Cleft Palate with Autosomal Recessive Transmission in Brittany Spaniels

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In the course of maintaining a large colony of Brittany spaniels for studying a dominantly inherited motor neuron trait, cases of sporadic complete cleft palate were observed. Without intervention, the pups with cleft palate that attempt to nurse, aspirate and die. In this study, we report on the incidence of cleft palate in this dog kindred, describe the gross morphologic characteristics of the cleft, and present a morphometric analysis of the skull of two of the cleft palate pups and one unaffected pup that died at birth. Our data thus far indicate 26.9% incidence of cleft palate in the colony. Pedigree analysis indicates that this cleft palate trait is inherited as an autosomal recessive. High resolution computed tomography scans of the pup heads were used in morphometric comparison of normal and cleft palate pups. We found specific morphologic differences between the cranial base and palate of normal and cleft palate pups. Plans for future studies of the genetics and growth and development of this animal model are discussed. This canine cleft palate trait provides an ideal model for studying a malformation common in humans.

KEY WORDS: *animal models, Brittany spaniels, cleft palate, computed tomography, genetics*

Cleft palate (CP) is a frequent clinical problem that requires complex management; it occurs in white and black individuals in 1/2000 to 1/2500 live births (Owens et al., 1985). Clefts involving both hard and soft palate show a 2:1 female:male frequency (Gorlin, 1993). Combined cleft lip and palate, which, with varying severity, is about twice as common as isolated cleft lip (Vanderas, 1987).

The concordance for isolated CP in humans is 22% for monozygotic twins and 4.6% for dizygotic twins (Gorlin et al., 1990). The best current model for combined cleft lip and palate is based on a multifactorial process involving a threshold (Fraser, 1974). However, the occurrence of CP is not well explained by that model and this may reflect etiologic heterogeneity (Shields et al., 1981). Thus, genetic counseling for CP must be based on empiric risk figures (Tolarovà, 1972). Importantly, 20 to 50% of human CP is associated with other congenital anomalies (Gorlin, 1993). Shprintzen et al. (1985) estimated that approximately 5% of facial clefting is syndromic and about half of these are simple Mendelian traits. Gorlin et al. (1990) estimate that there are now over 250 cleft associated syndromes in humans.

Considerable effort has been directed at developing animal models for clefting. Riboflavin deficiency can lead to CP in rats (Warkany and Schraffenberger, 1943) as can excess vitamin A (Deuschle et al., 1954). Clefting in the mouse following cortisone injection (Walker and Fraser, 1957) has become a valuable study system. Different strains of mice differ in their susceptibility to both spontaneous and teratogen-induced clefting (Walker and Fraser, 1956; Staats, 1972; Biddle and Fraser, 1976). Steroid-derived and other teratogens also can induce CP in hamsters (Shah, 1984). There is also a report of various combinations of nasal fissures, cleft lips, and CP in 11 of 26 pups sired by a male Dürrbächler dog with a median nasal fissure (Weber, 1959).

We have studied an informative CP trait in Brittany spaniels. In the course of maintaining a large colony of these dogs for studies of other traits (Cork et al., 1979; Winkelstein et al., 1981; Sack et al., 1984), we have observed CP in stillborn pups as well as in those with neo-

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and perinatal death. The aim of this study was to document the genetic background of our colony as it relates to the incidence of CP and to offer preliminary morphologic descriptions of the skulls of these puppies. These observations are combined with a preliminary morphometric comparison of the CP and noncleft palate (NC) pups (see below) to establish this occurrence of CP as a potentially important trait for further study. Our analysis of the incidence of CP and the pedigrees of affected individuals indicate that the trait is inherited as an autosomal recessive which may be associated with a male lethality.

METHODS

Breeding Program

In 1975, a breeding program was initiated to characterize and perpetuate a dominantly inherited motor neuron trait in a kindred of Brittanies. Genetic analysis of the motor neuron trait required intensive inbreeding (Sack et al., 1984). Since the colony was founded, we have maintained detailed records and have observed sporadic cases of complete CP in several newborn pups (Fig. 1). Because this trait was unexpected and, therefore, undiagnosed at birth, the pups born to this colony with this specific form of CP aspirated and died when they attempted to nurse.

The breeding colony was established using a Brittany bitch and her progeny which were sired by her father (father X daughter mating). All subsequent matings were between the descendants of these original dogs except for the following: matings to three purebred, purpose bred beagles from a commercial breeder, and one mating to a Brittany which was related to the dam of the original bitch. Dogs were housed individually or in compatible groups and fed and watered *ad libitum*. Pairs selected for breeding were housed together; artificial insemination was used occasionally.

