



**HAL**  
open science

## **Experimental staphylococcal mastitis in bitches: Clinical, bacteriological, cytological, haematological and pathological features**

H.N. Ververidis, V.S. Mavrogianni, I.A. Fragkou, D.C. Orfanou, D.A.  
Gougoulis, A. Tzivara, L. Athanasiou, C.M. Boscoc, G.C. Fthenakis

### ► To cite this version:

H.N. Ververidis, V.S. Mavrogianni, I.A. Fragkou, D.C. Orfanou, D.A. Gougoulis, et al.. Experimental staphylococcal mastitis in bitches: Clinical, bacteriological, cytological, haematological and pathological features. *Veterinary Microbiology*, 2007, 124 (1-2), pp.95. 10.1016/j.vetmic.2007.03.029 . hal-00532232

**HAL Id: hal-00532232**

**<https://hal.science/hal-00532232>**

Submitted on 4 Nov 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: Experimental staphylococcal mastitis in bitches:  
Clinical, bacteriological, cytological, haematological and  
pathological features

Authors: H.N. Ververidis, V.S. Mavrogianni, I.A. Fragkou,  
D.C. Orfanou, D.A. Gougoulis, A. Tzivara, L. Athanasiou,  
C.M. Boscas, G.C. Fthenakis



PII: S0378-1135(07)00157-5  
DOI: doi:10.1016/j.vetmic.2007.03.029  
Reference: VETMIC 3634

To appear in: *VETMIC*

Received date: 30-1-2007  
Revised date: 5-3-2007  
Accepted date: 22-3-2007

Please cite this article as: Ververidis, H.N., Mavrogianni, V.S., Fragkou, I.A., Orfanou, D.C., Gougoulis, D.A., Tzivara, A., Athanasiou, L., Boscas, C.M., Fthenakis, G.C., Experimental staphylococcal mastitis in bitches: Clinical, bacteriological, cytological, haematological and pathological features, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.03.029

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 Experimental staphylococcal mastitis in bitches: clinical,  
2 bacteriological, cytological, haematological and pathological features

3

4 H.N. Ververidis<sup>a</sup>, V.S. Mavrogianni<sup>a</sup>, I.A. Fragkou<sup>a</sup>, D.C. Orfanou<sup>a</sup>, D.A. Gougoulis<sup>a</sup>,

5 A. Tzivara<sup>a</sup>, L. Athanasiou<sup>a</sup>, C.M. Boscos<sup>a</sup>, G.C. Fthenakis<sup>a\*</sup>

6 <sup>a</sup> *Veterinary Faculty, University of Thessaly, P.O. Box 199, 43100 Karditsa, Greece*

7 <sup>b</sup> *School of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Macedonia, Greece*

8

9

10 \* Corresponding author. Tel.: +30 24410 66070; fax: +30 24410 66077

11 E-mail address: gcf@vet.uth.gr (G.C. Fthenakis)

12

13

14 **Abstract**

15 The objectives of the work were to study the features of experimentally induced canine mastitis and to  
16 present hypotheses regarding the pathogenesis of the disease. The right caudal abdominal mammary gland of  
17 six bitches was inoculated on day 8 after whelping with *Staphylococcus intermedius* to induce mastitis; adjacent  
18 mammary glands were used as controls. Clinical examination, bacteriological and cytological (Whiteside Test,  
19 Giemsa) examination of mammary secretion, as well as haematological tests were performed from 5 d before  
20 until 34 d after challenge. Mastectomy was sequentially performed 1, 2, 4, 18, 26 and 34 d after challenge in  
21 each of the bitches, in order to carry out a pathological examination of mammary glands. All animals developed  
22 clinical mastitis: challenged glands became painful, hot, enlarged and oedematous; secretion was brownish,  
23 purulent, with flakes or clots, subsequently becoming yellowish and thick. Staphylococci were isolated from all  
24 inoculated glands (up to 22 d). WST was positive in 41/46 samples from inoculated glands and 66/138 samples  
25 from control glands; neutrophils predominated during the acute stage. Blood leukocyte counts increased, whilst  
26 platelet counts decreased. Gross pathological findings initially included congestion, purulent discharge and  
27 subcutaneous oedema; then abscesses, brownish areas and size decrease were seen. Salient histopathological  
28 features were neutrophilic infiltration, haemorrhages, destruction of mammary epithelial cells and alveoli, as

1 well as infiltration by lymphocytes, shrunken alveoli, loss of glandular architecture and fibrous tissue  
2 proliferation. We conclude that in bitches, intramammary inoculation of *S. intermedius* can induce clinical  
3 mastitis, followed by subclinical disease. The disorder is characterized by bacterial isolation and leukocyte  
4 influx in challenged glands, by leukocyte presence in adjacent mammary glands, by increased blood leukocyte  
5 counts and by destruction of mammary parenchyma.

6

7 *Keywords:* mastitis / pathogenesis / dog / bitch / *Staphylococcus*

8

9

## 10 **1. Introduction**

11

12 Canine mastitis has not been studied in detail. There are very few references in the literature  
13 regarding the disease, most of which are clinical reports. To the best of our knowledge, no  
14 experimental studies into the pathogenesis of the disease have been described. However, all previous  
15 investigators agree that the disease is of importance for the post-parturient bitch (Van Walser and  
16 Henschelchen, 1983; Olson and Olson, 1984; Wheeler et al., 1984).

17 The disease occurs primarily during the post-partum period and more frequently, from 6th to  
18 10th day after whelping (Wheeler et al., 1984; Biddle and Macintire, 2000). It can also occur during  
19 pseudo-pregnancy, as well as after early weaning of puppies. Usually mammary infection is  
20 ascending, with haematogenous spread from other infection sites (e.g. uterus) also possible. Various  
21 bacteria have been incriminated as aetiological agents of the disease (e.g. *Staphylococcus* spp.,  
22 *Streptococcus* spp., *Escherichia coli*); however, no microorganisms could be isolated from some of  
23 the cases of clinical mastitis (Osbaldiston, 1978; Van Walser and Henschelchen, 1983).

24 Abnormal mammary secretion and enlarged, painful, hot mammary gland(s) with red- to  
25 purple-coloured abdominal skin, have been described as the salient clinical features of the acute  
26 phase; systemic signs (e.g. depression, fever) may also be evident. At a later stage, intramammary  
27 abscesses may develop; these often open, exuding pus. Intramammary presence of nodules or diffuse

1 hardness may be another finding during the long-standing phase (Wheeler et al., 1984; Johnston et al.,  
2 2001).

3 The objectives of the work described in this paper were i. to study the features of  
4 experimentally induced canine mastitis and ii. to present hypotheses regarding the pathogenesis of the  
5 disease.

6

## 7 **2. Materials and methods**

8

### 9 *2.1. Experimental overview*

10

11 Six (6) primiparous Beagle-breed bitches, from an animal colony which breeds dogs  
12 specifically for experimental work, were used in this study. The work was carried out under a licence  
13 for experimental procedures obtained from the Greek Ministry of Agriculture.

14 The animals were mated during their first oestrus. Ultrasonography was used to confirm  
15 pregnancy and help to predict a possible date for whelping. Two weeks before the expected day of  
16 whelping, each pregnant bitch was transferred into an individual pen. Regular examinations were  
17 carried out during lactation, as detailed in 2.4..

