negative, and other gram-negative bacteria as oxidase-positive. To identify species, lactose ferment reaction on Mac Conkey agar, and other biochemical characteristics (carbohydrate fermentation, indole and hydrogen sulfide production, urease activity) were assessed on commercial media incorporating biochemical tests and/or miniaturized methods (API 20E and API 20NE Identification System, BioMérieux).

*2.3. Potentially pathogenic bacteria*

*Staphylococcus pseudintermedius, Staphylococcus aureus*, and *Streptococcus canis* were examined for Gram-positive bacteria, and *Escherichia coli* for Gramnegative ones, respectively, as potentially pathogenic bacteria. Both types are part of the normal microbial flora in the dam’s vagina and milk but, according to evidence in the literature, may become opportunistic pathogens, causing local inflammation and septicemic illness, in immunocompromised puppies and newborns [3,7–10,12,13,16,25].

*2.4. Antimicrobial susceptibility test*

Antibiotic susceptibility tests were performed with the Kirby-Bauer disk diffusion method in Mueller-Hinton agar (Sclavo Diagnostics International, Siracusa, Italy) according to the guidelines of the Clinical Laboratory Standards Institute [23]. Gram-positive strains were tested with the following antimicrobial drugs: penicillin G (10 IU), ampicillin (10 µg), amoxicillin-clavulanic

acid (10 and 20 µg), gentamycin (10 µg), oxacillin (1 µg), cephalotin (30 µg), cefuroxime (30 µg), spiramycin (100 µg), streptomycin (10 µg), tetracycline (30 µg), tilmicosin (15 µg), tylosine (30 µg), enrofloxacin (5 µg),rifampicin (30 µg), tiamulin (30 µg), and trimethoprimsulfamethoxazole

(1.25 and 2.75 µg). Gram-negative strains were tested with sulfisoxazole (300 µg), trimethoprim-sulfamethoxazole (1.25 and 2.75 µg), streptomycin (10 µg), kanamycin (30 µg), gentamycin (10 µg), aminosidine (60 µg), ampicillin (10 µg), cephalosporin III (30 µg), amoxicillin/clavulanic acid (10 and 20 µg), tetracycline (30 µg), colistin (10 µg), nalidixic acid (30 µg), enrofloxacin (5 µg), spectinomycin (100 µg), and apramycin (30 µg). The criteria for interpreting zone size limits and quality control of material and reagents were those of the Clinical Laboratory Standards Institute [22,23] or, when necessary, according to literature data; the strains were then classified as being susceptible, intermediate, or resistant to drugs.

*2.5. Analysis of data*

The number of whelpings with stillbirths and neonatal mortality events of the total number of parturitions was analyzed by Pearson \_2 test. Stillbirths and neonatal mortality were defined as “within-litter stillbirths” and “within-litter neonatal deaths” and were analyzed, according to kennel category, by the Kruskal-Wallis nonparametric test. Data on site of isolation, Gram-positive and Gramnegative species and strain resistance were analyzed by Pearson \_2 test, taking into account:● frequency of isolation of potentially pathogenic bacteria according to kennel category and site of isolation; frequency of multiresistance of Gram-positive and Gram-negative bacteria according to kennel category; multiresistance was defined as in vitro resistance toward at least 4 antimicrobials [22]. Statistical significance was set at P \_ 0.05. All analyses were carried out with the SPSS statistical package (SPSS 12.0.1, SPSS, Inc., Chicago, IL, USA).

**3. Results**

*3.1. Stillbirths and neonatal deaths*

A total of 535 puppies were born from 92 whelpings. Forty-eight of 535 puppies (9%) were stillborn, and 106 (19.8%) died in the first 2 wk after birth. Kennel category was not associated with a significantly different number of stillbirths or neonatal deaths (Table 2). Taking into account only those whelpings in which there were stillbirths and neonatal deaths (Fig. 1), “within litter stillbirths” and “within-litter neonatal deaths” were not significantly different among kennel categories.

*3.2. Bacterial isolation*

A total of 84 vaginal specimens, 59 prepartum mammary secretions and 76 postpartum milk specimens were collected. A total of 313 bacterial strains was isolated and 173 were identified as *Staphylococcus pseudintermedius* (N= 88), *Streptococcus canis* (N =30), and *Escherichia coli* (N = 55), respectively. No *Staphylococcus aureus* was isolated. The frequency of isolation of commensal bacterial flora (CB) and potentially pathogenic bacteria (PB) in each kennel category is listed in Table 3, and did not differ significantly. In all three kennel categories (Table 3), prepartum mammary swabs yielded the lowest frequency of isolation of PB (χ2 =24.09; df =2; P < 0.001).

*3.3. Antimicrobial resistance*

Antibiotic susceptibility tests were performed on 168 strains of potentially pathogenic bacteria. *Staphylococcus pseudintermedius* isolates (N = 88) showed the following resistance percentages: penicillin G, 93.2%; ampicillin, 90.9%; spiramycin, 89.8%; tetracycline, 50%; and streptomycin, 44.3%. *Streptococcus canis* isolates (N = 30) gave the following percentages: streptomycin, 58.6%, tetracycline, 44.8%, sulfamethoxazol-trimethoprim, 41.4%; and spiramycin, 31%. *Escherichia coli* strains (N=50) were as follows: sulfisoxazole, 24%, streptomycin, 24%, ampicillin, 24%, trimethoprim-sulfamethoxazole, 22%, and tetracycline, 20%.