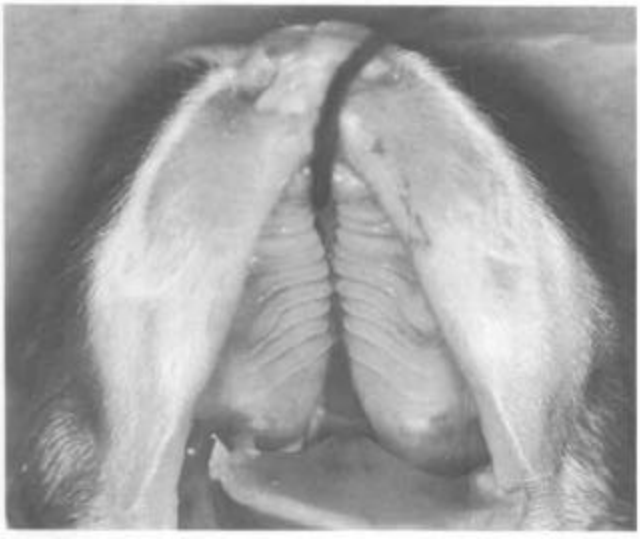


FIGURE 1 Intraoral view of a CP pup to show the cleft palate trait, inferior view.

Individual health records are maintained for each dog from the day of birth. Dogs are assigned an identification number and temporarily marked until they are large enough to be tattooed. The dogs used in these studies were cared for and housed under protocols approved by the Institutional Animal Care and Use Committee of The Johns Hopkins School of Medicine and in accordance with municipal, state, and federal laws governing animal welfare.

Morphometric Comparison

To determine the difference in craniofacial morphology between CP and NC pups, we performed a morphometric analysis. The method used, Euclidean Distance Matrix Analysis (Lele, 1991, 1993; Lele and Richtsmeier, 1991, 1992), enabled us to localize the morphologic differences between CP and NC pups in three-dimensional (3D) space. We used high resolution computed tomography (CT) images of two CP and one NC pup in this analysis. The cost of CT studies, and the potential dangers of anesthesia required for CT scanning of additional live NC pups, resulted in our small sample sizes. Our analysis and results provide information regarding the potential of these dogs for further analysis of the CP trait.

The two CP pups analyzed here died when they first attempted to nurse and were therefore, not more than a few hours old. A single NC pup, found dead in a litter not long after birth, was also analyzed. Both the CP and NC pups were perfused and stored in paraformaldehyde.

The heads of the fixed pups were scanned using the GE Highlight Advantage, housed in the Department of Radiology at The Johns Hopkins University. Three-dimensional coordinates of selected landmarks were recorded from these CT scans by studying individual slice images using the program IMAGE (version 1.47, Wayne Rasband, National Institutes of Health). Once the slice image was identified containing a landmark of interest, the landmark was located in X and Y space according to pixel column and row (Fig. 2). The Z coordinate for each landmark was assigned according to table position. Since each slice was 1.5 mm thick and pixel size was 0.21 mm, precision was disproportionately greater along the X (mediolateral) and Y (anteroposterior) axes as compared to the Z (superoinferior) axis (Corner et al., 1992). We have recently completed a validation study of 3D coordinates of landmark locations collected from CT scans using the same equipment and methods. These data Richtsmeier et al., 1994 showed that landmarks can be located with extremely high precision (i.e., the average intraobserver error in locating a landmark is .8 mm). Validation of the CT data against data collected using a Polhemus Navigation 3Space digitizer (Corner et al., 1992) show the average error on any linear distance to be approximately one

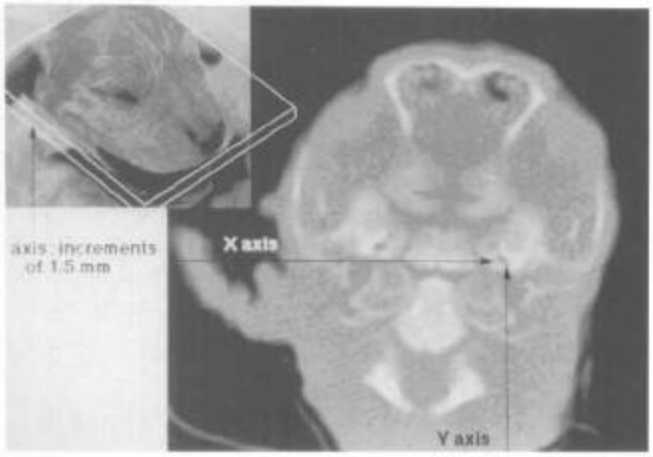


FIGURE 2 A single CT slice image (right) through the cranial base of a pup. Orientation of the slices shown in insert at left. To locate a landmark in 3D space using CT slice images, the slice image containing the landmark of interest is identified. Each slice image measures 512 x 512 pixels and is 1.5 mm thick. X and Y coordinates of the landmark are located according to pixel column and row on the image (for the CT scans used in this study a pixel = .21 mm). The Z coordinate for the landmark is assigned according to the table position of the slice in which the landmark is located. Slices are numbered sequentially as the scanner moves over the subject.

millimeter. In our case, this means that the location of a landmark may be misplaced, on average, by one or two pixels. Figure 3 shows the anatomical location of the 18 landmarks used.