18 Animals were inoculated on the 8th day after whelping (D0), with an isolate of  
19 *Staphylococcus intermedius*, which had been recovered from a case of clinical mastitis in a bitch. The  
20 right 2nd abdominal (caudal abdominal / penultimate) mammary gland of each bitch ("CH" gland)  
21 was inoculated. The left 2nd abdominal gland ("OP"), the right inguinal (ultimate) gland ("C-1") and  
22 the right 1st abdominal (cranial abdominal) ("C+1") were used as controls.

23 Throughout the study, bitches suckled their puppies. Finally, on D1, D2, D4, D18, D26 and  
24 D34 after challenge, mastectomy of the four mammary glands described above, was carried out for  
25 detailed pathological examination.

26

27

1    2.2. *Preparation of inocula and inoculation procedure*

2

3           For inoculation, the isolate was grown on Columbia blood agar and checked for purity; then it  
4 was inoculated into Soy-broth (BioMerieux, Marcy-l'-Etoile, France) and incubated aerobically at 37  
5 °C for 5 h. Serial dilutions of the broth culture into PBS were carried out; finally, 0.6 ml of the desired  
6 dilution was withdrawn with a 1 ml syringe. The suspension contained  $10^6$  c.f.u. ml<sup>-1</sup>, as estimated by  
7 the method of Miles and Misra (1938).

8           Puppies of each experimental bitch were allowed to suck, in order to empty the mammary  
9 glands. The bitch was then sedated by intramuscular administration of acetylo-promazine  
10 (Acepromazinum<sup>®</sup>, dose rate: 0.05 mg kg<sup>-1</sup> bw; Streuli Pharma, Uznach, Switzerland); intravenous  
11 administration of thiopental sodium (Pentothal<sup>®</sup>, dose rate: 6.0-8.0 mg kg<sup>-1</sup> bw; Abbott Laboratories  
12 Inc., Abbott Park, IL, USA) was used to induce general anaesthesia, which was maintained by 2%  
13 halothane. The bitch was placed in dorsal recumbency on a surgical table and prepared as for midline  
14 abdominal surgery, by clipping all hairs and disinfecting the skin and the teats by povidone iodine;  
15 sterile surgical screens were used to allow access only to the teat to be inoculated.

16           The person carrying out the inoculation, who wore sterile surgical gloves, held and stabilized  
17 the teat between the thumb and index fingers. A sterile plastic fine catheter (Abbcath<sup>®</sup>; Abbott) 26 G  
18 (19 mm long) was inserted through one of the teat orifices into the teat duct as far as possible; in order  
19 to avoid damage to the teat duct mucosa, the catheter stylet had been slightly withdrawn, so that its  
20 pointed end did not protrude through the plastic, but was nevertheless maintained within the catheter  
21 to provide adequate rigidity. As soon as the catheter was inserted into the teat, the stylet was  
22 withdrawn and the syringe with the inoculum was attached to the catheter. Finally, 0.2 ml of the  
23 bacterial suspension was inoculated inside the mammary gland.

24           The syringe was then disengaged and the catheter withdrawn. A new catheter was used and  
25 inserted into another of the teat orifices; the same procedure was repeated and another 0.2 ml of  
26 bacterial suspension was inoculated inside the mammary gland. Finally, the procedure was repeated  
27 for a third time into a third orifice.

1           The same technique was used to inject equal quantities of sterile PBS into the "C+1"  
2 mammary gland, whilst no injection or any other intervention was made in the "C-1" and "OP"  
3 mammary gland. After recovery of the bitch, she was held in a post-operative pen and 1 h after  
4 challenge, she joined her litter.

5

### 6 *2.3. Mastectomy procedure*

7

8           Food was withheld 12 h before each planned mastectomy operation. Each animal was sedated  
9 by intramuscular administration of 2% xylazine (Rompun<sup>®</sup>, dose rate: 1.0 mg kg<sup>-1</sup> bw; Bayer,  
10 Leverkusen, Germany); general anaesthesia was induced as described previously.

11           The four mammary glands ("CH", "OP", "C-1", "C+1") were excised by using a standard  
12 mastectomy procedure (Hedlund, 1997). In order to avoid inadvertent tissue damage, only mosquito-  
13 type forceps (rather than electro-cauterization) were used before ligation of vessels. The four  
14 mammary glands, the respective teats, the overlying skin and the right inguinal lymph nodes were  
15 removed *en bloc* and under aseptic conditions for pathological examination.

16           Appropriate subcutaneous and skin sutures were performed. Injectable antibiotics  
17 (amoxycillin plus clavulanic acid; Sunylox<sup>®</sup>, 0.05 ml kg bw<sup>-1</sup>; Pfizer, New York, NY, USA) and  
18 analgesics (morphine hydrochloride inj. sol., 0.35 mg kg bw<sup>-1</sup>) were administered post-operatively.  
19 Following recovery, the bitch was held in a post-operative pen for 4 h after operation, after which she  
20 was allowed to join her litter. All animals recovered uneventfully.

21

### 22 *2.4. Examinations and samplings*

23

24           Examinations and samplings, as detailed below, were carried out on the 3rd day after  
25 whelping (W3), on W5 and on W8 (before challenge). Subsequently, examinations and samplings  
26 were carried out 12 h after challenge (D0) and then, on D1, D2, D3, D4, D6, D8, D11, D14, D18,  
27 D22, D26, D30, D34 (unless of course, mastectomy had been performed to an animal before).

28

1    2.4.1. *Clinical examination*

2

3           A standard detailed general clinical examination of the animals and their puppies was carried  
4 out. Subsequently, examination of the mammary glands and teats was performed.

5           The four mammary glands of each animal were observed, palpated and compared to each  
6 other. Collection of mammary secretion was performed with the bitch standing on an examination  
7 table and restrained. Approximately, 3 to 5 minutes prior to collection, the bitch was intramuscularly  
8 injected with 3 IU of oxytocin.

9           Abdominal hair covering the mammary glands and the teats was clipped by using fine  
10 scissors. The teat and the abdominal skin adjacent to the teat were disinfected twice, by using cotton  
11 swabs moistened with sterile water and povidone iodine scrub (Betadine<sup>®</sup>; Mundipharma Medical  
12 Company, Basel, Switzerland). Subsequently, a sterile cotton gauze also moistened with sterile water  
13 and povidone iodine scrub, was used for a final disinfection; then, the area was swabbed with a sterile  
14 gauze to dry. After that, the procedure was carried out rather quickly, in order to avoid any accidental  
15 contamination (e.g. by the tail or the thigh skin) of the disinfected area.

16           The base of the teat was squeezed between the thumb and the index finger of the gloved hand  
17 of an investigator. A few (2 to 4) drops of secretion were expressed on the gloved hand of the  
18 investigator and examined for presence of abnormalities. Subsequently, further 2 to 3 drops of  
19 secretion were expressed directly onto a sterilised bacteriological loop. Finally, a quantity of 1.0 ml of  
20 secretion was collected into a plastic miniature tube.

21           Secretion collected on the bacteriological loop was immediately spread onto Columbia blood  
22 agar. The appearance of the secretion was recorded.

