ERYTHRITOL AND THREITOL IN CANINE PLACENTA: POSSIBLE IMPLICATION IN CANINE BRUCELLOSIS

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1. Introduction

Erythritol is a preferred nutrient and growth stimulant for brucella species [1]. In brucellosis of the pregnant cow, ewe, goat and sow the prolific growth of the bacteria in placental tissues which culminates in abortion correlates with the presence, only in these tissues, of high concentrations of erythritol; furthermore erythritol is absent from the placental tissues of man, rat, rabbit and guinea-pig and these species are resistant to the acute placental form of brucellosis [2, 3]. From this and other evidence a causal relationship between the presence of placental erythritol and susceptibility to brucella abortion has been inferred [3, 4]. Recently, Carmichael and Kenney [5] showed that a fulminating placentitis preceded abortion in pregnant bitches infected with *Brucella canis*. Evidence that erythritol and threitol exist in the canine placenta is presented here; erythritol may therefore be implicated in the placentitis of canine brucellosis.

2. Materials and methods

2.1. *Extraction, detection and estimation of tetritol*

Canine placenta tissue (placenta plus attached membranes) was obtained by caesarian section at term from 8 bitches of various breeds, and human placenta was collected post-partum from three normal pregnancies. The tissues were stored at −20° until use. Non-ionic material, including any free tetritol present, was obtained from a weighed sample of each tissue (100 g wet wt) by maceration of the sample in phosphate-buffered saline (100 ml, containing NaCl 7.50, Na_2HPO_4 2.13, KH_2PO_4 0.70 g/l; pH 7.4), centrifugation of the macerate, dialysis of the resultant supernatant (extract) against distilled water and removal of ionic components from the diffusate by ion-exchange resins (Zeokarb 225-H^+ form, Amberlite IR45 and IRA400-OH^- forms) using the methods described by Williams et al. [6]. Estimation by a periodate/chromotropic acid method [7] showed that about 70% of a sample of authentic erythritol (2 mg; supplied by G.T. Gurr Ltd. and recrystallised from moist acetone) could be recovered from the ion-exchange resins.

The non-ionic material recovered was freeze-dried and redissolved in water (1.5 ml). Aliquots (10 µl) were applied to Whatman No. 1 paper alongside authentic erythritol and subjected to descending paper chromatography in (a) iso-amyl alcohol:pyridine:water (4:3:2 by vol), (b) butanol:acetic acid:water (4:1:5 by vol), and (c) butan-2-one:acetic acid:4% boric acid (9:1:1 by vol) solvent systems. Solvent flowed for either 15 hr (solvent systems a and b) or 6 hr (solvent system c); the papers were then dried at room temp. and stained by 1) alkaline silver oxide reagent [8] or 2) periodate/*p*-anisidine spray [9]. Stain 1 in conjunction with solvent system a and stain 2 with solvent system c routinely detected 2 µg of erythritol.

The concentration of tetritol in a placental extract was assessed by visual comparison of the intensities of spots produced from known amounts of erythritol (2–10 μg) with the intensity of the corresponding spot produced by an aliquot of the extracted non-ionic material run on the same chromatogram in solvent system c and stained with stain 2.

2.2. *Identification of extracted tetritol*

The bulked non-ionic material (20 mg dry wt) from 3 samples of canine placenta was chromatographed as before using solvent system a, but on a preparative scale. The component indistinguishable from erythritol was eluted from the paper and the procedure repeated until a check chromatogram failed to show contamination with adjacent spots; the method was essentially that described by Pearce et al. [10].

A portion of the purified material (final yield 2 mg) was trimethylsilylated by dissolution in pyridine (250 μl) and treatment with trimethylsilyl chloride (50 μl) and hexamethyldisilazane (100 μl). After 10 min at 37°, the solution was analysed by gas-liquid chromatography with silicone ester 30 (10% on Celite) as stationary phase and nitrogen as carrier gas. Runs were made isothermally at 160° and a flame-ionisation detection system was employed. Erythritol was used as a standard.