We used Euclidean Distance Matrix Analysis (EDMA) to compare the morphology of CP and NC pup skulls. This method, detailed in the papers cited above, is described here briefly. The method can be used to statistically compare samples, but because our specimens are so few we could not study them statistically. Instead, we used EDMA to quantify and localize the differences in morphology between the two CP and single NC pup.

Once the 18 landmarks are identified and located according to their X, Y, Z coordinates on each specimen, the form of the skull is defined according to the relative location of these landmarks in 3D space. These landmark data can be written as an 18 (number of landmarks) by 3 (number of dimensions) matrix representing the form of the pup skull as defined by the landmarks. These data can be expressed equivalently as a matrix consisting of all possible inter-landmark distances. There are 153 unique linear distances among the 18 landmarks.

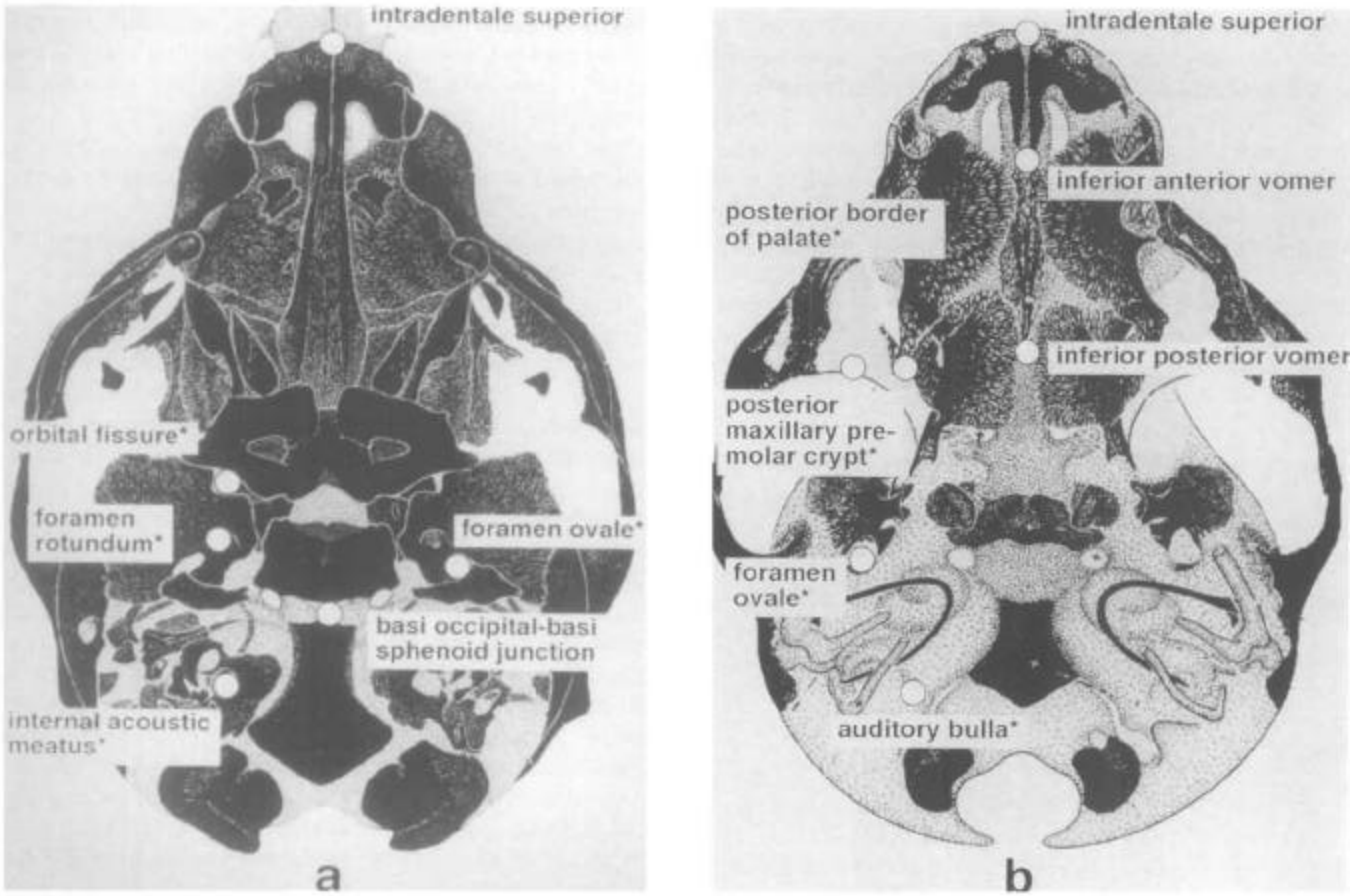


FIGURE 3 Eighteen landmarks located on the computed tomography images and used in analysis as shown on a generalized neonatal pup skull (adapted from Evans and Christensen (1979), Figs. 2–27B and 2–40). Landmarks that occur bilaterally are marked with an asterisk. The neurocranium has been removed from the superior view of the dog skull (A). Note that some of the landmarks (i.e., inferior anterior vomer) plotted on the inferior view (B) are actually located within the skull and not on the surface visualized. The inferior view (B) represents a 45-day beagle fetus. Consequently, the dental crypts are not as fully formed in this drawing as they are at birth (60 days).

