STUDIES IN BACTERIAL ADHERENCE TO CANINE AND FELINE CORNEOCYTES

By

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Thesis submitted for the degree of Master of Veterinary Medicine in the Faculty of Veterinary Medicine, University of Glasgow

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ABSTRACT

In the first chapter of this thesis a three-year retrospective survey showed 41% of dogs referred to the University of Glasgow Veterinary School for investigation of skin disease were diagnosed as suffering from pyoderma with 62% of these cases being associated with atopic dermatitis. In contrast, pyoderma was rarely diagnosed in the cat. The study confirmed the importance of canine pyoderma and its common association with atopic dermatitis. Although canine pyoderma due to *Staphylococcus intermedius* is common, it is poorly understood. Adhesion by staphylococci has been suggested as a potentially important factor in the establishment of infection. As many assays to quantify adhesion by bacteria are both tedious and time consuming this has hampered studies in this interesting area.

Chapters two and three detail the development of a simple adhesion assay to quickly quantify bacterial adhesion to canine and feline corneocytes. As far as the author can determine there is no published data on the size and surface area of canine and feline corneocytes therefore morphometric studies were undertaken in chapter 2. The mean canine and feline corneocyte diameter and surface area were found to be approximately 38-48 μm and 1100-1800 μm^2 respectively. Of three body sites examined the inner aspect of the canine and feline pinna proved to be most suitable for the collection of corneocytes for an adhesion assay.

In chapter 3 a simple adhesion assay is described. Corneocytes were collected from the inner aspect of the pinna using adhesive discs (D-squame® discs), and incubated in a moist chamber with bacteria under test. Image analysis software employing a script (macro) written by the author was used to count adherent bacteria. Adhesion by bacteria was found to be dependent on time, temperature, and bacterial concentration. The assay was both repeatable and reproducible. Using the adhesion assay so developed the pathogenic staphylococci (*S. aureus*, *S. hyicus* and *S. intermedius*) all adhered well to canine and feline corneocytes. *S. hominus* and a Micrococus species adhered poorly.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

PROCEEDINGS AND PRESENTATIONS

Lu, Y. and McEwan, N.A. (2003). Morphometric analysis of canine and feline corneocytes. British Veterinary Dermatology Study Group Proceedings. Spring meeting. 2nd April 2003. 49-50.

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AUTHOR'S DECLARATION

The work presented in this thesis was carried out by the author in the Department of Veterinary Clinical Studies, University of Glasgow.

Signed: ________________________________

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Date: 18. JUNE, 2004

CHAPTER 1

A Three Year Retrospective Study Of The Prevalence Of Canine And Feline Skin Diseases With Special Reference To Pyoderma

LITERATURE REVIEW

Introduction

The epidermis of mammalian skin forms a protective barrier which provides physical, chemical, and microbial defence (Lloyd, 1976; Roth and James, 1989; Hill and Moriello, 1994). The outer most layer of the epidermis, the stratum corneum has been shown to be an important barrier to the penetration of micro-organisms and their metabolites from the skin surface (Sulzburger, 1960; Lai a Fat and Van Furth, 1974; Lloyd and Jenkinson, 1980). Hair forms the first line of physical defence which protects against the contact of pathogens with the skin, but it may also harbour bacteria, especially staphylococci (White, Ihrke, Stannard, Sousa, Reinke, Rosser, Jr. and Jang, 1983; Cox, Hoskins, Newman, Turnwald, Foil, Roy and Kearney, 1985; Allaker, Lloyd and Simpson, 1992; Harvey, 1994; Harvey and Lloyd, 1995).

Canine and feline skin flora

The normal skin microflora contributes to skin defence mechanisms. Bacteria are located in the superficial epidermis and in the infundibulum of the hair follicles, where sweat and sebum provide nutrients (Lloyd, 1980a; Scott, 1992). The normal flora is a mixture of bacteria that live in symbiosis, probably exchanging growth factors. The flora may change with different cutaneous environments. These are affected by factors such as heat, pH, salinity, moisture, albumin level, and fatty acid level (Mason, 1996; Chesney, 1997). The close relationship between the host and microorganisms enables bacteria to occupy microbial niches and to inhibit colonization by invading organisms. In addition, many bacteria (*Bacillus* spp., *Streptococcus* spp., and *Staphylococcus* spp.) are capable of producing antibiotic substances, and some bacteria can produce enzymes (e.g. β-lactamase) that inhibit antibiotics (Saijonmaa-Koulumies and Lloyd, 1995). Bacteria that are isolated from the skin are commonly divided into three categories: resident organisms, transient organisms, and common pathogenic organisms (nomads) (Price, 1938; Somerville-Millar and Noble, 1974; Scott, Miller and Griffin, 2001b). Resident bacterial organisms can be routinely and repeatedly isolated and cultured from the surface of the skin or haircoat without causing clinical disease. Transient bacteria may on occasion be cultured from the skin but are of no significance unless they become involved in a pathologic process as secondary invaders. However, there is still controversy as to which microorganisms are truly residents on the canine skin. Studies describing the canine 'resident bacterial flora' have been based on short-term studies that fail to describe the dynamic situation of the microflora on the skin and thus do not provide evidence of the persistence of the individual bacterial species (Saijonmaa-Koulumies and Lloyd, 1996). In addition, both methods and anatomical sites employed for sampling have varied considerably in different studies (Krogh and Kristensen, 1976; Ihrke, Schwartzman, McGinley, Horwitz and Marples, 1978; Allaker, Lloyd and Simpson, 1992; Harvey and Lloyd, 1994; Harvey and Lloyd, 1995). Transient bacteria (e.g. *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas* spp., *Corynebacterium* spp., and *Bacillus* sp.) do not multiply on the skin of healthy dogs (Krogh and Kristensen, 1976; Kristensen and Krogh, 1978; Krogh and Kristensen, 1981). The resident bacteria of canine skin include coagulase-negative staphylococci (*Staphylococcus epidermidis*, *S. cohnii*, *S. saprophyticus*, *S. hominis*, *S. haemolyticus*, *S. capitis*, *S. warneri*, *S. xylosus*, *S. simulans*, and *S. sciuri*); coagulase-positive staphylococci (*S. intermedius*); *Micrococcus* spp.; alpha-hemolytic streptococci; *Actinobacter* sp., *Clostridium* spp. and *Propionibacterium acnes* (Cox, Hoskins, Roy, Newman and Luther, 1984; Cox, Hoskins, Newman, Foil, Turnwald and Roy, 1988; Woldehiwet and Jones, 1990; Lloyd and Allaker, 1991;

Harvey, 1993; Greene and Lammler, 1993; Harvey and Lloyd, 1995; Saijonmaa-Koulumies and Lloyd, 1996; Scott, Miller and Griffin, 2001b). In cats, the resident flora includes *Micrococcus* spp.; coagulase-negative staphylococci, especially *Staphylococcus simulans*; α-hemolytic streptococci; and *Acinetobacter* spp. (Scott, 1980; Medleau, Long, Brown and Miller, 1986). Coagulase-positive staphylococci, including both *Staphylococcus aureus* and *S. intermedius*, are also commonly isolated from normal cat skin and should be considered residents (Devriese, Nzuambe and Godard, 1984; Cox *et al*, 1985; Medleau and Blue, 1988). In Krogh and Kristensen's study (1976), 50% of the cultures from cats were negative. Household cats have a higher frequency of isolation of coagulase-negative staphylococci (*S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. sciuri*, and *S. warneri*) and coagulase-positive staphylococci (*S. aureus* and *S. intermedius*) compared with the case in cattery cats, suggesting that these organisms may be transferred from humans (Scott, Miller and Griffin, 2001b).

Pathogenic skin microorganisms

Nomads are able to colonize and reproduce only for short periods. They are able to take advantage of changing conditions at the skin surface and, if such changes favour proliferation on a long-term basis, clinical infection may result (Lloyd, 1995). Primary pathogenic organisms are capable of tissue invasion and creating disease. They are usually coagulase-positive staphylococci (*S. intermedius*, *S. aureus*, *S. hyicus*), and in the dog *S. intermedius* is the single most common isolate from canine skin infections (Phillips, Jr. and Kloos, 1981; Berg, Wendell, Vogelweid and Fales, 1984; Biberstein, Jang and Hirsh, 1984; Cox, Newman, Roy and Hoskins, 1984; Ihrke, 1987). The pathogenesis of canine pyoderma is poorly understood. Various virulence factors can be produced by *S. intermedius* but studies have failed to correlate this with pathogenicity (Greene and Lammler, 1993; Burkett and Frank, 1998). In the light of this, it has been suggested that host factors, such as poorly developed epidermal defences, are very important (Mason, 1999). Cats infrequently experience pyoderma but commonly have subcutaneous abscesses from biting wounds. Therefore, the mouth flora of the cat is an important factor, which includes *Pasteurella multocida*, β-hemolytic streptococci, *Corynebacterium* spp., *Actinomyces* spp., *Bacteroides* spp., and *Fusobacterium* spp. (Scott, Miller and Griffin, 2001b). *Staphylococcus felis* is increasingly isolated from cats with otitis externa, subcutaneous abscesses, paronychia, and other skin infections (Igimi, Kawamura, Takahashi and Mmitsuoka, 1989; Aarestrup and Jacobeson, 1993; Patel, 2003).

Canine and feline pyoderma

Skin infections are traditionally classified as primary or secondary infections to reflect the absence or the presence of an underlying cause (Scott, Miller and Griffin, 2001b). It is generally accepted that most pyodermas occur as a sequel to other cutaneous, metabolic, or immunologic abnormalities (Scott, Miller and Griffin, 2001b), such as hypersensitivity, endocrinopathies, or defects in keratinisation which allow the pathogen to multiply and result in pyoderma (Ihrke, 1987; Hill and Moriello, 1994).

The importance of staphylococcal carriage on the skin, hair and mucous membranes of dogs and the role of staphylococci in canine pyoderma is well documented (Mason and Lloyd, 1989; Mason and Lloyd, 1990; Allaker, Lloyd and Simpson, 1992; Allaker, Lloyd and Bailey, 1992; Harvey and Lloyd, 1994; Saijonmaa-Koulumies and Lloyd, 1996). In dogs, pyoderma is commonly diagnosed (Scott and Paradis, 1990), while in cats, there are few reports on staphylococcal skin disease (Cox *et al*, 1985; Medleau and Blue, 1988; White, 1991; Espinola and

Lilenbaum, 1996; Patel, 2002). The few published clinical reports (Raychaudhuri and Raychaudhuri, 1993; Scott, Miller and Griffin, 2001b) have in the main identified underlying immunosuppressive conditions such as feline leukaemia virus (FeLv), feline immunodeficiency virus (FIV), and toxoplasmosis as predisposing factors. *S. aureus* and *S. intermedius* have been cultured from feline skin lesions (Medleau and Blue, 1988; White, 1991) and have been implicated as pathogens, however, their exact role in the pathogenesis of feline skin disease remains unknown. *S. felis* is a coagulase-negative species which was first identified from clinical specimens (Igimi *et al*, 1989) and has subsequently been implicated in the pathogenesis of otitis externa (Higgins and Gottchalk, 1991), paronychia (Aarestrup and Jacobeson, 1993) and skin disease (Patel, Lloyd, Howell and Noble, 2002). The most recent study of the prevalence of cutaneous staphylococci in feral cats, healthy cats and pet cats with skin lesions suggested that *S. felis* may be a resident on cat skin and *S. intermedius* may play a role in perpetuating skin disease in cats (Patel, 2002).

The stratum corneum as a barrier

The stratum corneum consists of tightly packed keratinised cells, which are permeated by an emulsion of sebum, sweat, and intercellular cement substance. The cells and emulsion function as an effective physical and chemical barrier to potential pathogens (Lloyd, 1980a; Berg *et al*, 1984; Mason and Lloyd, 1993; Saijonmaa-Koulumies and Lloyd, 1996). The intercellular material of the stratum corneum is likely to be an important component of this barrier. However, it would appear from studies that less is present in dog skin than has been reported in cattle and man. This may partly explain the high incidence of pyoderma in comparison with other species of domestic animal (Scott, 1988; Scott, Miller and Griffin, 2001b). In cattle and sheep, scanning electron microscope studies have shown that the hair follicle infundibula are protected by layers of squames permeated with sebaceous lipid; this material fills the infundibula forming a raised conical mass (Lloyd, Dick and Jenkinson, 1979). In dogs, similar techniques have shown that smaller amounts of this material are present and the infundibula are seen as pits in the epidermal surface (Lloyd and Garthwaite, 1982). Examination of the skin surface topography in freeze-fracture specimens produced similar results (Mason and Lloyd, 1993). The effects of these differences are unknown but it is possible that the relatively unprotected hair follicles and thinner, more compact corneum of dogs are more permeable and therefore make them more susceptible to pyoderma. This hypothesis is supported by the results of previous studies (Mason and Lloyd, 1990) in which tritiated staphylococcal protein A was shown to pass through the canine epidermis and could be detected within the superficial dermis of normal dog skin 5 hours after topical application. In man, percutaneous absorption of protein A is impeded by the presence of an intact stratum corneum (White and Noble, 1980).

Predisposing factors for infection

The factors that may affect bacterial colonization on skin are environment (humidity and temperature) (Marples, 1965; Bibel and Lebrun, 1975; McBride, Duncan and Knox, 1977; Lloyd, 1980b; Ihrke, 1987; McBride, 1993), host factors (the hair coat and skin secretions) (McGinley, Leyden, Marples and Kligman, 1975; Ihrke *et al*, 1978; Lloyd and Garthwaite, 1982; Garthwaite, Lloyd and Thomsett, 1983; White *et al*, 1983; Allaker, Lloyd and Simpson, 1992; Mason and Lloyd, 1993; McEwan Jenkinson D., 1993; Harvey and Lloyd, 1994; Harvey and Lloyd, 1995; Scott, Miller and Griffin, 2001a), and bacterial factors (adherence and interaction between bacteria) (Murphy, 1975; Aly, Shinefield, Strauss and Maibach, 1977; Bibel, Aly, Shinefield, Maibach and Strauss, 1982;

Bibel, 1982; Feingold, 1986; Mason and Lloyd, 1989; McEwan, 1990; Romero-Steiner, Witek and Balish, 1990; Allaker and Noble, 1993; Aly and Bibel, 1993; Mason and Stewart, 1993; Harvey and Noble, 1994; Saijonmaa-Koulumies and Lloyd, 1995).

Bacterial adherence to host tissue is one of the prerequisite steps in the colonization and infection of the skin (Beachey, 1981; Aly and Bibel, 1993). It is a multifactorial process, and is influenced by host susceptibility and tissue tropism (Gibbons and van Houte, 1971; Aly *et al*, 1977). Both *S. aureus* and *S. intermedius* are known to adhere avidly to human (Ofek and Doyle, 1994; Lowy, 1998) and canine (McEwan, 2000) corneocytes respectively and this is likely to be a factor in the development of staphylococcal skin disease.

Conclusions

Canine bacterial pyoderma is one of the most common infections seen in small animal practice and *S. intermedius* appears to be involved in the majority cases (Berg *et al*, 1984). It is clear that bacterial skin disease is very common in the dog but apparently rare in the cat. In general cutaneous infections with *S. intermedius* are secondary to changes in the skin or are a complication of some other dermatosis. One factor in the development of infection may be the ability of *S. intermedius* to adhere to corneocytes. Recent studies have shown that *S. intermedius* adheres to canine corneocytes and that this may be important in the development of bacterial skin disease in canine atopic dermatitis (McEwan, 2000; Forsythe, Hill, Thoday and Brown, 2002; Saijonmaa-Koulumies and Lloyd, 2002).

INTRODUCTION

The literature review has shown bacterial skin disease to be common in the dog but rare in the cat. As a prelude to studies on bacterial adherence to canine and feline corneocytes a study to determine the frequency and types of skin disease within the referral clinic population at the University of Glasgow Veterinary School Hospital (UGVS) was conducted.

AIMS

1. To identify the prevalence of canine and feline skin disease over a three-year period by retrospective study of case records held by the UGVS Small Animal Hospital.
2. To identify cases of canine and feline pyoderma and any associated skin diseases referred to the UGVS over the three-year period.
3. To determine the breed, age and sex distributions (population denominators) of all dogs and cats referred to the UGVS Hospital over the same three-year period.
4. To identify any breed predisposition and disease trends by comparison of the study populations.

MATERIAL AND METHODS

Dermatology cases

Over a three-year period (2000 to 2002), all dogs and cats referred to the UGVS for investigation of skin disease were included in the study. Cases were referred mostly by veterinary surgeons in Glasgow and the surrounding areas but also from further afield, in Scotland and northern England. All cases were investigated by veterinary surgeons that hold the

Royal College of Veterinary Surgeons Diploma in Veterinary Dermatology. Cases underwent standard dermatological approaches to investigation and diagnosis as described by McEwan (2002a). Bacterial skin disease was diagnosed using the following criteria (McEwan, 2002b).

I. The presence of clinical findings characteristic of bacterial pyoderma
II. Culture of a significant micro-organism
III. A clinical response to antibiotic treatment

General hospital population

The computerised data base system (DataFlex 3.05b. Server edition. Data Access Corporation, Miami, Florida, USA) is used to record patient information at the UGVS. Information for the various dog breeds referred for the period 2000 to 2002 was obtained by interrogation of this data base system. This information so obtained was used to compare the breed frequency of skin disease during this period with that of the general referral hospital population.

Statistical analysis

Statistical analysis was carried out using computerised software GraphPad Prism, version 4 (GraphPad Software Inc. San Diego CA). A level of $p < 0.05$ was considered to indicate statistical significance. The Chi-Square test was used to compare sex and breed by analysing data in a 2 x 2 contingency table. The unpaired t-test was used to compare ages when two groups were compared. The Mann-Whitney rank sum test was employed when the data was drawn from a non-normal population.

RESULTS

Retrospective study of canine and feline skin disease referred to the University of Glasgow Veterinary School (2000-2002)

Canine skin disease

Signalment

During the 3 year period studied a total of 330 dogs were examined for skin disease. Of the 330 cases, 132 (40.00%) were entire male, 60 (18.18%) neutered male, 43 (13.03%) entire female and 94 (28.48%) neutered female. In 1 case the sex was not recorded. This gave a male to female ratio of 1.4: 1.

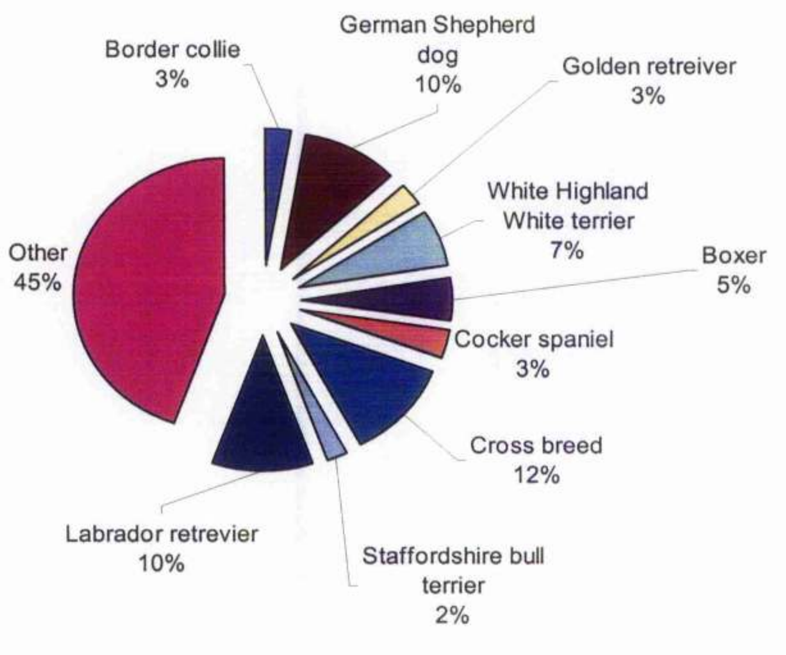
The mean age (±standard deviation) at examination was 4.01 ± 2.81 years (minimum and maximum age: 0.33 and 14.00 years; median age 3.21 years). In 6 cases the age was not recorded.

The most common breeds examined were Cross breed (38), German Shepherd dog (34), West Highland White terrier (22), Labrador retriever (34), Boxer (17), Cocker spaniel (11), Golden retriever (9), Border collie (9) and Staffordshire bull terrier (8). Overall there were 73 different pure breed dogs but 9 breeds accounted for approximately half (42.47%) of the cases examined (Table 1; Figure 1; Appendix A).

Table 1. Most common breeds referred for skin disease (2000-2002)

Breed	Number	Percentage
Cross breed	38	12
German Shepherd dog	34	10
Labrador retriever	34	10
West Highland White terrier	22	7
Boxer	17	5
Cocker spaniel	11	3
Golden retriever	9	3
Border collie	9	3
Staffordshire bull terrier	8	2
Others	148	45

Figure 1. Most common breeds referred for skin disease (2000-2002)



Canine skin diseases diagnosed

There were 340 primary skin diseases diagnosed in the 330 dogs examined. The most common skin condition diagnosed was atopic dermatitis (192 cases; 58.18 %). Other common skin disorders were: allergy not fully diagnosed, 24 cases (7.27%); primary bacterial pyoderma, 23 cases (6.97%); flea allergic dermatitis, 21 cases (6.36%), and parasitic dermatoses, 18 cases (5.45%). (Table 2, Figure 2, Appendix B).

Secondary infections were common. Superficial pyoderma was presented in 94 (28.5%) cases. Deep pyoderma was recorded in 18 (5.5%) cases. Deep and superficial pyoderma occurred together in 6 (0.2%) cases. There was a single primary *Malassezia* dermatitis case and secondary *Malassezia* dermatitis was identified in 44 (13.3%) cases.

Table 2. Summary of the skin diseases diagnosed over a 3-year period at the UGVS (2000-2002)

Diagnosis		Totals
Allergy	Atopic dermatitis	192
	Flea allergic dermatitis	21
	Food sensitivity	4
	Allergy not diagnosed *	24
Immune mediated	Pemphigus foliaceus	5
	Pemphigus vulgaris	1
	Cutaneous lupus erythematosus	3
	Erythema multiforme	1
Parasitic	Cheyletiellosis	6
	Demodicosis	8
	Sarcoptic mange	4
Endocrine	Hyperadrenocorticism	1
	Hypothyroidism	2
Acquired alopecia	Follicular dysplasia	1
	Pattern baldness	1
	Flank alopecia	1
Infections	*Malassezia* dermatitis (primary)	1
	Primary pyoderma	23
Neoplasia	Cutaneous lymphoma	1
Keratinisation defect	Primary seborrhoea	1
	Schnauzer comedone syndrome	1
Miscellaneous	Others	38

*History and clinical investigations suggested an allergic dermatosis but specific allergy testing and / or food trials not conducted due to cost and / or owner wishes.

Full list of diagnoses in Appendix B

Figure 2. The most common skin diseases diagnosed at the UGVS (2000 - 2002)

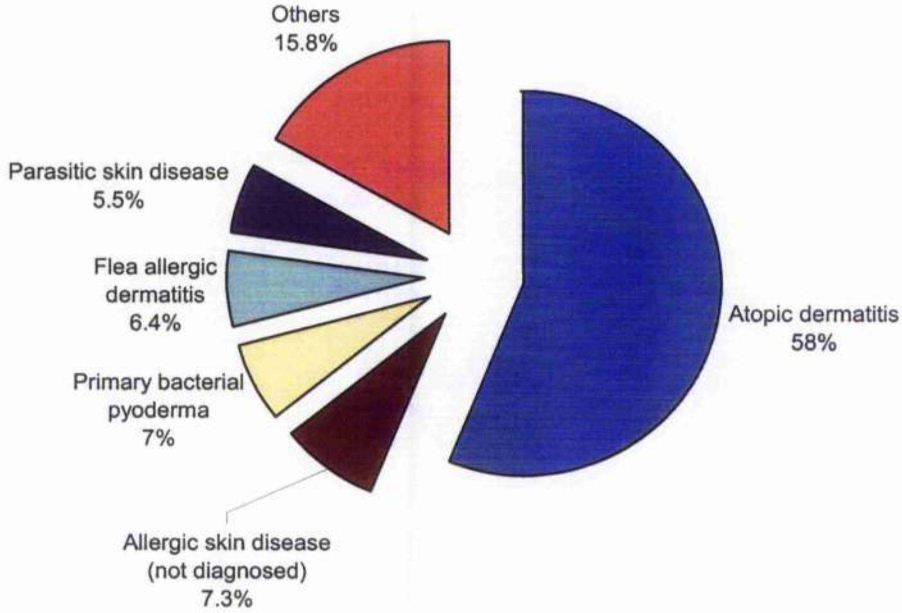


Table 3. Secondary bacterial and *Malassezia* infections associated with primary diagnoses

Secondary Infections			Associated primary diagnosis	
Type		Total cases	Type	Total cases
Pyoderma	Superficial	94	Atopic dermatitis	60
			Flea allergic dermatitis	5
			Cheyletiellosis	3
			Others	26
	Deep	18	Atopic dermatitis	9
			Demodicosis	3
			Others	6
	Superficial and deep	12	Atopic dermatitis	3
			Others	9
Malassezia		44	Atopic dermatitis	34
			Others	10

General canine hospital population at UGVS (2000-2002)

Excluding dogs referred for skin disease, a total of 4505 dogs were examined over the three-year period, 2000 to 2002. There were 991 (21.99%) entire female, 1064 (23.62%) neutered females, 1724 (38.27%) entire males and 664 (14.74%) neutered males. In 62 cases the sex was not recorded. This gave a male female ratio of 1.2: 1. There was no significant statistical difference in the overall sex ratios between the skin disease population and the general hospital population but there were significantly fewer intact females in the skin disease group compared to general hospital population ($P<0.001$). The mean (± standard deviation) age at examination was 5.17 (±3.82 years) with a median age of 4.80 years. The minimum age was 0.01 years and maximum age was 16.99 years. In 66 cases the age was not recorded. The age of the canine skin disease population was statistically significantly lower ($P<0.001$) when compared to the general canine hospital population by using Mann-Whitney Rank Sum Test.

The most common breeds seen in the general canine hospital population were Cross breed (483 cases; 10.72%), Labrador retriever (338 cases, 7.50%), German Shepherd dog (308 cases; 6.84%), Retriever (317 cases, 7.04%), Border collie (204 cases, 4.53%), Boxer (178 cases; 3.95%), Cocker spaniel (178 cases, 3.95%), Cavalier King Charles spaniel (174 cases, 3.86%), Golden retriever (158 cases, 3.51%), West Highland White terrier (116 cases, 2.57%), English Springer spaniel (134 cases, 2.97%), and Yorkshire terrier (96 cases, 2.13%). (Figure 3; Table 4)

When the breeds with skin disease were compared to the general hospital population using the Chi squared test, the West Highland White terrier was significantly over-represented in the dermatological population while there was no statistical difference between dermatological population and general hospital population for the other breeds. (Table 4)

Figure 3. Most common dog breeds in general hospital population (2000-2002)

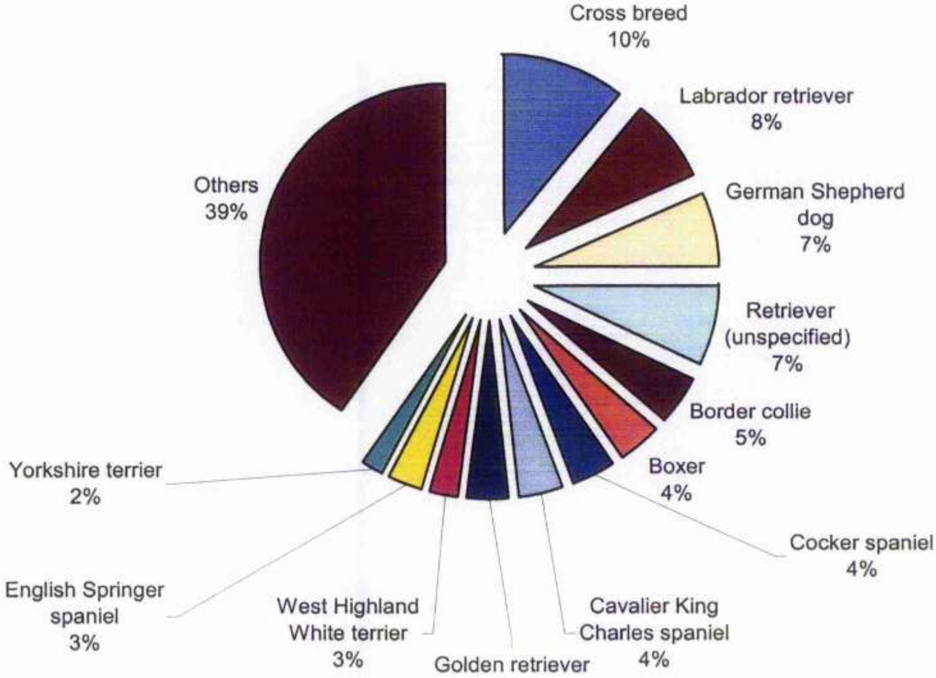


Table 4. Most common breeds of general hospital (GH) and skin disease (SD) populations

Breed	SD*	GH*	P value	Relative Risk
Cross breed	1	1	0.8703	0.9563
German Shepherd dog	2	3	0.0171	1.543
Labrador retriever	2	2	0.1104	1.344
West Highland White terrier	4	6	<0.0001	2.492
Boxer	5	5	0.3741	1.281
Cocker spaniel	6	5	0.6655	0.8427
Golden retriever	7	5	0.5410	0.7792
Border collie	7	4	0.2174	0.6401
Staffordshire bull terrier	8	9	0.3722	1.467
Cavalier King Charles spaniel	9	5	0.0786	0.4762
English Springer spaniel	10	7	0.1690	0.5168
Yorkshire terrier	11	8	0.3448	0.5777

*The order of the breeds in total population (e.g. 1: the most common breed in the population.)

