Induction of onion-induced haemolytic anaemia in dogs with sodium *n*-propylthiosulphate

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The haemolytic effect of sodium *n*-propylthiosulphate, which had been isolated from boiled onions, was studied to determine whether it could be one of the agents responsible for induced haemolytic anaemia in dogs. The oral administration of 500 μmol/kg bodyweight of the compound to dogs resulted in a haemolytic anaemia associated with an increase of Heinz body formation in erythrocytes, which was more severe in dogs with the hereditary condition which results in erythrocytes with high concentrations of reduced glutathione and potassium than in normal dogs. In the affected dogs there was a 10-fold increase in the concentration of oxidised glutathione in their erythrocytes 12 hours after the administration of the compound, whereas in normal dogs there was almost no change.

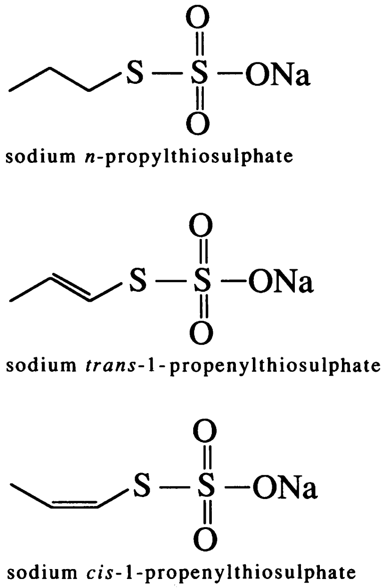

FIG 1: Molecular formulae of three sulphur-containing compounds derived from boiled onions, which caused an increase in the concentration of methaemoglobin in canine erythrocytes in vitro

ONIONS and other plants of the Allium family have been a cause of Heinz body haemolytic anaemia, so-called 'onion poisoning', in cattle (Goldsmith 1909), sheep (Stevens 1984) and horses (Thorp and Harshfield 1939). Haemolytic anaemia has also been reported in onion-fed dogs (Sebrell 1930) and cats (Kobayashi 1981). Since Gruhzit (1931b) reported that *n*-propyl disulphide caused haemolytic anaemia in dogs, the compound has been considered to be the agent responsible for onion-induced haemolytic anaemia.

Farm animals may develop the anaemia through the infestation of their pastures with such plants as wild onions or garlic. In contrast, dogs have developed clinical signs after eating cooked onions. Cooked onions contain fewer disulphides than fresh onions because disulphides are volatile, and it may therefore be doubted whether *n*-propyl disulphide is the main causative agent of onion poisoning in dogs.

Recently, the authors isolated three compounds (sodium *n*-propylthiosulphate, sodium *trans*-1-propenylthiosulphate and sodium *cis*-1-propenylthiosulphate [Fig 1]) from boiled onions, which caused oxidative damage to canine erythrocytes in vitro (Yamato and others 1994). The purpose of the present study was to investigate whether the compounds might be causative agents of onion-induced haemolytic anaemia in dogs. Because it has already been demonstrated that dogs with the hereditary condition which results in erythrocytes with high concentrations of reduced glutathione (GSH) and potassium (designated HK dogs) are more susceptible to the oxidant action of onions than normal dogs (Yamato and Maede 1992), the haemolytic effect of *n*-propylthiosulphate was compared in HK dogs and normal dogs.

Materials and methods

Synthesis of n*-propylthiosulphate*

The compound was synthesised from propane chloride and sodium thiosulphate, using trimethylbenzylammonium chloride as a catalyst (Chapelet and others 1980, Yamato and others 1994). The product was chromatographed on silica gel (Kieselgel 60; Merck) with methanol-chloroform (45:55) as the eluent, and further purified by high performance liquid chromatography in a μBondapak C18 column (19 x 300 mm; Waters), using 30 per cent aqueous methanol as a solvent. No impurities were found on the nuclear magnetic resonance spectrum of the purified *n*-propylthiosulphate.

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Dogs

Three clinically normal and three mixed-breed HK dogs were studied. The mean (sd) bodyweight of the normal dogs was 11·7 (0·8) kg, and that of the HK dogs was 11·4 (1·5) kg. The HK dogs were members of a family that has been kept in the authors' laboratory since 1982 (Maede and others 1982).