To compare two objects A and B, each defined by the 3D coordinates of 18 landmarks and expressed as a matrix of inter-landmark distances, the ratios of homologous linear distances are calculated (Fig. 4). EDMA describes the difference between two forms as a matrix of ratios of like linear distances measured on each of the two forms. Since there are 153 unique linear distances among the 18 landmarks, there are 153 ratios that describe the relative difference between the forms. Each ratio represents the relative difference between a single linear distance in the two forms being studied. Any ratio that equals 1 indicates that the two forms are similar for that particular linear distance. If one is constant throughout the entire matrix, the two forms are the same. If the matrix of ratios is a constant that is not equal to 1, then the difference between the two forms is a matter of scale.

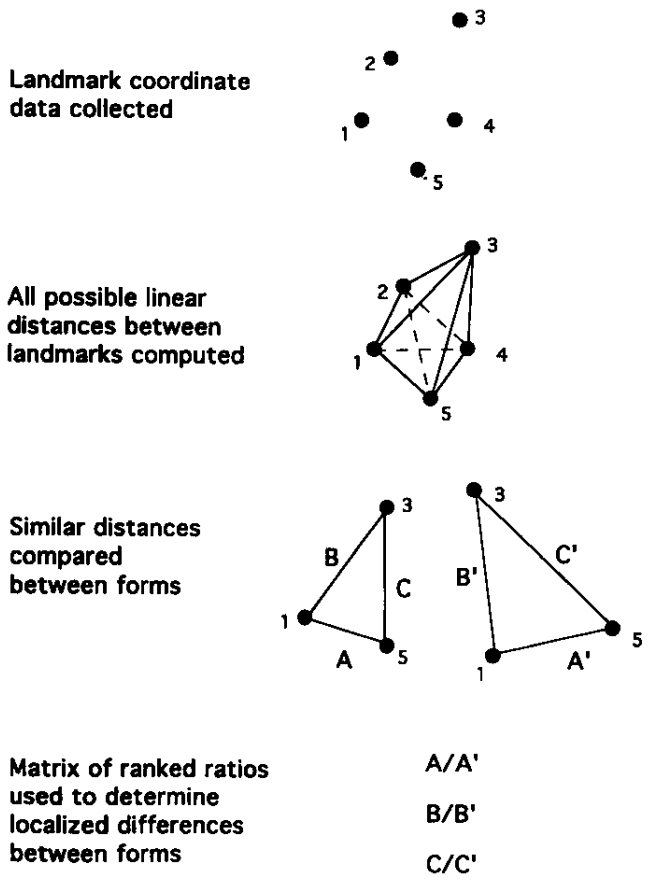


FIGURE 4 Outline of how forms are compared using EDMA. Three-dimensional coordinate data are collected from all specimens to be compared. All linear distances among these landmarks are then calculated. Homologous linear distances from two forms are compared by computing ratios of like linear distances. To keep this diagram simple, only three of the ten possible linear distances are shown at the bottom half of this figure, but an EDMA analysis, by definition, uses all possible linear distances calculated from the landmarks used in analysis.

RESULTS

Genetic and Breeding Studies

In 12 litters consisting of 52 pups, we recognized 14 pups with CP (26.9% incidence) (Fig. 5). The midline cleft extends through all but the most anterior aspect of the entire rostral-to-caudal length of the hard palate (Fig. 6A) and involves all hard tissues of the hard palate (Fig. 6B). This condition was found in all but one instance of CP in the colony. In the exceptional case, CP was associated with a cleft lip.

Postmortem dissections have not revealed additional midline defects or anomalies in CP pups. In ten litters (44 pups) where sex ratios were recorded, the male:female ratio was 15:29, and the ratio of male:female CP pups was 2:9. The size of litters containing CP pups was 4.3; the mean litter size of the remainder of the colony (36 litters with 170 pups) in which no CP pups were identified was 4.7. It should be noted, however, that several litters had neonatal deaths or apparent stillbirths. In these cases, the presence of CP may have been overlooked, particularly during the earlier phases of this project when the trait was not anticipated. If this is so, and CP was not detected among some of the "stillborns" or neonates in several of the smaller litters, the estimated average size of the litters without CP pups may be artificially low.

