Preventive Veterinary Medicine 116 (2014) 209–213

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Short communication

Inadequate passive immune transfer in puppies: definition, risk factors and prevention in a large multi-breed kennel

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ARTICLE INFO

Article history:
Received 22 July 2013
Received in revised form 7 May 2014
Accepted 8 May 2014

Keywords:
Puppy
Colostrum
Immunoglobulin G
Mortality
Passive immune transfer
Supplementation

ABSTRACT

The prevalence of neonatal mortality is high in the canine species and far from well-studied. In most domestic neonates, an appropriate colostrum intake is a key element of the control of neonatal mortality. The aim of this study was to evaluate the impact of passive immune transfer on puppy mortality, assessed through serum immunoglobulin G (IgG) concentration at 2 days of age. Factors impacting passive immune transfer and the value of an oral immunoglobulin supplementation to prevent it were also analyzed. A total of 149 puppies from 34 litters (12 breeds) within one breeding kennel were included. Blood samples were collected at 2 days of age and colostrum was collected from their dams 1 day after whelping to assay IgG concentration. Puppies were weighed at birth and at 2 days of age for calculation of growth rate. Mortality was recorded until 3 weeks of age. Seventy randomly assigned puppies were orally supplemented with hyper-immunized adult plasma twice within the first 8 h of life. IgG concentration at 2 days of age was significantly correlated with weight gain during the first 2 days of life. The multivariable model with litter as a random effect demonstrated that neonatal mortality was not influenced by breed size, sex, supplementation, litter size, nor colostrum IgG concentration, but by puppy IgG concentration at 2 days of age. According to the ROC curve, the minimal IgG concentration at and below which puppies were at higher risk of death was determined at 230 mg/dl. Puppy IgG concentration was significantly associated with growth rate, but not with breed size, sex, supplementation, litter size or colostrum IgG concentration in a multivariable model with litter as a random effect. This study demonstrates that neonatal mortality in puppies is related to the quality of passive immune transfer. The oral supplementation with hyper-immunized canine plasma neither decreased risk of mortality, nor improved serum IgG concentration at 2 days of age in puppies. Attention must thus be paid to early colostrum intake to control the neonatal mortality in puppies.

1. Introduction

Neonatal mortality in the canine species (within the first 3 weeks after birth) is highly prevalent ranging between 17 and 26% (Bowden et al., 1963; Nielen et al., 1998; Indrebø et al., 2007). Infectious diseases are described as the primary cause of death in puppies born alive

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http://dx.doi.org/10.1016/j.prevetmed.2014.05.001
0167-5877/

(Nielen et al., 1998; Van der Beek et al., 1999) with *Escherichia coli*, *Staphylococcus* sp., *Streptococcus* sp., canine herpesvirus type 1 most frequently isolated from neonates dying within the first week after birth (Münnich, 2008; Dahlbom et al., 2009).

Puppies, as well as calves, piglets, foals and kittens, are born agammaglobulinemic or hypo-gammaglobulinemic (Bouchard et al., 1992). In many species, it has been demonstrated that adequate transfer of maternal immunity through colostrum is crucial for survival and control of infectious diseases in the newborn (Tyler et al., 1998; Christley et al., 2003; Vallet et al., 2013). Blood immunoglobulin G (IgG) concentration in neonates is the routine criteria used to evaluate the quality of passive immune transfer (Beam et al., 2009). Transfer of immunoglobulins from dam to a newborn is influenced by numerous maternal factors (age of the dam, parity, maternal behavior, quantity and quality of colostrum), together with several neonatal factors (litter size, birth weight, vitality, time and quantity of ingested colostrum). Despite the high prevalence of lethal infections in canine species, the direct link between mortality and passive immune failure has been poorly evaluated in puppies.