Each of the six dogs received 500 μmol/kg bodyweight of *n*-propylthiosulphate administered orally in gelatine capsules. Blood samples (about 5 ml) were taken at the time the compound was administered, and three, six, 12, 24, 72, 96 and 120 hours later, from the saphenous vein of each dog into tubes containing disodium ethylenediaminetetra-acetate. One normal and one HK dog were used as controls and received empty gelatine capsules.

Haematological examinations

The erythrocyte (RBC) count, haemoglobin concentration and haematocrit were determined with an automatic cell counter (System 9000; Baker Instruments). Methaemoglobin concentration was measured as described by Hegesh and others (1970). Lipid peroxidation of blood was determined by estimating thiobarbituric acid-reactive substances (TBARS) according to the method of Ohkawa and others (1979); malondialdehyde was used as a standard. Heinz bodies in erythrocytes were detected by vital staining with 0·5 per cent brilliant green, and recorded as the percentage of RBC that had one or more Heinz bodies by the examination of 1000 RBC. Reticulocytes were detected by vital staining with 0·5 per cent new methylene blue, and recorded as the percentage of reticulocytes by the examination of 1000 RBC. The concentration of GSH in erythrocytes was determined by the mea-

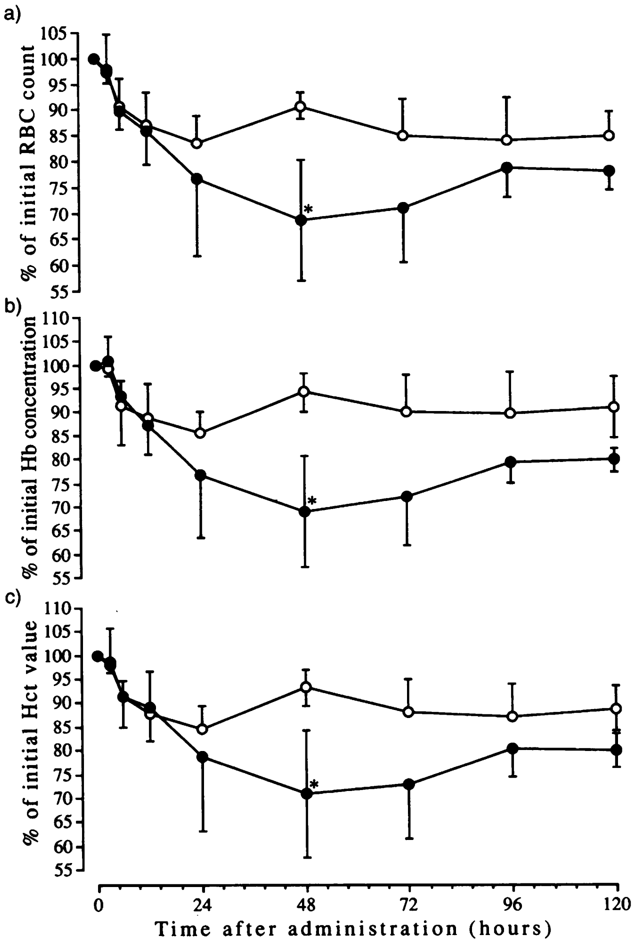

FIG 2: In vivo effect of sodium *n*-propylthiosulphate on mean (sd) erythrocyte (RBC) count (a), haemoglobin (Hb) concentration (b) and haematocrit (Hct) value (c) in three normal dogs (○) and three dogs with hereditary high concentrations of reduced glutathione and potassium in erythrocytes (HK dogs, ●). The values are all expressed as percentages of the initial values. *P<0·05