23

24    2.4.2. *Bacteriological examination*

25

26           The loopful from each secretion sample was plated onto Columbia blood agar. The media  
27 were incubated aerobically and anaerobically at 37 °C for up to 72 h. Organisms isolated were  
28 identified by using conventional techniques (Barrow and Feltham, 1993; Euzéby, 1997).



1 Identification of staphylococci was carried by means of API-Staph SYSTEM quick identification  
2 strips (BioMerieux) and a complete series of biochemical tests. The API-Staph SYSTEM was carried  
3 out in all staphylococcal isolates recovered during this study; profiles of isolates were compared.

4

#### 5 *2.4.3. Cytological examination*

6

7 The Whiteside Test (WST) was carried out in each secretion sample as described by Schalm  
8 and others (1971). Five drops of secretion (approximately 0.1 ml) were deposited on a clean slide and  
9 then, a drop of 1 N NaOH solution was added. The mixture was swirled by using a bacteriological  
10 wire. The resulting clot formation was scored as described by Schalm et al. (1971). Secretion films  
11 made by directly smearing 20 µl from each sample on a microscope objective plate, were stained by  
12 the Giemsa method. The percentage of leukocyte subpopulations was determined by distinguishing  
13 types present in the films.

14 The WST had been validated against the Microscopic cell counting method (Mccm), which is  
15 the method recommended by the International Dairy Federation (International Dairy Federation,  
16 1984). In ruminants, it is generally well established that WST results are reliable proxy measurements  
17 for somatic cell counts (SCCs) [cows: Schalm et al. (1971); sheep: Fthenakis (1995)]. Comparison of  
18 canine milk cell counting results obtained by the Mccm with WST scores (n=138), showed a  
19 Spearman's rank correlation coefficient  $r_s = +0.211$  ( $P = 0.013$ ). Comparison of results of  
20 bacteriological examination (isolation / no isolation of bacteria) with WST scores (n=138), by using  
21 the Fisher's exact test also showed an excellent correlation ( $P < 0.001$ ).

22

#### 23 *2.4.4. Haematological examination*

24

25 Jugular vein blood samples were collected into heparin- or EDTA-containing vials. Standard  
26 full haematological tests were carried out by using a blood-analyser (QBC Vet Autoreader; IDEXX  
27 Laboratories Inc., Westbrook, USA); measurement of albumin and total serum protein concentration  
28 was carried out in a biochemical analyzer (Vet Test 8008; IDEXX).

1    2.4.5. *Pathological examination*

2

3           Dissection of the four mammary glands started immediately after removal from the animal  
4 and was carried out by using aseptic technique. The mammary parenchyma was exposed and  
5 dissected. Lesions were described and samples were obtained for bacteriological and histological  
6 examination.

7           All samples obtained were plated onto Columbia blood agar. The media were incubated  
8 aerobically and anaerobically at 37 °C for up to 72 h. Identification of bacteria was carried out as  
9 described above.

10          Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax,  
11 using conventional techniques. Haematoxylin and eosin (H&E) standard staining procedures were  
12 performed for histopathological studies.

13

14    2.5. *Data management and analysis*

15

16          Initially, differences in frequency of bacterial recoveries from mammary glands were  
17 examined using the Fisher's Exact test. Then and for calculation purposes only, results of WST and of  
18 haematological-biochemical tests were arranged into four periods: period 1 (P1) from W3 to W8,  
19 period 2 (P2) from D0 to D4, period 3 (P3) from D6 to D11 and period 4 (P4) from D14 to D34.

20          Numerical values were given to WST scores as follows: score "-" = 0, score "±" = 1, score  
21 "1" = 2, score "2" = 3, score "3" = 4, score "4" = 5. Median values were obtained for each of the  
22 above periods; when abnormal secretion was recorded, it was given a score "4". Initially, the Kruskal-  
23 Wallis and the Mann-Whitney tests were used to test for differences between periods. Then, the  
24 Friedman non-parametric test followed by the Wilcoxon signed-rank test were employed to compare  
25 results between glands, within each of the above periods.

26          Results of haematology and biochemistry tests were initially tested for the homogeneity of  
27 variance of data (Levene's test). Then, one-way ANOVA followed by Bonferroni test or the Kruskal-

1 Wallis - Mann-Whitney tests were used as appropriately, in order to test for differences between  
2 periods.

3 Statistical analysis was performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA).  
4 Statistical significance was defined as  $P < 0.05$ .

5

### 6 **3. Results**

7

#### 8 *3.1. Pre-inoculation findings*

9

10 No pathological findings were detected in the mammary glands of the bitches before  
11 inoculation. The glands were soft with pale pink coloured skin. The secretion appeared normal. On  
12 W1, it was thick and yellowish, subsequently becoming "milky".

13 No bacteria were isolated from secretion samples (0/72) (Table 1). The majority of WST  
14 values (66/72) were "-" or "±" (Table 2). In Giemsa-stained films, macrophages predominated, with  
15 fewer neutrophils also present.

16 Mean haematological values were as follows: haematocrit 32.8%, leukocyte counts 18,964  
17 cells  $\mu\text{l}^{-1}$  (82% neutrophils) and platelet counts 596,000 particles  $\mu\text{l}^{-1}$  (Table 3).

18

#### 19 *3.2. Post-inoculation clinical findings*

20

21 All animals developed clinical mastitis within 12 h after challenge. The inoculated ("CH")  
22 gland became enlarged, hot, painful and oedematous (up to D3). Reddish colour of the overlying  
23 abdominal skin was evident. The mammary secretion was initially brownish, purulent, with flakes or  
24 clots (up to D3), subsequently becoming yellowish and thick. From D8 onwards, progressive  
25 reduction in the size of the inoculated glands was seen; this became particularly prominent after D21.  
26 In one bitch, an abscess developed (D8) near the base of the teat; however, the mammary secretion  
27 appeared normal.

1 In the bitch operated on D4, the "OP" gland was enlarged and harder than normal. No  
2 abnormal findings were seen in the other control glands.

3 Occasionally, mild systemic signs were observed: fever up to 40.4 °C (3 of 6 animals), mild  
4 depression (3/6) and decreased appetite (2/6). Their epimeletic ("care giving") behaviour did not  
5 change. The systemic signs were transient and did not last further than D2.

6

### 7 3.3. Post-inoculation bacteriological findings

8

9 *S. intermedius* was isolated from secretion samples of the inoculated glands ("CH"),  
10 consistently up to D11 and intermittently up to D22, when last isolation occurred: in total, 38/46  
11 recoveries. *S. intermedius* was isolated from secretion samples of the "OP" glands: in total, 8/46  
12 recoveries. Finally, no bacteria were isolated from the other two glands sampled ("C-1", "C+1"): in  
13 total 0/46 recoveries. All *S. intermedius* recovered after challenge had an API-Staph SYSTEM profile  
14 similar to that of the challenge strain. The difference in proportion of recoveries between "CH" gland  
15 and any of the three control glands was statistically significant ( $P < 0.001$ ); that difference between  
16 "OP" gland and any of the other two control glands was also statistically significant ( $P = 0.006$ ).  
17 Details of bacteriological results are presented in Table 1.