Feline skin disease population

Signalment

During the 3 year period studied a total of 40 cats were examined for skin disease. Of the 40 cases, there were no entire males, 19 (47.50%) neutered male, 2 (5.00%) entire female and 17 (42.50%) neutered female. In 2 cases the sex was not recorded. This gave a male to female ratio of 1:1.

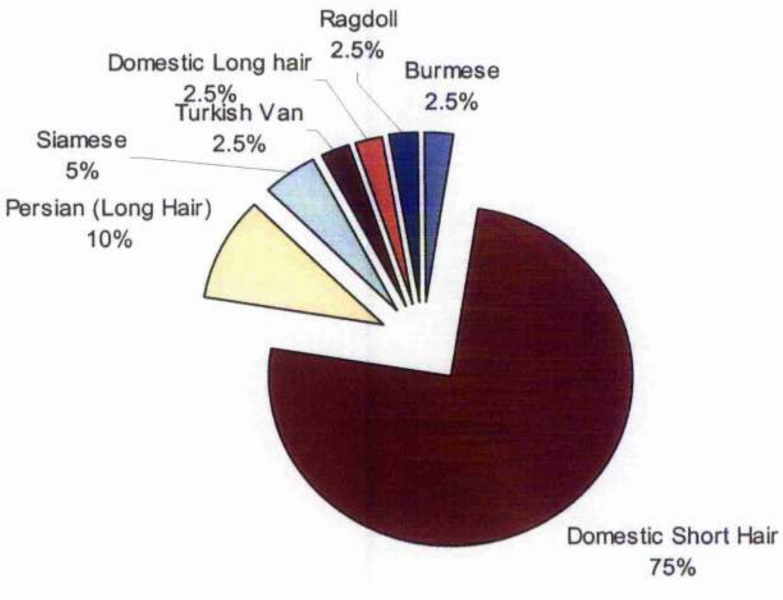
The mean age (± standard deviation) at presentation was 5.00 ± 3.21 years (minimum and maximum age: 0.58 and 13.00 years; median age 4.25 years).

There were in total 5 different breeds. They were Domestic short hair (30), Persian (long hair) (4), Siamese (2), Burmese (1), Domestic long hair (1); Ragdoll (1) and Turkish Van (1). (Table 5; Figure 4)

Table 5. Cat breeds referred for skin disease (2000-2002)

Breed	Number	Percentage
Domestic short hair	30	75
Persian (long hair)	4	10
Siamese	2	5
Ragdoll	1	2.5
Domestic long hair	1	2.5
Burmese	1	2.5
Turkish Van	1	2.5

Figure 4. Breeds referred for skin disease (2000-2002)



Feline skin diseases diagnosed

There were 42 primary skin diseases diagnosed in the 40 cats examined. The most common skin condition diagnosed was atopic dermatitis (12 cases; 28.6 %). Other common skin disorders, in descending order were: 9 cases (21.4%) of flea allergic dermatitis, 5 cases (11.9%) allergy not fully diagnosed, 4 cases (9.5%) of facial dermatitis and 2 cases (4.8%) of pemphigus foliaceus. (Table 6; Figure 5; Appendix C)

Secondary infection in cats was rarely diagnosed. There was only one case of superficial pyoderma in a cat with food sensitivity and a cat with feline acne had a deep pyoderma.

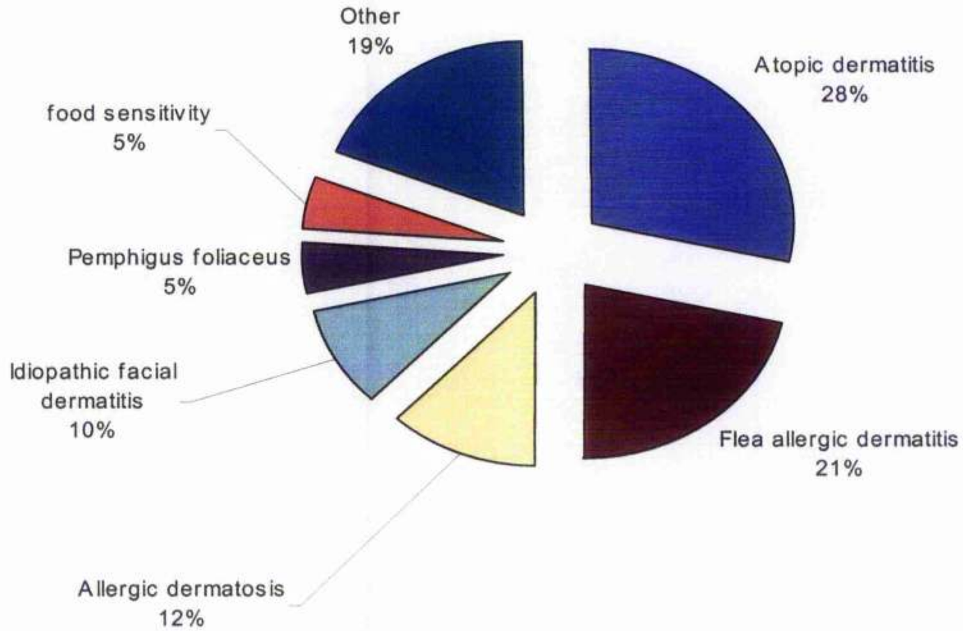
Table 6. Summary of the feline skin diseases diagnosed over a 3-year period at the UGVS (2000-2002)

Diagnosis		Totals
Allergy	Atopic dermatitis	12
	Flea allergic dermatitis	9
	Food sensitivity	2
	Allergy not diagnosed*	5
Immune mediated	Pemphigus foliaceus	2
Neoplasia	Ceruminous gland adenoma	1
Miscellaneous	Symmetrical alopecia (idiopathic)	1
	Eosinophilic granuloma complex	1
	Idiopathic facial dermatitis	4
	Feline acne	1
	Plasma cell pododermatitis	1
	Psychogenic alopecia	1
	Cutaneous asthenia	1
	Chin oedema	1

*History and clinical investigations suggested an allergic dermatosis but specific allergy testing and / or food trials not conducted due to cost and / or owner wishes.

Full list of diagnoses in Appendix C

Figure 5. The most common feline skin diseases diagnosed at the UGVS (2000 - 2002)



General feline hospital population at UGVS (2000-2002)

Excluding cats referred for skin disease, a total of 912 cats were examined over the three-year period, 2000 to 2002. There were 105 (11.51%) entire female, 328 (35.96%) neutered females, 60 (6.58%) entire males and 405 (44.41%) neutered males. In 14 cases the sex was not recorded. This gave a male female ratio of 1.07: 1. There was no statistical difference between the sex of skin disease population and general hospital population. The mean (± standard deviation) age at examination was 7.20 (± 4.92 years) with a median age of 7.00 years. The minimum age was 0.06 years and maximum age was 18.00 years. In 31 cases the age was not recorded. The age of the skin disease population was significantly lower than the general hospital population (P=0.024). The most common breeds seen in the general hospital population were Domestic short hair (664 cases; 72.81%), Persian (Long hair) (98 cases; 10.75%), Domestic long hair (43 cases; 4.71%), Siamese (22 cases; 2.41%), Exotic (13 cases; 1.43%), and Burmese (12 cases, 1.32%). (Figure 6; Table7)

Figure 6. Most common cat breeds in general hospital population (2000-2002)

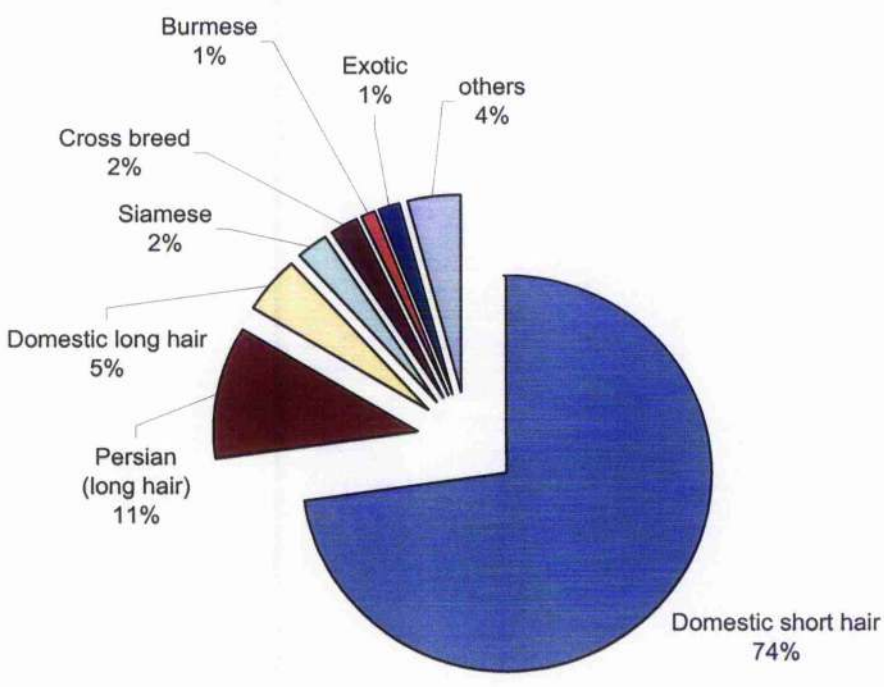


Table.7 Most common cat breeds of general hospital (GH) and skin disease (SD) populations

Breed	SD*	GH*	P value	Relative Risk
Domestic short hair	1	1	0.5888	1.274
Persian (long hair)	2	2	1.0000	0.9259
Siamese	3	4	0.2672	2.035
Turkish Van	4		0.0823	12.18
Domestic long hair	4	3	1.0000	0.5291
Burmese	4	5	0.4297	1.852
Exotic		5	1.0000	0

*The order of the breeds in total population (e.g. 1: the most common breed in the population.)

DISCUSSION

Canine skin disease

The overall prevalence of skin disease compared to the general hospital population of dogs at UGVS was 7.37% for the three-year period of 2000-2002. This figure is similar to a previous study of prevalence rates for canine skin disease in a ten-year (1990 – 1999) retrospective study undertaken in the same hospital conducted by McEwan (2002) where 7.69% prevalence for canine skin disease was found. There are few detailed published studies of the prevalence of canine skin disease but several studies and text books have estimated that between 20% and 75% of small animals examined in the average practice have skin problems as a chief or concurrent owner complaint (Schwartzman and Orkin, 1962; Nesbitt, 1983; Wilkinson, 1985; Ihrke, Stannard, Ardans and Griffin, 1985; Grant, 1991). In a comprehensive study conducted in a 50% referral and 50% first opinion practice Scott and Paradis (1990) found a prevalence of 18.8% for canine skin diseases. The variation in prevalence rates between various studies may reflect different case origins and in the resources available at the study hospitals. The study presented here was conducted in an entirely referral population while other studies have been in first opinion practice or a combination of first opinion and referral practice. Referral populations are unlikely to reflect the prevalence of skin disease in the general dog population but may reflect the number and types of chronic skin complaints in the general dog population.

The Mean age of dogs with skin disease in this study (4.01 years) was statistically significantly younger when compared to the general hospital population. The Mean age of general hospital population (5.17 years) was approximately one year older than the skin population. This may be due to the high prevalence of atopic dermatitis in the skin disease group. There was no overall sex predilection for skin disease found in this study, which is consistent with the findings of McEwan (2002) and Scott and Paradis (1990). Entire female dogs appeared to be at increased risk for skin disease in this study but the reasons for this are not clear.

When the most common breeds referred for skin disease in the study were examined, it was found that the West Highland White terrier was over-represented in the study presented here. In the ten-year study conducted in the same hospital by McEwan (2002) both West Highland White terrier and the Boxer were found to be predisposed to skin disease. There is considerable information available on apparent breed predilections for specific canine dermatoses (Halliwell and Schwartzman, 1971; Scott, 1981; McDonald and Kunkle, 1983; Scott, Walton, Slater, Smith and Lewis, 1987a; Scott, Walton, Slater, Smith and Lewis, 1987b; Grant, 1991; Scott, Miller and Griffin, 2001b). Ihrke and Franti (1985) reported that 31 breeds of dogs showed an elevated risk in northern California whereas only three breeds appeared to be at increased risk in Scott and Paradis' study, which were Boxer, Dachshund, and Bichon Frise. Of these three breeds only the Dachshund was also at increased risk in the northern California study. There appears to be variation in breed prevalence for skin disease reported. This may be due to differences in the genetic pools and possibly due to geographic variations in certain dermatoses. In referral centres particular interest in a specific breed or dermatosis may influence both the breed prevalence and prevalence of specific dermatoses.

The most common main diagnosis was atopic dermatitis (58%). As the condition tends to be chronic and so very likely to be referred, this finding is not surprising. This finding is consistent with a recent study

(McEwan, 2002a) while Scott and Paradis (1990) found atopic dermatitis was their second most common diagnosis.

Secondary bacterial skin disease was found to be very common with 28.5 % of dogs in the study being diagnosed with a superficial pyoderma and 5.5% having a deep pyoderma. These figures are very close to those found by McEwan (2002) 23.6% (superficial pyoderma), 4.2% (deep pyoderma) and Scott and Paradis (1990) who recorded a prevalence of 25.3% for bacterial skin disease (folliculitis/ furunculosis). Slightly over 50% of the cases of secondary pyoderma in this study were associated with atopic dermatitis, which is again consistent with McEwan (2002) and Scott and Paradis (1990).

Feline skin disease

The prevalence of feline skin disease was 4.39% during 2000-2002 compared to the general feline hospital population. There is little information available concerning the demographics of feline skin disorders compared to canine. The figure for this study is much lower than the study of Scott and Paradis (1990) where 15.2% of cases were feline skin diseases. Again this may reflect the different case resources between these two studies.

The two most common feline dermatologic disorders in this study were atopic dermatitis and flea allergic dermatitis. A survey conducted in 1981 by the American Academy of Veterinary Dermatology revealed parasitic dermatoses and miliary dermatitis to be common. Miliary dermatitis is not a specific diagnosis but rather a skin reaction pattern that may result from several potential causes. Scott & Paradis 1990 found abscess (18.5%), otodectic mange (12.9%), atopic dermatitis (5.6%) to be common skin disorders. The differences between studies probably reflect that different populations, geographical location, type of veterinary practice, and clients' economic consideration may influence the recorded prevalence of skin diseases. As in the dog the Mean age of cases of feline skin disorders (5 years) was significantly lower than the general hospital population of cats (7.20 years). Again this would be consistent with the high prevalence of atopic dermatitis found.

There was no sex predilection for skin disease found and the most common breeds were the Domestic short hair, Persian, Domestic long hair, Siamese and Burmese.

Although atopic dermatitis was a relatively common diagnosis compared to dogs, there were very few cases of feline bacterial skin disease. In general bacterial skin disease appears to be rare in the cat.

Conclusions

The result of this study and the findings of the literature reviewed emphasize the importance of dermatology in small animal practice. This study and others show that allergic skin disease is common in both the cat and dog (Ihrke and Franti, 1985; Sischo, Ihrke and Franti, 1989; Lund, Armstrong, Kirk, Kolar and Klausner, 1999; Nagata and Sakai, 1999). In the dog, and particularly in referral practices, atopic dermatitis is very common (Ihrke and Franti, 1985; Sischo, Ihrke and Franti, 1989; Scott and Paradis, 1990; Lund *et al*, 1999; Nagata and Sakai, 1999; McEwan, 2002a). In dogs, bacterial skin disease due to *S. intermedius* is common (Ihrke and Franti, 1985; Sischo, Ihrke and Franti, 1989; Scott and Paradis, 1990; Lund *et al*, 1999; Nagata and Sakai, 1999; McEwan, 2002a). Much of this is secondary to other skin diseases, especially atopic dermatitis (Ihrke, Halliwell and Deubler, 1977; White and Ihrke, 1987; Scott and Paradis, 1990; McEwan, 2002a). In man, *Staphylococcus aureus* skin infections are common in atopic dermatitis patients (Jones, Schmitz, Fluit, Acar, Gupta and Verhoef, 1999; Sader, Pfaller, Jones, Doern, Gales, Winokur and Kugler, 1999; Jones, Karlowsky, Draghi, Thornsberry, Sahm and Nathwani, 2003). Several theories have been advocated as to why both canine and human atopic dermatitis patients suffer from staphylococcal skin infections. One theory suggests that increased adhesion by staphylococci to skin could be a factor (Feingold, 1986; Roth and James, 1989; McEwan, 1990; Scott, Miller and Griffin, 2001b; Saijonmaa-Koulumies and Lloyd, 2002). A major problem when assessing bacterial adhesion to corneocytes is that many assays are tedious and time consuming to perform. A simple test to quantify adhesion by bacteria to corneocytes would be a useful tool. In the next two chapters the development of a simple assay to determine bacterial adhesion is described.

CHAPTER 2

A study of Canine and Feline Corneocyte Morphometrics

LITERATURE REVIEW

Introduction

The microscopic anatomy and physiology of the skin of the dog and the cat has been the subject of several studies (Lovell and Getty, 1957; Strickland and Calhoun, 1963; Kral and Schwartzman, 1964; Lyne and Short, 1965; Montagna, 1967; Schwarz, 1979; Schummer, 1981; Dyce, 1987; Suter, 1987; Evans and Christensen, 1993; Yager and Scott, 1993; Scott, Miller and Griffin, 2001b). The outer part of the skin is the epidermis, which is divided into stratum corneum (horny layer or cornified cell layer), stratum granulosum (granular layer), stratum spinosum (prickle cell layer or spinous cell layer), and stratum basale (basal layer). The epidermis rests on and is attached to the dermis, which in turn lies on the subcutis or hypodermis. The stratum corneum is the outer layer of terminally differentiated keratinocytes that is constantly being shed (Webb and Calhoun, 1954; Strickland and Calhoun, 1963; Schwarz, 1979; Scott, 1980; Greene and Lammler, 1993). It is a multilayered zone of corneocytes suspended in an extracellular lipid matrix, often likened to a series of bricks (corneocytes) with mortar (lipids) (Leigh, 1994). Corneocytes, which contain a variety of humectants and natural sunscreens synthesized from proteins, are the flattened, anuclear eosinophilic cells in stratum corneum which have no true cell membrane (Priestley, 1993). The stratum corneum varies in thickness from 3 to 35 μm in cats and from 5 to 1500 μm in dogs (Scott, Miller and Griffin, 2001b).

Epidermal function

The general functions of the epidermis are as follows (Moriello and Mason, 1995): (1) Barrier. In order to prevent loss of water and electrolytes and limit the penetration of bacterial, viruses, and fungal organisms and their metabolites. (2) Sensory perception. Free nerve endings and sensory end organs (Schwarz, 1979; Scott, 1980; Scott, 1984; Lever and Schaumburg-Lever, 1990). (3) Pigmentation, which can protect against UV radiation. (4) Immuno-regulation and defence. Protection against infection. Langerhans' cells, keratinocytes, epidermotropic T lymphocytes, and draining peripheral lymph nodes are thought to form collectively an integrated system of skin-associated lymphoid tissue (SALT) that mediates cutaneous immuno-regulation (Thoday and Friedmann, 1986). Langerhans' cells, which are mononuclear dendritic cells located suprabasally (Lever and Schaumburg-Lever, 1990), stimulate the proliferation of relevant helper T lymphocytes by the presentation of antigen; they also induce cytotoxic T lymphocytes directed to allergens and modified self-determinants, produce interleukin (IL) 1 and other cytokines, contain numerous enzymes, and are phagocytic (Thoday and Friedmann, 1986).

The stratum basale, spinosum and granulosum are known as the living epidermis while the stratum corneum constitutes the terminally differentiated non-living epidermis. The normal mammalian epidermis is a primary barrier against microbial invasion. Anatomical features of this region impede the penetration of microorganisms or their potentially toxic secretions and metabolites into the host. The arrangement of inert squames in a lipid-rich matrix prevents the penetration of aqueous substances. The keratinocyte also plays an important role in the immune function of epidermis (Thoday and Friedmann, 1986). Keratinocytes can no longer be regarded as simply the 'building blocks' of the tissue. They are phagocytic and can process antigens (Thoday and Friedmann, 1986). They produce IL-1 constitutively and, following antigenic stimulation, can produce a wide range of cytokines (e.g., IL-1, IL-3, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-18, IL-20, prostaglandins, leukotrienes, interferon, and tumor necrosis factor α (TNF-α)), which influence the immune responses (Grone, 2002). In addition, keratinocyte-derived IL-7 and 15 are considered to be significant in T-cell trafficking, possibly even in the pathogenesis of cutaneous T-cell lymphoma (Grone, 2002).

Keratinocyte differentiation

Normal epidermal homeostasis requires a finely tuned balance between growth and differentiation of keratinocytes (Suter, 1987; Suter, Crameri, Olivry, Mueller, von Tscharner and Jensen, 1997). This balance must be greatly shifted in the direction of proliferation in response to injury and then must return to a state of homeostasis with healing. In addition, epidermal keratinocytes have important functions as regulators of cutaneous immunity and inflammation. The epidermis is ectodermal in origin and normally undergoes an orderly pattern of proliferation, differentiation, and keratinisation. As the primary cell type, keratinocytes define the structure and organization of the epidermis through their program of terminal differentiation. Keratinocytes are arranged in overlapping layers, which are indicative of their states of differentiation. The basal layer of keratinocytes, which abuts the basement membrane, is the least differentiated, proliferative layer. The next layer, stratum spinosum, is composed of keratinocytes derived from its basal layer. With increasing differentiation, the keratinocyte continues to migrate upwards. When it reaches the upper zone of the granular layer, the keratinocyte undergoes a type of programmed cell death, leading to dissolution of the nucleus and other organelles and formation of the cross-linked envelope characteristic of squames. Finally, the squames are lost from the surface of the skin, and the end product is the fully keratinised corneocyte. In healthy Beagle dogs, similar to human keratinocytes, the time required for a keratinocyte to migrate from the basal layer to the surface of the skin is 22 days (Baker, Maibach, Park, McFarland and O'Brien, 1973; Kwochka and Rademakers, 1989). In normal epidermis there is a tight coordination between vertical migration and differentiation state, with characteristic molecules expressed and morphology manifested at each level. Keratinisation is the term used to describe the maturation of a keratinocyte from the stratum basale to the stratum corneum (Moriello and Mason, 1995).

Corneocyte morphometrics

The human stratum corneum constitutes less than 10% of the cells of the intact epidermis and is made of about 15 layers of a tightly packed syncytium of flattened non-nucleated keratinised cells known as corneocytes (Christophers and Kligman, 1964; Amer, Mostafa, Tosson and Nasr, 1996). The canine stratum corneum consists of approximately 50 cell layers (Lloyd and Garthwaite, 1982). The studies that have been carried out to investigate the morphology of corneocytes show that human corneocytes are extremely flat, but very large cells lacking nuclei (Goldschmidt and Kligman, 1967a; Blair, 1968) with their largest diameter measuring between 30-50μm (Amer *et al*, 1996; Mihara, 1997). Usually, corneocytes have a regular pentagonal or hexagonal shape with a surface area of 900-1200 μm^2 (Plewig and Marples, 1970). As a result of keratinization of cells, and transepidermal water loss, corneocytes become progressively flatter and are finally desquamated at the skin surface. The rate of desquamation in normal skin matches the rate of cell production so epidermal thickness is maintained. This physiolological desquamation goes unnoticed, but it results in visible scales when the rate of keratinisation is pathologically raised, such as in parakeratosis (Amer *et al*, 1996). Morphological variations of corneocytes depend on biologic variables, such as body region, age, season, race and sex. (1) Regional difference. Corneocytes vary in cell size and thickness with topographical site (Plewig, 1970). In man large cells are found in the axilla, on the trunk, thigh, and upper arm. These cells also tend to be thin. Experimental cytologic examinations of the trunk and arms revealed statistically significant variations of cell sizes even when corneocytes were removed from regions only 10 cm apart (Holzle and Plewig, 1977; Haidl and Plewig, 1988). (2) Effects of age. Cell surface area of corneocytes increases with age (Plewig and Marples, 1970; Marks, 1980; Toyoshima, 1989). The mean surface area of corneocytes taken from the chest area in infancy is 900 μm^2. In adults the surface area increases with the largest values occurring in old age (1200 μm^2). An age-dependent increase in size, often significant, is also seen in most other regions, with the exception of the forehead and mucous membrane of the tongue. (3) Seasonal changes. Morphological variations of corneocytes are seen as a consequence of climatic fluctuations, such as temperature and humidity. It is known that increased epidermopoiesis leads to a decrease in corneocyte cell surface areas. Stratum corneum turnover is faster in summer than in winter. In one study, the corneocyte surface area and cell counts were measured in four groups of ten subjects monthly throughout the year (Herrmann, Scheuber and Plewig, 1983). The paraumbilical region and the lower lateral portion of the right leg were used as sampling sites. In the warm season during the months from March to September, the corneocytes were smaller than in the cold season. The cell counts increased correspondingly. The seasonal changes were most pronounced on lower legs in both older and younger women. (4) Sex and racial differences. It was found that the size of corneocytes in females was larger than those of males in 8 out of 10 different collection sites. The exceptions being the popliteal fossa and the heel (Lee and Lee, 1986). However, there was no significant difference between black, white and oriental skin (Corcuff, Lotte, Rougier and Maibach, 1991).

The cause of the difference in size according to the site of the body might be due to the difference in: (1) the turnover time of the stratum corneum, (2) intercorneal cohesion, and (3) the thickness and shape of the corneocytes (Marks and Barton, 1983). In a study of mammalian corneocytes using hairless guinea pigs (HL-GP) and normal-haired guinea pigs (HD-GPs), it was concluded that HL-GP skin has a greater similarity to human skin than that of HD-GPs and other rodents (Sueki, Gammal, Kudoh and Kligman, 2000). In this same study, the measurements of the mean projected area showed that guinea pig corneocytes are slightly larger than human corneocytes. The mean corneocyte surface area of the guinea pig varied between 1034 and 1415 μm^2. However, since corneocyte size varies depending on the body site and age this should be taken into consideration.

Collection of corneocytes and their study

Most of the literature concerning skin structure pertains to the human species and rodents (mice and rats) (Mackenzie, 1969; Christophers, 1971; Menton and Eisen, 1971a; Menton and Eisen, 1971b; Mackenzie, 1972; Goerttler, Reuter and Stahmer, 1973; Mackenzie and Linder, 1973; Allen and Potten, 1974). Several techniques have been developed over the past decades for the study of the human stratum corneum, including potassium hydroxide mount (Lookingbill, 1985), Tzanck smear (Tzanck, 1947), biopsy (Lookingbill, 1985) , skin replica methods (Thomson, 1953; Sarkany, 1962; Makki, Barbenel and Agache, 1979), tape stripping (Pincus, 1951; Keddie and Sakai, 1965), liquid non-drying adhesive stripping (Goldschmidt and Kligman, 1967b), cryanoacrylate adhesive specimens (Keddie, Shadomy and Shadomy, 1963; Goldschmidt and Kligman, 1967b; Marks and Dawber, 1971; Marks and Dawber, 1972; Marks, 1972; Holland and Roberts, 1974; Tring and Murgatroyd, 1974; Tring and Murgatroyd, 1975; Marks, 1980; Mills, Jr. and Kligman, 1983; Katz, Prawer, Hien and Mooney, 1985), scanning electron microscope (SEM) (Hashimoto and Kanzaki, 1975), transmission electron microscope

(TEM) (Hashimoto and Kanzaki, 1975), fluorescent microscope (Christophers, 1971), confocal laser scanning microscope (Schatzlein and Cevc, 1998), and atomic force microscope (Kashibuchi, Hirai, O'Goshi and Tagami, 2002). The adhesive D-Sqame® disc (CuDerm Corp., Dallas, TX, USA), is a circular crystal clear foil 25 mm in diameter coated with a special adhesive substance that has been used as a convenient harvesting method for the superficial desquamating layer of the stratum corneum in several research studies (Serup, Winther and Blichmann, 1989; Jemec and Serup, 1992; Serup, 1992a; Serup, 1992b; Pierard and Pierard-Franchimont, 1993; El Gammal, Pagnoni, Kligman and el Gammal, 1996; Kawakami, Callicott and Zhang, 1998; De Paepe, Janssens, Hachem, Roseeuw and Rogiers, 2001; Yoon, Baik and Oh, 2002; Hill and Edwards, 2002).