The economic importance of correct passive immune transfer in large animals has led to the development of numerous colostrum replacers and immunoglobulin supplements (calves: Vega et al., 2011; piglets: Yokoyama et al., 1992; foals: Franz et al., 1998). In contrast, few studies have investigated IgG supplementation in small animal neonates. In experimental conditions, adult dog serum, administrated either orally or subcutaneously to colostrum-deprived newborn puppies, proved to be an alternative source of immunoglobulins (Bouchard et al., 1992). However, there is no data in the literature regarding the improvement of passive immune transfer in puppies under natural conditions and with unlimited access to colostrum, with the aim to control mortality.

The first objective of this study was to evaluate the impact of passive immune transfer through puppy blood IgG concentration at 2 days of age on mortality within the first 3 weeks after birth; subsequently to determine a critical threshold below which the risk of neonatal mortality is significantly increased. The second objective was to identify factors influencing IgG concentration at 2 days of age. A third objective was to estimate the efficacy of an oral immunoglobulin supplementation within the first hours after birth on passive immune transfer.

2. Materials and methods

2.1. Animals

The experiment was conducted in a French breeding kennel from March to June 2012. All canine neonates born during this period ($n = 195$) and their dams ($n = 39$) were included in the study. All bitches in the kennel were routinely vaccinated annually (EURICAN CHPPI2, Merial, Lyon, France). Puppies were housed with their dam in a single, heated whelping box (2–4 m^2 of surface) from birth until 56 days of age and were allowed to suckle freely. Both bitches and their offspring at weaning were fed a dry balanced diet for growing dogs (Starter, Royal Canin, Aimargues, France) ad libitum. From a total of 195 puppies born in the kennel, 149 puppies from 34 litters which survived with no abnormalities until the time of blood collection were included in the study. Depending on the average adult body weight of their breed, puppies were divided into small breed dogs (body weight < 25 kg: Poodle ($n = 10$), Cocker Spaniel ($n = 16$), Bichon Frise ($n = 9$), Bichon Maltese ($n = 15$), Lhasa Apso ($n = 20$), Shih Tzu ($n = 17$), West Highland White Terrier ($n = 12$), Jack Russell Terrier ($n = 3$), Pomeranian ($n = 4$)) and large breed dogs (body weight ≥ 25 kg: Labrador Retriever ($n = 10$), Golden Retriever ($n = 28$), German Shepherd Dog ($n = 5$)).

2.2. Data collection and immunoglobulin G assay

Immediately after birth, each neonate was identified by a colored woolen collar and its breed and sex were recorded. Puppies were weighed at birth and at 2 days of age to calculate a growth rate over the first 2 days of life ((weight at 2 days – weight at birth)/weight at birth). For each litter, the total number of puppies born was recorded (litter size). Mortality over the first 3 weeks after birth was also recorded. One milliliter of blood was collected into a plain tube from each puppy at 2 days of age (between 36 and 48 h) from the jugular vein. One day after whelping onset, 0.5–1 ml of colostrum was collected from the dams. Milk and blood were stored at −20 °C until IgG assay in duplicate using a previously described and validated ELISA method (Dog IgG-Quantitation Kit, Bethyl Lab, Montgomery, USA) (Schäfer-Somi et al., 2005).

2.3. Immunoglobulin supplementation

Seventeen large breed non pregnant bitches from the same kennel, which were not included in the protocol described above, were vaccinated against canine herpesvirus type 1 (EURICAN Herpes 205, Merial) and *Bordetella bronchiseptica* (PNEUMODOG, Merial). The same vaccination was repeated 2 weeks later combined with a polyvalent vaccine (EURICAN, Merial). Two weeks after the last vaccination, blood (7.5 ml/kg body weight) was collected from each bitch into heparinized containers and centrifuged. Plasma from the 17 bitches was pooled and aliquoted before storage at −20 °C. Plasma IgG concentration was assayed as previously described. Ensuring equal distribution of individuals, in terms of birth weight and breed size, puppies were assigned to 2 different groups within each litter. In the first group (non-supplemented, $n = 79$), puppies did not receive hyper-immunized plasma. In the second group (supplemented, $n = 70$), puppies received 2 doses of hyper-immunized plasma before intestinal barrier closure: the first dose was administrated a maximum of 4 h after birth by a feeding tube (1.5 ml/100 g body weight); the second administration, using the same method and dose, was performed 4 h later, at a maximum of 8 h after birth. Both supplemented and non-supplemented puppies were allowed to suckle their dam during the entire experiment. The dose of hyper-immunized plasma administrated to puppies was chosen as the best compromise between maximal volume for administrated supplement