18

### 19 3.4. Post-inoculation cytological findings

20

21 In samples from inoculated glands, WST values increased already 12 h after challenge (5/6  
22 with score  $\geq 1$ ", median value: score "3") and remained high throughout lactation: in total 41/46 with  
23 score  $\geq 1$ " ( $P < 0.001$  throughout lactation) (Fig. 1). In samples from control glands, WST values also  
24 increased already 12 h after challenge (14/18 with score  $\geq 1$ ", median value: score "1"); they  
25 remained increased in the immediately post-challenge period (Period 2): in total 44/75 with score  
26  $\geq 1$ "; subsequently however (Periods 3 and 4), they decreased again: in total 22/63 with score  $\geq 1$ " ( $P$   
27  $< 0.01$  throughout lactation for each of the three glands). Details of cytological findings are presented  
28 in Table 2 and Figure 1.

1 In secretion films from inoculated glands, neutrophils predominated (>75%) at the initial  
2 stages, but their proportion was reduced subsequently to D11. Lysed neutrophils were evident from  
3 D3 onwards. Lymphocytes were seen in a marked proportion (>20%) after D6 and were predominant  
4 from D14 onwards.

5 Similar findings were seen in secretion films from control glands: neutrophils predominated  
6 (>75%) at the initial stages, with their proportion reduced from D14 onwards. However, macrophages  
7 and lymphocytes were seen in a marked proportion (>25%), but less numbers, at the later stages,  
8 coinciding with reduced WST values after D8.

### 9 10 3.5. Post-inoculation haematological findings

11  
12 Haematocrit values did not change significantly immediately after challenge, but increased  
13 towards the end of the experimental period and with the advancement of the puerperium.

14 There was a significant overall increase of leukocyte counts after challenge ( $P = 0.021$ ).  
15 Nevertheless, it is noteworthy that 12 h after challenge there was a decrease of leukocyte counts,  
16 followed by a sharp increase: median value on W8 = 17,000 cells  $\mu\text{l}^{-1}$ , on D0 = 12,800 cells  $\mu\text{l}^{-1}$ , on  
17 D1 = 23,000 cells  $\mu\text{l}^{-1}$ . Leukocyte counts returned to pre-challenge values after D6 (Table 3, Fig. 2).

18 There was a significant overall increase of total neutrophil counts after challenge ( $P < 0.05$ ).  
19 Initially there was a decrease, followed by a sharp increase: median value on W8 = 14,061 cells  $\mu\text{l}^{-1}$ ,  
20 on D0 = 11,490 cells  $\mu\text{l}^{-1}$ , on D1 = 19,038 cells  $\mu\text{l}^{-1}$ , on D2 = 21,584 cells  $\mu\text{l}^{-1}$ . The proportion of  
21 immature neutrophils was as follows: W8 = 6.1% of total neutrophils, on D0 = 16.7%, on D1 =  
22 14.2%, on D2 = 6.3%. Total neutrophil counts returned to pre-challenge values after D6. The  
23 proportion of immature neutrophils became <3% after D11 (Table 4, Fig. 2).

24 There was a significant decrease of platelet counts after challenge, evident already 12 h after  
25 challenge ( $P = 0.010$ ). This was followed by an increase after D4 ( $P = 0.002$ ): median value on W8 =  
26 471,000 platelets  $\mu\text{l}^{-1}$ , on D0 = 412,000 platelets  $\mu\text{l}^{-1}$ , on D5 = 642,000 platelets  $\mu\text{l}^{-1}$  (Table 3).

27 There were no significant changes in the concentration of globulins and albumins during the  
28 experiment (Table 3).

1

2 *3.6. Pathological findings*

3

4 *3.6.1. Bacteriological findings*

5

6 *S. intermedius* was isolated from tissue samples of the "CH" glands on D1, D2, D4 and D26.

7 It was also isolated from one sample from an "OP" gland on D4, but not from any other control  
8 gland. All *S. intermedius* recovered after challenge had an API-Staph SYSTEM profile similar to that  
9 of the challenge strain.

10

11 *3.6.2. Macroscopic findings*

12

13 Inoculated glands. In the bitch operated on D1, there was mild congestion in the parenchyma,  
14 which was reddened with multifocal dark red to brownish areas sharply demarcated from adjacent  
15 tissue; the veins were distended; blood exuded at sectioning; secretion clots were seen on the intima.  
16 In the bitch operated on D2, there was extensive subcutaneous oedema and generalised congestion;  
17 copious thick haemorrhagic secretion exuded at sectioning; dark brown areas and petechiae were  
18 clearly distinguished within the affected parenchyma. In the bitch operated on D4, similar extensive  
19 lesions as before were recorded; furthermore, the ducts were plugged by threads of fibrin and clots of  
20 milk; brown masses of exudates with fibrin and debris were obvious. In the bitch operated on D18,  
21 there were focal light brown irregular areas in the parenchyma. In the bitch operated on D26, there  
22 were focal light brown irregular areas inside the parenchyma; furthermore, there was a distinct focal  
23 (1 cm) whitish lesion characteristic of a small abscess. In the bitch operated on D34, there was a  
24 markedly reduced (approx. 35%) size of the gland with presence of brown irregular areas; apparently  
25 normal secretion was evident at sectioning.

26 Control glands. In the bitch operated on D1, there were no abnormal findings. In the bitch  
27 operated on D2, the three control glands were mildly swollen and congested; however, clear  
28 mammary secretion was evident. In the bitch operated on D4, there was mild subcutaneous oedema,

1 with congestion of the "OP" gland. In each of the bitches operated on D18, on D26 or D34, no  
2 abnormal findings were observed.

3

#### 4 *3.6.3. Histopathological findings*

5

6 Inoculated glands. In the tissue sample obtained on D1, a massive neutrophilic inter- and  
7 intra-alveolar infiltration was evident; the alveolar epithelium appeared intact and some areas of  
8 mammary tissue were free of cellular infiltration (Fig. 3 i). In the samples obtained on D2 and D4,  
9 neutrophilic infiltration was again the predominant feature; lysis of neutrophils, degeneration of  
10 epithelial cells, as well as areas of complete alveolar destruction with extravasation were seen. In the  
11 tissue sample obtained on D18 and on D26, features of chronic inflammation predominated:  
12 infiltration by lymphocytes, alveolar destruction, early stages of fibrous tissue proliferation with  
13 development of fibroblasts; in some areas of the samples, the glandular elements had been replaced  
14 almost entirely by fibrous tissue; this developed characteristically around the alveoli. Finally, in the  
15 tissue sample obtained on D34, extensive presence of fibrous tissue with lymphocytic infiltration  
16 were seen; shrunken or involuting alveoli and thickened interlobular tissue were evident (Fig. 3 ii and  
17 iii). It is noteworthy that in all samples areas of healthy tissue interspaced with areas of pathological  
18 tissue.

19 Control glands. In the bitch operated on D2, there was mild neutrophilic infiltration sparsely  
20 present in the parenchyma. In the bitch operated in D4, there was moderate neutrophilic infiltration  
21 and characteristic congestion in the "OP" gland; there was also sparse mild neutrophilic infiltration in  
22 the other two glands ("C+1", "C-1"). No other pathological features were evident.