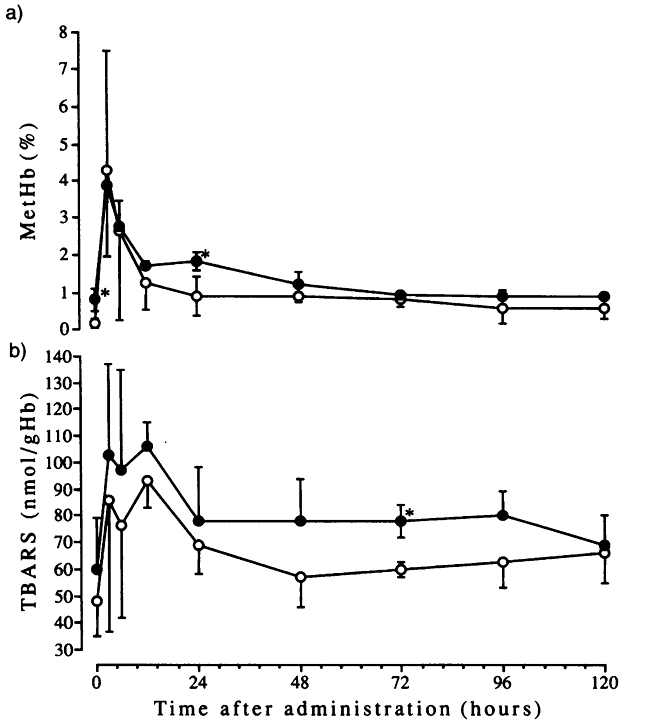

FIG 3: In vivo effect of sodium *n*-propylthiosulphate on the mean (sd) concentrations of methaemoglobin (MetHb) (a) and thiobarbituric acid reactive substances (TBARS) (b) in three normal dogs (○) and three dogs with hereditary high concentrations of reduced glutathione and potassium in erythrocytes (HK dogs, ●). *P<0·05

surement of the 5,5′-dithiobis-(2-nitrobenzoate) derivative (Beutler and others 1963). The concentration of oxidised glutathione (GSSG) in erythrocytes was measured by the method of Srivastava and Beutler (1968).

Determination of haemoglobinuria

Urine was collected through a catheter, and haemoglobinuria was determined by means of a urinary test paper (BM Test 6-III; Boehringer Mannheim).

Analysis of data

The data were analysed by means of Student's *t* test.

Results

The changes in the RBC count and haemoglobin concentration in the normal and HK dogs after the administration of *n*-propylthiosulphate are shown in Fig 2. By 24 hours after the administration, the RBC count had decreased on average to 83·6 per cent of the initial value in the normal dogs, and to 76·8 per cent in the HK dogs. The RBC count in the normal dogs had returned to 90·8 per cent of the initial value after 48 hours, whereas that in the HK dogs continued to decrease to 68·7 per cent of the initial value at 48 hours (Fig 2a). The haemoglobin concentration (Fig 2b) and haematocrit (Fig 2c) of the normal and HK dogs changed in a similar way to the RBC count. The RBC count, haemoglobin concentration and haematocrit of the HK dogs were significantly (P<0·05) lower than those of the normal dogs at 48 hours. Reddish plasma and haemoglobinuria were observed at three to 24 hours after the administration in both the normal and HK dogs, but the changes were more prominent in the HK dogs. The control dogs showed no significant changes in the RBC count, haemoglobin concentration or haematocrit during the experimental period.

The concentration of methaemoglobin increased rapidly and reached a maximum mean (sd) concentration of 4·3 (3·4) per cent in the normal dogs and 3·9 (1·9) per cent in the HK dogs after three hours, and then declined in both groups (Fig 3a). A significant (P<0·05) increase in the concentration of TBARS was observed at 12 hours in both groups of dogs (Fig 3b).

The appearance of Heinz bodies within erythrocytes was observed at three hours in the HK dogs and at six hours in the normal dogs. The Heinz body count in the HK dogs then increased rapidly to 45·2 per cent and was maintained at 45 to 55 per cent, a higher level than in the normal dogs, until 72 hours (Fig 4a), although there was no significant difference in Heinz body formation between the normal and HK dogs. The reticulocyte count also increased gradually after the administration of *n*-propylthiosulphate and reached 8·7 (4·0) per cent at 96 hours in the HK dogs, compared with 4·7 (4·3) per cent in the normal dogs at 72 hours. In the HK dogs, there was a marked increase in the reticulocyte count between 72 and 96 hours after the administration (Fig 4b), which seemed to correspond to the increase in the RBC count during the same period (Fig 2a).

The concentration of erythrocyte GSH in the normal and HK dogs increased significantly (P<0·05) after the administration, and reached peaks of 6·65 (0·85) μmol/g haemoglobin in the normal dogs and 35·2 (4·89) μmol/g haemoglobin in the HK dogs at 12 hours (Fig 5a). The concentration of GSSG in the HK dogs had increased by about 10-fold at 12 hours, whereas that in the normal dogs increased only slightly at three to six hours (Fig 5b). The maximum value of erythrocyte GSSG in the HK dogs was 1·11 (0·66) μmol/g haemoglobin at 12 hours, compared with the initial value of 0·11 (0·02) μmol/g haemoglobin.