and maximal volume for ingested colostrum, as volume of stomach in newborn puppy is 5 ml/100 g of body weight.

2.4. Statistical analyses

Statistical analyses were performed with the SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA) to determine the risk factors for mortality and factors influencing puppy IgG concentration. Spearman's rho correlation coefficient was used to evaluate the correlation between puppy IgG concentration at 2 days of age, IgG concentration in colostrum, litter size and growth rate.

Firstly, a generalized linear mixed model (proc GLIMMIX) with mortality as a binary outcome (logit transformation) was used to assess the following fixed effects: sex (male/female), breed size (small/large), supplementation (yes/no), litter size, colostrum IgG and puppy IgG concentrations. Litter was modeled as a random effect. Two way interactions between sex and supplementation were investigated, as it improved the quality of the model. As it was found that growth rate and puppy IgG concentration were correlated; puppy IgG concentration was chosen to be included into described model, as evaluation of its effect on mortality was of primary interest. Subsequently, a receiver operating characteristic (ROC) curve was drawn based on the final logistic model. The Hosmer and Lemeshow Goodness-of-Fit test was used to assess the quality of this model. Youden's index was used to define the best cutoff from the model for high and low mortality risk populations. Finally, a Kaplan–Meier plot and the Log-rank test were used to assess the survival between these two groups.

Secondly, a linear mixed model (proc MIXED) was performed to determine the variables affecting puppy IgG concentration at 2 days of age. As residuals of this multivariable model were not normally distributed, a non-parametric analysis was performed (rank transformation of the outcome). This model included as fixed effects: breed size, sex, supplementation with their related interactions (encoded as described for previous model). Moreover, the effects of litter size, colostrum IgG and growth rate were added as covariates. Litter was modeled as a random effect.

Quantitative data are presented as medians with interquartile range (IQR).

3. Results

3.1. Study population

The total mortality in puppies within the first 3 weeks of life was 31.3% (61/195). Among all puppies 28.7% (56/195) died within the first week after birth. IgG concentration was assayed on sera from 149 puppies which survived until the blood collection (Day 2) and on colostrum from their 34 dams. Eighteen out of 149 puppies died before 3 weeks of age. The IgG concentration in puppies at 2 days of age was 610 mg/dl (360; 975 mg/dl). The colostrum IgG concentration was 1940 mg/dl (1500; 2290 mg/dl). IgG concentration of the pooled hyper-immunized plasma was 1430 mg/dl, i.e. 73.7% of median IgG concentration in colostrums collected in this experiment. The growth rate between birth and 2 days of life was 3.1% (−5.2; 10.9%) and was significantly correlated with puppy IgG concentration ($\rho = 0.7$; $p < 0.001$). The other variables in correlation matrix were not correlated.

Table 1
Risk factor assessment for mortality in puppies between 2 and 21 days after birth evaluated by a generalized linear mixed model ($n = 149$).