Taken together, these observations suggest that the CP trait may be lethal for males or may be associated with a trait causing reduced male viability. Pedigree analysis (see Fig. 5) indicates that this CP trait is inherited as an autosomal recessive. Based on this pattern analysis, we have assigned carrier status to earlier dogs as indicated, although testing for this trait could not be performed currently. This trait appears to have been present in the Brittany lineage as we identified pups with CP in purebred Brittanies before outcrosses were made to beagles.

Description and Qualitative Comparison

The CP pups appeared grossly normal with no apparent, external anomalies of the head and neck. As noted above, autopsy and dissection confirmed that the cleft palate was not indicative of a more generalized midline defect. Upon intraoral inspection, an obvious midline cleft of the hard palate is visible (see Fig. 1). The most anterior portion of the palatal shelves of the incisive bones were intact and a midline suture was present. However, the bony processes of the incisive bone that normally abut one another at the midline and separate the right from the left incisive fissures were not present (see Fig. 6A). The most medial edge of the palatal shelves of the maxilla and palatine bones were also absent. The vomer and pterygoid plates were present. The vomer may have been only partially present, however, and histological studies are required to confirm this. The cleft then, involves the following bones

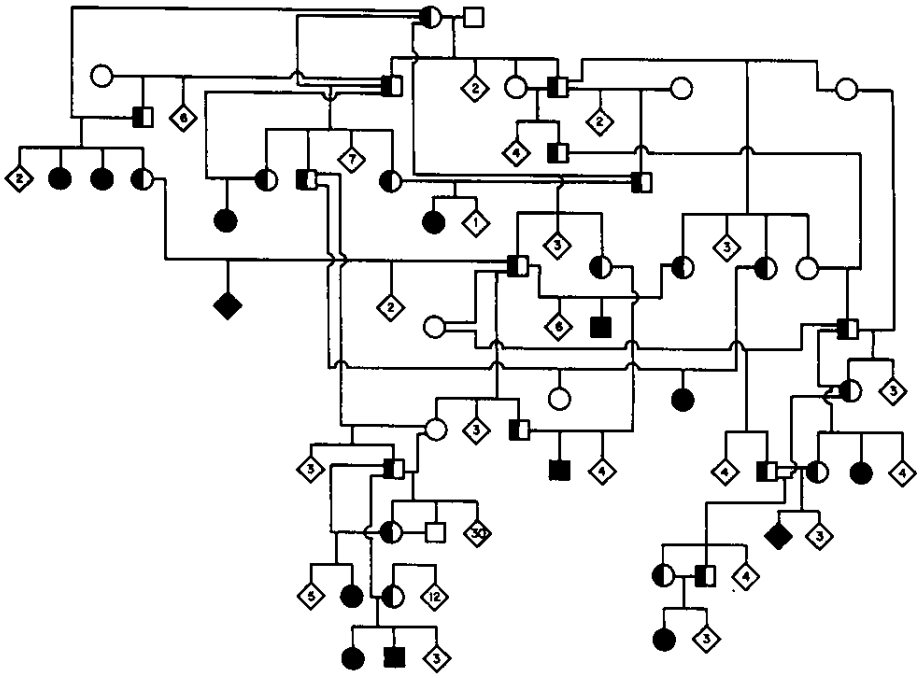
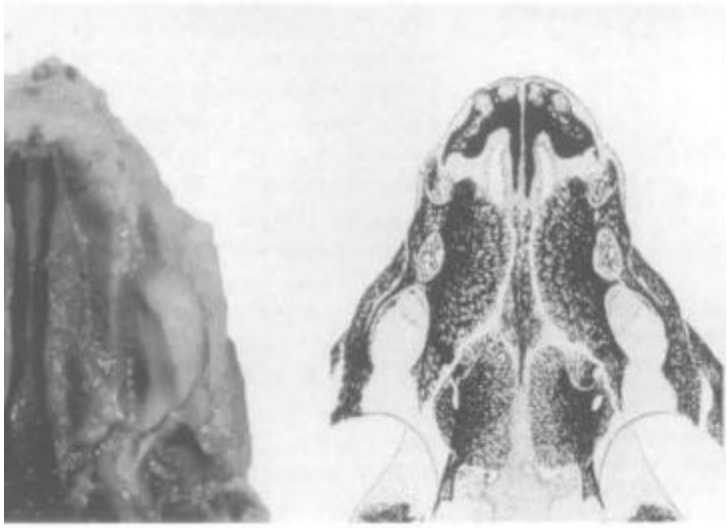


FIGURE 5 Pedigree of CP pups. This pedigree is simplified from the larger study to include only lines implicated in transmitting CP on the basis of the analysis described. Note that it is possible to trace the carrier status to the original Brittanies. Once the autosomal recessive pattern was proposed several of the matings shown were initiated with results consistent with the hypothesis. ◯ designates female, □ designates male, and ◇ designates additional siblings (N is given inside diamond), sex not reported. Filled characters are clinically affected with CP.