23

24

25

26

#### 1 4. Discussion

2

3 The dearth of information about canine mastitis was the stimulus to carry out an experimental  
4 study on the disease. Kuhn and others (1991) considered staphylococci as non-pathogenic or of low  
5 pathogenicity for the mammary gland of bitches; furthermore, existence of subclinical mastitis in bitches  
6 has been questioned (Wheeler et al., 1984). The pathogenicity of the microorganism was suggested by the  
7 initial isolation of *S. intermedius* from clinical mastitis.

8 Initially, clinical signs developed; the features of the experimental disease resembled those that  
9 observed in natural cases of canine mastitis. Mild systemic signs, abnormal secretion, mammary  
10 enlargement and abscess formation have all been reported in the literature and have been observed in our  
11 clinical experience. Subsequently, the disease reverted to subclinical form, confirmed by the consistent  
12 isolation of bacteria, the demonstration of increased WST scores and the presence of leukocytes in  
13 secretion films; finally, bacterial presence and inflammatory reaction were recorded during the  
14 histopathological examination. This experimental model can be considered useful for further studies on  
15 the subject.

16 *S. intermedius* is part of the skin flora of dogs (Allaker et al., 1992a; Harvey and Noble, 1998;  
17 Woldehiwet and Jones, 1990); it is isolated from their skin, hairs, buccal cavity, nasopharynx, anus and  
18 vagina (Allaker et al., 1992b). Bitches harbouring the organism, transmit it to their puppies during the  
19 nursing period (Allaker et al., 1992c). The organism is involved in suppurative canine infections (Ihrke,  
20 1987), by producing an exfoliative toxin (Hesselbarth et al., 1994; Terauchi et al., 2003); presence of a  
21 leukocidin responsible for tissue necrosis has also been reported (Prevost et al., 1995; Piemont, 1997).  
22 Finally, tissue adherence by the bacterium contributes to its pathogenicity (Cree and Noble, 1995;  
23 Saijonmaa-Koulumies and Lloyd, 2002).

24 The organism has been isolated occasionally from cases of bovine (Roberson et al., 1996) or  
25 caprine (Hugnet, 2002) mastitis. Nevertheless, specific toxic effects of *S. intermedius* on mammary cells  
26 have not been reported. Based on the histological findings, we can postulate that necrosis of mammary  
27 cells could be the effect of leukocidin production. In order to rule out a facilitative effect of the presence



1 of milk in the mammary glands on bacterial proliferation, given that milk is a good substrate, the  
2 mammary glands were emptied before challenge.

3 As in ruminant mastitis, there was an influx of inflammatory cells (indicated by the increased  
4 WST scores and the abundance of neutrophils in Giemsa-stained films) into the affected mammary gland  
5 as soon as 12 h after challenge. Subsequently, WST scores remained high for a long-time after challenge.  
6 The increase was coupled with a transient decrease of blood leukocyte counts, followed by a subsequent  
7 sharp increase. Counts increased and exceeded 20,000 cells  $\mu\text{l}^{-1}$ , a value considered normal in bitches  
8 during late pregnancy and early lactation (Bush, 1993). During the subacute phase of the disease (P3)  
9 blood counts decreased, but those of immature neutrophils remained above 300 cells  $\mu\text{l}^{-1}$ , a threshold  
10 considered the upper limit of normality (Bush, 1993). The latter finding indicates an active inflammatory  
11 reaction in the host and persistence of high "demand" of neutrophils (Bush, 1993) and is consistent with  
12 the inflammation areas observed on all histological sections. Persistent mild mammary inflammation is  
13 characteristic of staphylococcal mastitis in ruminants [cows: Shibahara and Nakamura (1999), Brouillette  
14 and Malouin (2005); ewes: Fthenakis and Jones (1990), Bergonier and others (2003); goats: Contreras  
15 and others (1997), Bergonier and others (2003)].

16 However in contrast to ruminants, we found neutrophils also in the three control mammary  
17 glands. WST scores were found to increase 12 h after challenge also in samples from control glands and  
18 concurrently to the challenged gland. In control glands, either injected with PBS ("C+1") or uninoculated  
19 ("OP" and "C-1"), there was a significant difference between WST scores recorded in P1 and scores  
20 recorded in P2 ( $P < 0.002$ ); furthermore in P2, but not in P1, there was a difference between WST scores  
21 from challenged and control glands ( $P < 0.001$  and  $P < 0.05$ , respectively). These findings suggest that  
22 during the acute phase of the disease, leukocytes immigrated from blood into all mammary glands of the  
23 animal.

24 In relation to platelet values, their initial decrease is consistent with the extensive mammary  
25 haemorrhages observed during pathological examination. Subsequently, their values returned to counts  
26 expected for the pre- and post-partum stage (Bush, 1993).

27 During the pathological examination, extensive tissue destruction of the mammary gland was  
28 evident. In ruminants, where similar lesions have been reported (Van de Putte van Messom et al., 1993;

1 Shibahara and Nakamura, 1999), mastitis causes significant decrease in milk yield, possibly leading to  
2 detrimental growth of newborns. Hence, one can postulate that growth retardation of puppies in cases of  
3 mammary disorders, as reported by Wheeler and others (1984), may also be due to the same sequence of  
4 events. However, the fact that in our study no abnormal effects on puppy growth were seen, may be due  
5 to only one mammary gland being implicated in the infection process, as well as the number of puppies  
6 reared by each experimental bitch. One may postulate that no growth retardation would be expected if, for  
7 instance, a bitch rearing 4 puppies has mastitis in only two glands.

8 Finally, the reduction in mammary size observed clinically at the final stages of the experiment  
9 coincides with partial involution features seen histologically. In fact, such process might be a defensive  
10 mechanism of the mammary gland (Lucker et al., 2000).

11 We conclude that in bitches (a) intramammary inoculation of *S. intermedius* is useful to study  
12 mastitis, (b) *S. intermedius* can be pathogenic for the canine mammary gland, (c) clinical mastitis may  
13 lead to subclinical disease within 18 to 26 days, (d) bacterial isolation and leukocyte increase (in blood  
14 and in mammary secretion) are the main features of the disease, (e) in contrast to ruminants, increase of  
15 leukocytes is present in adjacent mammary glands and (f) mastitis results to destruction of mammary  
16 parenchyma.

17  
18

## 19 **Acknowledgements**

20

21 The project was funded by a grant from the Research Committee of the University of Thessaly.  
22 The help of Sue Rodway (Department of Pathology and Infectious Diseases, The Royal Veterinary  
23 College, London) for performing the histological sections and of Athina Tzora (Department of Animal  
24 Production, TEI Epirus) for confirming the bacterial identifications, is gratefully acknowledged.

25  
26  
27  
28

1 **References**

2

3 Allaker, R.P., Jensen, L., Lloyd, D.H., Lamport, A.I., 1992a. Colonization of neonatal puppies by  
4 staphylococci. *Br. Vet. J.* 148, 523-528.

5 Allaker, R.P., Lloyd, D.H., Bailey R.M., 1992b. Population sizes and frequency of staphylococci at  
6 mucocutaneous sites on healthy dogs. *Vet. Rec.* 130, 303-304.