Factor	*P* value	Odd ratio	95% confidence interval	
Breed size	0.386	0.3	0.0	4.8
Sex	0.765	0.8	0.2	3.1
Supplementation	0.851	0.8	0.1	5.0
Litter size	0.382	1.2	0.8	2.0
Colostrum IgG	0.728	1.0	0.9	1.2
Puppy IgG at Day 2	0.018	0.7	0.5	0.9

3.2. Factors influencing mortality

In the generalized linear mixed model, risk of neonatal mortality was influenced by puppy IgG concentration at 2 days of age and litter effect as a random term ($p = 0.018$; $p = 0.003$; respectively). Puppies with lower IgG concentrations presented higher risk of death than other puppies (Table 1). IgG concentration in puppies dying between 2 and 21 days was 172 mg/dl (42; 508 mg/dl) vs. 669 mg/dl (430; 1030 mg/dl) in puppies still alive at Day21. None of the other factors tested (sex, breed size, supplementation, colostrum IgG concentration and litter size) demonstrated any influence on neonatal mortality (Table 1). The optimal cutoff value of IgG concentration to assess predictive likelihood of mortality was 230 mg/dl (odd ratio = 0.7; 95% confidence interval: 0.5; 0.8) with a sensitivity of 66.7% and a specificity of 87.8%. Among the 149 puppies which survived until the blood collection, 18.1% (27/149) presented a blood IgG concentration at 2 days of age below or equal to 230 mg/dl. The survival analysis indicated significant differences in survival kinetics between puppies above or under the cutoff value for IgG concentration ($\chi^2 = 30.33$; $p < 0.001$; Fig. 1). Among puppies with IgG concentration ≤230 mg/dl at 2 days of age, 44.4% (12/27) died, while it

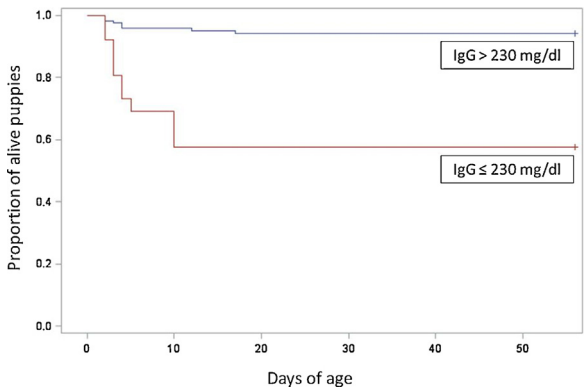

Fig. 1. Kaplan–Meier plot of survival kinetics in puppies above ($n = 123$) and under ($n = 26$) the critical threshold of puppy IgG concentration at 2 days of age (230 mg/dl) for higher risk of death ($\chi^2 = 30.33$; $p < 0.001$).

was the case only for 4.9% (6/122) of puppies with higher IgG concentration.

3.3. Factors influencing blood IgG concentration in puppies at Day 2

Growth rate between 0 and 2 days after birth and the litter effect as a random term had an impact on puppy IgG concentration at 2 days of age ($p < 0.001$; $p = 0.007$, respectively). None of the other factors tested in this linear mixed model (breed size, sex, supplementation, colostrum IgG concentration, litter size) demonstrated any influence on IgG concentration at 2 days of age.

4. Discussion

4.1. IgG concentration and mortality

The percentage of neonatal mortality (over the first 3 weeks of life) was higher in this study (31.3% of all born puppies) compared with other published data (Nielen et al., 1998; Indrebø et al., 2007). Among the puppies that died during the first 3 weeks after birth, 91.8% died within 7 days, similar to other studies (Nielen et al., 1998; Tønnessen et al., 2012). In many species, passive immune transfer through colostrum is known to be crucial for the control of neonatal mortality (i.e. in bovine Tyler et al., 1998; in porcine Vallet et al., 2013).

We evidenced in this study a strong relationship between passive immune transfer (IgG concentration assayed in serum at 2 days of age) and neonatal mortality in puppies. In our experiment puppy IgG concentration below or equal to 230 mg/dl defines the deficit of passive immune transfer in the canine species associated with decreased survival rate. The reliability of this threshold has to be evaluated in other kennels, with different management and infectious conditions. Dam, as one of a random term for all puppies coming from the same litter, displayed a significant impact on canine neonatal mortality in this study. Maternal factors influencing colostrum ingestion in other species are maternal behavior, quantity of colostrum produced and anatomic morphology of teats (more or less easy to be suckled) (Rooke and Bland, 2002). In this study, neither breed size nor litter size had an influence on neonatal mortality, although Tønnessen et al. (2012) previously reported higher mortality rates in puppies from large litters and puppies belonging to large or giant breeds.