In conclusion while there are occasional reports on the microanatomy of the skin of domestic large animals (Lloyd, Sasiak, Kitson, McEwan and Elder, 1993), and that of the dog and cat (Webb and Calhoun, 1954; Baker *et al*, 1973) there is little information on corneocyte morphometrics in these species. In man and rodents several different methods have been employed to collect corneocytes for study. The D-Squame® disc has been shown to be a valuable tool for the collection of corneocytes and would be potentially suitable for use in studies in the dog and cat.

INTRODUCTION

The literature reviewed shows that there is little published information on the size and shape of canine and feline corneocytes. Prior to developing an adhesion assay to study bacterial adhesion to corneocytes, studies were undertaken to determine size and surface area of canine and feline corneocytes collected from different body sites.

AIMS

The aims of the study were:

1. To determine the size and shape of canine and feline corneocytes
2. To assess three body sites for the collection of corneocytes that would be suitable for use in an assay for bacterial adherence using image analysis

MATERIAL AND METHODS

STUDY 1. CORNEOCYTE MORPHOMETRICS

Populations studied

Three groups of animals were studied:

1. Normal dogs. Normal dogs were obtained from the following sources: blood donor dogs held in the University of Glasgow Veterinary School, pet dogs belonging to Veterinary School staff and dogs referred with minor medical complaints. All dogs in this group were given a full clinical examination and were free from skin disease.

2. Atopic dermatitis dogs. These dogs fulfilled the critera for diagnosis as described by Willemse (1986) and were cases referred to the UGVS dermatology clinic.
3. Normal cats. Normal cats were obtained from the following sources: pet cats belonging to Veterinary School staff, cats undergoing screening studies for polycystic kidney disease or arthritis and cats referred with minor medical complaints. All cats in this group were given a full clinical examination and were free from skin disease.

None of the animals used in the studies had received either systemic or topical treatments for a period of at least three weeks prior to corneocyte sampling.

The breed, sex and age of individual animals used in the study are documented in Appendices D-I.

Body sites sampled

Three body sites were sampled from each of 5 animals from each group studied (atopic dogs, normal dogs and cats). The body sites sampled were:

1. Inner aspect of the base of the pinna (Figure 7)
2. Inner thigh in the femoral triangle area (Figure 8)
3. The left lateral thorax at the 10/11th intercostal space (Figure 9)

Figure 7. Ear sampling area

Figure 8. Groin sampling area

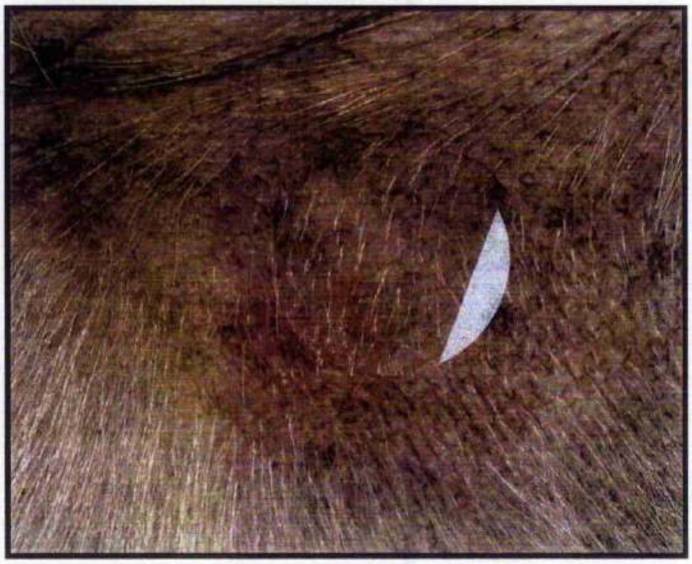
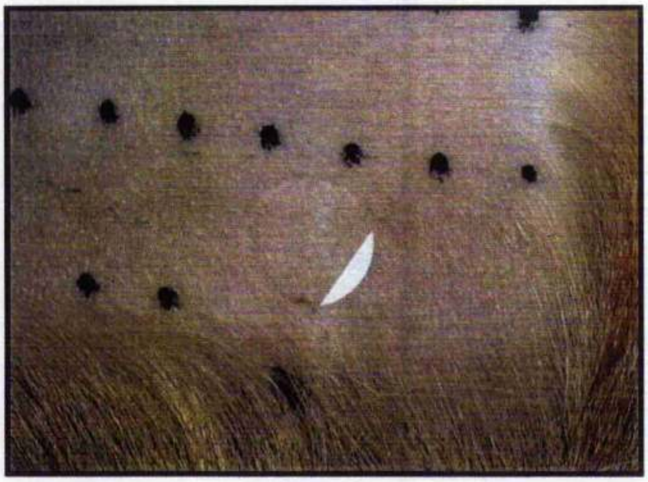


Figure 9. Thoracic sampling area



Felt pen marks can be seen marking sites for intradermal skin testing

Hair was removed by gently clipping (electric clippers; size 40 blade) from the thorax and groin area. In the atopic dog group a total of five dogs were studied and for each dog all three sites sampled. For the normal dogs and cats due to owner constraint on clipping of some sample sites it was not possible to obtain samples from all three sites in each individual animal. Seven normal dogs and ten cats were used in the study.

Corneocyte collection

Prior to sampling, surface debris was removed from the skin by applying five successive adhesive tape strips (Sellotape® Original). Corneocytes were then collected using 25 mm diameter adhesive discs (D-squame®, CuDerm Corporation, Dallas, Texas USA). From each collection site five consecutive samples were obtained using five D-squame® discs. Each disc was applied to the sample site once.

Corneocyte staining and Image acquisition

Corneocytes were stained using 2.5% rhodamine B and 0.75% methylene blue (Sueki *et al*, 2000) for 30 minutes, rinsed in tap water then air dried. A Leica DMLS microscope with a Leica ICCA digital video camera was used to acquire images. Images were obtained from each disc at a microscope magnification of x400 and saved as TIFF files onto the hard drive of a Viglen computer (Viglen Genie Pentium® 4). From each body site five D-Squame® discs were collected and from each disc ten corneocytes were measured giving a total of 50 corneocytes examined from each collection site per animal.

Calibration and measurements

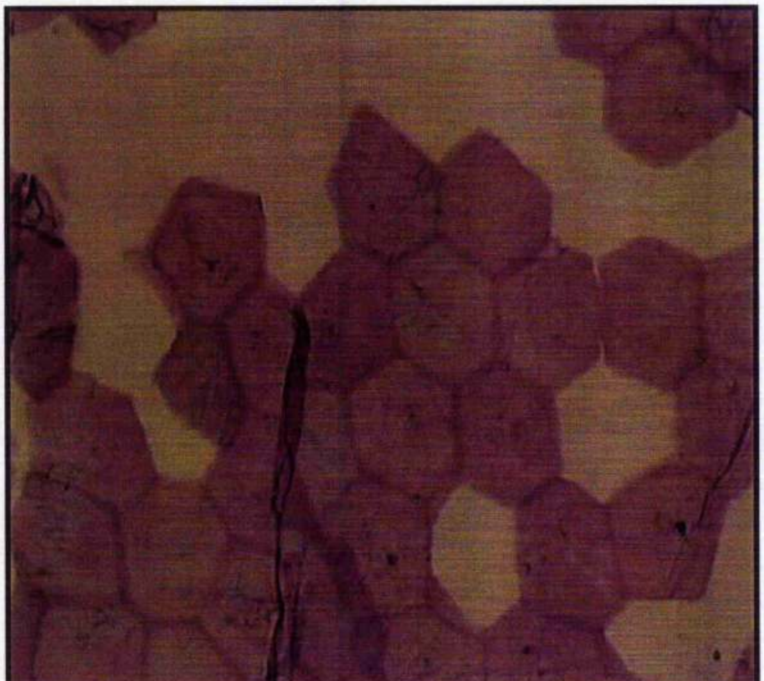
Measurements were carried out using an image processing and analysis program (UTHSCSA ImageTool Version 3.0). Prior to measurement the image analysis program measuring tools were calibrated by use of a stage micrometer (Agar Scientific Ltd., Essex, UK).

Preliminary studies showed two distinct populations of corneocytes obtained from dog and cat skin. For the purposes of study these were designated type I and type II corneocytes. Type I corneocytes (Figure 10) were small, elongated and deeply staining. Type II corneocytes (Figure 11) were poorly staining and polygonal. The diameter and surface area of polygonal type II corneocytes were calculated and analysed. The diameter was calculated as an Mean of two measurements (longest and shortest breadth) for each corneocyte. The programme software automatically calculated the surface area after outlining the perimeter of each corneocyte using the software draw tool.

Figure 10. Type I corneocytes from canine thorax

Type I corneocytes (*) predominate. Rhodamine B and methylene blue staining. Original magnification x400.

Figure 11. Type II corneocytes from canine ear



Rhodamine B and methylene blue staining. Original magnification x400.

STUDY 2. COMPARISON OF CORNEOCYTES FROM HAIRED AND NON-HAIRED SKIN

It was observed that type I corneocytes were plentiful in samples from densely haired regions and type II corneocytes more readily obtained from less haired regions. In order to investigate the origin of the two populations of corneocytes, a study of haired and non-haired skin was undertaken.

Corneocyte collection

Five normal dogs and five normal cats were used in this study. D-Squame® discs were used to collect corneocytes from three sites on the inner aspect of the right pinna from the base of pinna to the more haired apex (Figure12). Prior to corneocyte collection surface debris was removed using successive adhesive tape strips as described above. Corneocytes were stained using the method detailed above.

Figure 12. Comparison of haired and non-haired skin

Counting method

From each D-Squame® disc, five images were collected at x400 magnification and saved as TIFF files. Using the image analysis software (ImageTool) a script (macro program) was written that produced a box out line 400 x 400 pixels (approximately 13000μm^2) in size centrally positioned over the image to be studied (Appendix J). The numbers of the two types of corneocytes were counted manually within the centrally positioned counting area. Any corneocytes on the edge of counting box were counted if they were at least 50% within the square counting area.

STATISTICAL ANALYSIS

Statistical analysis and graphical presentation of data was conducted using computerised statistical packages, GraphPad Prism, version 4 (Graphpad Software Inc. San Diego CA). Comparisons between groups were conducted using simple one-way analysis of variance (ANOVA), where data was not normally distributed; Kruskal-Wallis ANOVA on ranks was used. To isolate the group or groups that differed from the others a multiple comparison procedure (Dunn's method) was employed. A value of $p < 0.05$ was considered to be significant.

RESULTS

Corneocyte morphometrics

Two broad populations of corneocytes could be identified. One consisted of small, single, irregular and often fragmented cells that stained darkly (type I corneocytes Figure 10) and a second population of lightly pink-staining, transparent and polygonal cells that formed a honeycomb pattern with slightly overlapping edges (type II corneocytes Figure 11). Type I corneocytes appear to arise from hair follicles (tables 16 and 17; figures 16 and 17).

A summary of the results for the mean diameter and surface area of type II corneocytes collected from normal dogs, atopic dermatitis dogs and normal cats are shown in tables 8 and 9, below together with a statistical analysis comparing data between animals and between discs for each site sampled. The mean diameter of corneocytes from the ear, thorax and groin of normal dogs was 37.92μm, 42.82μm and 43.47μm. For atopic dermatitis dogs equivalent figures were 38.38μm, 43.24μm and 40.19μm. For cats the figures were 40.41μm, not done and 49.92μm. The mean surface area of corneocytes from the ear, thorax and groin of normal dogs was 1091.96μm^2, 1399.40 μm^2 and 1435.72μm^2. For atopic dermatitis dogs equivalent figures were 1160.86μm^2, 1439.67 μm^2 and 1249.75μm^2. For cats the figures were 1222.17μm^2, not done and 1870.71μm^2 . In general cat corneocytes were larger than dogs (both normal and atopic) and corneocytes from the groin appear to be larger than form the ear or thorax.

When corneocytes were from each set of five discs were compared from an individual animal there was generally little within animal difference between each of the five discs (tables 10-15).

Differences were noted when corneocytes were compared between individual animals of the same group (tables 10-15).

Studies were not conducted on samples obtained from cat thorax as there were insufficient type II corneocytes obtained for this site to count. The results of the mean findings for each body site with statistical summaries are showed in tables 10a – 15c. Figures 13a – 15b depict box plots of the corneocyte diameters and surface areas from each body site studied. Appendices D-I contain the raw data for these studies.

Table 8. Summary results of corneocyte diameter

	Ear		Thorax		Groin	
	Mean (μm) ± SD	Median (μm)	Mean (μm) ± SD	Median (μm)	Mean (μm) ± SD	Median (μm)
Normal dogs	37.92 ± 4.27	38.30	42.82 ± 4.36~~	42.61	43.47 ± 4.32~	43.19
Atopic dogs	38.38 ± 5.44	38.40	43.24 ± 4.61##	43.40	40.19 ± 6.42**	40.80
Cats	40.41 ± 4.74*	40.19	Not Done	Not Done	48.48 ± 7.32#	49.92

* Significantly larger than normal and atopic ear corneocytes
\# Significantly larger than normal and atopic groin corneocytes
~ Significantly larger than atopic groin corneocytes
Groin corneocytes from normal and cat significantly bigger than ear for equivalent groups
**Atopic groin corneocytes significantly bigger than atopic ear corneocytes
\#\#Atopic thorax corneocytes significantly bigger than atopic groin and ear corneocytes
~~Normal thorax corneocytes significantly bigger than normal ear corneocytes

Table 9. Summary results of corneocyte surface area

	Ear		Thorax		Groin	
	Mean (μm^2) ± SD	Median (μm^2)	Mean (μm^2) ± SD	Median (μm^2)	Mean (μm^2) ± SD	Median (μm^2)
Normal dogs	1091.96 ± 208	1109.44	1399.40 ± 29.60##	1411.58	1435.72 ±236.87‡	1415.36
Atopic dogs	1160.86 ±72.28#	1142.51	1439.67 ± 74.80**	1450.63	1249.75 ±368.55	1236.29
Cats	1222.17 ± 39.02*	1197.70	Not Done	Not Done	1772.11 ± 461.11~	1870.71

* Significantly larger than normal and atopic ear corneocytes
# Significantly larger than normal ear corneocytes
~ Significantly larger than normal and atopic groin corneocytes
‡ Significantly larger than atopic groin corneocytes
All groin corneocytes significantly bigger than ear corneocytes for equivalent groups
**Atopic thorax corneocytes significantly bigger than atopic ear and groin corneocytes
##Normal thorax corneocytes significantly bigger than normal ear corneocytes

Box plots of corneocyte morphometrics

Figure 13a. Ear diameter

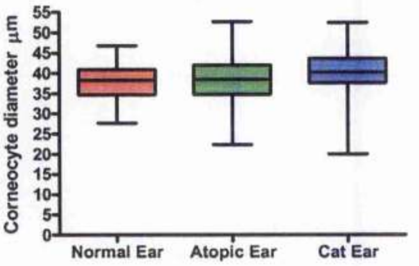


Figure 13b. Ear surface area

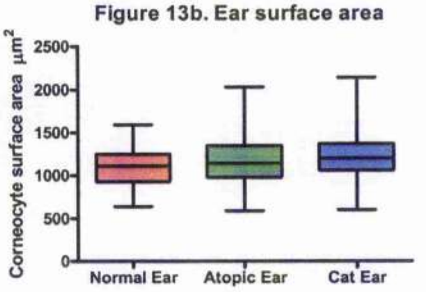


Figure 14a. Thoracic diameter

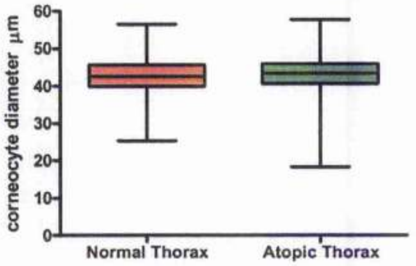


Figure 14b. Thorax surface area

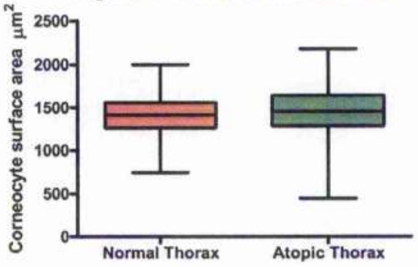


Figure 15a. Groin diameter

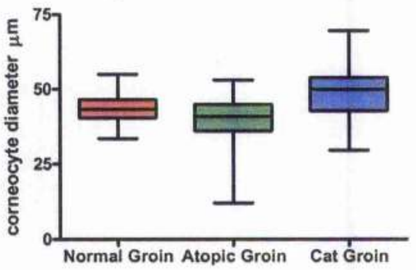
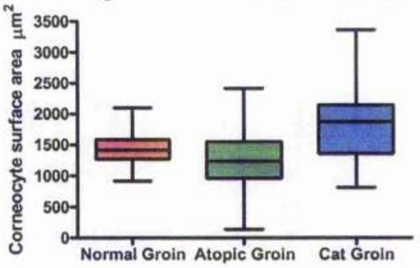


Figure 15b. Groin surface area



Box and whisker plots. The horizontal line in the middle shows the median of the sample. The top and bottom of the box show the 75th and 25th percentiles and the top and bottom of the whiskers show the maximum and minimum values.

Table 10a. Corneocyte diameter ears

	Normal dogs ears (Mean±SD) (μm)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
N1	40.00± 2.72	35.85± 5.34	39.55± 5.20	37.84± 2.62	41.09± 2.75	**38.99± 4.03**
N2	40.00± 3.93	39.90± 2.18	38.01± 3.50	36.95± 1.96	42.30± 4.04	**39.16± 3.86**
N3	39.70± 4.75	38.41± 2.14	39.07± 3.78	39.63± 4.69	40.31± 4.60	**39.41± 3.86**
N4	35.36± 2.92	38.72± 3.03	37.42± 2.64	40.45± 2.84	39.46± 4.47	**38.47± 3.55**
N5*	34.91± 2.73	32.41± 2.72	32.64± 2.55	35.66± 3.52	32.89± 2.70	**33.57± 3.06**
All	**37.99± 4.11**	**37.24± 4.08**	**37.18± 4.44**	**38.03± 3.67**	**39.16± 4.84**	**37.92± 4.27**

No significant difference between discs 1-5

* N5 significantly smaller than N1, 2, 3 and 4

Table 10b. Corneocyte diameter ears

	Atopic dogs ears (Mean±SD) (μm)					
	Disc 1*	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
AD1	36.76± 2.3	37.21± 2.44	40.03± 4.49	38.19± 2.88	40.32± 2.04	**38.68± 3.54**
AD2**	32.31± 3.46	35.44± 3.74	31.41± 3.98	32.75± 4.68	35.11± 2.64	**33.37± 4.01**
AD3	35.47± 2.42	39.40± 3.73	43.59± 3.19	37.58± 3.61	41.68± 3.24	**39.58± 4.17**
AD4	29.50± 4.40	39.55± 2.89	38.42± 4.68	37.76± 2.98	40.57± 2.54	**37.59± 5.72**
AD5#	41.56± 2.88	45.16± 3.60	43.99± 4.29	45.11± 4.45	37.30± 4.86	**42.65± 5.00**
All	**35.12± 5.14**	**39.44± 4.65**	**39.50± 6.16**	**38.53± 5.64**	**39.30± 4.27**	**38.38± 5.44**

* Disc 1 significantly smaller that discs 2, 3 and 4

** AD2 significantly smaller than AD1, 3, 4 and 5

AD5 significantly bigger than AD1, 2, 3 and 4

Table 10c. Corneocyte diameter ears

	Cat ears (Mean±SD) (μm)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
C1#	40.93± 2.89	40.69± 2.94	43.06± 3.87	41.88± 2.46	42.47± 3.02	**41.81± 3.08**
C2	39.62± 4.95	37.40± 4.18	36.53± 2.59	38.12± 4.23	38.82± 3.33	**38.10± 3.93**
C3*	40.14± 4.39	43.25± 4.24	41.70± 4.08	45.05± 3.99	45.06± 5.49	**43.04± 4.70**
C4	37.01± 6.77	37.09± 7.23	36.47± 3.74	36.81± 3.72	37.86± 2.98	**37.05± 5.00**
C5	40.82± 3.10	41.49± 4.22	43.18± 4.22	42.79± 3.60	42.09± 3.11	**42.09± 3.63**
All	**39.70± 4.67**	**39.99± 5.17**	**40.19± 4.73**	**40.93± 4.65**	**41.28± 4.43**	**40.41± 4.74**

No significant differences between discs 1-5
* C3 significantly bigger than C2, and 4
# C1 significantly bigger than C2, and 4

Table 11a. Corneocyte diameter thorax

	Normal dogs thorax (Mean±SD) (μm)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
N1	40.95± 2.34	41.24± 2.23	41.14± 2.63	40.20± 3.88	41.48± 2.89	**40.83± 3.05**
N2	37.41± 6.31	41.08± 4.91	40.28± 3.51	42.68± 5.96	42.46± 4.98	**40.82± 5.18**
N3	48.41± 2.86	46.68± 4.38	48.58± 2.83	48.63± 2.56	45.84± 2.38	**47.53± 3.13**
N4*	45.501± 5.94	47.39± 4.26	43.02± 3.29	43.24± 4.52	46.10± 3.61	**45.05± 4.57**
N5#	46.60± 3.71	43.81± 2.88	43.25± 2.16	43.70± 2.84	44.79± - 3.53	**44.63± 3.18**
All	**42.91± 5.56**	**42.84± 4.41**	**42.20± 3.36**	**42.58± 4.18**	**43.58± 4.04**	**42.82± 4.36**

No significant differences between discs 1-5
* N4 significantly bigger than N1, and 2
# N5 significantly bigger than N1 and 2

Table 11b Corneocyte diameter thorax

	Atopic dogs thorax (Mean±SD) (μm)					
	Disc 1	Disc 2*	Disc3	Disc 4	Disc 5	Disc1-5
AD1	41.20± 3.75	43.79± 2.80	41.29± 3.97	38.83± 5.08	37.62± 8.18	**40.69± 5.14**
AD2	40.71± 2.74	43.10± 1.90	44.07± 1.78	41.66± 3.10	41.20± 1.92	**42.27± 2.75**
AD3	42.23± 3.08	41.57± 4.68	43.58± 4.26	41.11± 3.27	40.72± 3.05	**41.79± 3.96**
AD4**	44.42± 4.24	47.04± 3.41	44.83± 3.64	49.74± 3.47	43.10± 3.25	**46.11± 4.28**
AD5#	45.36± 5.25	43.98± 3.89	46.60± 2.76	46.04± 5.86	45.21± 3.67	**45.33± 4.18**
All	**42.78± 4.17**	**44.14± 3.74**	**44.00± 4.14**	**43.57± 5.63**	**41.71± 4.87**	**43.24± 4.61**

* Disc 2 significant bigger than disc 5
** AD4 significantly bigger than AD1, 2 and 3
# AD5 significantly bigger than AD1, 2 and 3

Table 12a. Corneocyte diameter groin

	Normal dogs groin (Mean±SD) (μm)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
N1	40.54± 4.46	39.38± 3.85	42.74± 4.11	43.63± 2.67	39.93± 2.67	**41.21± 3.88**
N2#	43.11± 3.32	46.43± 2.54	46.24± 5.10	42.73± 2.60	42.79± 2.72	**44.40± 3.68**
N3*	48.41± 2.86	46.62± 4.13	48.08± 3.11	48.28± 2.65	46.25± 2.60	**47.53± 3.13**
N4~	44.89± 4.30	41.51± 2.19	42.30± 1.83	44.49± 3.89	44.98± 3.97	**43.73± 3.46**
N5	39.36± 2.96	41.16± 4.82	40.72± 3.47	42.17± 2.52	39.37± 3.74	**40.46± 3.48**
All	**43.26± 4.77**	**43.08± 4.64**	**44.09± 4.45**	**44.26± 3.47**	**42.64± 4.09**	**43.47± 4.32**

No significant differences between discs 1-5
* N3 significantly bigger than N1, 2, 4 and 5
# N2 and ~N4 significantly bigger than N1 and 5

Table 12b. Corneocyte diameter groin

	Atopic dogs groin (Mean±SD) (µm)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
AD1~	33.79± 4.95	39.33± 3.55	39.17± 4.61	39.39± 11.08	41.03± 2.67	**38.56± 6.17**
AD2	32.64± 4.33	31.67± 3.57	30.97± 2.61	30.57± 4.40	36.62± 2.48	**32.43± 4.11**
AD3#	45.10± 3.71	41.74± 5.70	42.82± 4.48	44.08± 3.76	45.42± 3.00	**43.84± 4.13**
AD4*	46.63± 4.23	45.59± 5.35	46.23± 4.79	45.32± 3.18	44.97± 2.27	**45.86± 3.84**
AD5	41.07± 3.10	37.42± 4.52	38.32± 1.67	41.18± 3.00	36.62± 2.48	**38.93± 3.39**
All	**39.87± 7.00**	**39.28± 6.63**	**40.78± 6.24**	**40.00± 7.53**	**40.42± 3.67**	**40.19± 6.42**

No significant differences between discs 1-5
* AD4 and # AD3 significantly bigger than AD1, 2 and 5
~ AD1 significantly bigger than AD2

Table 12c. Corneocyte diameter groin

	Cats groin (Mean±SD) (µm)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
C1~	52.03± 3.96	52.81± 4.55	55.83± 2.56	53.96± 4.52	48.47± 0.99	**52.41± 4.42**
C2‡	37.49± 3.98	41.25± 4.86	47.00± 4.75	43.07± 3.43	47.25± 5.92	**43.16± 5.75**
C3	37.78± 3.21	39.19± 3.67	43.10± 3.16	42.41± 3.70	38.19± 5.10	**40.13± 4.29**
C4#	54.03± 2.98	53.03± 5.87	51.18± 3.14	52.66± 4.54	53.51± 3.31	**52.88± 4.07**
C5*	54.39± 5.68	51.85± 2.45	55.07± 3.36	51.75± 3.56	55.94± 5.76	**53.80± 4.53**
All	**47.14± 8.80**	**47.49± 7.48**	**50.44± 5.98**	**48.69± 6.20**	**48.62± 7.62**	**48.48± 7.32**

No significant differences between discs 1-5
* C5, #C4 and ~C1 significantly bigger than C2 and 3
‡ C2 significantly bigger than C3

Table 13a. Corneocyte surface area ears

	Normal dogs ears (Mean±SD) (μm²)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
N1	1,230.14± 191.36	1,085.03± 237.09	1,166.60± 146.13	1,128.03± 170.36	1,161.00± 122.19	**1,154.16± 177.14**
N2	1,194.79± 116.14	1,234.44± 145.99	1,018.23± 178.31	1,082.06± 200.16	1,260.86± 160.13	**1,158.07± 181.91**
N3	1,356.17± 130.4	1,152.23± 145.34	1,121.83± 172.71	1114.94± 126.38	971.47± 165.26	**1143.33± 189.68**
N4	967.14± 107.10	1,191.92± 193.93	1,221.67± 161.28	1,287.91± 150.24	1,110.81± 101.54	**1,155.89± 179.50**
N5*	908.17± 99.59	786.00± 130.78	786.46± 112.97	888.87± 96.21	872.35± 93.82	**848.37± 115.68**
All	**1,131.28± 212.25**	**1089.93± 232.81**	**1,062.96± 215.44**	**1100.36± 195.05**	**1,075.30± 188.06**	**1091.96± 208.95**

No significant differences between discs 1-5

* N5 significantly smaller than N1, 2, 3 and 4

Table 13b. Corneocyte surface area ears

	Atopic dogs ears (Mean±SD) (μm²)					
	Disc 1	Disc 2#	Disc3*	Disc 4	Disc 5	Disc1-5
AD1	1,063.87± 158.63	1,032.97± 108.77	1,260.56± 169.43	1,080.58± 165.67	1,179.29± 142.8	**1,133.56± 169.71**
AD2 ‡	856.95± 82.07	1,117.51± 150.66	737.28± 99.18	906.41± 140.43	936.84± 125.39	**910.17± 164.11**
AD3	1,23.54± 157.11	1,182.24± 161.83	1,518.94± 98.99	1,159.32± 177.09	1,276.74± 254.22	**1,252.17** ±222.61
AD4	699.51± 112.93	1,115.27± 173.01	1,219.25± 167.2	1,154.43± 117.14	1,274.17± 129.27	**1,112.18± 267.84**
AD5~	1,429.91± 149.66	1,505.46± 214.2	1,456.65± 243.24	1,482.13± 264.44	1,064.95± 104.84	**1,396.22± 259.56**
All	**1,034.76± 282.64**	**1192.65± 223.93**	**1243.64± 318.73**	**1,166.64± 273.91**	**1,166.59± 213.93**	**1,160.86± 272.28**