as shown in Figure 6B: the incisive, maxillary, and palatine bones, and potentially the vomer. The rudimentary palatal shelves seen in gross dissection (see Fig. 6A) are not visible on the computed tomography scans. The 3D reconstruction (Fig. 7) shows a total lack of hard palate. There are two possible explanations: (1) the medial edge of the palatal projections (see Figs. 1 and 6A) are composed of nonosseous membrane; or (2) the medial border of the palatal shelves is composed of bone that is too thin to be captured by the 3D CT reconstruction that was windowed for bone. Histologic studies are required to determine the composition of the palatal shelves.



Morphometric Analysis

We performed a morphometric analysis of the two CP and one NC pup that were fixed in paraformaldehyde and CT scanned. These data were analyzed to address three questions: (1) Are there substantial differences in shape and/or size of the skull of the two CP pups? (2) Are there substantial differences in shape and/or size between the CP and NC pups? (3) Do the CP pups show any degree of facial asymmetry?

We compared the two CP pups using EDMA and found that although these pups were not identical in shape, 85% of 153 linear distances were similar (ratios of like linear distances ranged from .92 to 1.07). The linear distances

FIGURE 6 Intraoral view of CP pup after partial dissection and removal of palatal mucosal lining (A). Note apparent incisive-maxillary suture and maxillary-palatine suture. At right, (B), a line drawing of a 45-day beagle fetus (beagles have approximately a 60-day gestation period) with lower jaw removed (adapted from Evans and Christensen (1979) Fig. 2–27B) to show the extent of the palate and alveolar surface at this age. Note the individual osseous elements affected by the clefting observed in the pedigree.

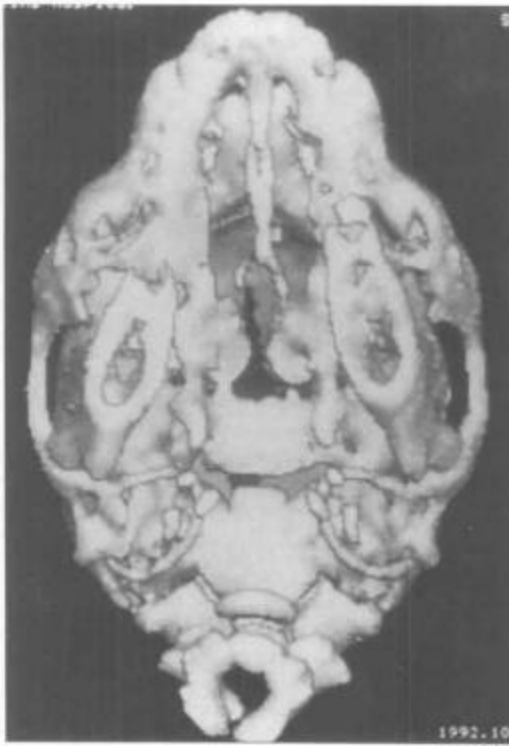


FIGURE 7 Three-dimensional CT reconstruction of CP pup skull, inferior view. The base of the posterior body and ascending ramus of the mandible are visible, but the anterior body of the mandible has been removed by computer. Note the paucity of osseous material on the palatal shelves (compare to Figs. 1 and 6A).

that differed substantially between the two CP pups suggest that one has a relatively longer anterior and middle cranial base, and longer muzzle. We suggest that these differences between the CP pups expresses ordinary individual variability.

Comparison of the CP pups with the NC pup showed that both CP pups differed from the noncleft pup in similar ways. Several linear distances that extend from right to left side of the pup's skull are shorter in the NC pups (Figs. 8A and B). These include width of the posterior palate, distance between the orbital fissures, and the distance between the foramina rotunda. The relatively wider posterior palate and cranial base of the CP pups may be directly related to the cleft. The anteroposterior length of the vomer is shorter in the NC pups as is the distance measured from the internal acoustic meatus to foramen rotundum. However, certain linear distances are more than two times longer in the NC pup as compared to the CP pups (Figs. 8C and D). These include distances measured from foramen ovale to foramen rotundum and from points located on the posterior palate to the orbital fissure landmark. The other anteroposterior oriented linear distances (see Figs. 8C and D) are 40 to 80% longer in the NC pup. Despite these local differences in anteroposterior lengths, the NC and CP pups are similar in total skull length.

Finally, we checked for asymmetry by using EDMA to compare all midline and right sided landmarks to all midline and left sided landmarks within each animal. Surprisingly, the animal with the most notable asymmetry was the NC pup. The largest asymmetries amounted to differences of little more than a millimeter between measures taken on the right and left sides and these differences could represent measurement error. Approximately 9% of the linear distances in the CP pups show asymmetry between right and left sides of about a millimeter, but the majority (91%) of the measures show the right and left sides of the pup skulls to be similar.