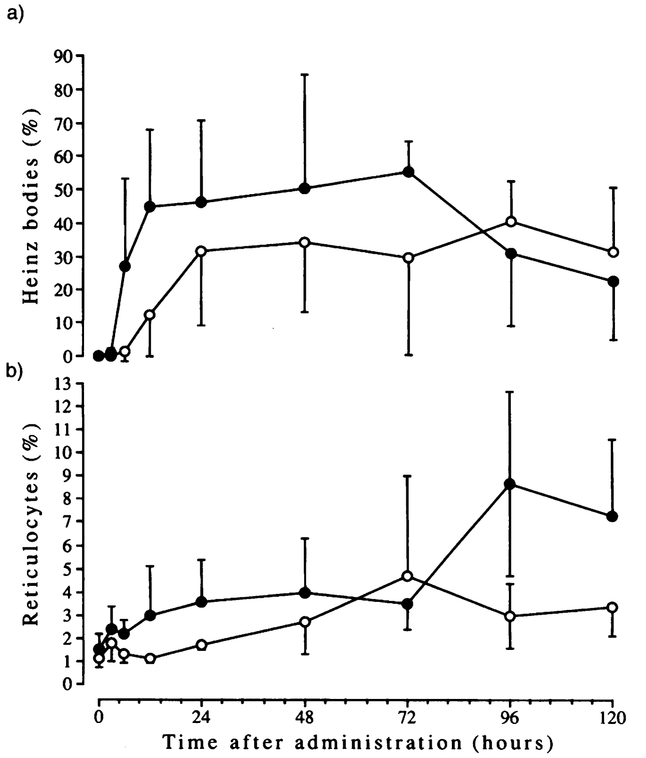

FIG 4: In vivo effect of sodium *n*-propylthiosulphate on the mean (sd) percentages of Heinz bodies (a) and reticulocytes (b) in the erythrocytes of three normal dogs (○) and three dogs with hereditary high concentrations of reduced glutathione and potassium in erythrocytes (HK dogs, ●)

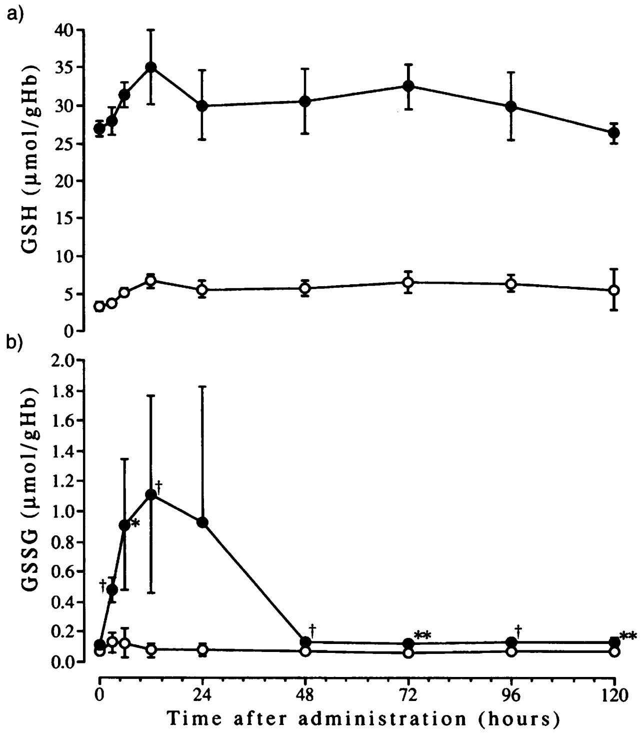

FIG 5: In vivo effect of sodium *n*-propylthiosulphate on the mean (sd) concentrations of reduced glutathione (GSH) (a) and oxidised glutathione (GSSG) (b) in the erythrocytes of three normal dogs (○) and three dogs with hereditary high concentrations of reduced glutathione and potassium in erythrocytes (HK dogs, ●). *P<0·05, **P<0·01, †P<0·005. The data for GSH concentration were not analysed statistically