* Disc3 and #Disc 2 significantly bigger than Disc 1

~ AD5 significantly bigger than AD1, 2 and 4

‡ AD2 significantly smaller than AD1, 3, 4 and AD5

Table 13c. Corneocyte surface area ears

Cats ears (Mean±SD) (μm^2)						
	Disc 1*	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
C1	1,017.67± 196.74	1,209.12± 171.39	1,183.39± 129.59	1,311.74± 142.05	1,281.46± 218.66	**1,200.68± 197.07**
C2	1,069.80± 129.1	1,184.45± 224.72	1,043.28± 296.05	1,113.28± 210.38	1,248.98± 122.63	**1,121.13± 211.12**
C3#	1,229.81± 188.69	1,418.24± 332.37	1,309.68± 192.65	1,446.31± 207.14	1,553.25± 305.95	**1,379.85± 257.57**
C4	1,121.60± 117.89	1,128.86± 100.79	1,065.57± 136.1	1,072.90± 153.49	1,011.76± 230.32	**1,093.85± 158.5**
C5~	1,161.38± 224.34	1,357.03± 226.80	1,391.12± 281.11	1,430.52± 147.75	1,236.57± 180.27	**1,315.32± 231.35**
All	**1,120.59± 188.16**	**1,258.35± 237.13**	**1,202.17± 242.46**	**1,271.43± 231.92**	**1,258.21± 264.62**	**1,183.33± 232.73**

* Disc 1 significantly smaller than Discs 4 and 5
# C3 significantly bigger than C1, 2 and 4
~ C5 significantly bigger than C2 and 4

Table 14a. Corneocyte surface area thorax

Normal dogs thorax (Mean±SD) (μm^2)						
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
N1	1,251.51± 217.31	1,126.04± 239.45	1,246.77± 150.78	1,336.07± 131.35	1,251.93± 220.61	**1,242.46± 200.2**
N2	1,051.62± 160.85	1,251.35± 182.28	1,391.06± 128.68	1,377.73± 243.49	1,426.28± 172.15	**1,299.61± 222.44**
N3~	1,413.21± 171.24	1,418.87± 228.79	1,467.52± 165.32	1,286.50± 170.9	1,486.63± 202.44	**1,414.55± 194.67**
N4*	1,583.76± 219.17	1,464.43± 299.48	1,503.64± 162.65	1,540.98± 298.04	1,531.76± 131.93	**1,524.91± 226.44**
N5#	1,617.90± 91.06	1,531.92± 196.43	1,490.79± 142.41	1,431.00± 161.48	1,505.71± 125.42	**1,515.47± 154.3**
All	**1383.60± 273.44**	**1,358.52± 268.98**	**1,419.96± 173.28**	**1,394.45± 219.8**	**1,440.46± 195.69**	**1,399.40± 229.6**

No significant differences between discs 1-5
* N4 significantly bigger than N1, 2 and 3
#N5 and ~N3 significantly bigger than N1 and 2

Table 14b. Corneocyte surface area thorax

	Atopic dogs thorax (Mean±SD) (μm²)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
AD1	1,247.35± 323.74	1,437.98± 255.95	1,101.52± 237.27	1,110.08± 335.17	1,100.57± 317.79	**1,210.99± 303.05**
AD2	1,307.85± 191.7	1,391.32± 222.68	1,458.90± 118.92	1,390.47± 129.96	1,329.60± 148.58	**1,387.97± 180.37**
AD3~	1,399.20± 195.93	1,333.94± 261.45	1,543.14± 179.08	1,466.59± 334.75	1,313.97± 134.3	**1,398.96± 248.13**
AD4#	1,597.22± 204.3	1,723.51± 178.12	1,577.18± 190.2	1,772.55± 239.01	1,502.05± 157.39	**1,636.83± 207.55**
AD5*	1,708.66± 197.94	1,466.21± 165.7	1,456.38± 254.58	1,558.77± 207.07	1,592.68± 135.84	**1,563.59± 203.98**
All	**1,452.06± 281.1**	**1,485.15± 245.14**	**1,428.74± 274.53**	**1,456.91± 318.97**	**1,375.49± 246.21**	**1,439.67± 274.8**

No significant differences between discs 1-5
* AD5 and #AD4 significantly bigger than AD1, 2 and 3
~AD3 significantly bigger than AD1

Table 15a. Corneocyte surface area groin

	Normal dogs groin (Mean±SD) (μm²)					
	Disc 1	Disc 2	Disc3	Disc 4	Disc 5	Disc1-5
N1	1,364.14± 183.34	1,308.43± 202.53	1,316.14± 132.84	1,300.40± 184.94	1,271.71± 123.6	**1,312.17± 164.19**
N2*	1,721.94± 254.8	1,641.21± 259.83	1,630.60± 179.65	1,794.27± 116.99	1,602.62± 176.6	**1,678.13± 208.67**
N3#	1,457.36± 173.94	1,522.01± 114.98	1,498.72± 249.31	1,430.90± 242.92	1,433.21± 150.98	**1,468.44± 189.12**
N4~	1,405.62± 126.13	1,344.22± 144.26	1,299.91± 136.6	1,608.70± 302.87	1,509.36± 171.02	**1,433.56± 212.74**
N5	1,296.18± 161.04	1,309.61± 210.15	1,226.31± 176.47	1,297.67± 175.70	1,301.76 221.11	**1,286.30± 184.89**
All	**1,449.05± 230.61**	**1,425.10± 229.3**	**1,394.34± 228.42**	**1,486.39± 281.97**	**1,423.73± 207.05**	**1,435.72± 236.87**

No significant differences between discs 1-5
* N2 significantly bigger than N1, 3, 4, and 5
# N3 and ~ N4 significantly bigger than N1 and 5

Table 15b. Corneocyte surface area groin

Atopic dogs groin (Mean±SD) (μm^2)						
AD1 ‡	965.22± 254.25	1,063.21± 413.20	1,138.99± 232.39	1,214.99± 272.78	1,085.60± 188.08	**1,099.52± 273.18**
AD2	780.05± 87.72	812.26± 84.51	796.41± 103.83	661.57± 88.41	1,055.70± 168.28	**825.10± 165.97**
AD3#	1,656.34± 130.09	1,333.17± 227.49	1,414.81± 290.97	1,460.66± 221.23	1,474.47± 188.27	**1,476.10± 227.48**
AD4*	1,688.51± 224.54	1,667.44± 408.53	1,569.39± 245.69	1,518.54± 286.16	1,585.86± 142.48	**1,631.91± 276.75**
AD5~	1,181.22± 233.71	1,130.46± 193.17	1,098.88 213.65	1,181.74± 181.51	1,058.43± 158.89	**1,216.13± 268.10**
All	**1,254.27± 413.76**	**1,223.05± 421.48**	**1,212.79± 348.01**	**1,213.02± 367.94**	**1,259.67± 287.72**	**1,249.74± 368.55**

No significant differences between discs 1-5
* AD4 significantly bigger than AD1, 2, 3, and 5
# AD3 significantly bigger that AD1, 2 and 5
~AD5 and ‡AD1 significantly bigger than AD2

Table 15c. Corneocyte surface area groin

Cats groin (Mean±SD) (μm^2)						
C1~	2,044.77± 208.86	2,103.23± 130.24	2,080.69± 423.21	2,022.30± 181.32	1,680.96± 177.64	**1,986.75± 276.04**
C2 ‡	1,103.03± 111.22	1,222.70± 140.85	1,513.67± 206.2	1,338.72± 176.3	1,753.24± 229.82	**1,404.73± 295.85**
C3	1,148.41± 215.11	1,170.70± 153.42	1,199.26± 234.6	1,327.68± 176.57	1,257.95± 196.67	**1,227.36± 195.21**
C4#	2,238.97± 273.52	2,003.97 387.64	2,021.59± ,181.27	2,130.68± 285.27	2,053.71± 172.52	**2,077.76± 269.7**
C5*	2,205.50± 109.27	2,098.67± 206.3	2,175.93± 153.73	2,139.09± 173.76	2,177.81± 236.42	**2,163.95± 257.58**
All	**1,755.41± 540.77**	**1,719.31± 464.64**	**1,796.40± 452.1**	**1,778.60± 416.23**	**1,810.82± 434.98**	**1,772.11± 461.11**

No significant differences between discs 1-5
* C5 significantly bigger than C1, 2 and 3
# C4 and ~C1 significantly bigger than C2 and 3
‡ C2 significantly bigger than C3

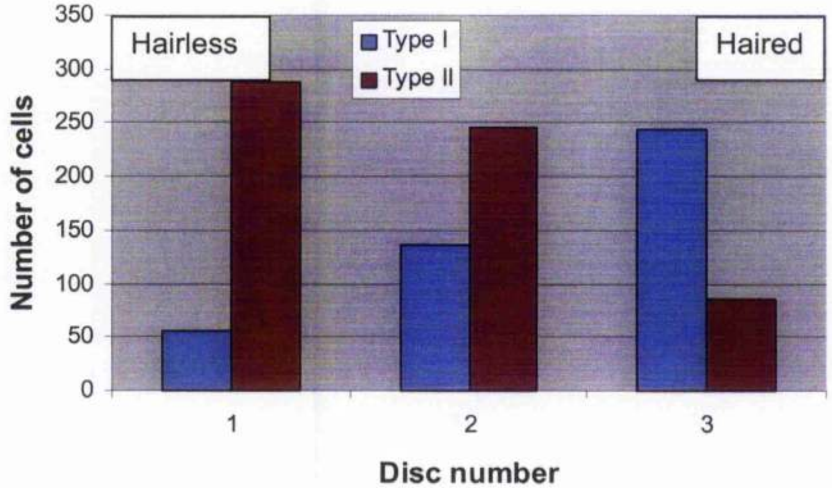
Comparison of corneocytes from haired and nonhaired sites

The numbers of polygonal type II corneocytes were highest in the sparsely haired sampling site 1 at the base of the pinna becoming progressively less common in discs 2 to 3. The converse finding was obtained for type I corneocytes.

Table 16. Canine corneocytes from haired area to unhaired area

Dog	Corneocyte						
1	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	10	2	1	7	1	21
	II	13	7	12	8	13	53
Disc2	I	12	16	13	14	7	62
	II	4	5	5	7	2	23
Disc3	I	26	40	12	27	31	136
	II	5	1	2	2	2	12
2	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	1	0	0	1	2	4
	II	14	11	17	22	23	87
Disc2	I	5	3	2	0	1	11
	II	24	17	17	22	7	87
Disc3	I	1	4	9	6	1	21
	II	4	2	5	2	5	18
3	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	2	2	2	3	5	14
	II	9	9	12	5	9	44
Disc2	I	2	6	9	4	4	25
	II	9	9	5	5	15	43
Disc3	I	4	8	4	3	4	23
	II	2	7	1	3	2	15
4	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	1	4	0	1	3	9
	II	13	3	13	14	8	51
Disc2	I	1	4	6	0	3	14
	II	10	19	7	22	9	67
Disc3	I	12	5	5	7	5	34
	II	8	11	2	1	1	23
5	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	0	1	3	1	2	7
	II	24	8	11	7	3	53
Disc2	I	7	7	5	3	3	25
	II	3	3	2	5	12	25
Disc3	I	9	4	4	5	8	30
	II	3	2	2	2	9	18

Figure 16. Histogram of numbers of type I and type II corneocytes collected from canine ear

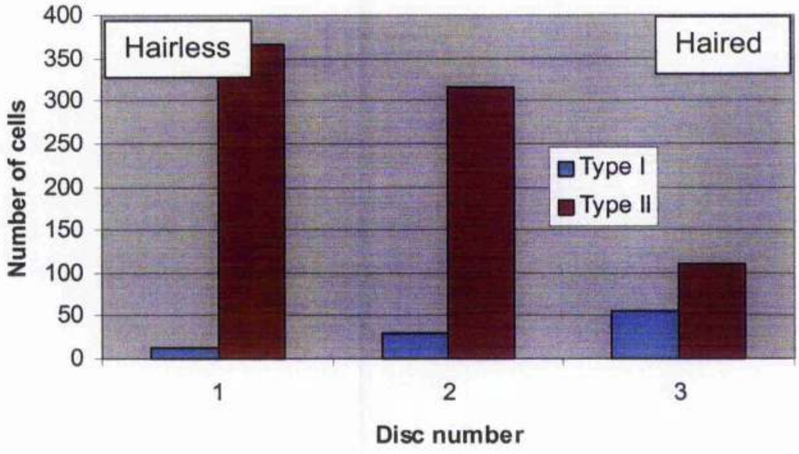


Columns represent the total counts for 5 dogs from each of three sites.

Table 17. Feline corneocytes from haired area to unhaired area

Cat 1	Corneocyte Type	Image1	Image2	Image3	Image4	Image5	Total
Disc1	I	1	1	0	2	0	4
	II	7	10	5	14	17	53
Disc2	I	0	1	3	4	3	11
	II	5	22	15	8	8	58
Disc3	I	4	3	0	1	2	10
	II	11	3	7	14	3	38
2	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	1	3	1	0	2	7
	II	19	18	11	18	7	73
Disc2	I	0	1	3	1	5	10
	II	20	10	5	5	1	41
Disc3	I	1	2	3	4	3	13
	II	2	0	2	4	1	9
3	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	0	0	0	0	0	0
	II	14	14	25	12	21	86
Disc2	I	0	0	4	0	0	4
	II	14	20	11	19	27	91
Disc3	I	0	5	2	4	6	17
	II	26	1	4	1	3	35
4	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	0	0	0	0	0	0
	II	24	4	28	15	18	89
Disc2	I	0	0	0	1	0	1
	II	6	9	3	14	12	44
Disc3	I	4	0	1	1	2	8
	II	4	1	1	6	0	12
5	**Type**	**Image1**	**Image2**	**Image3**	**Image4**	**Image5**	**Total**
Disc1	I	0	1	0	0	0	1
	II	12	6	9	24	15	66
Disc2	I	1	1	0	1	0	3
	II	21	17	17	14	12	81
Disc3	I	2	1	1	1	2	7
	II	1	3	11	2	0	17

Figure 17. Histogram of numbers of type I and type II corneocytes collected from feline ear



Columns represent the total counts for 5 cats from each of three sites.

DISCUSSION

The average diameter of canine and feline corneocytes in this study was approximately 38-48 μm. The equivalent figures for surface area were found to be 1100-1800 μm^2. This is similar to that reported for man (El Gammal, Kligman and el Gammal, 1997)(30-40μm) and larger than that reported for the mouse (Schatzlein and Cevc 1998) (25-30μm). This study appears to be the first to define the size and shape of canine and feline corneocytes. Because not all sites were sampled from each individual animal and some sites required clipping before sampling it was not possible to directly compare the various sampling sites of the different animal groups studied. This was not the major purpose of the study but in general, cat corneocytes were larger than those from dogs. Corneocytes from the groin tended to be largest and corneocytes from the ears tended to be smaller than those from the other two sites. There was therefore some species and site differences appreciated which may justify further larger studies. There was little variation in the size of corneocytes between each of the five discs collected from the same site suggesting that samples from the same site would be suitable for comparative studies in an adhesion assay and that the corneocytes do not vary in size between the layers sampled.

In man corneocyte size has been shown to be determined by the epidermal turn over rate which in turn may be influenced by body site, sex and inflammatory skin disease (Fluhr, Kao, Jain, Ahn, Feingold and Elias, 2001; Kashibuchi *et al*, 2002). Because inflamed skin is likely to have an increased epidermal turnover time it could be anticipated that corneocytes from atopic dogs would be smaller than those from normal dogs. There were individual atopic dogs that did appear to have small corneocytes but in general there was no overall tendency for atopic corneocytes to be smaller than normal ones. In general there were significant variations in size between individual animals, which may suggest possible breed, sex or age influences. Much larger studies would be required to identify any such differences positively.

The two types of corneocytes identified based on shape are of interest. The elongated type I corneocytes appeared to be more common in haired skin areas suggesting that their origin is the hair follicle. The lighter staining and polygonal type II corneocytes are more typical of those identified in human skin and are likely to originate from the interfollicular skin area.

Type II corneocytes being flat and lightly staining are better suited for use in adhesion assays and particularly those that employ image analysis as a counting tool. When counting bacteria using image analysis good distinction between the bacteria to be counted and the background is necessary. Sheets of type II corneocytes could be most readily obtained from the inner pinnae of both the dog and cat and this site was considered to be most suited for the collection of corneocytes to be used in an adherence assay.

CHAPTER 3

An Adhesion Assay To Quantify Bacterial Adherence To Canine And Feline Corneocytes

LITERATURE REVIEW

Introduction

Although bacteria were observed to adhere to red blood cells as far back as 1908, it was not until the 1970's that bacterial adherence was studied in any detail (Gibbons and van Houte, 1971; Gibbons and Houte, 1975). Adhesion is known to be essential for maintaining the normal microflora in or on their host, and it is also the crucial first stage in any infectious disease. It is now recognized that bacterial adhesion is a prerequisite to colonization and infection (Feingold, 1986; Roth and James, 1989). Over the last twenty years, research has revealed the enormous complexity underlying the phenomenon of bacterial adhesion. The initial research goal was to understand the mechanism of attachment and its effects on bacteria as well as the host. As research progressed, however, it became evident that many different attachment mechanisms exist. Various studies have shown that certain species of bacteria will adhere preferentially to particular tissues whilst other bacteria fail to adhere. For example, *Staphylococcus mitis* adheres well to the surface of teeth and to cheek epithelium while *Escherichia coli* adheres well to intestinal epithelium but not to oral tissues (Gibbons and van Houte, 1971; Gibbons and Houte, 1975; Aly and Bibel, 1993). Similarly, host species specificity can be shown (Gibbons, Spinell and Skobe, 1976; Valentin-Weigand, Chhatwal and Blobel, 1988). These diverse forms of adhesion are the results of numerous evolutionary pressures, and each may be part of a larger survival strategy. This suggests that bacteria have developed specific adhesion molecules to allow them to colonise and flourish in specific niches on specific host animal species. The ability of bacteria to adhere has obviously evolved as an important factor and is one of the significant initial steps in the process of colonization (Feingold, 1986).

Mechanisms of bacterial adherence and infection

Bacterial adhesion is a complex process influenced by both the host and the organism. Bacteria adhere only to complementary substrata. They adhere by ionic or Coulombic interactions, by hydrogen bonding (Pimentel and McClellan, 1960), by the hydrophobic effect (Duncan-Hewitt, 1990), and by coordination complexes involving multivalent metal ions. Adhesion is highly stereospecific in that a bacterium will bind to a substratum only if the substratum possesses a certain kind of receptor. Bacteria possess surface adhesion molecules, which influence their ability to bind to cells. For those bacteria that possess pilli and fimbriae such as the *Enterobacteriaceae* those structures have adhesins at their tips that enables specific binding to receptors. Of the staphylococci *S. aureus* has been the most studied and here teichoic acids, fibronectin binding proteins, protein A, collagen binding proteins and capsular polysaccharide have all been shown to be involved in adhesion (Van Belkum, Kools-Sijmons and Verbrugh, 2002).

Several studies have shown that adhesion is dependent on time, temperature, and concentration of bacteria as well the influence of certain disease states (Berg *et al*, 1984; Harvey and Noble, 1994; Forsythe *et al*, 2002; Saijonmaa-Koulumies and Lloyd, 2002). Although there is evidence that adhesion by staphylococci may play a part in bacterial infections in both human and canine atopic dermatitis (Cole and Silverberg, 1986; McEwan, 2000) there is currently little evidence that the ability to adhere by *S. intermedius* can be considered a virulence factor. Using *S. intermedius* strains isolated from normal and infected canine skin Cree and Noble (1995) found increased adherence by pyoderma strains while Saijonmaa-Koulumies and Lloyd (2002) found no such relationship. It may be better to think of adhesion as an indirect virulence factor in that after attachment direct virulence factors can be delivered at close proximity to host cells (Van Belkum, Kools-Sijmons and Verbrugh, 2002).

A number of approaches have been used to examine the relationship between adhesion and infectivity. One method was to compare the infectivity phenotypes or genotypes expressing or not expressing adhesions. Another method to assess the role of adhesion in the infectious process is to compare the sensitivity or resistance to infection by hosts that either over express or do not express, respectively, adhesin receptors. In general, there is a positive correlation between the ability of the tissue cells to bind the bacterial pathogen and susceptibility of the host to develop infection caused by the pathogen. Ofek and Beachey (1980) reviewed the bacterial binding capacity of epithelial cells from infection-prone people finding it to be greater than that from similar cells of people not prone to infection. One piece of convincing evidence described the relationship between resistance to infection and the capacity of host cells to bind pathogenic bacteria (Sellwood, Gibbons, Jones and Rutter, 1975). Intestinal epithelial cell brush borders isolated from piglets that were resistant to the diarrhoeal disease failed to bind *E. coli* K88 bacteria in vitro, whereas those from susceptible animals were able to bind the organisms in high numbers. (Mouricout, Petit, Carias and Julien, 1990) were able to block the adhesion by *E. coli* K99 to calf intestine, and hence disease, by administration of glycoprotein glycans derived from bovine plasma. Thus demonstrating that adhesion is important in the infection process and its study can lead to potentially novel treatments.

There are a number of reasons to support the view that adhesion is important in infectivity. One of the most widely accepted is that bacterial adhesion endows the pathogen with the ability to withstand cleansing mechanisms operation on mucosal and endothelial surfaces

(Ofek and Beachey, 1980; Beachey, 1981). It has been pointed out (Freter, 1978; Freter, O'Brien and Halstead, 1978; Freter, 1980) that successful association of the pathogen with mucosal surfaces such as intestinal cells is a complex process. Adhesion to animal cells seems to confer at least three main advantages in addition to the ability to withstand mechanical cleansing mechanisms, including growth advantages, toxin targeting, and escape from antimicrobial or antitoxin activities (Ofek and Doyle, 1994).

Methods of assessing bacterial adherence

Several methods have been used to quantify microbial adhesion to cells (Evans-Hurrell, Adler, Denyer, Rogers and Williams, 1993; Ofek and Doyle, 1994), including direct cell counting using light microscopy (Tavendale, Jardine, Old and Duguid, 1983; Miyake, Kohada, Fujii, Sugai and Suginaka, 1989; Miyake, Sugai, Kohada, Minagi and Suginaka, 1990; Romero-Steiner, Witek and Balish, 1990), plate counting (Cooper, Jeffery-Wiseman, Bortner, Wheat and Wadstrom, 1990), and radiometric assays (Jordens, 1990; Verbrugh, 1990; Wyatt, Poston and Noble, 1990). Generally, cells are incubated with the microbe under test either *in vivo* or *in vitro*. Non-adherent bacteria are commonly removed by either filtration or by differential centrifugation. Adhesion is then quantified either by direct visualisation under light or electron microscopy or quantitatively by the use of markers such as fluorescent dyes or radioactivity (Bartelt and Duncan, 1978; Bibel, Aly, Lahti, Shinefield and Maibach, 1987; Valentin-Weigand, Chhatwal and Blobel, 1987; Kanzaki, Morishita, Akiyama and Arata, 1996). The major limitations of these methods include extended time for analysis, operator fatigue, subjectivity, and in the case of radiometric assays, an inability to discriminate between bacteria specifically adhering to the epithelial tissue and those attached to the inert support (Evans-Hurrell *et al*, 1993). Experimental design is also important and several studies have shown that fluorescein used as a marker may significantly alter bacterial adherence, presumably by modification of bacterial adhesins (Evans-Hurrell *et al*, 1993; Barthelson, Hopkins and Mobasseri, 1999).

As expected, adherence is dependent on temperature, bacterial concentration and also pH (Valentin-Weigand, Chhatwal and Blobel, 1987; Saijonmaa-Koulumies and Lloyd, 1996). However, one study showed that too high a bacterial concentration may result in cytotoxic effects, which may increase or decrease adhesion by exposing, inducing or decreasing binding sites (Charboneau and Rubins, 1998).

Staphylococcal adherence in human and canine atopic dermatitis

There is a growing body of evidence to suggest that all of the pyogenic Gram-positive cocci rely on multiple adhesins in order to adhere avidly to substrata (Hasty, Ofek, Courtney and Doyle, 1992). The most successful pyogenic cocci have the ability to adhere to and grow on mucosal tissues without causing symptoms. This is commonly known as the carrier state.

In human and canine atopic dermatitis patients, bacterial skin disease is a very common and important complication. The major microbial pathogens involved are the pathogenic staphylococci; *Staphylococcus aureus* in man (Leyden, Marples and Kligman, 1974; Dahl, 1983; Hanifin and Homburger, 1986; Masenga, Garbe, Wagner and Orfanos, 1990) and *Staphylococcus intermedius* in dogs (Phillips, Jr. and Kloos, 1981; Berg *et al*, 1984; Biberstein, Jang and Hirsh, 1984; Cox *et al*, 1984; Ihrke, 1987). In human atopics, the normal microflora changes with an increase in density and carriage of pathogenic staphylococci on the skin (Aly *et al*, 1977). A similar situation is likely to exist in the dog

(Kristensen and Krogh, 1978). Mason and Lloyd (1989) investigated the hypothesise that changes in the skin surface defence mechanisms in atopic dogs led to their susceptibility to invasion by *S. intermedius* and suggested that the pathogenesis of pyoderma secondary to hypersensitivity may be via an effect on epidermal permeability, promoting penetration of staphylococcal antigens from the stratum corneum which then cause the lesions of pyoderma.

Cole and Silverberg (1986) were the first to hypothesise that human atopic dermatitis patients may be susceptible to *S. aureus* infections due to increased adherence to atopic corneocytes. Foster and Höök (1998) reviewed *S. aureus* adherence to host extracellular matrix. They concluded that adherence is mediated by protein adhesions of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, such as fibronectin-binding, fibrinogen-binding and collagen-binding proteins. For adhesion to the skin the closely related fibronectin-binding proteins (FnBPA and FnBPB) appear to be important. Most strains of *S. aureus* express both fibronectin-binding proteins which are encoded by two closely linked genes (Greene, McDevitt, Francois, Vaudaux, Lew and Foster, 1995; Johansson, Flock and Svensson, 2001). More recent work has identified that *S. aureus* does bind to fibrinogen and fibronectin in skin (Kanzaki *et al*, 1996; Cho, Strickland, Boguniewicz and Leung, 2001), who also provided further evidence of enhanced binding by *S. aureus* to human atopic skin compared to normal human skin.

In atopic dogs, there are few published studies on the adherence by *S. intermedius*. Adherence by *S. intermedius* to normal canine corneocytes was shown to depend on duration of incubation, temperature of incubation and the concentration of bacteria used (Saijonmaa-Koulumies and Lloyd, 2002). McEwan (2000, 2002c) found a statistically significantly greater adherence by *S. intermedius* to corneocytes from atopic dogs and dogs suffering from pyoderma when compared with a normal group. Forsythe *et al.* (2002) suggested that *S. intermedius* adheres to canine corneocytes by a specific receptor-ligand interaction and there may be a breed predisposition for adherence and that different body sites also influence bacterial adherence.

From the available evidence of the literature the ability of staphylococci to adhere to corneocytes is likely to be important in the development of pyoderma. In both human and canine atopic dermatitis pathogenic staphylococci adhere well to corneocytes. A major limitation of adherence studies is the time consuming and tedious method of many adherence assays. A simple and quick method to assess bacterial adherence to corneocytes would be a useful tool for the investigation of this phenomenon, and this was the goal of the studies that follow.

AIMS

The aims of the following studies were to:

1. Develop a simple assay to quantify bacterial adherence to canine and feline corneocytes
2. To develop image analysis techniques to count bacteria adherent to canine and feline corneocytes

MATERIAL AND METHODS

Populations studied

Three groups of animals were used as sources of corneocytes:

1. Normal dogs
2. Dogs suffering from atopic dermatitis
3. Normal cats

These groups were defined as detailed in Chapter 2 of this thesis. None of the animals used in the studies had received either systemic or topical treatments for a period of at least three weeks prior to corneocyte sampling.

Corneocyte collection

Corneocytes were collected in a similar fashion to that described in Chapter 2 of this thesis. The skin area to be sampled was first prepared by removing surface debris by five successive applications of ordinary adhesive tape. Corneocyte samples for the adhesion assay were collected by applying a 25mm D-Sqaume® disc that was gently pressed onto the skin surface once. Preliminary studies (study 4) indicated that up to 5 consecutive D-Squame® discs from the same area would sample corneocytes with similar adhesive properties.