7 Allaker, R.P., Lloyd, D.H., Simpson, A.I., 1992c. Occurrence of *Staphylococcus intermedius* on the hair  
8 and skin of normal dogs. *Res. Vet. Sci.* 52, 174-176.

9 Barrow, G.I., Feltham, R.K.A., 1993. *Manual for the Identification of Medical Bacteria*, 3rd edn.  
10 Cambridge University Press, Cambridge.

11 Bergonier, D., De Cremoux, R., Rupp, R., Lagriffoul, G., Berthelot, X., 2003. Mastitis of dairy small  
12 ruminants. *Vet. Res.* 34, 689-716.

13 Biddle, D., Macintire, D.K., 2000. Obstetrical emergencies. *Clin. Tech. Small. Anim. Pract.* 15, 88-93.

14 Brouillette, E., Malouin, F., 2005. The pathogenesis and control of *Staphylococcus aureus*-induced  
15 mastitis: study models in the mouse. *Microb. Infec.* 7, 560-568.

16 Bush, B.M., 1993. *Interpretation of Laboratory Results for Small Animal Clinicians*. Blackwell, Oxford.

17 Contreras, A., Corrales, J.C., Sanchez, A., Sierra, D., 1997. Persistence of subclinical intramammary  
18 pathogens in goats throughout lactation. *J. Dairy Sci.* 80, 2815-2819.

19 Cree, R.G., Noble, W.C., 1995. In vitro indices of tissue adherence in *Staphylococcus intermedius*. *Lett.*  
20 *Appl. Microbiol.* 20, 168-170.

21 Euzeby, J.P., 1997. List of bacterial names with standing in nomenclature: a folder available on the  
22 internet. *Int. J. Syst. Bacteriol.* 47, 590-592. (List of Prokaryotic Names with Standing in  
23 Nomenclature. Last full update September 07, 2006. URL: <http://www.bacterio.net>).

24 Fthenakis G.C., 1995. California Mastitis Test and Whiteside Test in diagnosis of subclinical mastitis of  
25 dairy ewes. *Small Rumin. Res.* 16, 271-276.

26 Fthenakis, G.C., Jones, J.E.T., 1990. The effect of inoculation of coagulase-negative staphylococci into  
27 the ovine mammary gland. *J. Comp. Pathol.* 102, 211-219.

- 1 Harvey, R.G., Noble, W.C., 1998. Aspects of nasal, oropharyngeal and anal carriage of *Staphylococcus*  
2 *intermedius* in normal dogs and dogs with pyoderma. *Vet. Dermatol.* 9, 99-104.
- 3 Hedlund, C.S., 1997. Surgery of the reproductive and genital systems. In: Fossum T.W., Hedlund C.S.,  
4 Johnson A.L., Seim H.B. III, Willard M.B., Carroll G.L. (Eds), *Small Animal Surgery*. Mosby, St  
5 Louis, pp. 517-574.
- 6 Hesselbarth, J., Flachsbarth, M.F., Amtsberg, G., 1994. Studies on the production of an exfoliative toxin  
7 by *Staphylococcus intermedius*. *J. Vet. Med. B.* 41, 411-416.
- 8 Hugnet, C., 2002. Mastitis by *Staphylococcus intermedius* and domestic flies in goat herd. *Bull. GTV* 15,  
9 163-164.
- 10 Ihrke, P.J., 1987. An overview of bacterial skin disease in the dog. *Br. Vet. J.* 143, 112-118.
- 11 International Dairy Federation, 1984. Recommended methods for somatic cell counting in milk. *Bull.*  
12 *Internatl. Dairy Feder.* 168.
- 13 Johnston, S.D., Root Kustritz, M.V., Olson, P.N.S., 2001. *Canine and Feline Theriogenology*. Saunders,  
14 Philadelphia.
- 15 Kuhn, G., Pohl, S., Hingst, V., 1991. Investigations on bacteria in the milk of healthy lactiferous bitches.  
16 *Berlin. Munchen. Tierarztl.* 104, 130-133.
- 17 Lucker, E., Mazurek, S., Eigenbrodt, E., 2000. Apoptosis and mammary health. *Tierarztl. Prax. G. N.* 28,  
18 182-188.
- 19 Miles, A.A., Misra, J.S., 1938. The estimation of the bactericidal power of the blood. *J. Hyg. Camb.* 38,  
20 732-749.
- 21 Olson, P.N., Olson, A.L., 1984. Cytologic evaluation of canine milk. *Vet. Med. Small. Anim Clin.* 79,  
22 641-646.
- 23 Osbaldiston, G.W., 1978. Bacteriological studies of reproductive disorders of bitches. *J. Am. Anim.*  
24 *Hosp. Assoc.* 14, 363-367.
- 25 Piemont, Y., 1997. Synergo-hymenotropic toxins from staphylococci. *Med. Maladies Infect.* 27, 135-142.
- 26 Prevost, G., Bouakham, T., Piemont, Y., Monteil, H., 1995. Characterisation of a synergohymenotropic  
27 toxin produced by *Staphylococcus intermedius*. *FEBS Lett.* 376, 135-140.

- 1 Roberson, J.R., Fox, L.K., Hancock, D.D., Gay, J.M., Besser, T.E., 1996. Prevalence of coagulase-  
2 positive staphylococci, other than *Staphylococcus aureus*, in bovine mastitis. *Am. J. Vet. Res.* 57,  
3 54-58.
- 4 Saijonmaa-Koulumies, L.E., Lloyd, D.H., 2002. Colonization of neonatal puppies by *Staphylococcus*  
5 *intermedius*. *Vet. Dermatol.* 13, 123-130.
- 6 Schalm, O.W., Carroll, E.J., Jain, N.C., 1971. *Bovine Mastitis*. Lea and Febiger, Philadelphia.
- 7 Shibahara, T., Nakamura, H., 1999. Pathology of acute necrotizing mastitis caused by *Staphylococcus*  
8 *aureus* in a dairy cow. *Jarq-Jap. Agric. Res. Q.* 33, 139-142.
- 9 Terauchi, R., Sato, H., Hasegawa, T., Yamaguchi, T., Aizawa, C., Maehara, N., 2003. Isolation of  
10 exfoliative toxin from *Staphylococcus intermedius* and its local toxicity in dogs. *Vet. Microbiol.* 94,  
11 19-29.
- 12 Van de Putte van Messom, G., Burvenich, C., Roets, E., Massartleen, A.M., Heyneman, R., Kremer,  
13 W.D.J., Brand, A., 1993. Classification of newly calved cows into moderate and severe responders  
14 to experimentally induced *Escherichia coli* mastitis, *J. Dairy Res.* 60, 19-29.
- 15 Von Walser, K., Henschelchen, O., 1983. Etiology of canine mastitis in the bitch, Berlin. Munchen.  
16 *Tierarztl.* 96, 195-197.
- 17 Wheeler, S.L., Magne, M.L., Kaufman, J., Husted, P.W., Allen, T.A., Olson, P.N., 1984. Postpartum  
18 disorders in the bitch, *Comp. Cont. Educ. Pract.* 6, 493-500.
- 19 Woldehiwet, Z., Jones, J.J., 1990. Species distribution of coagulase-positive staphylococci isolated from  
20 dogs, *Vet. Rec.* 126, 485.