Discussion

Onion-induced haemolytic anaemia in dogs was first recognised in 1930 (Serbell 1930). The following year, Gruhzit (1931a) investigated the characteristics of the anaemia induced by experimental onion feeding in dogs, and observed that a similar haemolytic anaemia developed after the oral administration of *n*-propyl disulphide, a compound present in distilled oil of onions (Gruhzit 1931b). Since then, it has been suggested that *n*-propyl disulphide is the agent responsible for onion-induced haemolytic anaemia. However, in several cases of clinical onion poisoning, the affected dogs developed anaemia after eating cooked onions such as onion soufflé (Spice 1976) or boiled onions (Maede 1977, Lees and others 1979) although the concentration of *n*-propyl disulphide decreases during cooking. Volatile disulphides such as *n*-propyl disulphide are major components of fresh onion flavour, being derived enzymatically from a number of involatile precursors (Fenwick and Hanley 1985).

In a previous study (Yamato and others 1994), three novel sulphur-containing compounds were isolated from boiled onions, and it was demonstrated that these compounds caused an increase in the concentration of methaemoglobin in canine erythrocytes in vitro, indicating that they had an oxidative effect on the erythrocytes. In the present study, the oral administration of *n*-propylthiosulphate, one of the compounds, to dogs resulted in a decrease in the RBC count accompanied by increases in the concentrations of methaemoglobin and TBARS and Heinz body formation in the erythrocytes of the dogs. Haemoglobinuria was also observed. These results clearly indicate that the compound caused oxidative damage to the erythrocytes, resulting in the development of haemolytic anaemia, because TBARS is one of the major secondary products of peroxidised polyunsaturated fatty acids, and because Heinz body formation indicates the extent of the oxidative denaturation of haemoglobin in erythrocytes. Lipid peroxidation and Heinz body formation in erythrocytes result in the destruction of the cells (Rifkind 1965, Jacob and others 1968, Stocks and Dormandy 1971).

Furthermore, *n*-propylthiosulphate caused more severe oxidative injury to the erythrocytes in the HK dogs than to those in the normal dogs. The formation of Heinz bodies and the increase in the concentration of GSSG were more prominent in the HK dogs than in the normal dogs, suggesting that the accumulation of GSSG in HK erythrocytes may accelerate the formation of Heinz bodies. The propensity to form a mixed disulphide between haemoglobin and glutathione is likely to lead to Heinz bodies (Srivastava and Beutler 1970). In patients with hereditary pyropoikilocytosis, increases in erythrocyte GSSG and Heinz body formation, which could conceivably lead to haemoglobin precipitation, have been observed by Prchal and others (1982). The mechanism of the increase in erythrocyte GSSG observed in the HK dogs is not clear. However, the results obtained in the present study are in agreement with the previous observation that HK dogs are more susceptible to onion-induced oxidative damage to erythrocytes than normal dogs (Yamato and Maede 1992). As a result, *n*-propylthiosulphate was considered to be one of the haemolytic agents responsible for onion-induced anaemia in dogs. If the relationship between the haemolytic effect and the concentration of the compound in onions is clarified, it might provide additional evidence that the compound is active in inducing haemolytic anaemia.

It has not been possible to demonstrate the haemolytic effect of the other two compounds, sodium *trans*-1-propenylthiosulphate and sodium *cis*-1-propenylthiosulphate, because the compounds have not been synthesised. In a previous study (Yamato and others 1994), it was observed that the mixture of the three compounds had a 10-fold greater oxidative activity than synthetic *n*-propylthiosulphate indicating that the two other compounds could be largely responsible for the haemolytic anaemia in dogs induced by feeding onions. This possibility, however, remains unelucidated.

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Short Communications

ELISA detection of antibodies to glycoprotein E of bovine herpesvirus 1 in bulk milk samples

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A GLYCOPROTEIN E (gE) deleted strain of bovine herpesvirus 1 (BHV-1) provides the basis for efficacious and safe attenuated and inactivated marker vaccines (Kaashoek and others 1994, Bosch and others 1996). Use of these vaccines in the Dutch BHV-1 eradication programme will be compulsory commencing in 1998. The companion diagnostic test to differentiate between infected and vaccinated cattle is a blocking ELISA that detects only antibodies directed against gE of BHV-1. Hence antibodies against wild-type BHV-1 and conventional non-gE deleted whole BHV-1 vaccines are detected, whereas antibodies against the gE-deleted marker vaccine are not (Van Oirschot and others 1997). The BHV-1-gE ELISA used in this study was a commercially available product (Idexx). The principle of the test is based on the use of one monoclonal antibody (Mab 66) whose binding can be blocked by antibodies in the test sample. This ELISA has a relative sensitivity and specificity of 99 per cent as compared with a BHV-1-gE ELISA using two monoclonal antibodies (Kaashoek 1995).