Bacteria

All staphylococci used in the study were collected from clinical cases. Specific species of staphylococci were identified by a microtray method (API Staph. System. BioMerieux, Lyons, France). The *Micrococcus* sp. used was isolated from normal human skin and identified by conventional microbiological techniques.

1. *Staphylococcus intermedius.* Isolated from purulent material collected from a case of canine deep pyoderma (muzzle furunculosis)
2. *Staphylococcus hominis.* Isolated from a bovine nasal swab
3. *Staphylococcus aureus.* Isolated from a case of bovine mastitis
4. *Staphylococcus hyicus.* Isolated from a case of bovine mastitis
5. *Micrococcus* sp. Isolated from human skin

All bacteria were cultured on sheep blood agar, subcultured into liquid medium (Oxoid Nutrient Broth. No. 2. Unipath Ltd., Basingstoke, Hampshire, England) and frozen at -70° C in one millilitre aliquots.

Suspensions of *S. intermedius* and *S. hominis* increasingly diluted in sterile phosphate buffered saline (PBS) were made at optical densities (OD) ranging from 0.2-0.8 at 570 nanometers (Cole and Silverberg, 1986) on a spectrophotometer (Cecil instruments, Cambridge, England) and plated on sheep blood agar at ten fold dilutions to determine the number of colony forming units (CFU) per ml. By extrapolation an OD_{570} of 0.15

was found to give approximately $1x10^6$ CFU per ml and this dilution was used for each bacterial suspension in the final assay.

When required, a frozen aliquot of staphylococci was thawed and plated on sheep blood agar and incubated at 38° C for 24 hours. Colonies were harvested and placed in a centrifuge tube with 10 millilitres of sterile PBS. The cocci were washed in twice in PBS by centrifugation and the resulting suspension adjusted to an OD of approximately 0.15 at 570 nanometers. Preliminary studies (study 1) indicated that an incubation time of 45 minutes and a bacterial concentration of 0.15 at OD_{570} was suited to the adhesion assay used.

Adhesion assay

A 25mm D-Squame® disc with adherent corneocytes was placed corneocyte surface uppermost on a microscope slide. A 0.5 ml bacterial suspension in PBS was placed over the corneocyte layer and incubated at 38°C for 45 minutes in a moist chamber held within a water bath (figures 18 and 19). After incubation the disc was washed in tap water, stained for 10 seconds with crystal violet and washed to remove excessive stain (figure 20). Washing and staining procedures followed a standard protocol. After washing the disc was placed on a glass microscope slide and air-dried.

Figure 18. Moist chamber within water bath

Figure 19. D-Squame® discs and bacteria suspension prepared for incubation

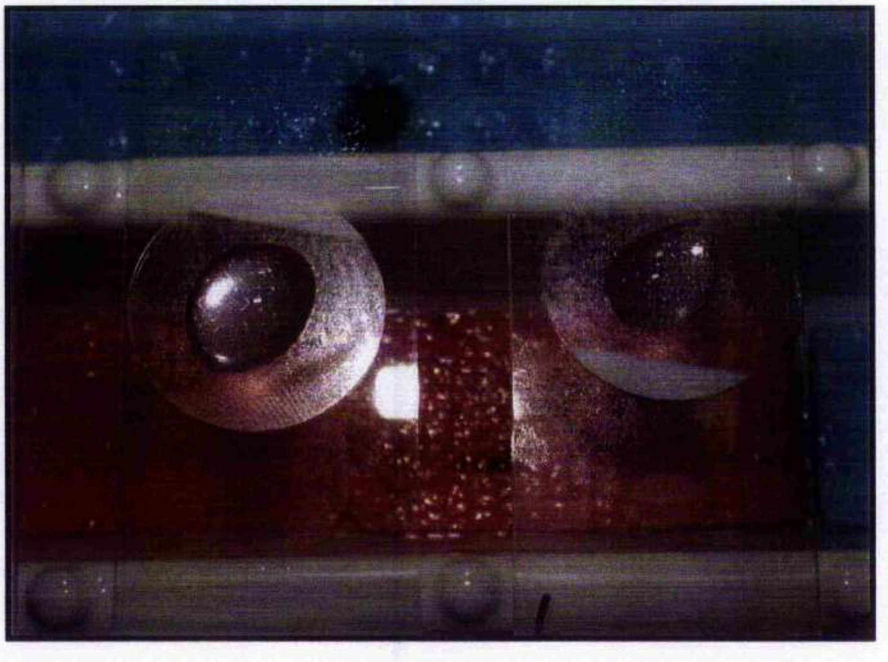


Figure 20. Slides after washing and staining

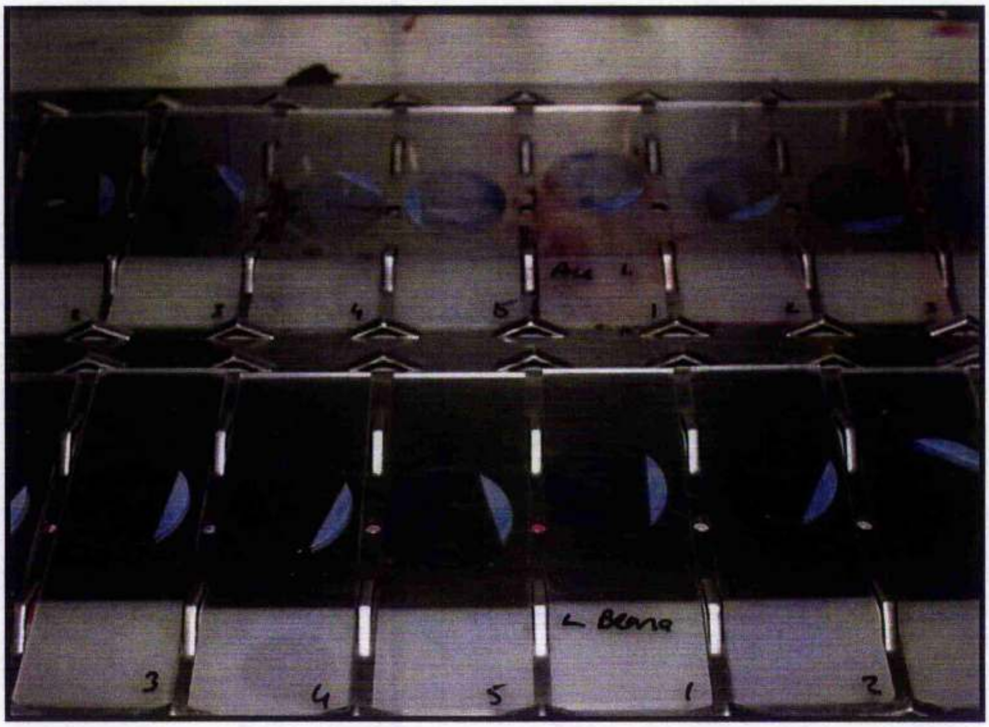


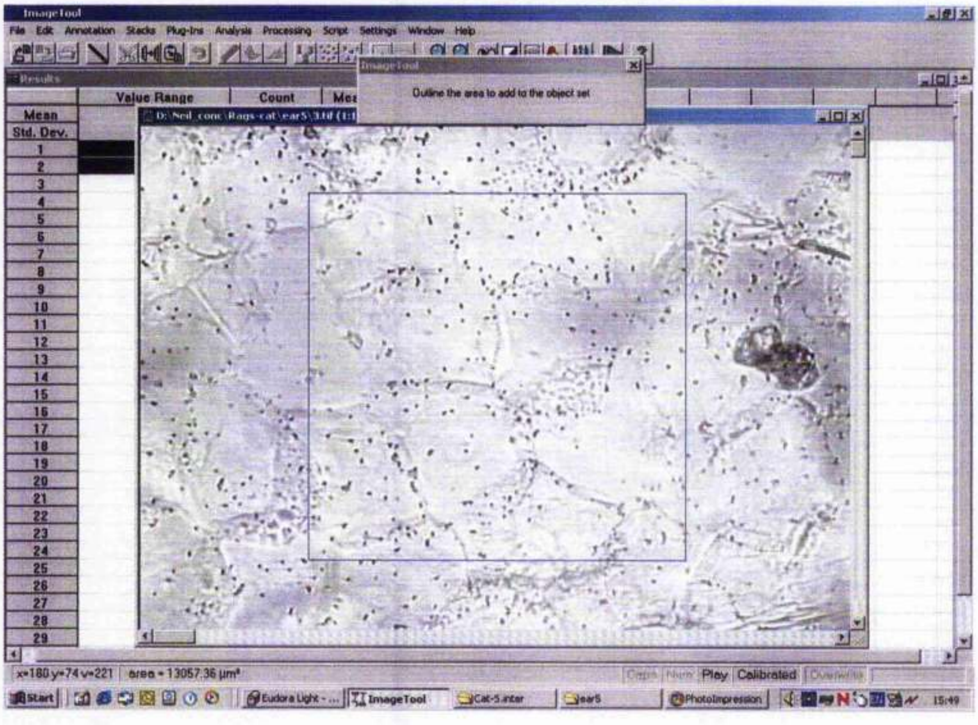
Image acquisition and bacteria counting

Adherent bacteria were counted by an image processing and analysis program (UTHSCSA ImageTool Version 3.0 and The Image Processing Tool Kit v.5, Reindeer Graphics Inc., Asheville, NC). UTHSCSA ImageTool is an image processing and analysis program for Microsoft Windows available from the following web site http://ddsdx.uthscsa.edu/dig/itdesc.html.

From each D-Squame® disc ten images at x 630 magnification were collect and saved as black and white TIFF files. The process of image thresholding and counting was semi-automated by a macro or script written by the author (Appendix J). This script was identified as "Bacteria Count".

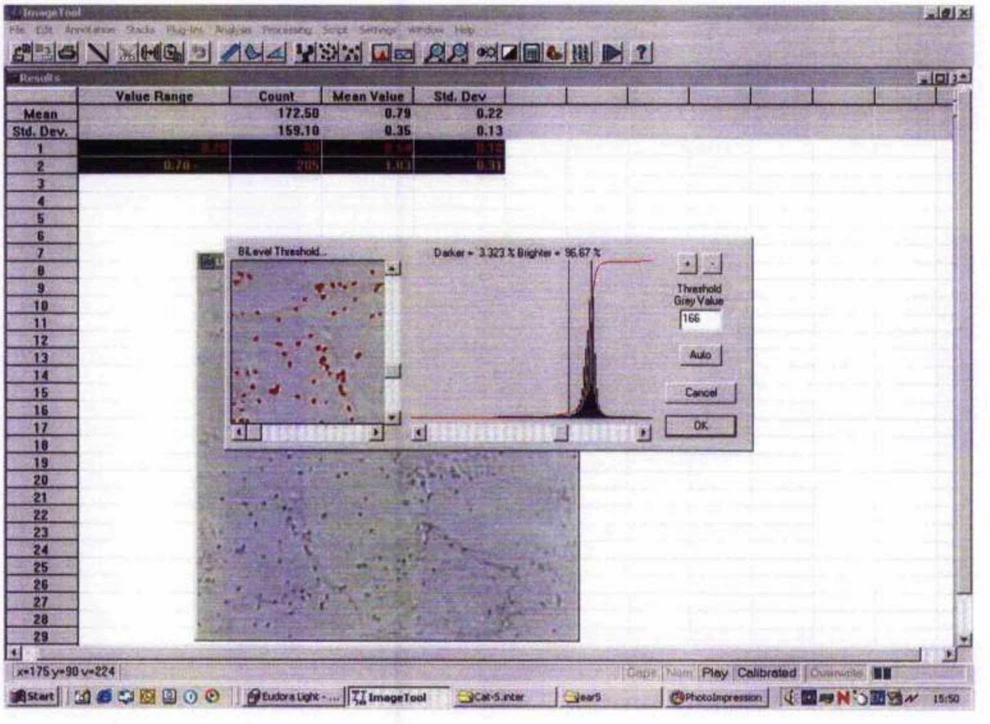
The various steps in the image analysis bacteria counting are depicted in figures 21 - 24. Figure 21 shows the initial image collected from the disc and the creation of a 400 x 400 pixel box (an area of approximately 13060 μm^2, approximately the surface area of 10 corneocytes), which could then be manually placed over a selected counting area. Figure 22 shows the thresholding process. Automatic bilevel thresholding using the plugin provided generally produced good separation of bacteria from corneocytes but the program also allowed for manual selection of the threshold value. Next (figure 23) a round filter was applied which allowed better selection of bacteria from "background noise" created by debris and corneocyte folds. Finally the total count for the area was displayed (figure 24). For each study a total of ten images were collected and counted from each disc this gave a final count, which equated to a surface area equivalent to 100 corneocytes.

Figure 21. Selection of the counting area



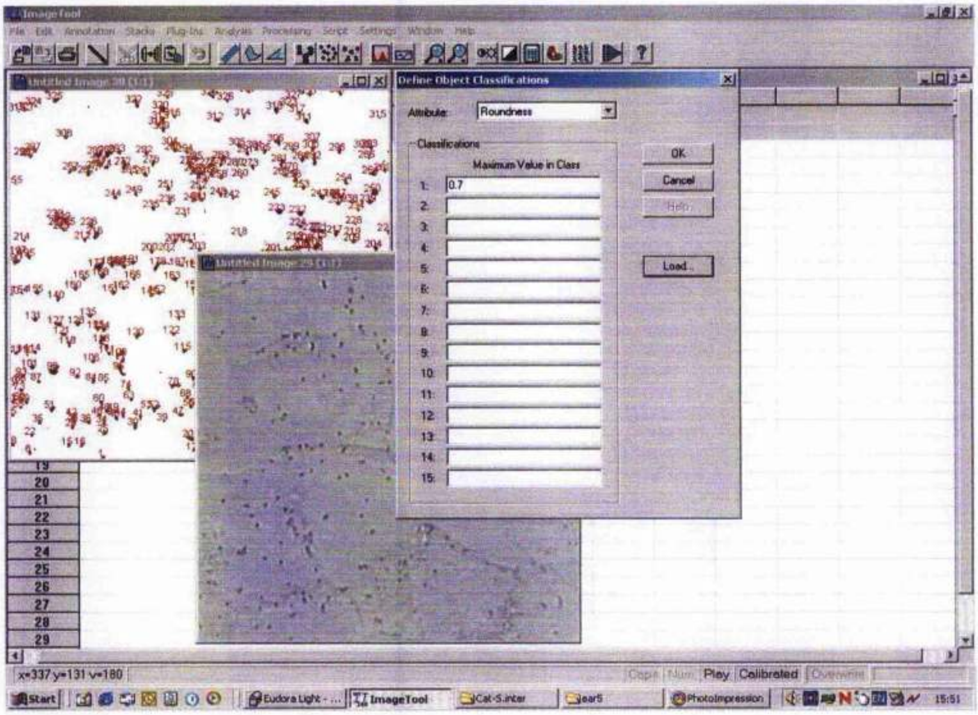
A 400 x 400 pixel box is generated which can be manually placed over the desired counting area.

Figure 22. Image thresholding



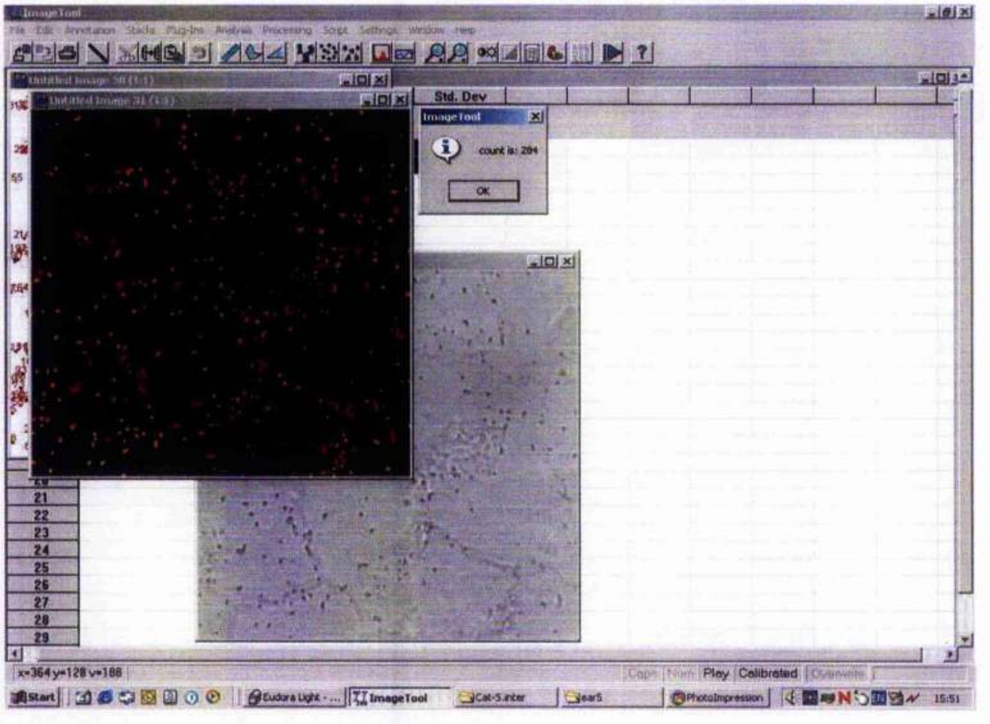
A greyscale threshold value is selected that separates bacteria from background to produce a binary image.

Figure 23. Application of a round filter



A round filter was chosen to differentiate bacteria cocci from non-round background “noise”

Figure 24. The final count



After application of the round filter the final count is displayed.

For each study control discs were included that consisted of discs incubated with sterile PBS and processed as for the discs incubated with bacteria. Control discs were scanned for adherent bacteria and were subjected to image analysis and counting.

Study 1. The effects of time, temperature and bacterial concentration on adhesion

Time study

A set of 5 D-Squame® discs with adherent corneocytes were obtained from the right pinna of 3 dogs and 2 cats. All animals were normal and free of skin disease apart from Cat 2, which had been diagnosed as suffering from atopic dermatitis. Corneocytes were incubated with either *S. intermedius* or *S. hominis* for 15, 30, 45 and 60 minutes. From each disc 10 images were obtained and stored as TIFF files for counting. The number of adherent bacteria was determined using image analysis and the "Bacteria Count" script.

Dog 1. Three-year-old male Greyhound
Dog 2. Ten-year-old female neutered Labrador retriever
Dog 3. Four-year-old male Greyhound
Cat 1. Eight-year-old female neutered domestic shorthair
Cat 2. Ten-year-old male neutered domestic shorthair

Temperature study

Two dogs and one cat were used in this study. All animals were normal with no skin disease. Samples were collected from the right and left ears of all three animals and incubated at 38°C and 4°C as described above. Control samples were incubated with sterile PBS at 38°C and 4°C. Bacteria were counted using the method described above.

Dog 1. Ten-year-old female neutered Labrador retriever
Dog 2. Four-year-old male Cross breed
Cat 1. Three-year-old FN Domestic shorthair

Concentration study

Bacterial suspensions of *S. intermedius* and *S. hominis* were made in sterile PBS over a range of optical densities (570nm) from 0.045 to 1.156 and were incubated at 38°C with corneocytes using the assay technique as above. Two cats and three dogs were used in this study. All animals were normal with no skin disease.

Cat 1. A six-year-old female neutered domestic shorthair
Cat 2. A three-year-old male neutered domestic shorthair
Dog 1. A nine-year-old male Bull terrier
Dog 2. An eleven-year-old female neutered German Shepherd dog
Dog 3. A three-year-old male Greyhound

Study 2. Comparison of visual counting and counting by image analysis

Ten images were randomly selected from each of the following four groups:

1. Cat corneocytes incubated with *S. intermedius*
2. Cat corneocytes incubated with *S. hominis*
3. Dog corneocytes incubated with *S. intermedius*
4. Dog corneocytes incubated with *S. hominis*

Using the image analysis software a centrally placed rectangular counting area 200 x 200 pixels (3265 μm^2) was generated (Appendix J). The numbers of cocci in each counting area, including those at the edge of the counting area, were then calculated by firstly direct visual (manual) counting by two operators using the image analysis program count and tag tool and then by the image analysis program using the Image Tool script “Bacteria Count”.

Study 3. Repeatability study

Twelve D-Squame® discs (six incubated with *S. intermedius* and six incubated with *S. hominis*) were randomly selected from a pool of discs that had been prepared for previous studies. For each D-Squame® disc the operator selected and stored 10 images as TIFF files. The number of bacteria in each image was then calculated using the image analysis program and the script “Bacteria Count”. The same operator repeated this entire procedure (selected different images) a second time after a time interval of at least one week. The operator was blinded as to the identity of individual discs. The two sets of data were then compared to assess repeatability of the counting method.

Study 4. A comparison of corneocytes collected from the ear and groin

Corneocytes were collected from the pinna and groin area in each of four dogs for comparison. In two cats corneocytes were collected from the pinna only. From each site five consecutive D-Squame® discs were obtained. The corneocytes so collected were incubated with either *S. intermedius* or *S. hominis.* A sixth disc was collected from each site and used, as a control disc and incubated with sterile PBS. D-Squame® discs and adhesion assays were performed as detailed above. The operator was blinded as to the identity of individual discs.

Dog 1. Two years and 2 month old male neutered Bull terrier suffering from atopic dermatitis

Dog 2. Three-year-old male neutered West Highland white terrier suffering from atopic dermatitis

Dog 3. Normal four years and 4 months old Labrador retriever

Dog 4. Normal five-year-old Grey hound

Cat 1. Normal three-year-old male neutered Domestic shorthair

Cat 2. Normal six-year-old female neutered Domestic shorthair

Study 5. Comparison of adherence by 5 different staphylococcal species and a *Micrococcus* to canine and feline corneocytes

Five species of staphylococcus and one *Micrococcus* were incubated with canine and feline corneocytes as described above. The species of bacteria used and their origin are detailed above. Five consecutive D-Squame® discs were obtained from the right pinna of five normal cats and dogs. A sixth disc was included as a control. The corneocytes collected from each animal were incubated with each of the five bacteria species and the adherent bacteria counted as described above. The operator was blinded as to the identity of individual discs.

Dog 1. Four-year-old male Greyhound

Dog 2.Three-year-old female neutered Greyhound

Dog 3. Ten-year-old female neutered Labrador retriever

Dog 4. Six-year-old female neutered Cocker spaniel

Dog 5. 4-year-old female neutered Cross breed

Cat 1. Five-year-old male neutered Domestic shorthair

Cat 2. One year and 2 months old female entire Exotic shorthair

Cat 3. Six-year-old female neutered Domestic shorthair

Cat 4. Two years and 3 month old male entire Exotic shorthair

Cat 5. Eleven-month-old female entire Exotic shorthair

STATISTICAL ANALYSIS

Statistical analysis and graphical presentation of data was conducted using computerised statistical packages, GraphPad Prism, version 4 (Graphpad Software Inc. San Diego CA). Comparisons between groups of more than two were conducted using simple one-way analysis of variance (ANOVA), where data was not normally distributed; Kruskal-Wallis ANOVA on ranks was used. To isolate the group or groups that differed from the others a multiple comparison procedure (Dunn's method) was employed. Where two groups were compared the paired t test was used (comparison of ear and groin, table 23). Comparison between two or more groups was also assessed by linear regression and calculation of the correlation coefficient. Bland-Altman plots were used to compare methods for assessing bacteria adherence. A value of $p < 0.05$ was considered to be significant.

RESULTS

Effect on time on adhesion to corneocytes (table 18 and figure 25)

For *S. intermedius* there was a clear increase in the number of adherent cocci with time. In contrast there appeared to be little increase in the numbers of adherent over time shown by *S. hominis*.

Table 18. Effect on time on adhesion to corneocytes (mean values ± standard deviation)

	15 minutes	30 minutes	45 minutes	60 minutes	Control
S. intermedius					
Dog 1	162.80± 36.11	185.20± 24.01	197.60± 32.15	194.20± 37.46	36.20± 22.88
Cat 1	108.30± 21.38	131.30± 43.72	248.90± 42.42	282.00± 48.02	24.80± 22.71
Dog 3	186.40± 34.35	332.20± 51.98	385.80± 91.35	498.50± 26.58	38.70± 25.16
Mean	**152.50**	**216.23**	**277.43**	**324.90**	**33.23**
S. hominis					
Dog 4	47.30± 22.49	55.30± 36.33	53.80± 24.68	56.20± 38.22	28.50± 26.31
Dog 2	45.00± 23.78	51.40± 23.78	50.90± 19.35	48.50± 9.63	8.80± 7.63
Mean	**46.15**	**53.35**	**52.35**	**52.35**	**16.97**

Figure 25. Effect of time on bacterial adhesion

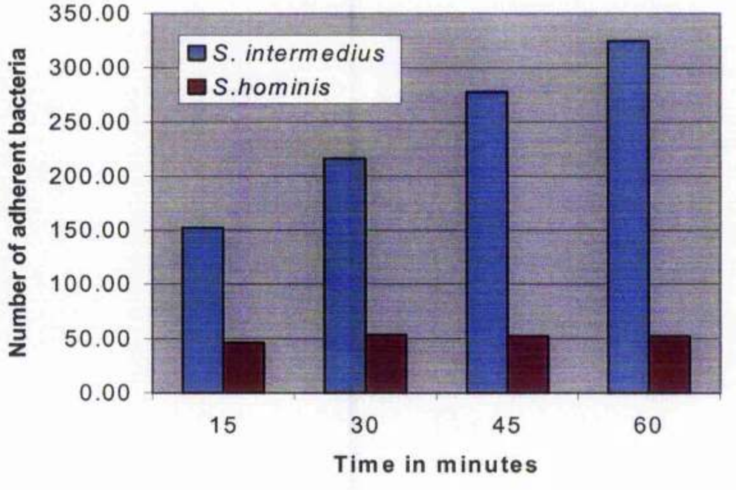
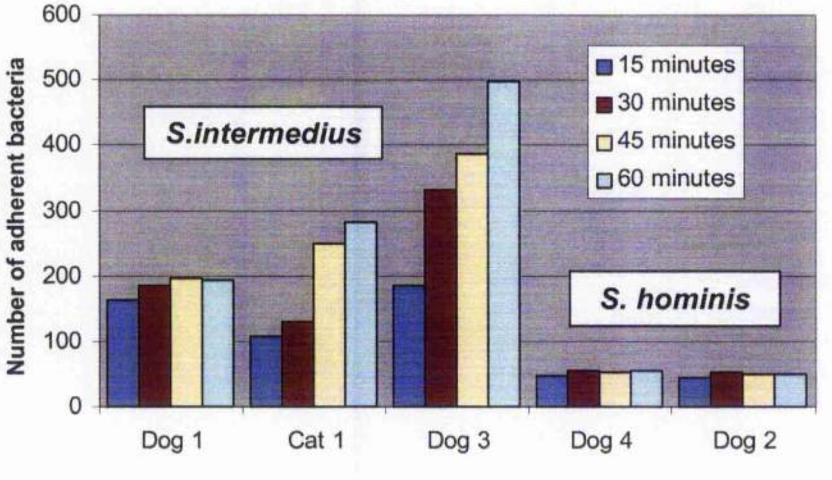


Figure 26. Effect of time on bacterial adhesion – individual animals



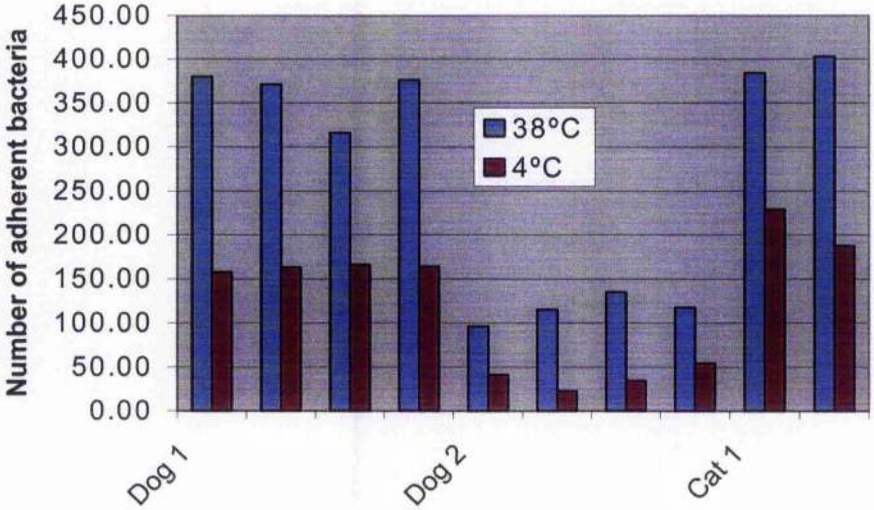
Comparison of adherence at 38°C and 4°C (table 19 and figure 27) (mean values ± standard deviation)

For both *S. intermedius* and *S. hominis* there was a clearly increased adherence at 38°C compared to 4°C.