DISCUSSION

Natsume and co-workers monitored major breeders of beagles for experiments in Japan to establish the rate of occurrence of cleft lip with or without cleft palate in these beagles. Their goal was to establish a line of beagles in which unilateral cleft lip and palate is spontaneous (Natsume et al., 1994). Data from their 9 year long survey indicate that the rate of occurrence of cleft lip and palate was 1.1 per 1000 beagles, but the phenotype and mode of inheritance has not yet been clearly defined for this animal model. In this study, we have summarized our findings of isolated cleft palate in a colony of Brittany spaniels, a condition that is etiologically distinct from cleft lip with or without cleft palate (Melnick, 1990). The primary palate is formed from fusion of the median nasal prominence and the maxillary part of the mandibular arch bilaterally (Gorlin, 1993). The secondary palate begins its development as swellings on the maxillary prominences that eventually form the palatal shelves. Natsume (1994) and the study presented here provide two animal models that may eventually be useful in clarification of the etiologic distinctions between cleft lip with or without cleft palate, and isolated cleft palate. The potential development of these different, though complementary animal models may allow detailed analysis of the role of various factors such as the embryologic appearance of specific tissues, tissue movement and positioning, and developmental timing of morphogenetic events in the production of the two types of cleft.

In our affected litters, an incidence of CP slightly greater than 25% was shown which would be expected for typical autosomal recessive Mendelian inheritance. Almost twice as many females as males were born in litters in which CP occurred. The relatively high numbers of females in litters in which CP has occurred, the relatively small sizes for litters with CP, and the paucity of males with CP compared to females (only 2 of 9) are also compatible with reduced viability in CP males.

According to Larsen (1993), failure of the human palatine shelves to fuse during development may result from a variety of errors. These include inadequate growth of the palatine shelves, failure of the shelves to elevate at the