The testing of bulk milk has been shown to be of value in screening herds for bovine brucellosis (Knosel and others 1991) and in estimating the prevalence of BHV-1 (Hartman and others 1997, Van Wuijckhuise and others 1998). The aim of this study was to examine whether the BHV-1-gE ELISA is suitable for detecting antibodies directed against BHV-1 in bulk milk, and whether testing of bulk milk could give an indication of the percentage of infected animals in unvaccinated herds and in herds that were vaccinated with the marker vaccine.

To evaluate the specificity of the BHV-1-gE ELISA, Danish negative bulk milk samples were collected from 500 randomly chosen BHV-1-free herds. These freeze-dried bulk milk samples were dissolved in 1·0 ml of deionised water and analysed undiluted in the BHV-1-gE ELISA and in the BHV-1-gB ELISA, which was used as the standard test (Kramps and others 1994). The quality of each BHV-1 ELISA batch was first checked and only approved batches were used in this study (Wellenberg and others 1998). All Danish bulk milk samples were negative in both ELISA systems. This indicated that for the detection of antibodies directed against BHV-1 in bulk milk samples the relative specificity of the BHV-1-gE ELISA was 100 per cent as compared with the BHV-1-gB ELISA.

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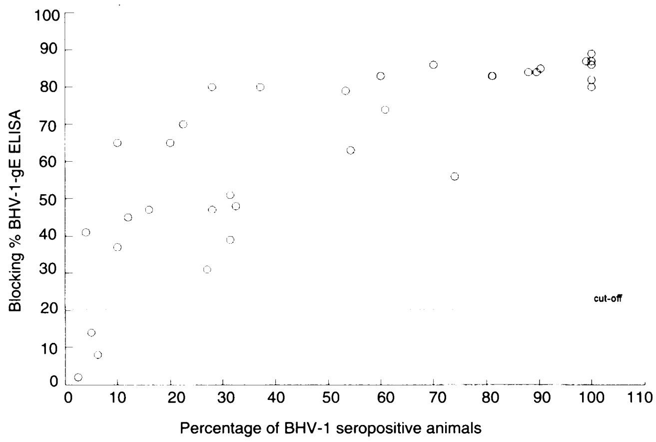

FIG 1: Blocking percentages of bulk milk samples versus the percentages of BHV-1-gE-seropositive cattle in unvaccinated herds

To evaluate the sensitivity of the BHV-1-gE ELISA, Dutch bulk milk samples were collected from unvaccinated herds with a BHV-1 history, and from herds with a BHV-1 history that had been vaccinated three times with the attenuated gE-deleted marker vaccine. The bulk milk samples obtained from vaccinated herds were collected five to six months after the third vaccination. All the herds had participated in a field trial for vaccine efficacy where they were randomly assigned to either an unvaccinated placebo group or to a vaccine group. From each unvaccinated (n = 35) and vaccinated herd (n = 34) one bulk milk sample was collected. On the same day as bulk milk samples were collected, individual serum samples (n = 4297) were collected from all animals older than two years, including those that were dry. The bulk milk samples collected from unvaccinated herds were analysed undiluted in the BHV-1-gE ELISA and the BHV-1-gB ELISA. The bulk milk samples collected from vaccinated herds were analysed undiluted in the BHV-1-gE ELISA only. All bulk milk samples were analysed without concentrating the immunoglobulin fraction as described by Von Forschner and others (1986). The concentration of bulk milk is too laborious for routine screening (Frankena and others 1997). The serum samples were analysed in a 1/2 dilution in the BHV-1-gE ELISA according to the instructions of the manufacturer. The blocking percentages of the bulk milk samples were plotted against the percentages of BHV-1-gE-seropositive cattle per unvaccinated (Fig 1) or per vaccinated herd (Fig 2).

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Notes