Table 19. Comparison of adherence at 38°C and 4°C

		Test samples		Control	
		Mean bacteria count ± SD			
		38°C	4°C	38°C	4°C
Dog 1 *S. intermedius*	Right Ear 1	379.90± 71.90	157.50± 30.74	30.30± 13.50	29.10± 24.35
	Right Ear 2	371.00± 33.54	163.10± 26.30		
	Left Ear 1	315.50± 92.77	165.90± 30.37		
	Left Ear 2	376.00± 74.42	164.00± 26.06		
Dog 2 *S. hominis*	Right Ear 1	95.90± 31.48	41.20± 22.44	13.30± 8.97	18.50± 11.64
	Right Ear 2	115.30± 42.35	22.80± 5.94		
	Left Ear 1	135.00± 31.76	34.60± 9.96		
	Left Ear 2	117.30± 30.97	54.20± 16.7		
Cat 1 *S. intermedius*	Right Ear 1	384.00± 61.98	229.20± 52.97	30.80± 24.43	26.00± 23.40
	Right Ear 2	402.70± 53.34	187.60± 44.09		

Figure 27. Histogram of temperature effects



Effects of bacterial concentration on adhesion to corneocytes (table 20, figures 28 and 29)

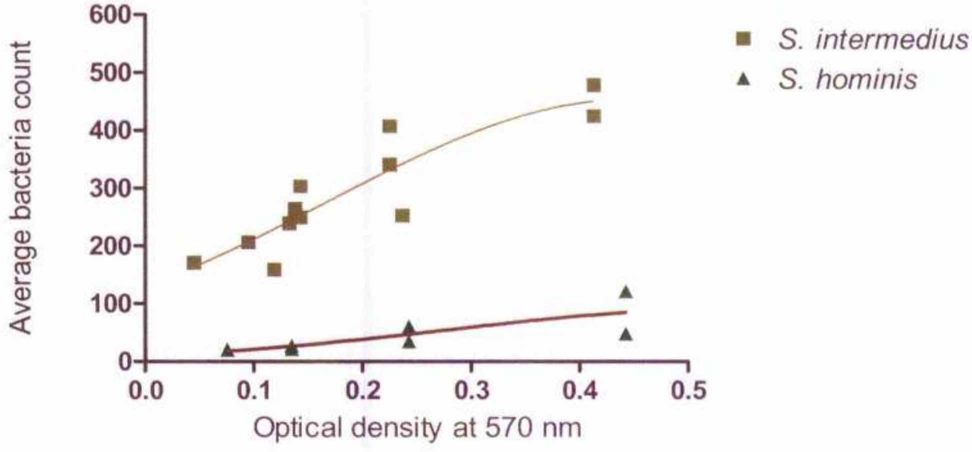
In general bacteria adherence increased with increasing concentration. At higher bacteria concentrations (optical densities above 0.200) clumping of bacteria was commonly encountered. Clumping was rarely encountered at an OD of under 0.200.

Table 20. Effects of bacterial concentration on adhesion

		OD at 570 nm	Mean bacteria count ± SD			
			Count		Control	
Cat 1 R ear	*S. intermedius*	0.698	Clumped		28.70±	26.80
		0.433	Clumped			
		0.321	Clumped			
		0.133	239.30±	51.18		
Cat 2 R ear	*S. intermedius*	0.476	Clumped		54.4±	26.72
		0.303	Clumped			
		0.148	Clumped			
		0.138	264.30±	30.43		
		0.119	158.90±	47.12		
Dog 1 R ear	*S. intermedius*	0.448	Clumped		21.3±	22.47
		0.237	252.60±	42.16		
		0.095	206.40±	51.70		
		0.045	170.50±	47.21		
Dog 2 R ear	*S. intermedius*	1.156	Clumped		24.3±	14.46
		0.810	Clumped			
		0.413	424.00±	56.20		
		0.225	407.20±	47.06		
		0.143	303.40±	53.00		
Dog 2 L ear	*S. intermedius*	0.810	Clumped		36.6±	19.42
		0.413	478.10±	51.61		
		0.225	340.60±	58.45		
		0.143	249.40±	60.70		
Dog 3 R ear	*S. hominis*	0.443	121.00±	37.72	14.6±	9.97
		0.243	60.60±	16.86		
		0.135	27.00±	9.13		
		0.076	20.70±	5.42		
Dog 3 L ear	*S. hominis*	0.443	47.90±	22.08	14.5±	10.12
		0.243	34.70±	14.38		
		0.135	21.00±	7.41		
		0.076	19.50±	4.84		

OD – optical density

Figure 28. Concentration of bacteria and adherence



Comparison of bacterial counts by image analysis and visual counting

Image analysis correlated well with the visual counting by both operator 1 (R= 0.955) and operator 2 (R=0.835). Bland-Altman plots for both operators 1 and 2 when compared to image analysis showed similar results in that discrepancies between the two methods became larger when the bacterial counts were high.

Table 21. Comparison of bacterial counts by image analysis and visual counting

Dog		Image analysis	Operator 1	Operator 2	Cat	Image analysis	Operator 1	Operator 2
S. hominis	1	6	9	8		32	32	40
	2	35	51	55		32	41	53
	3	13	15	16		7	6	10
	4	29	35	32		6	6	6
	5	6	10	11		5	5	9
	6	48	49	60		7	5	6
	7	13	15	17		7	5	6
	8	12	13	13		8	7	8
	9	5	3	3		24	21	20
	10	59	50	66		26	26	33
S. intermedius	1	85	60	63		109	129	204
	2	43	45	59		45	44	60
	3	90	97	111		79	81	101
	4	129	125	131		62	63	76
	5	97	98	88		111	137	180
	6	95	104	108		88	109	123
	7	108	120	112		70	84	119
	8	121	122	115		105	138	197
	9	86	90	89		51	65	73
	10	83	88	102		44	57	77

Figure 30. Comparison of Image analysis counting with manual count

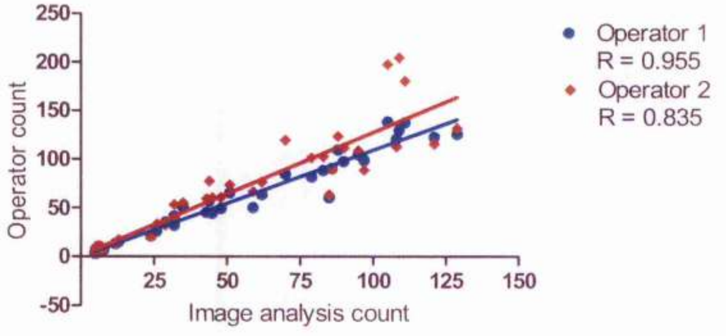


Figure 31. Bland-Altman Image analysis and Operator 1:Difference vs average

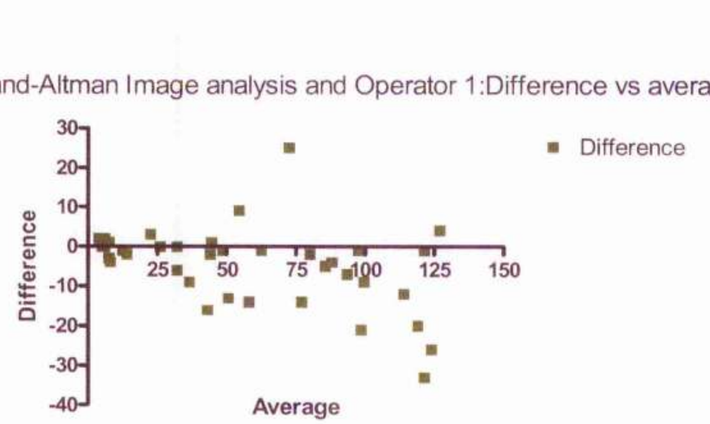
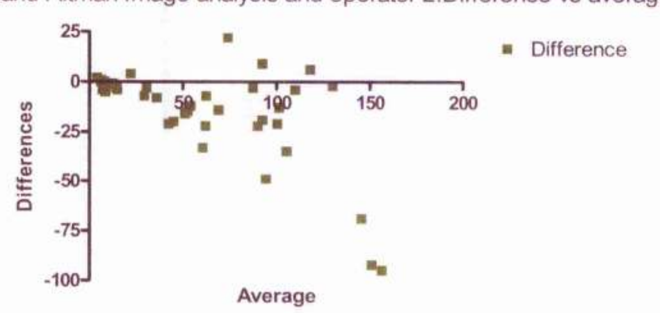


Figure 32. Bland-Altman Image analysis and operator 2:Difference vs average



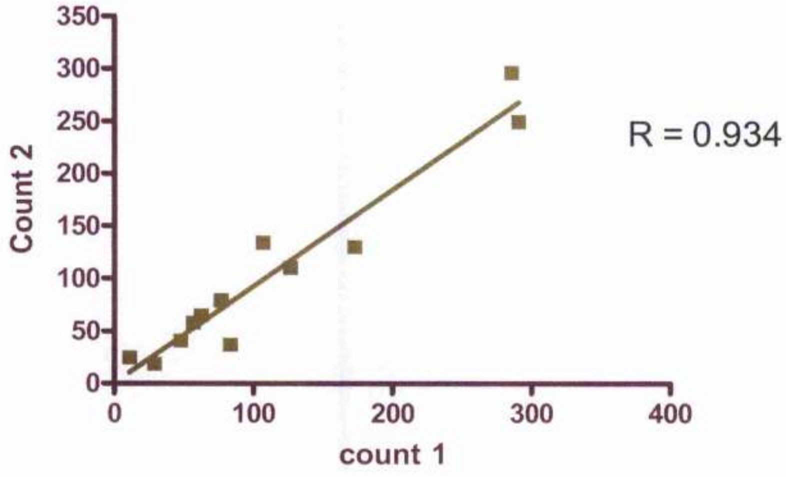
Repeatability of counting method (table 22, figure 33)

There was no significant statistical difference between the results of repeated counts of adherence on two separate occasions (paired t-test p = 0.228). The two counts also showed a high degree of correlation (R= 0.934).

Table 22. Repeatability of counting method

Case	Bacterium used	Count 1	Count 2
Dog 1	*S. intermedius*	290.8	249.3
Dog 2	*S. hominis*	56.7	58.3
Dog 3	*S. intermedius*	285.6	296.2
Dog 4	*S. hominis*	83.5	37.1
Dog 5	*S. intermedius*	126.5	110.2
Dog 6	*S. hominis*	10.9	24.8
Cat 1	*S. intermedius*	107.0	133.9
Cat 2	*S. hominis*	62.5	65.2
Cat 3	*S. intermedius*	76.7	79.4
Cat 4	*S. hominis*	28.7	18.7
Cat 5	*S. intermedius*	173.1	130.1
Cat 6	*S. hominis*	47.6	41.0

Figure 33. Comparison of count 1 and 2



Comparison of ear and groin corneocytes (table 23, figure 34)

In the dog the degree of adherence by both *S. intermedius* and *S. hominis* to corneocytes from the ear and groin were comparable (p = 0.407). The adherence counts for ear and groin were highly correlated (R = 0.976).

Figure 34. Linear regression Ear vs Groin
R = 0.976

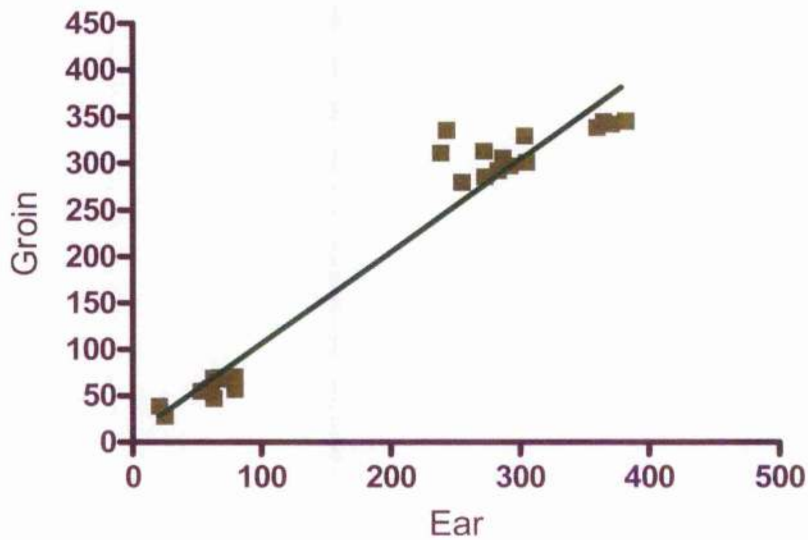


Table 23. Comparison of ear and groin corneocytes

	Disc	Mean ± SD Ear	Groin
Dog 1	1	286.50± 65.83	305.80± 58.50
S. intermedius	2	282.90± 55.02	291.80± 30.10
Right side	3	254.90± 21.87	279.60± 50.90
	4	272.60± 45.70	285.20± 22.86
	5	291.90± 23.61	297.30± 65.90
	Control	20.70± 6.33	38.90± 12.93
Dog 2	1	238.50± 43.71	311.10± 64.15
S. intermedius	2	242.90± 33.68	335.60± 56.73
Right side	3	*304.90± 38.00	301.10± 55.05
	4	272.20± 36.06	312.90± 27.81
	5	303.30± 78.48	329.30± 59.26
	Control	48.10± 11.59	72.90± 13.34
Dog 3	1	381.90± 85.07	345.60± 45.25
S. intermedius	2	382.00± 71.95	344.90± 25.19
Right side	3	359.80± 76.45	338.30± 26.19
	4	364.90± 82.19	344.70± 32.75
	5	370.70± 41.77	342.10± 29.78
	Control	28.60± 22.53	39.00± 26.13
Dog 3	1	60.80± 22.52	57.90± 15.95
S. hominis	2	53.10± 16.49	54.90± 24.91
Left side	3	62.90± 26.54	47.00± 15.16
	4	55.00± 15.71	55.20± 28.69
	5	58.50± 19.30	55.50± 13.28
	Control	25.10± 18.27	25.60± 16.68
Dog 4	1	72.00± 22.72	67.50± 22.53
S. hominis	2	79.10± 26.00	70.20± 32.48
Right side	3	79.20± 37.11	56.50± 24.98
	4	62.70± 21.95	69.00± 33.47
	5	67.50± 17.03	69.60± 17.89
	Control	25.10± 10.81	27.90± 17.19

*Dog2. Statistically significantly larger than 1 and 4 ($p < 0.05$) (one way ANOVA)

Paired t test ear v groin $p=0.407$ (not significant)

Comparison of bacterial adhesion to consecutive sampling from ear (table 24)

There were no statistical differences (ANOVA) between the adherence by *S. intermedius* or *S. hominis* on consecutive samples from the same individual animal. That is there was no within animal differences in the adhesion from each of five consecutive samples.

Table 24. Comparison of bacterial adhesion to consecutive sampling from ear

	Disc	Mean ± SD Ear
Cat 1	1	148.70± 33.26
S. intermedius	2	145.50± 43.62
Right ear	3	140.30± 68.33
	4	144.30± 38.21
	5	146.50± 50.45
	Control	34.20± 17.73
Cat 1	1	56.50± 15.06
S. hominis	2	57.60± 20.06
Left ear	3	50.70± 18.07
	4	52.50± 18.40
	5	52.00± 21.31
	Control	18.00± 8.00
Cat 2	1	184.20± 25.63
S. intermedius	2	181.90± 31.14
Right ear	3	183.70± 36.89
	4	175.30± 30.88
	5	187.80± 35.49
	Control	23.10± 13.52
Cat 2	1	48.00± 18.12
S. hominis	2	46.60± 19.71
Left ear	3	46.30± 24.17
	4	53.70± 17.19
	5	49.20± 27.23
	Control	23.00± 14.08

Adhesion by different bacteria species to canine and feline corneocytes (table 25, figure 35)

S. intermedius, *S. hyicus* and *S. aureus* all adhered well to both canine and feline corneocytes but in general was higher to canine corneocytes. *S. hominis* and a *Micrococcus* sp. Adhered poorly to both canine and feline corneocytes.

Table 25. Adhesion by different bacteria species to canine and feline corneocytes

	S. aureus	*S. hyicus*	*Micrococcus*	*S. hominis*	*S. intermedius*	Control
Cat 1	101.10± 31.64	184.60± 12.84	15.90± 9.69	62.50± 30.23	107.00± 36.20	23.50± 11.82
Cat 2	95.70± 38.86	116.70± 27.18	20.70± 8.39	32.20± 14.99	58.40± 30.21	24.60± 15.26
Cat 3	27.00± 15.06	108.00± 15.63	14.00± 10.62	28.70± 15.85	76.70± 47.78	19.10± 8.31
Cat 4	26.60± 9.88	130.70± 26.84	8.70± 7.12	7.50± 7.63	47.60± 15.89	20.40± 10.27
Cat 5	162.50± 73.42	285.80± 52.32	73.40± 27.06	76.90± 49.68	115.20± 51.36	16.90± 10.06
Mean	**82.58**	**165.16**	**26.54**	**41.56**	**80.98**	**20.90**
Dog 1	107.30 ± 57.02	251.60 ± 34.62	22.00 ± 21.57	35.60± 28.07	122.60± 45.24	21.80± 12.40
Dog 2	116.00 ± 70.33	227.20± 39.80	28.80 ± 14.34	40.10± 24.51	113.80± 32.27	29.70± 12.75
Dog 3	176.60± 38.31	257.00± 51.29	25.80± 18.67	10.90± 8.20	126.50± 94.26	19.20± 9.60
Dog 4	101.00± 48.26	198.00± 79.09	31.70± 17.78	32.70± 16.74	245.40± 45.24	25.10± 13.75
Dog 5	35.30± 25.21	166.50± 81.36	5.70± 4.30	16.80± 13.34	105.80± 63.30	22.20± 12.59
Mean	**107.24**	**220.06**	**22.80**	**27.22**	**142.82**	**23.6**

Figure 35. Histogram of adherence by different bacteria species to canine and feline corneocytes

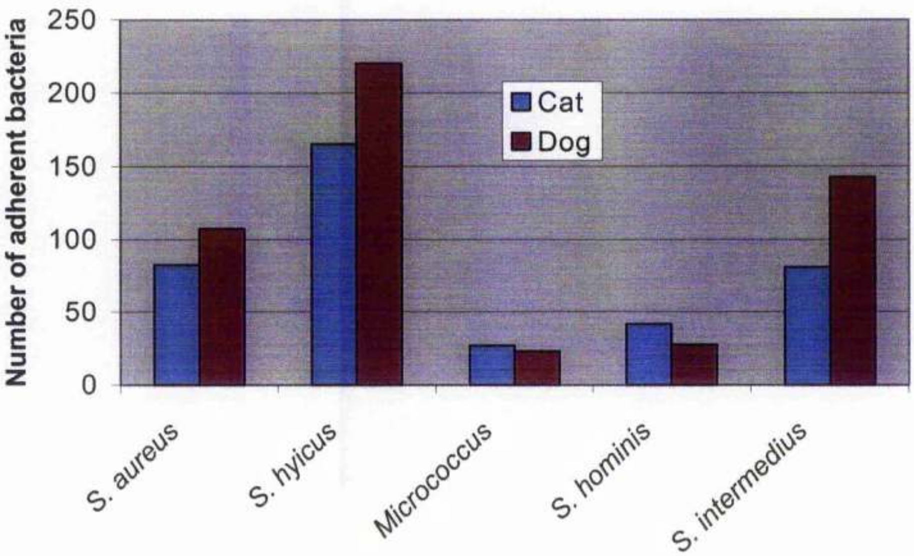
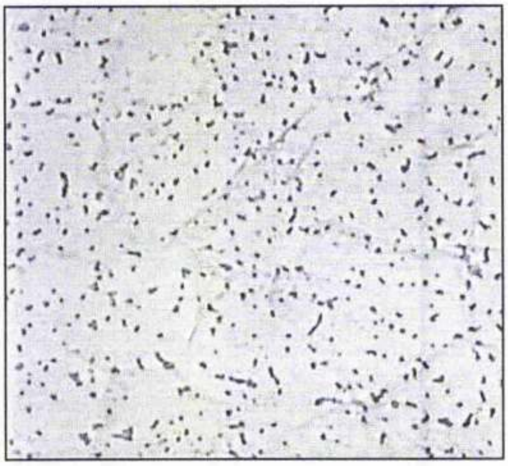
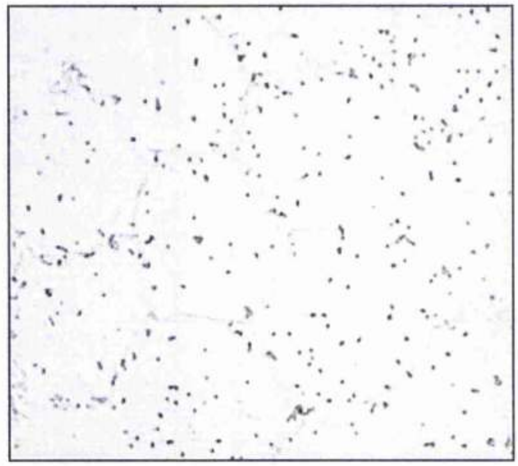


Figure 36. Adherence shown by different staphylococcal species

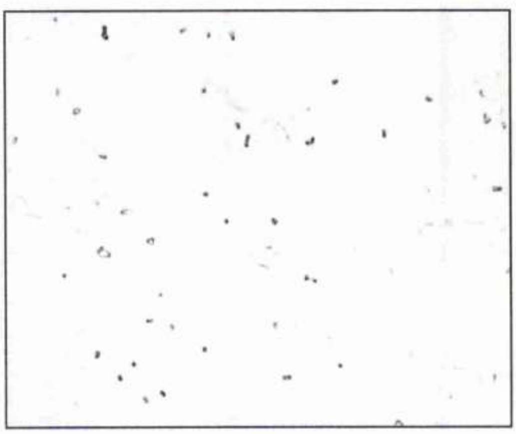
S. intermedius dog corneocytes



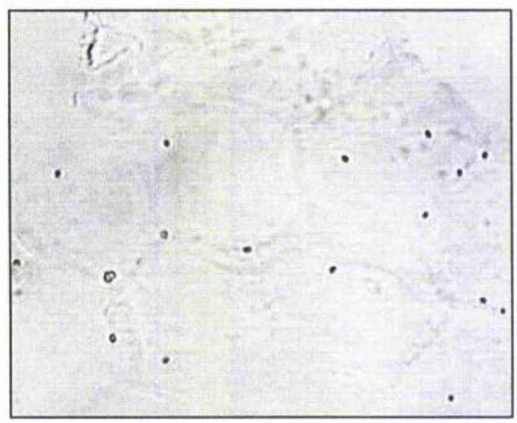
S. intermedius cat corneocytes



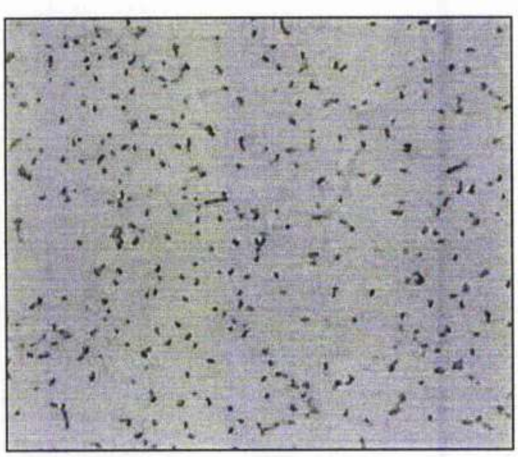
S. hominis dog corneocytes



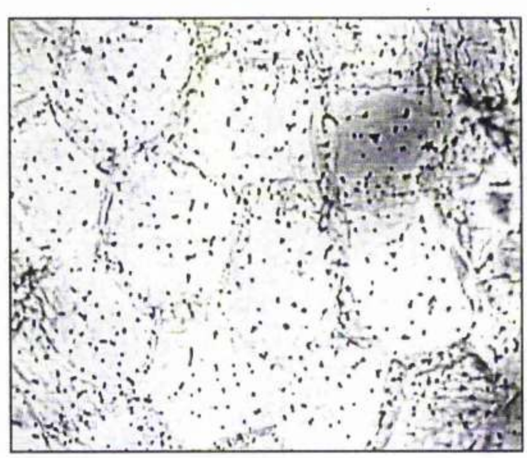
S. hominis cat corneocytes



S. hyicus dog corneocytes



S. hyicus cat corneocytes



DISCUSSION

The findings from this study indicate that the assay developed is a reliable and relatively quick method for the assessment of adhesion by bacteria cocci to both canine and feline corneocytes. The assay was able to differentiate between a known high adhering bacteria strain of *S. intermedius* and known low adhering bacteria stain of *S. hominis*. This is the first study of bacterial adherence to feline corneocytes.

The study on effects of temperature produced results similar to those of previous studies (Forsythe *et al*, 2002; Saijonmaa-Koulumies and Lloyd, 2002) in that bacterial adherence was higher at a higher temperature. The adherence shown by *S. intermedius* was also directly correlated with time, which too is similar to previous studies. The adherence shown by *S. hominis* did not appear to differ greatly over the time interval studied. This may suggest that *S. hominis* does not actively adhere to canine corneocytes and that the adherence shown was passive in nature. The findings of the temperature study in which the degree of adherence was higher at the higher temperature for both does not, however, support a passive mode of adhesion. A larger study of *S. hominis* over a longer period of time would provide information that may help to resolve this contradiction. The concentration study provided valuable information. As expected the higher the concentration of bacteria used the greater was the adherence but problems were encountered with clumping of bacteria at the higher concentrations. In general *S. intermedius* was very likely to clump at concentrations equivalent to an OD_{570nm} of 0.3 to 0.4 or above. Clumping of both *S. intermedius* and *S. hominis* was rare under an OD_{570nm} of 0.2. Clumping of bacteria made counting difficult or impossible when there was severe clumping. Bacterial clumping has been a problem recognised in other studies (Saijonmaa-Koulumies and Lloyd, 2002). It has been noted that staphylococci tend to form clumps in both a strain-dependent and growth-dependent manor (Van Wamel, Vandenbroucke-Grauls, Verhoef and Fluit, 1998; Van Belkum, Kools-Sijmons and Verbrugh, 2002). In the assay used by (Saijonmaa-Koulumies and Lloyd, 2002) visual enumeration of bacteria was used to assess adhesion. Clumping was encountered in this study but because the authors considered that it was not possible to count individual bacteria in a clump each clump was counted as a single unit. The problem of clumping was overcome by Forsythe *et al* (2002) by using image analysis to calculate the surface area of adherent bacteria rather than counting individual bacteria. It would appear, however, that it would be advisable to conduct preliminary studies to identify potential problems with bacterial clumping for any adherence assay being developed. An incubation time of 45 minutes at 38°C and a bacterial concentration equivalent to 0.15 at OD_{570nm} was chosen as being both suitable and practical for the adhesion assay developed.

There was a high degree of correlation between manual (direct visual) counting and counting by image analysis. This was shown for both observers. This does not, however, imply that the results of the two methods gave identical results. Bland-Altman plots are useful to compare two methods of measurement (Bland and Altman, 1986; Bland and Altman, 1999). The Bland-Altman plots obtained in this study indicate that both observers tended to produce higher counts than the results obtained by image analysis. There was a tendency for this discrepancy to become wider as the number of adherent bacteria increased. Both observers were able to recognise small bacterial clumps of 2-4 cocci and count these as individual bacteria while the image analysis program would either exclude these or count them as a single count. The two observers did not give identical results with again a tendency for greater discrepancy when counting large numbers of adherent bacteria. Observer 1 tended to count consistently lower than observer 2 which may suggest that there might be some subjectivity in assessing the numbers of adherent bacteria. More constant results may be obtained between observers with practice.

Since the images collected for analysis were individually selected by the operator there was the opportunity for variation here. The study comparing repeat analysis of the image selection and counting technique using image analysis showed that the method was repeatable.

Each control disc was visually scanned for the presence of bacteria and in all discs so examined no bacteria were seen. Control discs when counted using image analysis tended to give a count of approximately 20-30. After processing for image analysis and thresholding portions of the image that represented folds and edges of the corneocyte were visible and some of these were counted by the software program. Corneocytes and adherent bacteria were examined under a x 63 dry objective and the discs were not mounted. Mounting the discs in various mountants would largely abolish visible corneocyte edges and folds but had two disadvantages over the images obtained from unmounted discs. Firstly all mountants used resulted in the adhesive on the D-Squame® discs dissolving so that corneocytes and adherent bacteria tended to float loose in the preparation. Secondly when a mountant was used the image was cleared of corneocyte folds and edges but this made it impossible to identify clearly individual corneocytes. Using no mountant ensured that the distribution of bacteria adherent to corneocytes could be assessed.