*3.4. Multiresistance of Gram-positive and Gramnegative bacteria*

Table 4 lists the frequency of isolation of multiresistant Gram-positive and Gram-negative bacteria according to site of isolation and kennel category. Multiresistant Gram-positive bacteria were isolated more frequently than multiresistant Gram-negative ones(56/118 vs. 13/50; χ2= 6.68; df = 1; P < 0.01). Kennel category 3 showed a significantly higher frequency of isolation (86.7%) of multiresistant Grampositive bacterial strains (χ2 =25.62; df = 2; P <0.001); multiresistant Gram-negative strains were not significantly different in any category (χ2 =0.14; df = 2; P< 0.93) (Table 4).

**4. Discussion**

Neonatal mortality in dogs is highest in the first week of life [24] and, although it may depend on many factors, from inadequate environmental conditions to congenital malformations, genetic defects, parasitism, and infectious diseases [1], bacterial infections and septicemia are the prevailing causes [5]. Many dog breeders believe that routine treatment of periparturient bitches with antibiotics may help to prevent neonatal morbidity and mortality. In this study, we found that stillbirths were evenly distributed among kennels, independently of antibiotic use, because they were mostly due to pathological conditions which occurred before and during parturition. Kennel category 3 showed the lowest within-litter mortality but, also in this case, the difference was not statistically significant. Since the entire litter was not lost in those whelpings in which a death event occurred, breeders may have believed that antimicrobial administration was effective. This interpretation of the results is misleading, because misuse of antimicrobials, far from being permanently effective, leads to the selection of resistant strains which have potentially deleterious effects. The hypothesis that routine antimicrobial administration cannot and does not represent standard integration of whelping management is confirmed by our results. Our study did not show significant differences in the isolation of potentially pathogenic bacteria in the three kennel categories, despite the different use of antibiotics, but we did find a significantly higher antimicrobial resistance rate of Gram-positive potentially pathogenic bacteria in kennel category 3. The canine vagina normally harbors microorganisms [14]. Those colonizing the vaginal mucosa are usually in a state of balance both with the host and with one another, and may therefore protect the host from pathogens [12]. Antibiotic administration affects the vaginal flora: Ström and Linde-Forsberg [25] showed that treatment with ampicillin or trimethoprim-sulfametoxazole induces the selection of *E. coli* from vaginal cultures of bitches. The normal bacterial flora has the task of preventing overgrowth of bacterial species which may cause much local damage or be life-threatening for puppies during colonization. Many authors have observed that organisms from healthy bitches and from ones with vaginitis are qualitatively similar [13,16] but the quantitative count is higher in bitches with vaginal discharge [26]. Saijonmaa-Koulumies and Lloyd [27] report that the degree of colonization by *Staphylococcus pseudintermedius* in bitches affects the degree of colonization of their puppies, and high bacterial colonization may predispose to infection. The same observations apply to *E. coli* [2]. Our results confirm the hypothesis that, when antibiotics are not used, many bacterial species can be isolated from the same specimen, whereas extensive use of antibiotics reduces the variety of the bacterial flora and selects resistant strains. Our results also show that antibiotic administration to breeding bitches to protect their puppies from disease or death in the immediate postpartum period does not effectively reduce neonatal mortality. On the contrary, the pressure exerted by selective antimicrobials on microbial flora can induce multiresistance, as has been shown in food animals [28]. Resistant strains are difficult to control and may later represent a threat to puppies’ health.The association between excessive antimicrobial administration to dogs and development of resistant bacterial strains has previously been shown in dogs living in kennels [18] and in breeding kennels [19]. On the whole, the resistance rates of *Staphylococcus pseudintermedius, Streptococcus canis*, and *Escherichia coli* which we found were higher than those previously reported [29 –34]: in particular, resistance toward ampicillin, spiramycin, and penicillin G for *Staphylococcus pseudintermedius* [29 –33], and toward tetracycline for *Streptococcus canis* and *Escherichia coli* [29,31,32]. *Staphylococcus pseudintermedius* showed almost 90% resistance to spiramycin and even higher for penicillin G and ampicillin. Bacterial resistance and multiresistance is an emerging problem in hospitalized pets as a cause of nosocomial infections [35]. The possibility that pets may also represent a reservoir of resistant genes and the risk of zoonotic transmission between humans and companion animals are issues under discussion [36,37]. Breeders’ specific skills in obstetrics and husbandry and environmental hygiene are important factors which can reduce neonatal mortality. All hygienic precautions against bacterial overgrowth, such as temperature monitoring, ventilation and humidity control, environmental disinfection, organization of a nursery isolated from other dogs, strict procedures to be followed on entering the nursery (e.g., disinfection of shoes, etc.), should be adopted, with the awareness that antibiotic overuse does not permanently decrease neonatal mortality but, on the contrary, increases the risk of selecting resistant bacterial strains, thus decreasing the therapeutic properties of antimicrobials day by day.

*4.1. Conclusions*

Excessive antimicrobial administration to breeding bitches during the peripartum period leads to a reduction in the variety of commensal bacteria flora without reducing the frequency of isolation of potentially pathogenic bacterial strains. On the contrary, potentially pathogenic bacteria show increased resistance to antimicrobials. Thus, puppies are exposed to higher risks of difficult-to-treat infections. An effective and permanent reduction in neonatal losses requires in-depth study to identify and correct predisposing structural factors [7]. More attention must be directed to the control of microbial flora through environmental hygiene and the adoption of strategies to prevent the selection of pathogenic bacteria due to drug misuse and uncontrolled treatments.

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