4.2. Factors influencing IgG concentration

In our study, growth rate over the first 2 days of life was highly associated with puppy serum IgG concentration. Both reflect colostrum intake, as this secretion plays not only an immune but also nutritional role. In piglets, the concentration of serum immunoglobulins was found to be associated with the quantity of colostrum ingested (Rooke and Bland, 2002). It suggests that routine weighing of newborns can be used to evaluate the colostrum intake and thus, indirectly the passive immune acquisition in puppies. Although colostrum provides energy together with immunological protection, absorption of immunoglobulins, unlike nutrients, depends on time elapsed from birth, because of the progressive intestinal barrier closure. In puppies, IgG absorption rate at 4 h of life is decreased two-fold compared to the level at birth and is almost null at 12–16 h of life (Chastant-Maillard et al., 2012). As observed for mortality, common effect within a litter, such as dam also influenced immunoglobulin transfer in puppies. Dystocia, inappropriate care of newborns or absence of colostrum secretion at whelping onset may decrease the passive immune transfer in puppies before the gut closure. In cattle and pigs, attention is paid to the immune quality of colostrum, since IgG concentration in colostrum is known to be a significant factor in the quality of passive immune transfer to the offspring (Beam et al., 2009; Devillers et al., 2011). In the present study, the IgG concentration in colostrum was significantly associated neither with mortality, nor with serum IgG concentration of puppies. The relationship between IgG level in colostrum and puppy serum could be nonexistent or masked by several factors of larger impact such as timing of colostrum intake and quantity of colostrum ingested by the newborn.

4.3. Hyper-immunized plasma supplementation

Supplementation was designed to optimize IgG absorption and decrease the risk of failure of passive immune transfer: plasma was administrated orally before intestinal barrier closure (within the first 8 h of life; Chastant-Maillard et al., 2012), and plasma immunoglobulins were protected from digestion since puppies were suckling colostrum providing antitrypsin. Nevertheless, in our experiment, no effect of supplementation on neonatal mortality has been observed, but a lack of statistical power cannot be ruled out. Since puppies had free access to the colostrum of their dam, the supplementation may have partially substituted spontaneously ingested colostrum. As the hyper-immunized plasma used in the experiment had an IgG concentration 30.5% lower than the colostrum, supplementation might even have decreased the total quantity of ingested immunoglobulins. In another study, puppies fed milk replacer and adult serum while being colostrum deprived, absorbed significantly less IgG than puppies fed colostrum (Bouchard et al., 1992). The efficacy of supplementation with higher IgG concentration in order to decrease the frequency of passive immune failure needs to be tested.

5. Conclusions

This study clearly demonstrates that neonatal mortality in puppies is related to passive immune transfer. Based on these data, obtained from one kennel, the deficit of immunoglobulin G in puppies, associated with higher risk of death, was defined at 230 mg/dl. More data have to be collected to confirm this cutoff value. However, our results show that attention has to be paid to the colostrum intake during the first 12 h of life in order to diminish the risk of passive immune failure and mortality in canine neonates. To date, at variance with some other species, no efficient alternative source of immunoglobulins is available for puppies. The design of an adequate immune booster

immediately after birth, to decrease high mortality rate in canine neonates remains to be established.

Conflict of interest

No product branded by Royal Canin has been tested in the experiment and authors belonging to Royal Canin staff have no conflict of interest to declare. Other authors also declare no conflict of interest.

Acknowledgments

This study was partially funded by Royal Canin SAS (Aimargues, France) (grant no. R3789 1/02/2012). Royal Canin SAS participated in the study design and statistical analyses. We would like to thank the owner of the kennel for his contribution to this work and Mr. Adrian Watson for the English revision of the manuscript.

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