Forsythe *et al* (2002) did find significant differences in the adhesion by *S. intermedius* to corneocytes collected from different body sites but this was not correlated to predilections sites for canine pyoderma. In this study both *S intermedius* and *S. hominis* appeared to adhere equally as well or poorly to corneocytes from the ear and groin of dogs. Superficial pyoderma is rarely encountered on the inner aspect of the canine ear but is common in the inner thigh and groin region. This study suggests that in the dogs studied adherence to the ear corneocyte samples reflects adherence to groin corneocytes. Apart from a single instance there were no statistical differences between the numbers of adherent bacteria from each of the five discs collected from a single site of either the ear (cat and dog) or the groin (dog). This suggests that up to five discs from the one site may be used in comparative studies into potential factors that may affect adhesion. In cats comparison between the ear and groin was not conducted, as it was difficult to obtain suitable corneocyte samples from haired body sites such as the groin.

The final study compared the adhesion by five different staphylococcal species and a *Micrococcus* species. Pathogenic staphylococci (*S. intermedius, S. aureus* and *S. hyicus*) adhered well to both canine and feline corneocytes. The non-pathogenic *S. hominis* and the *Micrococcus* species tested did not adhere well. The strain of *S. intermedius* and *S. hominis* had been shown to adhere well and poorly respectively to canine corneocytes in a previous study (McEwan, 2002a). The adhesion assay used by McEwan (2002c) employed incubating corneocytes and bacteria in a suspension of PBS. Both studies using the same strains of *S. intermedius* and *S. hominis* but different methods produced similar results suggesting that both assays accurately assessed adhesion. Saijonmaa-Koulumies and Lloyd (2002) found two strains of *S. aureus* to adhere poorly to canine corneocytes while Forsythe *et al* (2002) using a single strain of *S. aureus* also found poor adherence to canine corneocytes. The *S. aureus* strains used by both Forsythe *et al* (2002) and Saijonmaa-Koulumies and Lloyd (2002) were of human origin while the strain used in the study described here was of bovine origin. Strain variation for adherence is recognised for *S. aureus* (Wyatt, Poston and Noble, 1990; Peacock, Day, Thomas, Berendt and Foster, 2000). Strain variation may be one explanation for the different findings from different studies. Other factors may be the adherence assay methods used including the concentrations of bacteria, growth stage of the bacteria and possibly the media on which test bacteria were grown. The high adherence shown by *S. hyicus* to both dog and cat corneocytes is interesting. Since *S. hyicus* is not commonly recognised as a pathogen in the dog or cat this finding may suggest that the ability to adhere does not play a role in the development of infection or that it is but one factor in many that contribute to infection. Again larger studies using several strains are needed to clarify this situation.

In its present form the assay and counting procedure is a simple and reliable method for the assessment adhesion by cocci to canine and feline corneocytes. The inner aspect of the ear is a convenient sampling site that is tolerated well by both dogs and cats. Multiple samples from the same site can be obtained and used in studies to compare variables. Image analysis proved a useful and time saving method for bacteria counting. Further improvements in counting may be achievable by the use of more sophisticated software and through additional automation of the image manipulation and processing.

SUMMARY

The main findings of the various studies in this thesis are as follows:

- The three year retrospective survey of skin disease at the UGVS showed 62% of canine patients suffered from pyoderma, which was associated with atopic dermatitis, however, pyoderma was rarely diagnosed in the cat.
- The corneocyte morphometric studies appear to be the first study of its kind in the dog and cat.
- Two morphological types of corneocytes were identified. One a deeply staining elongated cell than probably arises from the hair follicle and a second mostly hexagonal cell that stained poorly.
- The mean canine and feline corneocyte diameter and surface area were found to be 38-48 μm and 1100-1800 μm^2.
- According to studies, corneocytes collected by D-squame® discs from pinna area of dogs and cats proved to be suitable for the collection of corneocytes for use in bacterial adhesion assays.
- Image analysis simplified more traditional methods for the counting of bacteria adherent to cells.
- *S. intermedius, S. hyicus* and *S. aureus* all adhered well to both canine and feline corneocytes while *S. hominis* and a *Micrococcus* sp. adhered poorly.

The studies conducted here provide a good starting point for several future studies. Larger studies could be usefully conducted to determine if there are truly differences in corneocytes from different body areas and animal breeds. The adherence assay opens the way to process large numbers of samples. Of particular interest here are identifying differences in strains of bacteria and methods for the blocking of adhesion by *S. intermedius* to canine skin.

Appendix A. Dog breeds examined for investigation of skin disease at the UGVS 2000-2002

Breed	Number	Breed	Number
Airedale terrier	2	Leonberger	1
Akita	4	Lhasa Apso	1
Alaskan Malamute	1	Lurcher	1
Basset hound	2	Maltese terrier	3
Beagle	4	Mastiff Neopolitan	1
Bearded collie	2	Miniature Poodle	1
Bernese Mountain Dog	1	Miniature Schnauzer	4
Bichon Frise	3	Munsterlander	1
Border Collie	9	Newfoundland	1
Border terrier	3	Old English sheepdog	1
Boxer	17	Papillon	1
Bull terrier	4	Pekinese	2
Bulldog	4	Pomeranian	2
Bullmastiff	2	Rhodesian ridgeback	2
Cairn terrier	1	Rottweiler	6
Cavalier King Charles spaniel	6	Rough collie	4
Chow Chow	2	Saint Bernard	1
Clumber spaniel	1	Saint Bernard	2
Cocker spaniel	11	Samoyed	1
Collie	1	Schnauzer	1
Dachshund (Short Hair)	1	Scottish terrier	1
Dalmatian	1	Shar Pei	5
Deerhound	1	Shetland sheepdog	5
Dobermann	6	Shih Tzu	2
Elkhound	1	Spinone	1
English setter	5	Spitz	1
English Springer spaniel	5	Staffordshire bull terrier	8
Field spaniel	2	Tibetan spaniel	1
Flat coated retriever	2	Tibetan terrier	3
French bulldog	1	Weimeraner	3
German Shepherd	34	White Highland White terrier	22
German Short Haired pointer	2	Wire Haired Fox terrier	1
German Wired Haired pointer	1	Yorkshire terrier	6
Golden retriever	9	X	15
Greyhound	3	Collie X	11
Husky	1	German Shepherd X	2
Irish setter	1	Jack Russell terrier X	1
Jack Russell terrier	4	Labrador X	5
Labrador retriever	34	Rottweiler X	1
Lakeland terrier	2	Terrier X	3

Appendix B. Main diagnosis of skin disease seen in dogs referred to the UGVS 2000-2002

Case	Breed	Sex	Age in years	Main diagnosis
141811	Border collie	M	5.00	Acral lick dermatitis
141894	German Shepherd dog	M	4.42	Acral lick dermatitis
141068	Labrador retriever	FN	2.00	Acral lick dermatitis
140514	Labrador retriever	M	6.58	Acral lick dermatitis
123121	Saint Bernard	M	2.50	Acral lick dermatitis
142082	Beared collie	M	7.42	Allergic dermatosis
144692	Bichon Frise	MN	10.00	Allergic dermatosis
145727	Boxer	M	0.67	Allergic dermatosis
142695	Boxer	F	2.50	Allergic dermatosis
143376	Cavalier King Charles spaniel	M	2.00	Allergic dermatosis
140543	Cocker spaniel	FN	7.58	Allergic dermatosis
140680	Dobermann	M	2.00	Allergic dermatosis
141369	English setter	M	0.92	Allergic dermatosis
200352	German Shepherd dog	FN	1.67	Allergic dermatosis
142007	German Shepherd dog	M	5.00	Allergic dermatosis
144732	German Shepherd dog	M	5.00	Allergic dermatosis
139570	Golden retriever	M	8.00	Allergic dermatosis
138744	Labrador retriever	FN	3.00	Allergic dermatosis
144010	Labrador retriever	M	5.67	Allergic dermatosis
144326	Labrador retriever	M	1.50	Allergic dermatosis
143415	Labrador retriever	M	8.00	Allergic dermatosis
139752	Labrador X	FN	5.00	Allergic dermatosis
139142	Schnauzer	F	4.00	Allergic dermatosis
145596	Shar pei	M	2.00	Allergic dermatosis
145663	Shar pei	MN	2.83	Allergic dermatosis
144161	White Highland White terrier	FN	1.83	Allergic dermatosis
141708	White Highland White terrier	M	1.00	Allergic dermatosis
140684	X	MN	6.00	Allergic dermatosis
200863	X	M	4.00	Allergic dermatosis
139000	Alaskan Malamute	M	3.00	Alopecia X
141097	Pomeranian	M	2.92	Alopecia X
139783	Pomeranian	MN	6.00	Alopecia X
145646	Lhasa Apso	MN	6.25	Arnold Chiari syndrome
142531	Airedale terrier	FN	3.17	Atopic dermatitis
145469	Airedale terrier	MN	2.42	Atopic dermatitis
144227	Beagle	FN	8.00	Atopic dermatitis
140972	Bernese Mountain Dog	M	4.50	Atopic dermatitis
200253	Bichon Frise	M	1.00	Atopic dermatitis
139314	Bichon Frise	FN	4.00	Atopic dermatitis
143973	Border collie	M	0.67	Atopic dermatitis
139424	Border collie	M	2.00	Atopic dermatitis

Case	Breed	Sex	Age in years	Main diagnosis
200350	Border collie	F	5.00	Atopic dermatitis
139841	Border terrier	FN	2.83	Atopic dermatitis
139674	Border terrier	MN	6.00	Atopic dermatitis
142207	Boxer	M	1.00	Atopic dermatitis
140336	Boxer	M	1.08	Atopic dermatitis
140790	Boxer	FN	1.42	Atopic dermatitis
139574	Boxer	M	1.67	Atopic dermatitis
141011	Boxer	M	2.00	Atopic dermatitis
138946	Boxer	M	2.00	Atopic dermatitis
200326	Boxer	MN	3.17	Atopic dermatitis
141004	Boxer	F	3.25	Atopic dermatitis
139987	Boxer	FN	3.58	Atopic dermatitis
144566	Boxer	M	4.50	Atopic dermatitis
141624	Boxer	FN	5.00	Atopic dermatitis
140879	Boxer	M	0.67	Atopic dermatitis
140122	Bull terrier	M	2.92	Atopic dermatitis
141634	Bull terrier	M	5.00	Atopic dermatitis
139837	Bull terrier	FN	6.17	Atopic dermatitis
144127	Bull terrier	M	7.58	Atopic dermatitis
145819	Bulldog	F	1.00	Atopic dermatitis
140353	Bulldog	F	1.00	Atopic dermatitis
143816	Bulldog	M	2.50	Atopic dermatitis
144520	Cairn terrier	MN	7.00	Atopic dermatitis
144282	Cavalier King Charles spaniel	M	3.00	Atopic dermatitis
145695	Cavalier King Charles spaniel	M	5.00	Atopic dermatitis
143746	Chow Chow	M	5.67	Atopic dermatitis
200250	Clumber spaniel	F	6.33	Atopic dermatitis
142191	Cocker spaniel	MN	3.00	Atopic dermatitis
143910	Cocker spaniel	F	3.00	Atopic dermatitis
140194	Cocker spaniel	M	3.00	Atopic dermatitis
141042	Cocker spaniel	F	4.00	Atopic dermatitis
143431	Cocker spaniel	RN	4.58	Atopic dermatitis
140073	Cocker spaniel	M	7.58	Atopic dermatitis
141977	collie X	FN	0.58	Atopic dermatitis
144909	collie X	M	4.08	Atopic dermatitis
139678	collie X	M	7.00	Atopic dermatitis
200473	collie X	FN	9.58	Atopic dermatitis
139006	Dobermann	M	1.50	Atopic dermatitis
140799	Dobermann	FN	2.00	Atopic dermatitis
145169	Dobermann	MN	2.00	Atopic dermatitis
141769	English setter	M	1.58	Atopic dermatitis
140278	English setter	MN	2.50	Atopic dermatitis
144338	English setter	FN	4.00	Atopic dermatitis
138856	English setter	FN	4.00	Atopic dermatitis

Case	Breed	Sex	Age in years	Main diagnosis
141369	English Springer spaniel	M	2.00	Atopic dermatitis
138793	Field spaniel	FN	7.00	Atopic dermatitis
141751	Flat coated retriever	MN	4.25	Atopic dermatitis
140032	Flat coated retriever	M	7.00	Atopic dermatitis
145831	French bulldog	M	1.00	Atopic dermatitis
143167	German Shepherd dog	M	1.75	Atopic dermatitis
141542	German Shepherd dog	FN	2.00	Atopic dermatitis
139872	German Shepherd dog	M	2.00	Atopic dermatitis
145094	German Shepherd dog	M	2.50	Atopic dermatitis
139658	German Shepherd dog	M	3.42	Atopic dermatitis
142089	German Shepherd dog	FN	4.00	Atopic dermatitis
141268	German Shepherd dog	MN	4.42	Atopic dermatitis
143991	German Shepherd dog	M	5.67	Atopic dermatitis
140929	German Shepherd dog	FN	6.00	Atopic dermatitis
145613	German Shepherd dog	FN	6.00	Atopic dermatitis
140862	German Shepherd dog	FN	7.00	Atopic dermatitis
200528	German Shepherd dog	M	7.75	Atopic dermatitis
142744	German Shepherd dog	M	7.92	Atopic dermatitis
125279	German Shepherd dog	MN	8.00	Atopic dermatitis
143142	German Shepherd dog	FN	8.00	Atopic dermatitis
145117	German Shepherd dog		8.00	Atopic dermatitis
143215	German Shepherd dog	M	0.75	Atopic dermatitis
200155	German Short Haired Pointer	M	1.00	Atopic dermatitis
143842	German Wired Haired Pointer	FN	2.75	Atopic dermatitis
139920	Golden retriever	M	2.00	Atopic dermatitis
143495	Golden retriever	MN	2.50	Atopic dermatitis
141924	Golden retriever	MN	2.75	Atopic dermatitis
138873	Jack Russell terrier	FN	4.00	Atopic dermatitis
144703	Jack Russell terrier	MN	4.92	Atopic dermatitis
141664	Jack Russell terrier X	MN	8.00	Atopic dermatitis
140711	Jack Russell terrier	MN	4.00	Atopic dermatitis
145450	Jack Russell terrier	M	6.00	Atopic dermatitis
139563	Labrador retriever	FN	2.25	Atopic dermatitis
144588	Labrador retriever	MN	4.25	Atopic dermatitis
145659	Labrador retriever	FN	5.17	Atopic dermatitis
141156	Labrador retriever	M	6.50	Atopic dermatitis
143287	Labrador retriever	M	7.83	Atopic dermatitis
145886	Labrador retriever	M	9.00	Atopic dermatitis
145740	Labrador retriever	M	5.00	Atopic dermatitis
144088	Labrador retriever	M	2.00	Atopic dermatitis
141728	Labrador retriever	M	4.00	Atopic dermatitis
138294	Labrador retriever	M	6.00	Atopic dermatitis
143218	Labrador retriever	M	5.58	Atopic dermatitis
143470	Labrador retriever	FN	1.50	Atopic dermatitis

Case	Breed	Sex	Age in years	Main diagnosis
143679	Labrador retriever	MN	1.00	Atopic dermatitis
141752	Labrador retriever	FN	2.00	Atopic dermatitis
140546	Labrador retriever	M	2.00	Atopic dermatitis
142096	Labrador retriever	FN	4.00	Atopic dermatitis
140761	Labrador retriever	MN	4.00	Atopic dermatitis
141749	Labrador X	M	7.00	Atopic dermatitis
200362	Lurcher	FN	4.00	Atopic dermatitis
142020	Maltese terrier	MN	3.00	Atopic dermatitis
143861	Maltese terrier	F	8.00	Atopic dermatitis
142900	Miniature Schnauzer	FN	2.17	Atopic dermatitis
144537	Miniature Schnauzer	MN	4.00	Atopic dermatitis
126905	Munsterlander	M	2.00	Atopic dermatitis
138788	Labrador retriever	M	1.83	Atopic dermatitis
140894	Labrador retriever	F	2.67	Atopic dermatitis
138912	Labrador retriever	M	4.00	Atopic dermatitis
139978	Labrador retriever	F	5.92	Atopic dermatitis
140503	Labrador X	M	5.00	Atopic dermatitis
140957	Rhodesian Ridgeback	F	1.17	Atopic dermatitis
142529	Rhodesian Ridgeback	FN	2.00	Atopic dermatitis
141731	Rottweiler	F	2.00	Atopic dermatitis
127740	Rottweiler	M	1.42	Atopic dermatitis
127740	Rottweiler	FN	7.00	Atopic dermatitis
143417	Rottweiler X	MN	2.00	Atopic dermatitis
145609	Rough collie	MN	5.50	Atopic dermatitis
142049	Rough collie	M	5.50	Atopic dermatitis
143465	Saint Bernard	F	2.75	Atopic dermatitis
140842	Shar Pei	F	0.58	Atopic dermatitis
139601	Shar pei	F	1.50	Atopic dermatitis
144763	Shetland Sheepdog	FN	3.17	Atopic dermatitis
144416	Shetland sheepdog	M	4.92	Atopic dermatitis
143661	Shetland Sheepdog	FN	7.00	Atopic dermatitis
145259	Shih Tzu	FN	4.00	Atopic dermatitis
142759	Saint Bernard	MN	2.00	Atopic dermatitis
145494	Staffordshire Bull terrier	M	0.92	Atopic dermatitis
145417	Staffordshire Bull terrier	F	1.83	Atopic dermatitis
141416	Staffordshire Bull terrier	F	1.92	Atopic dermatitis
138733	Staffordshire Bull terrier	FN	2.00	Atopic dermatitis
138733	Staffordshire Bull terrier	M	3.50	Atopic dermatitis
141864	Staffordshire Bull terrier	M	4.50	Atopic dermatitis
144071	Terrier	MN	4.00	Atopic dermatitis
200303	Terrier X	FN	1.25	Atopic dermatitis
141235	Terrier X	M	3.42	Atopic dermatitis
139598	Tibetan terrier	FN	2.00	Atopic dermatitis
142090	White Highland White terrier	M	0.67	Atopic dermatitis

Case	Breed	Sex	Age in years	Main diagnosis
143942	White Highland White terrier	F	0.67	Atopic dermatitis
144284	White Highland White terrier	MN	1.00	Atopic dermatitis
141029	White Highland White terrier	FN	1.75	Atopic dermatitis
141134	White Highland White terrier	MN	1.83	Atopic dermatitis
142297	White Highland White terrier	MN	2.17	Atopic dermatitis
144638	White Highland White terrier	M	4.50	Atopic dermatitis
141825	White Highland White terrier	FN	5.00	Atopic dermatitis
200866	White Highland White terrier	MN	5.00	Atopic dermatitis
143606	White Highland White terrier	FN	8.08	Atopic dermatitis
128055	X	M	0.75	Atopic dermatitis
144407	X	MN	1.00	Atopic dermatitis
141908	X	MN	1.58	Atopic dermatitis
139921	X	FN	2.00	Atopic dermatitis
200313	X	FN	2.50	Atopic dermatitis
141414	X	F	4.00	Atopic dermatitis
139750	X	FN	4.00	Atopic dermatitis
140344	X	FN	4.25	Atopic dermatitis
144302	Yorkshire terrier	M	0.83	Atopic dermatitis
145951	Yorkshire terrier	M	1.58	Atopic dermatitis
145487	Yorkshire terrier	FN	4.00	Atopic dermatitis
141015	Yorkshire terrier	FN	4.00	Atopic dermatitis
144981	Yorkshire terrier	FN	2.80	Atopic dermatitis
140347	Basset hound	M	1.00	Atopic dermatitis
145367	Beagle	FN	1.00	Atopic dermatitis
145541	Beagle	FN	7.08	Atopic dermatitis
145869	Bullmastiff	M	1.00	Atopic dermatitis
142404	Bullmastiff	M	1.67	Atopic dermatitis
145477	collie	FN	1.00	Atopic dermatitis
140372	collie X	MN	3.83	Atopic dermatitis
140243	German Shepherd dog	MN	2.00	Atopic dermatitis
144341	Golden Retreiver	M	3.00	Atopic dermatitis
140033	Labrador retriever	M	1.33	Atopic dermatitis
141455	Labrador retriever	M	1.00	Atopic dermatitis
142293	Maltese terrier	MN	3.25	Atopic dermatitis
141132	Mastiff Neopolitan	F	4.00	Atopic dermatitis
139227	Tibetan terrier	FN	2.00	Atopic dermatitis
143938	Tibetan terrier	M	2.00	Atopic dermatitis
141439	White Highland White terrier	FN	1.00	Atopic dermatitis
141547	White Highland White terrier	F	1.00	Atopic dermatitis
139300	White Highland White terrier	MN	3.00	Atopic dermatitis
200203	White Highland White terrier	F	3.00	Atopic dermatitis
145545	White Highland White terrier	MN	4.00	Atopic dermatitis
142794	White Highland White terrier	F	6.00	Atopic dermatitis
140921	collie X	FN	6.00	Atopic dermatitis / FAD

Case	Breed	Sex	Age in years	Main diagnosis
141326	White Highland White terrier	F	0.67	Atopic dermatitis / demodectic mange
140389	German Shepherd dog	FN	2.00	Atopic dermatitis / FAD
142862	German Shepherd dog	M	5.00	Atopic dermatitis / FAD
200251	Labrador retriever	FN	4.00	Atopic dermatitis / FAD
200332	X	FN	9.00	Atopic dermatitis / FAD
142693	Yorkshire terrier	F	1.00	Atopic dermatitis / FAD
140542	Labrador retriever	F	1.00	Atopic dermatitis / food sensitivity
143067	White Highland White terrier	MN	3.00	Atopic dermatitis / food sensitivity
137559	Greyhound	FN	2.00	Bald thigh syndrome
200410	Greyhound	FN	3.00	Bald thigh syndrome
141031	Rottweiler	M	6.58	Calcinosis cutis iatrogenic
144340	Elkhound	MN	6.92	Cheyletiellosis
127238	Cocker spaniel	FN	2.00	Cheyleteillosis / hypothyroidism
139066	Cocker spaniel	M	9.00	Cheyletiellosis
143412	Labrador retriever	M	3.33	Cheyletiellosis
144788	Spitz	MN	5.00	Cheyletiellosis
142766	Old English Sheepdog	FN	3.00	Cheyletiellosis / FAD
144684	German Short Haired Pointer	F	0.42	CLE (cutaneous lupus erythematosis)
143311	German Shepherd dog	FN	7.00	CLE (cutaneous lupus erythematosis)/ DLE
145111	Shetland sheepdog	M	11.00	Cutaneous lymphoma
145535	terrier X	FN	14.00	Cutaneous lymphoma
143304	Shetland sheepdog	FN	5.00	Cutaneous vesicular lupus erythematosus
139038	Rough collie	FN	1.08	Demodectic mange
142533	Staffordshire Bull terrier	F	0.50	Demodectic mange
140871	collie X	M	0.33	Demodectic mange adult
200595	German Shepherd dog X	FN	14.00	Demodectic mange adult
200123	Miniature Poodle	MN	9.00	Demodectic mange adult
144550	Newfoundland	M	4.25	Demodectic mange adult
140499	Pekinese	M	7.08	Demodectic mange adult
144153	Akita	M	0.67	Erythema multiforme / drug eruption
143866	Golden Retriever	FN	7.08	Exfoliative dermatosis / ichthyosis
145022	Border collie	M	0.83	FAD
139136	Border collie	FN	?	FAD
139340	Border collie	MN	1.25	FAD
142709	Cavalier King Charles spaniel	FN	3.00	FAD

Case	Breed	Sex	Age in years	Main diagnosis
144972	Collie X	F	1.00	FAD
138782	Field spaniel	F	6.58	FAD
142428	German Shepherd dog	F	4.00	FAD
142747	Pekinese	FN	10.92	FAD
138869	Samoyed	M	7.08	FAD
140200	Shih Tzu	FN	8.50	FAD
141038	Tibetan spaniel	M	2.00	FAD
143724	White Highland White terrier	M	0.50	FAD
141865	X	FN	4.00	FAD
145766	German Shepherd dog X	MN	6.00	FAD / anal furunculosis
144742	Boxer	MN	3.25	Flank alopecia
139616	Border collie	MN	7.25	Follicular dysplasia
145427	German Shepherd dog	M	2.50	Food sensitivity
144901	Rough collie	M	1.42	Food sensitivity
141590	German Shepherd dog	FN	1.67	Granulomatous dermatitis
140431	Collie X	FN	9.92	Hyperadrenocorticism
140500	Papillon	M	13.00	Hypothyroidism
145046	Lakeland terrier	M	7.83	Iatrogenic cushings
200138	Scottish terrier	M	7.00	Interdigital pyoderma / sterile granuloma
144375	Beagle	FN	8.00	Interdigital pyoderma / sterile granuloma
143936	Bulldog	FN	8.00	Interdigital pyoderma / sterile granuloma
143676	Golden retriever	M	11.50	Keratinsation defect
143938	Weimeraner	M	1.00	Microchip related dermatosis
142184	Bearded collie	MN	4.00	Nail dystrophy - symmetrical lupoid onchodystrophy
145126	Greyhound	M	8.00	Nail dystrophy - symmetrical lupoid onchodystrophy
140457	Boxer	FN	5.00	Nail dystrophy - symmetrical lupoid onchodystrophy
142816	Labrador retriever	MN	8.00	Nail dystrophy - symmetrical lupoid onchodystrophy
139525	German Shepherd dog	FN	4.00	Nodular dermatofibrosis
144082	Border terrier	MN	5.00	Otitis
144732	German Shepherd dog	FN	6.92	Otitis
139740	Lakeland terrier	M	7.75	Otitis
144459	Shar Pei	F	1.50	Otitis

Case	Breed	Sex	Age in years	Main diagnosis
143602	Cavalier King Charles spaniel	FN	13.00	Otitis (pseudomonas)
142899	Dachshund (Short Hair)	F	1.00	Pattern baldness
143703	Akita	FN	2.92	Pemphigus foliaceus
144307	Akita	MN	2.00	Pemphigus foliaceus
145658	Akita	M	4.92	Pemphigus foliaceus
143066	Dalmatian	MN	2.42	Pemphigus foliaceus
142332	German Shepherd dog	MN	7.08	Pemphigus foliaceus
140456	Collie X	M	1.00	Pemphigus vulgaris / bullous pemphigoud
200002	Basset hound	M	1.33	Primary malassezia dermatitis
139283	German Shepherd dog	M	0.67	Primary pyoderma
143587	Border collie	FN	3.00	Primary pyoderma
141885	Boxer	M	2.00	Primary pyoderma
143744	Chow Chow	M	1.00	Primary pyoderma
143290	Deerhound	F	3.08	Primary pyoderma
140764	Dobermann	F	1.25	Primary pyoderma
141464	Dobermann	F	1.25	Primary pyoderma
140281	English Springer spaniel	FN	0.00	Primary pyoderma
142052	German Shepherd dog	F	1.00	Primary pyoderma
142283	German Shepherd dog	M	1.17	Primary pyoderma
145396	Golden retriever	MN	2.00	Primary pyoderma
142196	Irish setter	FN	3.00	Primary pyoderma
125469	Leonberger	M	4.00	Primary pyoderma
138726	Miniature Schnauzer	FN	4.42	Primary pyoderma
139125	Staffordshire Bull terrier	M	1.08	Primary pyoderma
145256	Weimeraner	M	2.00	Primary pyoderma
143372	White Highland White terrier	FN	8.42	Primary pyoderma
145172	X	MN	7.50	Primary pyoderma
145809	X	M	9.00	Primary pyoderma
200017	X	MN	8.00	Primary pyoderma
139371	Cocker spaniel	F	0.92	Primary seborrhoea
140675	Cocker spaniel	FN	11.00	Primary seborrhoea
144018	Labrador retriever	M	3.00	Pyoderma secondary to trauma
143078	English Springer spaniel	M	6.00	Pyotraumatic folliculitis
143536	Golden retriever	F	1.17	Pyotraumatic folliculitis
141765	Rottweiler	M	6.42	Pyotraumatic folliculitis
141065	Rottweiler	M	6.42	Pyotraumatic folliculitis
143590	Collie X	MN	2.50	Sarcoptic mange
143110	English Springer spaniel	M	6.92	Sarcoptic mange
145288	English Springer spaniel	M	13.42	Sarcoptic mange
139800	X	FN	2.00	Sarcoptic mange
139335	Wire haired fox terrier	FN	3.00	Sebaceous hyperplasia

Case	Breed	Sex	Age in years	Main diagnosis
143109	Miniature Schnauzer	M	7.00	Schnauzer comedo syndrome
145885	Spinone	F	7.00	Traumatic wound
145648	Weimeraner	F	5.00	Urticaria
143560	Cavalier King Charles spaniel	FN	12.00	Vulval fold dermatitis
140185	Husky	M	1.25	Zinc responsive dermatosis