21  
22  
23

1 Fig. 1. Mean reaction scores of WST in mammary secretion of bitches before and after intramammary inoculation of  
2 *S. intermedius* (■: challenged mammary gland, ● control glands, grouped together for the purposes of this graph,  
3 bars show standard error of the mean).

4

5

6 Fig. 2. Mean leukocyte (straight line, ■) and total neutrophil (dotted line, ●) counts (cells  $\mu\text{l}^{-1}$ ) in blood of bitches  
7 before and after intramammary inoculation of *S. intermedius* (bars show standard error of the mean).

8

9

10 Fig. 3. (i) massive neutrophilic inter- and intra-alveolar infiltration, intact epithelium (H&E, X100, D1). (ii)  
11 histologic findings: fibrous tissue with lymphocytic infiltration, alveolar destruction (H&E, X200, D34). (iii)  
12 histologic findings: thickened interlobular tissue; shrunken or involuting alveoli (H&E, X200, D34).

13

14

15

16

Table 1

Sequential bacteriological findings in mammary secretion of bitches intramammarily challenged with *S. intermedius*

	W3 (D-5) <sup>b</sup>	W5 <sup>b</sup>	W8 <sup>b</sup>	D0 <sup>b</sup>	D1 <sup>b</sup>	D2 <sup>b</sup>	D3 <sup>b</sup>	D4 <sup>b</sup>	D6 <sup>b</sup>
CH <sup>a</sup>	0/6 <sup>c</sup>	0/6	0/6	6/6	6/6	5/5	4/4	4/4	3/3
OP <sup>a</sup>	0/6	0/6	0/6	0/6	1/6	2/5	2/4	1/4	0/3
C-1 <sup>a</sup>	0/6	0/6	0/6	0/6	0/6	0/5	0/4	0/4	0/3
C+1 <sup>a</sup>	0/6	0/6	0/6	0/6	0/6	0/5	0/4	0/4	0/3
	D8 <sup>b</sup>	D11 <sup>b</sup>	D14 <sup>b</sup>	D18 <sup>b</sup>	D22 <sup>b</sup>	D26 <sup>b</sup>	D30 <sup>b</sup>	D34 <sup>b</sup>	D0 to D30
CH	3/3	3/3	2/3	1/3	1/2	0/2	0/1	0/1	38/46 <sup>*</sup>
OP	0/3	1/3	1/3	0/2	0/2	0/2	0/1	0/1	8/46 <sup>*,†</sup>
C-1	0/3	0/3	0/3	0/2	0/2	0/2	0/1	0/1	0/46 <sup>*,†</sup>
C+1	0/3	0/3	0/3	0/2	0/2	0/2	0/1	0/1	0/46 <sup>*,†</sup>

<sup>a</sup> CH: challenged mammary gland, OP: gland opposite to challenged, C-1: gland behind challenged, C+1: gland in front of challenged. <sup>b</sup> W3, W5, etc. = days after whelping -

D0, D1, D2, etc. = days after challenge. <sup>c</sup> n/m = positive results out of total samples obtained. \* and † Differences statistically significant.

Table 2

Results of WST scores in mammary secretion of bitches intramammarily challenged with *S. intermedius*

	Period 1 (P1) <sup>b</sup>	Period 2 (P2) <sup>b</sup>	Period 3 (P3) <sup>b</sup>	Period 4 (P4) <sup>b</sup>	<i>P</i> = <sup>e</sup>
CH <sup>a</sup>	0 (0-2) <sup>c, d</sup> 1/18 <sup>c</sup>	4 (0-5) 23/25	2 (0-4) 7/9	2 (0-3) 11/12	<0.001*
OP <sup>a</sup>	0 (0-2) 3/18	2 (0-4) 17/25	2 (0-2) 5/9	0 (0-3) 4/12	0.001*
C-1 <sup>a</sup>	0 (0-2) 2/18	2 (0-4) 15/25	0 (0-3) 4/9	0 (0-3) 3/12	0.009*
C+1 <sup>a</sup>	0 (0-1) 0/18	1 (0-4) 12/25	0 (0-2) 4/9	0 (0-2) 2/12	0.003*
<i>P</i> = <sup>e</sup>	0.300	<0.001*	0.056	<0.001*	

<sup>a</sup> CH: challenged mammary gland, OP: gland opposite to challenged, C-1: gland behind challenged, C+1: gland in front of challenged. <sup>b</sup> Period 1: Pre-challenge (W3 to W8), Period 2: D0 to D4, Period 3: D6 to D11, Period 4: D14 to D34. <sup>c</sup> j (k-l) = median value (range), n/m = positive results (score  $\geq$  "1") out of total samples obtained. <sup>d</sup> For calculation purposes, WST scores were transformed as follows: score "-" = 0, score "±" = 1, score "1" = 2, etc. <sup>e</sup> In the column of periods: significance of differences between the 4 periods, in the row of glands: significance of differences between the 4 glands. \* Differences statistically significant



Table 3

Results of haematological and blood biochemical parameters in bitches intramammarily challenged with *S. intermedius*

	Period 1 (P1) <sup>a</sup> (N=18 <sup>b</sup> )	Period 2 (P2) <sup>a</sup> (N=25 <sup>b</sup> )	Period 3 (P3) <sup>a</sup> (N=9 <sup>b</sup> )	Period 4 (P4) <sup>a</sup> (N=12 <sup>b</sup> )	P= <sup>d</sup>
Haematocrit (%)	32.8±0.6 <sup>c</sup>	32.5±0.6	33.0±0.7	36.0±0.7	0.012*
Leukocyte counts (cells µl <sup>-1</sup> )	18,694±1,060	24,287±1,755	18,467±1,315	17,675±1,428	0.034*
Platelet counts (particles µl <sup>-1</sup> )	596,000±32,146	461,458±21,253	649,333±44,051	594,917±44,631	< 0.001*
Globulin (g dl <sup>-1</sup> )	3.60±0.10	3.56±0.09	3.62±0.15	4.00±0.25	0.139
Albumin (g dl <sup>-1</sup> )	2.11±0.12	1.96±0.04	2.16±0.11	1.97±0.10	0.344

<sup>a</sup> Period 1: Pre-challenge (W3 to W8), Period 2: D0 to D4, Period 3: D6 to D11, Period 4: D14 to D34. <sup>b</sup> "N" refers to number of samples. <sup>c</sup> n±m = mean ± standard error of the mean. <sup>d</sup> Significance of differences between the 4 periods. \* Differences statistically significant

Table 4

Results of differential leukocyte type (cells  $\mu\text{l}^{-1}$ ) in bitches intramammarily challenged with *S. intermedius*