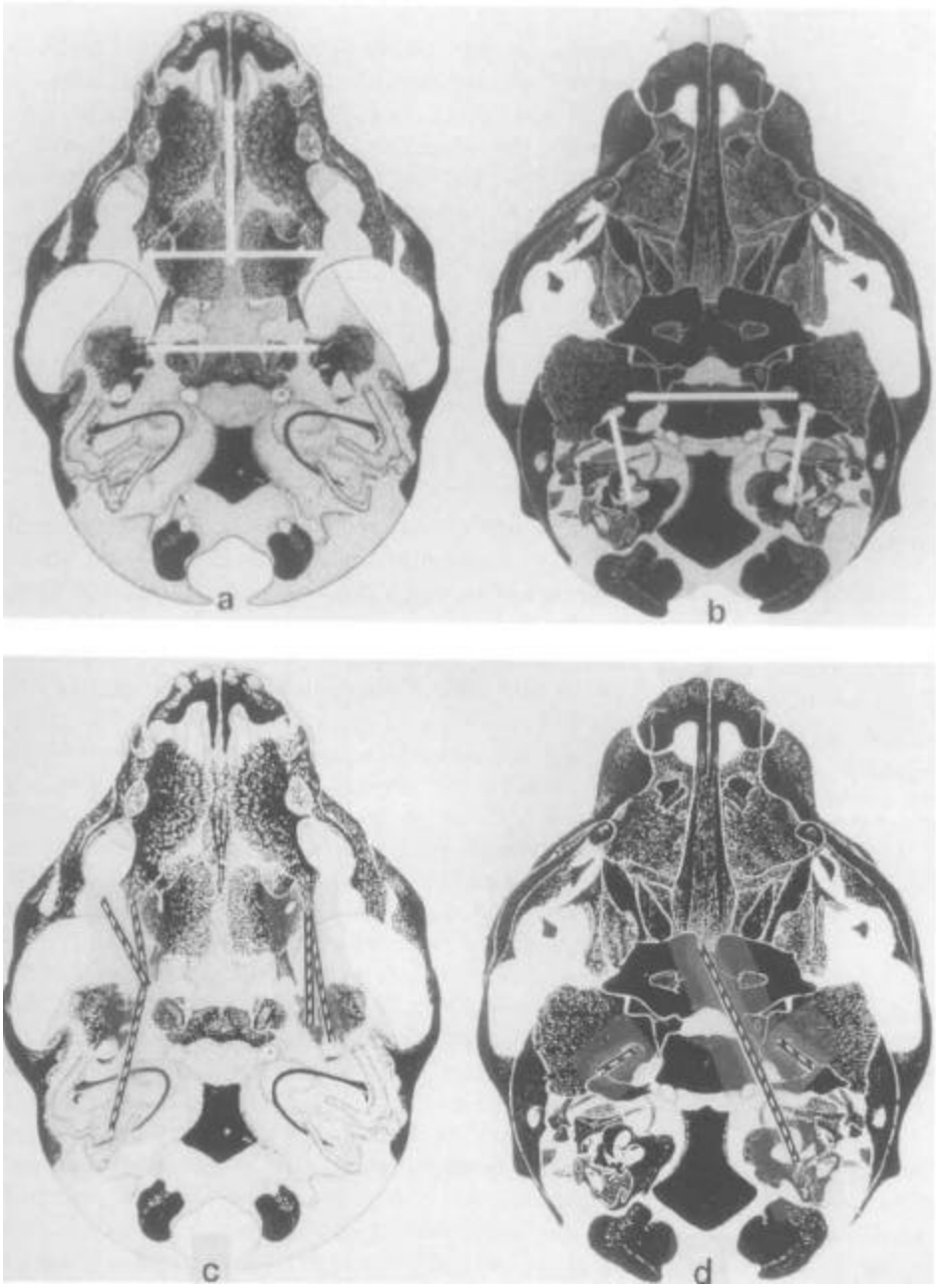


FIGURE 8 Linear distances that differ between CP and NC pups. The pup drawings are adapted from Evans and Christensen (1979) Figures 2–27B and 2–40 and represent a NC pup just before birth. Figures 8A and 8C show a ventral view of the skull with the mandible removed. Figures 8B and 8D show a dorsal view of the skull with the calvaria removed. Lines shown in white in Figures 8A and B are linear distances between specific landmarks that are relatively shorter in our NC neonatal pup compared with the CP pups. These linear distances include: inferior anterior vomer to inferior posterior vomer; left posterior border of the palate to right posterior border of the palate; left orbital fissure to right orbital fissure; left foramen rotundum to right foramen rotundum; foramen ovale to internal acoustic meatus (both sides). Lines shown in stipple on Figures 8C and D represent linear distances between landmarks that are relatively longer in the NC pups compared with the CP pups. These linear distances include: foramen ovale to foramen rotundum; orbital fissure to posterior border of palate; orbital fissure to posterior maxillary premolar crypt; orbital fissure to auditory bulla; inferior posterior vomer to internal acoustic meatus. Note that these linear distances are diagrammed on a two-dimensional representation of a three-dimensional object. Some linear distances project through the skull as shown.

correct time, an excessively wide head, failure of the shelves to fuse, and secondary rupture after fusion. Although sample sizes are inadequate to test any of these as hypotheses, our analysis underscores certain observations that have bearing on these potential causes. As noted earlier, several local mediolateral distances are larger in CP pups, but our analysis does not suggest that the CP skulls are generally wider than normal. If palatal shelf contact failure can result from excessive width of the midface secondary to a large tongue (Johnston and Bronsky, 1991), it will be important to sort local from regional measures of midfacial width in future analyses. We have no evidence concerning hypotheses of failure of the shelves to elevate at the correct time, simple failure of the shelves to fuse, and secondary rupture after fusion. Dissection and 3D CT reconstructions indicate that the palatal shelves have elevated and that there may be a reduced amount of osseous tissue along the medial border. Contact failure appears to be due to inadequate growth of the palatine shelves, perhaps due to failure or delay of local osteogenesis. Because the midline cleft that we have studied potentially involves many separate bones that make up the hard palate: incisive bones, maxilla, palatine bones, and vomer, failure of the tongue to exit from between the palatal shelves is a reasonable explanation for the presence of the cleft. Our observation that the posterior palate and cranial base were wider in the CP pups is consistent with delayed tongue removal. This kindred provides the potential for tracking this developmental event using fetal specimens, thus providing a developmentally based model

of canine cleft palate that may be useful in the study of cleft palate development in humans (Siegel and Mooney, 1990). The developmental expression of genes controlling the formation of the canine hard palate is unknown. Further genetic work may enable us to isolate the mechanism responsible for a hypothesized change in developmental events responsible for CP in this kindred.

This canine CP phenotype will be characterized in more detail as more pups become available. By using a feeding tube or prosthetic device, it may be possible to rear a CP pup and permit study of postnatal growth and maturation. As pointed out by one of our reviewers, successful rearing of CP pups might also provide the opportunity to study the effect of surgical repair of CP on growth. We plan to search for genetic markers to facilitate CP carrier detection using simple sequence repeat markers recently developed for dogs (Ostrander et al., 1993). Another mapping approach, which has considerable promise for comparative mapping, involves syntenic gene studies such as we have been using in studies of canine markers recognized by human DNA probes from chromosomes 5, 11, and 21 (Cork et al., 1991). Identifying syntenic markers could be useful in localizing susceptibility loci on the human gene map. The occurrence of CP in this colony of dogs provides an ideal opportunity to understand an important malformation and builds on current efforts to develop genetic linkage markers which are homologous with those in humans. This canine condition may help bridge the gap between the genetic and morphologic maps of an anomaly.

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