A portion (2.9 mg) of the freeze-dried non-ionic material from one extract of canine placenta was redissolved in water (100 μl) and fractionated on a column (bed vol 15 ml) of AG1 X 8 anion exchange resin, molybdate form, 200–400 mesh using water at a flow rate of 0.22 ml/min as described by Barker et al. [11]. The column eluate was continuously monitored automatically by a process involving periodate oxidation and spectrofluorimetric determination of any formaldehyde produced [12].

3. Results

Paper chromatography showed that extracts from each of the eight samples of canine placenta, representing 6 breeds of bitch, contained a component indistinguishable from erythritol. No such component was detectable in corresponding extracts of the 3 samples of human placenta which were employed as controls. The concentrations of the component in the samples, as revealed by visual comparison of stained chromatogram spots, are listed in table 1,

That the fraction was a tetritol was confirmed by gas-phase chromatography as the trimethylsilyl derivative. The retention time of the only peak on the chromatogram corresponded to that of erythritol and threitol. The two tetritols are inseparable by the paper and gas chromatographic methods used.

Table 1
Tetritol content of extracts of canine and human placenta*.

Source	Estimated concentration of tetritol** in placental extract (μg/ml)
Poodle	11
Miniature Dachshund	9
Corgi	9
Mongrel	8
Miniature Dachshund	7
Jack Russell Terrier	6
Corgi	3
Pekingese	3
Human (three samples)	0

* Term placenta together with attached membranes was obtained post-partum (human) or by caesarian section (canine) and extracted into phosphate-buffered saline (100 g wet wt/100 ml) by maceration.

** Tetritol content was estimated after removal of large molecular weight and ionic material from the extract by dialysis and treatment with ion-exchange resins: the intensity of the tetritol spot obtained by paper chromatography of an aliquot of the residue (equivalent to a known volume of the original extract) was visually compared with the intensities of spots developed from known quantities of erythritol run in parallel.

Table 2
Molybdate column differentiation of extracted tetritols.

Sample	Retention time (min)	Concentration** (μg/ml of extract)
Erythritol	103	–
A*	101	3.1
Threitol	40	–
B*	40	0.4

* Peak detected on fractionation of non-ionic material extracted from canine placenta.

** Calculations based on areas under curves and comparison with standard.

Specific identification of the tetritols is provided by the molybdate column fractionation technique which in this instance showed two peaks, one (A) corresponding to the elution position of erythritol, and one (B) corresponding to that of threitol (table 2).

4. Discussion

Threitol has seldom been detected in biological sources [13–18], and its occurrence in normal animal tissue has not previously been reported. Erythritol has been shown to be synthesized in the sheep placenta [19] but the significance of either tetritol in placental metabolism is not known.

Since the concentration of tetritol in placenta was assessed using the methods of Williams et al. [6] without significant modification, the results may be compared. Thus placental tetritol concentration near termination of pregnancy is about 3 to 10-fold lower in the bitch (based on the average of the 8 extracts) than in the ewe, cow, goat or sow. Erythritol, the predominant tetritol, might nevertheless be a significant factor in canine brucellosis; only small amounts (< 1 μg/ml of suitable medium) were required to increase the growth of *Brucella abortus*, *Brucella melitensis* and *Brucella suis in vitro* [3, 10, 20]. Furthermore, the local concentration in the intraplacental micro-environment of the infecting organism could be much higher than that observed in a crude extract.

Although *Br. canis* produces a typical localising brucellosis in the pregnant bitch, Jones et al. [21] were unable to show stimulation of growth by erythritol *in vitro*. However, the organism is able to catabolise this substrate [22] and is otherwise similar to the classical brucella species on the basis of the usual bacteriological tests and DNA homology studies [23].

Demonstration of the growth response of other brucellae to erythritol depends upon the composition of the basal medium; nutritionally 'poor' media are necessary, especially with *Br. melitensis* and *Br. suis* [3, 20], and excess glutamic acid has been found to prevent stimulation of *Br. abortus* in a synthetic medium [1]. Such medium dependence may therefore account for the reported failure of *Br. canis* growth to respond to erythritol.

Further examination of the effect of erythritol on the growth of *Br. canis* seems appropriate since we have here extended to the canine the correlation between the presence of placental erythritol and susceptibility to brucella abortion.

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