	Period 1 (P1) <sup>a</sup> (N=18 <sup>b</sup> )	Period 2 (P2) <sup>a</sup> (N=25 <sup>b</sup> )	Period 3 (P3) <sup>a</sup> (N=9 <sup>b</sup> )	Period 4 (P4) <sup>a</sup> (N=12 <sup>b</sup> )	<i>P</i> = <sup>f</sup>
Leukocyte counts (cells $\mu\text{l}^{-1}$ )	18,694±1,060 <sup>c</sup>	24,287±1,755	18,467±1,315	17,675±1,428	0.034*
Mature neutrophil counts (cells $\mu\text{l}^{-1}$ )	13,548±956 [72.5%] <sup>d</sup>	19,311±1,714 [79.5%]	13,231±1,018 [71.5%]	13,365±1,146 [75.6%]	0.033*
Immature neutrophil counts (cells $\mu\text{l}^{-1}$ )	1,450±425 [9.7%] <sup>e</sup>	1,879±396 [8.9%]	1,519±443 [10.3%]	150±63 [1.1%]	0.001*
Lymphocyte counts (cells $\mu\text{l}^{-1}$ )	3,148±392	2,712±430	3,229±568	3,208±336	0.783
Monocyte counts (cells $\mu\text{l}^{-1}$ )	547±251	385±73	488±132	952±240	0.148
Eosinophil counts (cells $\mu\text{l}^{-1}$ )	0±0	0±0	0±0	0±0	1.000
Basophil counts (cells $\mu\text{l}^{-1}$ )	0±0	0±0	0±0	0±0	1.000

<sup>a</sup> Period 1: Pre-challenge (W3 to W8), Period 2: D0 to D4, Period 3: D6 to D11, Period 4: D14 to D34. <sup>b</sup> "N" refers to number of samples. <sup>c</sup> n±m = mean ± standard error of the mean. <sup>d</sup> Proportion of neutrophils among leukocytes. <sup>e</sup> Proportion of immature neutrophils among total neutrophils. <sup>f</sup> Significance of differences between the 4 periods. \* Differences statistically significant

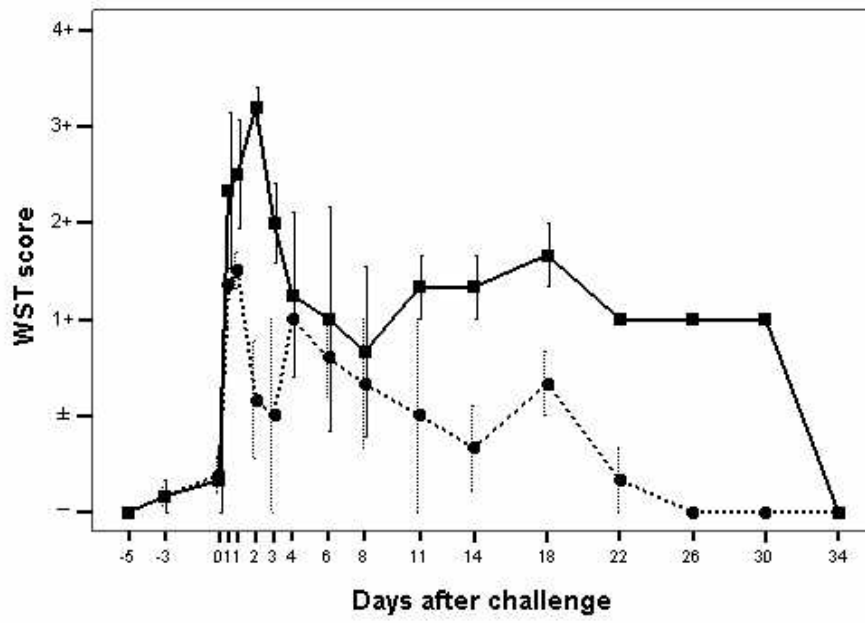


Figure 1.

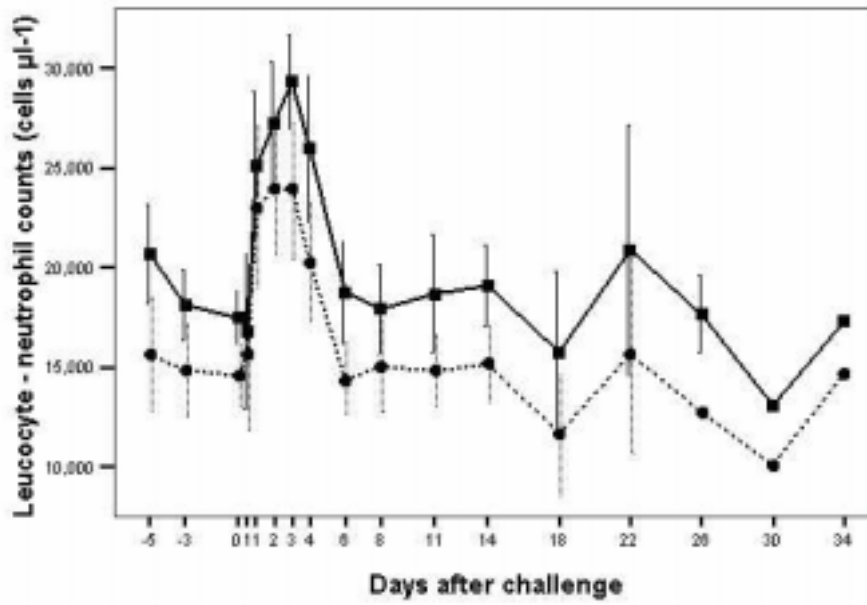


Figure 2.

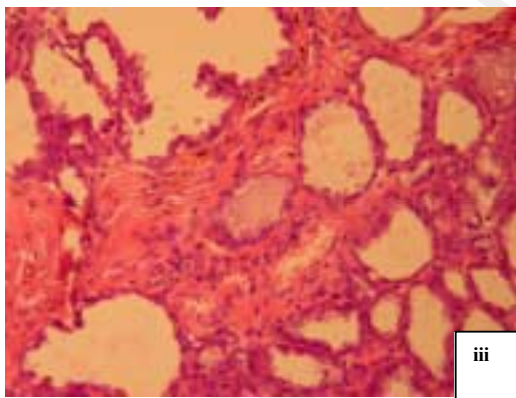
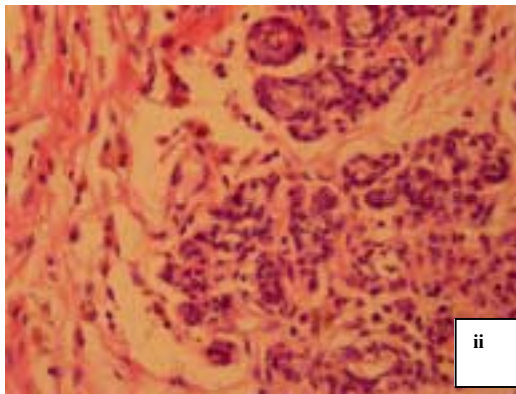
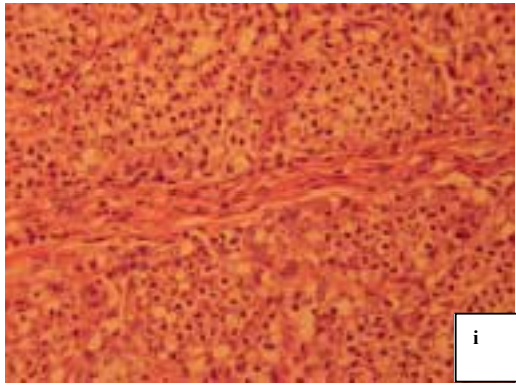


Figure 3.