Appendix C. Cats referred for investigation of skin disease at the UGVS 2000-2002

Case	Breed	Sex	Age in years	Diagnosis
145224	Domestic short hair	FN	2.92	Allergic dermatosis
139715	Domestic short hair	FN	2.92	Allergic dermatosis
141263	Domestic short hair	FN	2.92	Allergic dermatosis
142194	Domestic short hair	MN	5.00	Allergic dermatosis
140991	Domestic short hair	MN	7.00	Allergic dermatosis
142894	Domestic short hair	FN	7.00	Allergic dermatosis
139688	Domestic short hair	FN	5.00	Atopic dermatitis
139992	Domestic short hair	FN	8.00	Atopic dermatitis
145619	Domestic short hair	FN	3.17	Atopic dermatitis
141705	Domestic short hair	FN	5.00	Atopic dermatitis
142380	Domestic short hair	FN	3.00	Atopic dermatitis
140788	Domestic short hair	MN	7.00	Atopic dermatitis
140897	Domestic short hair	NR	3.00	Atopic dermatitis
142430	Domestic short hair	NR	4.00	Atopic dermatitis
141296	Persian (long hair)	F	2.00	Atopic dermatitis
140911	Siamese	MN	7.00	Atopic dermatitis
143545	Domestic long hair	FN	4.92	Atopic dermatitis / FAD
144905	Domestic short hair	FN	4.50	Atopic dermatitis / FAD
140446	Domestic short hair	MN	13.00	Ceruminous gland adenoma
141200	Domestic short hair	MN	6.00	Chin oedema
144022	Domestic short hair	MN	0.75	Cutaneous asthenia
143114	Domestic short hair	FN	4.00	Eosinophilic plaque/eosinophilic ulcers - idiopathic
141558	Ragdoll	MN	2.75	Facial dermatitis - idiopathic
141422	Domestic short hair	F	10.00	FAD
138942	Domestic short hair	FN	6.42	FAD
139354	Domestic short hair	FN	6.00	FAD
140214	Domestic short hair	FN	11.67	FAD
140907	Domestic short hair	MN	6.00	FAD
144168	Domestic short hair	MN	12.00	FAD
139700	Turkish Van	MN	1.25	FAD
142390	Domestic short hair	FN	1.58	Feline acne / folliculitits
143849	Burmese	MN	0.67	Food sensitivity
143675	Persian (Long Hair)	MN	2.58	Idiopathic facial dermatitis
145816	Persian (Long Hair)	MN	3.00	Idiopathic facial dermatitis
140787	Persian (Long Hair)	MN	2.00	Idiopathic facial dermatitis
143084	Domestic short hair	MN	9.00	Pemphigus foliaceus
143357	Domestic short hair	MN	10.00	Pemphigus foliaceus
145009	Domestic short hair	MN	2.80	Plasma cell pododermatitis
200759	Siamese	MN	3.42	Psychogenic alopecia
143094	Domestic short hair	FN	0.58	Symmetrical alopecia – idiopathic

Appendix D. Normal dogs corneocyte diameter (µm)

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
Ndog 1	37.94	40.05	42.75	40.00	37.93		38.36	39.63	33.69	46.59	35.60		38.54	35.98	46.49	43.50	38.10	
Labrador	34.37	46.26	39.44	38.05	38.90		40.12	39.35	42.01	43.32	45.05		47.75	39.86	41.73	40.50	42.21	
retriever	41.39	31.08	34.94	38.51	44.94		44.09	44.99	41.80	40.66	38.33		37.45	39.81	38.62	43.12	40.40	
10 years	39.28	30.65	38.09	40.99	39.62		39.91	39.43	43.84	41.98	40.11		47.47	35.93	40.45	47.87	35.92	
FN	39.84	34.11	45.72	40.83	37.57		37.61	41.80	38.29	42.22	44.94		42.43	39.68	49.57	46.46	37.80	
	45.04	40.71	46.72	37.52	41.40		41.24	43.38	38.02	36.11	38.66		41.99	33.41	41.37	42.56	35.93	
	40.21	33.11	31.42	39.89	42.58		41.96	37.86	39.51	41.20	38.38		37.79	36.20	45.29	43.61	41.55	
	41.13	40.23	44.74	33.31	40.72		44.61	42.31	41.35	32.64	41.78		40.98	46.05	48.62	39.70	42.47	
	41.18	34.49	38.36	34.78	38.72		42.44	41.84	45.98	38.46	45.61		36.41	40.24	38.64	45.67	42.01	
	39.61	32.05	36.56	36.67	45.37		39.17	40.25	39.47	45.23	41.12		34.66	43.27	40.40	43.20	41.11	
Mean	**40.00**	**35.85**	**39.56**	**37.84**	**41.09**	**38.99**	**40.95**	**41.24**	**41.14**	**40.20**	**41.48**	**40.83**	**40.54**	**39.38**	**42.74**	**43.53**	**39.93**	**41.21**
SD	**2.72**	**5.34**	**5.20**	**2.62**	**2.75**	**4.03**	**2.34**	**2.23**	**2.63**	**3.88**	**2.89**	**3.05**	**4.46**	**3.85**	**4.11**	**2.67**	**2.67**	**3.88**
mode	**40.00**	**34.11**	**38.36**	**37.84**	**40.72**	**39.52**	**40.95**	**41.24**	**41.14**	**40.66**	**41.12**	**41.16**	**40.54**	**39.68**	**41.37**	**43.20**	**40.40**	**41.04**
Ndog 2	45.64	40.52	32.10	29.99	40.82		43.67	49.67	45.61	43.63	46.93		44.52	49.56	49.03	41.31	45.45	
Cocker	39.25	39.45	39.23	34.36	40.58		45.84	46.50	41.51	44.67	46.67		42.98	45.57	42.33	39.14	46.23	
spaniel	39.44	40.78	33.18	38.29	46.07		46.16	41.73	45.46	45.95	47.74		40.49	43.12	42.81	48.06	45.51	
6 years	38.66	35.83	34.39	38.18	43.34		43.17	44.25	44.05	42.50	43.75		39.30	44.16	50.28	42.82	39.40	
FN	37.66	41.20	36.03	37.82	32.78		46.69	41.59	41.10	39.53	40.97		43.67	50.92	47.80	43.08	44.27	
	32.19	40.43	36.87	39.12	43.05		44.86	44.80	45.25	47.33	37.56		37.78	47.09	55.12	44.57	44.97	
	45.98	38.14	44.46	39.26	43.27		53.99	46.67	39.98	43.64	45.08		46.46	44.01	43.75	41.83	40.19	
	39.62	43.82	36.31	34.79	46.73		44.79	46.28	41.92	40.86	45.97		46.63	48.19	50.20	43.30	43.87	
	41.54	39.61	41.67	35.27	42.09		52.61	44.46	44.03	47.29	46.95		47.71	46.16	38.44	40.12	39.38	
	40.05	39.87	37.99	35.44	42.78		44.19	37.99	45.94	41.31	48.42		41.57	48.73	45.43	41.65	41.34	
Mean	**40.00**	**39.90**	**38.01**	**36.95**	**42.30**	**38.16**	**46.60**	**43.81**	**43.25**	**43.70**	**44.79**	**44.83**	**43.11**	**46.43**	**46.24**	**42.73**	**42.79**	**44.40**
SD	**3.93**	**2.18**	**3.50**	**1.96**	**4.04**	**3.86**	**3.71**	**2.88**	**2.16**	**2.84**	**3.53**	**3.18**	**3.32**	**2.54**	**5.10**	**2.60**	**2.72**	**3.68**
mode	**39.44**	**39.87**	**37.99**	**36.95**	**42.78**	**39.35**	**45.84**	**44.25**	**43.25**	**43.70**	**45.08**	**44.79**	**42.98**	**46.16**	**45.43**	**42.73**	**42.79**	**44.08**
Ndog 3	34.17	39.50	35.99	40.08	40.85								50.01	46.06	43.52	45.15	49.95	
German	39.51	38.06	42.38	42.15	39.51								47.27	49.97	48.50	48.84	49.47	
shepherd	44.30	37.03	41.42	29.76	44.30								48.45	48.80	47.61	49.11	42.87	
dog	40.98	37.56	44.73	42.54	40.98								45.65	47.83	49.95	51.12	42.91	
4 years	30.74	38.07	36.65	39.34	30.74								46.38	50.11	49.57	44.60	48.13	
M	35.47	40.29	37.47	35.10	35.47								43.20	45.00	44.61	48.82	44.76	
	41.58	35.71	31.83	39.56	41.58								48.87	49.07	45.19	52.27	44.95	
	44.00	41.12	38.16	45.43	44.00								51.30	46.57	49.56	47.44	48.12	
	44.86	36.18	40.84	40.16	44.85								49.99	35.89	54.20	44.96	46.72	
	41.38	41.67	38.21	42.65	41.38								52.50	46.91	48.09	50.35	44.65	
Mean	**39.70**	**38.41**	**39.07**	**39.63**	**40.31**	**39.41**							**48.41**	**46.68**	**48.59**	**48.63**	**45.84**	**47.53**
SD	**4.75**	**2.14**	**3.78**	**4.69**	**4.60**	**3.86**							**2.86**	**4.38**	**2.83**	**2.56**	**2.38**	**3.13**
mode	**40.98**	**38.06**	**38.21**	**39.63**	**40.98**	**40.12**							**48.41**	**46.91**	**48.50**	**48.82**	**44.95**	**48.11**
Ndog 4	30.96	40.86	39.64	41.85	42.70								45.23	44.97	41.75	46.31	44.81	
Labrador	37.36	35.59	40.15	36.59	46.25								44.96	40.53	42.75	45.54	45.94	
retriever	32.18	41.73	39.55	45.90	45.24								48.34	39.31	41.08	46.96	41.54	
6 years	38.59	39.94	32.97	41.46	36.90								51.08	42.31	41.58	48.90	47.72	
FN	35.19	38.84	39.44	38.77	37.59								39.90	42.02	41.24	42.90	38.70	
	36.00	41.77	38.52	41.39	42.36								49.31	39.93	45.78	39.64	43.49	
	39.49	40.24	35.67	37.64	41.46								38.11	46.27	39.51	44.86	46.47	
	37.54	41.30	39.24	39.51	34.19								47.92	40.85	43.46	50.55	45.81	
	32.54	34.83	34.26	42.93	35.33								41.55	42.93	41.66	40.75	52.49	
	33.75	34.29	36.72	39.90	35.83								42.54	39.48	43.69	40.35	42.71	
Mean	**35.36**	**38.72**	**37.42**	**40.45**	**39.46**	**38.47**							**44.89**	**41.51**	**42.30**	**44.49**	**44.88**	**43.73**
SD	**2.92**	**3.03**	**2.64**	**2.84**	**4.47**	**3.55**							**4.30**	**2.19**	**1.83**	**3.89**	**3.97**	**3.46**
mode	**35.36**	**38.84**	**37.42**	**39.90**	**37.59**	**38.88**							**44.89**	**40.85**	**41.66**	**44.49**	**44.98**	**42.91**

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
Ndog 5	35.13	33.11	27.75	34.93	31.40		27.01	41.40	42.95	41.52	42.64		37.16	40.87	38.38	41.84	37.34	
Greyhound	30.87	31.56	33.38	38.39	31.90		25.33	39.75	44.67	42.84	38.98		37.32	44.41	38.91	42.19	43.39	
3 years	34.40	34.07	27.61	35.95	38.11		41.73	35.56	41.26	56.52	38.81		35.57	52.27	43.18	41.52	35.48	
M	38.77	33.97	35.48	31.09	31.14		41.33	46.51	41.56	42.65	34.55		38.33	36.70	43.01	41.62	35.84	
	40.76	29.47	31.64	34.05	33.44		41.62	39.26	35.99	37.27	49.05		37.86	37.62	39.94	43.62	44.93	
	37.25	31.74	32.90	30.96	32.13		41.42	49.16	34.11	38.27	44.99		38.21	39.00	44.51	41.22	35.04	
	34.24	35.77	30.18	39.57	35.73		37.84	34.13	41.85	46.12	49.42		39.06	41.48	43.29	38.90	38.02	
	33.10	27.64	36.10	40.61	32.34		41.61	42.14	39.01	42.66	40.87		44.64	37.80	42.10	47.92	38.89	
	32.41	31.92	33.51	35.85	32.53		35.20	39.16	39.80	37.64	44.91		42.73	39.48	33.65	42.21	39.28	
	38.19	35.59	32.82	33.51	28.70		41.04	44.09	44.30	39.76	40.58		42.79	41.69	37.89	40.35	43.44	
Mean	**34.91**	**32.41**	**32.64**	**35.66**	**32.89**	**33.57**	**37.41**	**41.08**	**40.28**	**42.68**	**42.46**	**40.82**	**39.36**	**41.16**	**40.72**	**42.17**	**39.37**	**40.46**
SD	**2.73**	**2.72**	**2.55**	**3.52**	**2.70**	**3.06**	**6.31**	**4.91**	**3.51**	**5.96**	**4.98**	**5.18**	**2.96**	**4.82**	**3.47**	**2.52**	**3.74**	**3.48**
mode	**34.40**	**31.92**	**32.82**	**35.66**	**32.34**	**33.41**	**41.04**	**39.75**	**40.28**	**42.66**	**40.87**	**41.37**	**38.33**	**39.48**	**40.72**	**41.62**	**38.89**	**40.14**
Ndog 6							48.00	44.43	38.48	42.22	43.30							
Greyhound							46.31	39.51	48.36	43.45	43.61							
4 years							42.58	42.11	41.13	39.72	35.83							
M							43.59	35.52	43.67	37.61	46.23							
							41.28	36.17	45.03	41.25	42.77							
							47.83	37.79	38.66	46.25	44.91							
							42.81	44.62	47.23	45.86	45.12							
							49.19	43.93	42.50	41.85	41.00							
							40.63	38.74	45.37	48.97	49.29							
							39.02	37.48	44.96	38.72	42.23							
Mean							**44.12**	**39.76**	**44.12**	**42.61**	**43.44**	**42.78**						
SD							**3.49**	**3.11**	**3.00**	**3.81**	**3.76**	**3.59**						
mode							**42.81**	**38.74**	**44.12**	**41.65**	**43.44**	**42.79**						
Ndog 7							42.82	49.43	42.13	43.10	48.57							
Greyhound							38.18	49.50	47.24	39.10	50.41							
5 years							40.74	52.16	39.68	33.87	51.12							
FN							51.99	51.20	46.26	46.28	47.11							
							40.23	52.20	47.19	44.06	46.26							
							39.65	47.36	42.11	42.38	48.10							
							52.20	46.42	44.72	40.57	42.65							
							51.28	42.00	38.72	47.59	40.22							
							45.19	40.58	38.83	48.53	43.37							
							52.68	43.15	43.30	46.98	43.19							
Mean							**34.91**	**32.41**	**32.64**	**35.66**	**32.89**	**33.57**						
SD							**2.73**	**2.72**	**2.55**	**3.52**	**2.70**	**3.06**						
mode							**34.40**	**31.92**	**32.82**	**35.66**	**32.34**	**33.41**						

Appendix E. Normal dogs corneocyte surface area (μm^2)

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
Ndog 1	1311.76	1214.93	881.21	1320.13	1022.07		913.09	1374.92	1472.34	1481.91	925.64		1190.83	1272.51	1352.80	1368.14	1317.14	
Labrador	809.89	1472.74	1052.76	1136.84	1135.84		1322.92	1093.40	1269.52	1212.74	1243.22		1055.15	1671.98	1279.68	1501.43	1532.91	
retriever	1281.88	1074.87	1322.72	1155.36	1330.29		1190.63	830.81	1302.40	1419.94	1384.88		1463.38	1003.74	1310.96	1118.60	1188.63	
10 years	1093.80	898.55	1095.99	1208.16	1047.58		1246.01	1116.91	1063.91	1548.45	986.21		1473.34	1391.45	1268.33	1184.85	1400.02	
FN	1351.61	920.26	1161.14	723.02	1024.07		1390.43	998.17	1210.95	1141.61	1519.96		1280.48	1472.14	1571.96	1306.98	1223.10	
	1351.21	1174.29	1168.11	1318.53	1031.84		1259.56	874.84	1234.88	1190.03	1292.63		1682.34	1034.63	1207.96	1530.52	1208.56	
	1529.92	1042.20	1361.77	1131.45	1267.13		1320.29	913.29	1276.70	1337.06	1501.63		1427.71	1370.93	1207.96	1327.90	1282.67	
	1178.07	961.63	1306.18	1109.74	1286.86		1012.71	1544.07	1338.66	1405.20	1387.07		1523.35	1263.15	1114.32	917.67	1286.66	
	1216.33	1389.86	1085.03	1007.33	1243.42		1139.02	1383.48	1357.58	1283.87	1317.34		1252.59	1183.25	1447.24	1425.52	1106.94	
	1175.88	680.98	1231.07	1170.70	1220.91		1703.48	1130.46	940.79	1339.85	960.71		1292.24	1420.54	1400.22	1322.52	1166.51	
Mean	**1230.14**	**1085.03**	**1166.60**	**1128.03**	**1161.00**	**1154.16**	**1251.51**	**1126.04**	**1246.77**	**1336.07**	**1251.93**	**1242.46**	**1364.14**	**1308.43**	**1316.14**	**1300.40**	**1271.71**	**1312.17**
SD	**191.36**	**237.09**	**146.13**	**170.36**	**122.19**	**177.14**	**217.31**	**239.45**	**150.78**	**131.35**	**220.61**	**200.20**	**183.34**	**202.53**	**132.84**	**184.94**	**123.60**	**164.19**
mode	**1223.23**	**1058.54**	**1163.87**	**1133.65**	**1148.42**	**1169.41**	**1248.70**	**1105.16**	**1258.15**	**1336.56**	**1272.28**	**1273.11**	**1328.19**	**1290.47**	**1295.32**	**1314.75**	**1247.41**	**1299.61**
													1660.62	1576.94	1591.09	1708.24	1677.56	
Ndog 2	1101.37	1246.21	990.59	1171.50	1420.74		1489.68	1737.33	1670.78	1503.22	1403.01		1727.56	1806.46	1538.69	1911.46	1704.05	
Cocker	1268.13	1211.15	890.78	1241.23	1286.06		1548.85	1595.67	1577.54	1566.58	1485.09		1779.76	1616.79	1660.82	1841.33	1506.01	
spaniel	1275.70	1078.46	1111.13	1086.23	1523.15		1518.17	1476.13	1665.60	1618.38	1567.78		1643.88	2015.26	1881.17	1863.24	1440.66	
6 years	1248.20	919.07	663.65	1189.83	1170.50		1756.65	1314.15	1483.98	1383.29	1519.96		1203.78	1613.40	1571.76	1985.37	1463.98	
FN	1264.07	1353.40	1076.86	1364.76	1091.61		1691.10	1911.06	1390.66	1568.37	1417.35		2099.33	1479.91	1788.73	1844.71	1750.28	
	957.72	1340.05	1059.53	1236.05	1151.97		1583.72	1361.37	1399.03	1120.69	1636.11		2010.48	1368.14	1422.14	1737.72	1624.36	
	1201.78	1224.30	1146.79	1069.89	1333.88		1587.70	1453.02	1200.59	1400.82	1563.00		1909.67	1174.49	1875.40	1745.49	1800.28	
	1222.10	1260.56	1312.56	760.40	1173.09		1743.30	1335.47	1444.25	1565.98	1237.84		1526.93	1912.45	1339.25	1580.93	1266.73	
	1322.72	1432.70	863.08	885.20	1418.55		1649.46	1690.51	1545.46	1277.49	1571.96		1658.43	1848.30	1636.91	1724.18	1792.11	
	1066.10	1278.49	1067.30	795.54	1039.01		1610.41	1444.45	1550.04	1305.19	1655.04		**1721.94**	**1641.21**	**1630.60**	**1794.27**	**1602.62**	**1673.13**
Mean	**1194.79**	**1234.44**	**1018.23**	**1082.06**	**1260.86**	**1158.07**	**1617.90**	**1531.92**	**1490.79**	**1431.00**	**1505.71**	**1515.47**	**254.80**	**259.83**	**179.65**	**116.99**	**176.60**	**208.67**
SD	**116.14**	**145.99**	**178.31**	**200.16**	**160.13**	**181.91**	**91.06**	**196.43**	**142.41**	**161.48**	**125.42**	**154.30**	**1691.28**	**1615.10**	**1610.84**	**1769.88**	**1613.49**	**1690.81**
mode	**1211.94**	**1248.32**	**1038.88**	**1084.15**	**1216.97**	**1181.46**	**1599.06**	**1464.58**	**1477.39**	**1415.91**	**1512.84**	**1547.16**						
Ndog 3	1090.01	1082.84	1082.24	1136.04	1145.40								1296.82	1583.52	1455.68	1390.26	1465.17	
German	1284.66	1246.81	1258.37	1075.27	1112.92								1452.02	1509.00	1242.63	1249.20	1282.07	
shepherd	1393.05	913.49	722.23	862.29	806.50								1340.25	1535.90	1206.76	1140.22	1531.52	
dog	1325.51	1376.51	1325.11	1005.74	840.57								1603.64	1306.18	2085.99	1759.24	1424.33	
4 years	1325.31	1275.10	1038.61	1208.16	782.79								1570.37	1360.77	1454.01	1489.88	1467.96	
M	1378.11	1225.49	1115.32	1103.76	1032.04								1536.50	1650.46	1375.32	1938.95	1783.15	
	1483.10	1049.37	1240.43	1089.41	1061.62								1520.96	1602.24	1623.76	1324.31	1326.11	
	1588.90	1199.39	1269.72	1330.89	1179.47								1490.51	1653.05	1650.66	1359.38	1252.99	
	1384.48	1187.04	1109.14	1213.94	1022.67								1684.33	1479.12	1447.84	1277.69	1370.73	
	1308.57	966.29	1057.14	1123.80	730.79								1088.22	1539.88	1464.57	1379.90	1428.11	
Mean	**1356.17**	**1152.23**	**1121.83**	**1114.94**	**971.47**	**1143.33**							**1457.36**	**1522.01**	**1498.72**	**1430.90**	**1433.21**	**1468.44**
SD	**130.40**	**145.34**	**172.71**	**126.38**	**165.26**	**189.68**							**173.94**	**114.98**	**249.31**	**242.92**	**150.98**	**189.12**
mode	**1340.84**	**1169.64**	**1112.23**	**1109.35**	**997.07**	**1129.96**							**1468.84**	**1528.96**	**1450.93**	**1369.64**	**1426.22**	**1459.29**
Ndog 4	1000.76	1125.68	1495.26	1576.34	1146.79								1648.07	1410.18	1078.06	1737.72	1479.32	
Labrador	1070.29	705.29	1109.39	1131.25	1131.05								1352.21	1385.68	1200.79	1920.42	1410.18	
retriever	785.58	1401.42	1221.51	1299.21	1071.68								1270.32	1393.65	1316.74	1997.92	1302.20	
6 years	1175.88	1379.30	1124.08	1471.35	984.02								1376.71	1258.37	1243.02	1975.81	1697.28	
FN	916.28	1276.30	1353.20	1244.42	1253.78								1249.60	1367.75	1276.70	1105.75	1286.46	
	993.59	1237.84	1201.78	1223.90	1185.84								1527.13	1387.07	1234.46	1304.79	1409.78	
	1001.55	1257.97	1396.63	1181.26	1173.09								1323.12	1035.42	1231.67	1400.59	1590.89	
	899.94	1126.27	1229.28	1399.82	936.00								1440.27	1456.61	1506.21	1669.78	1816.41	
	924.85	1198.99	982.82	1118.30	1032.43								1351.81	1546.66	1406.80	1391.65	1617.59	
	902.73	1210.15	1012.51	1233.26	1193.42								1516.97	1201.78	1504.62	1522.56	1481.51	
Mean	**967.14**	**1191.92**	**1221.67**	**1287.91**	**1110.81**	**1155.89**							**1405.62**	**1344.22**	**1299.91**	**1608.70**	**1509.36**	**1433.56**
SD	**107.10**	**193.93**	**161.28**	**150.24**	**101.54**	**179.50**							**126.13**	**144.26**	**136.60**	**302.87**	**171.02**	**212.74**
mode	**946.00**	**1204.57**	**1211.65**	**1238.84**	**1120.93**	**1178.57**							**1364.46**	**1376.72**	**1259.86**	**1565.62**	**1480.42**	**1400.23**

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
Ndog 5	928.03	708.88	755.50	738.58	838.78		1337.46	1496.25	1210.15	1475.33	1397.63		1132.25	1123.09	924.45	1424.33	1169.51	
Greyhound	753.70	1021.67	701.31	887.79	739.36		998.17	1200.59	1626.36	1658.63	1656.24		1442.06	1447.04	1506.61	1347.22	1618.58	
3 years	1023.67	778.41	971.27	844.56	843.56		1020.68	1330.49	1259.56	1547.65	1525.14		1328.50	1656.83	1378.90	1306.78	1407.19	
M	806.50	686.16	812.68	867.99	868.86		981.43	1498.24	1450.80	1098.56	1416.76		1141.22	1234.66	1351.61	1076.06	937.40	
	944.97	676.40	654.09	809.89	926.44		1256.56	1226.69	1411.38	1298.61	1268.13		1329.89	1422.73	1316.14	1282.87	1225.29	
	932.02	740.75	651.70	1090.01	779.41		1095.39	1286.86	1466.76	1778.97	1530.52		1269.52	985.41	1290.24	1160.54	1453.02	
	800.92	762.47	826.82	990.20	780.80		1040.80	1306.16	1395.44	1089.41	1285.86		1143.01	1200.59	1019.28	1212.74	1019.68	
	916.08	634.56	698.32	910.10	971.67		1019.68	1212.94	1257.17	1163.53	1696.28		1646.87	1114.12	1190.43	1543.27	1477.92	
	1074.87	670.46	932.02	874.04	1028.05		742.35	896.75	1498.04	1195.21	1181.66		1194.01	1514.38	1168.51	1061.32	1225.29	
	900.94	980.23	860.89	855.51	946.56		1021.67	1058.53	1327.90	1471.35	1304.59		1334.47	1397.23	1116.91	1559.61	1483.70	
Mean	**908.17**	**786.00**	**786.46**	**888.87**	**872.35**	**848.37**	**1051.62**	**1251.35**	**1391.86**	**1377.73**	**1425.28**	**1299.61**	**1457.36**	**1522.01**	**1498.72**	**1430.90**	**1433.21**	**1468.44**
SD	**99.59**	**130.78**	**112.97**	**96.21**	**93.82**	**115.68**	**160.85**	**182.28**	**128.68**	**243.49**	**172.15**	**222.44**	**173.94**	**114.98**	**249.31**	**242.92**	**150.98**	**189.12**
mode	**912.13**	**751.61**	**770.98**	**880.92**	**856.21**	**850.84**	**1021.18**	**1239.02**	**1393.25**	**1338.17**	**1407.20**	**1292.74**	**1468.94**	**1528.96**	**1450.93**	**1369.64**	**1426.22**	**1459.29**
Ndog 6							1488.68	1561.40	1202.50	1298.81	1594.67							
Greyhound							1080.65	1376.11	1584.51	1396.63	1505.62							
4 years							1364.96	1076.66	1646.28	1403.01	1554.23							
M							1235.65	1056.14	1603.84	1407.59	1554.83							
							1563.79	1434.89	1289.65	1158.15	1017.49							
							1338.46	1571.96	1494.86	1366.75	1475.53							
							1351.21	1552.83	1487.49	1493.66	1263.15							
							1633.92	1757.45	1282.87	971.07	1544.27							
							1505.02	1547.85	1411.78	1319.33	1696.68							
							1569.77	1253.38	1671.38	1049.97	1659.82							
Mean							**1413.21**	**1418.87**	**1467.52**	**1286.50**	**1486.63**	**1414.55**						
SD							**171.24**	**228.79**	**165.32**	**170.90**	**202.44**	**194.67**						
mode							**1389.09**	**1426.88**	**1477.51**	**1309.07**	**1524.95**	**1455.21**						
Ndog 7							1195.81	1508.80	1568.50	1220.11	1661.54							
Greyhound							1836.94	1912.05	1319.73	1080.65	1602.64							
5 years							1522.15	1807.66	1552.24	1439.67	1469.16							
FN							1886.95	1674.97	1815.62	1257.37	1387.27							
							1624.76	1438.27	1433.27	1460.76	1414.96							
							1481.11	1442.66	1339.45	1995.53	1507.01							
							1355.39	1336.66	1671.98	1716.41	1547.45							
							1770.40	1501.63	1364.96	1764.42	1463.76							
							1688.71	932.22	1564.19	1619.98	1436.08							
							1475.33	1089.41	1381.49	1848.90	1807.66							
Mean							**1583.76**	**1464.43**	**1503.64**	**1540.98**	**1531.76**	**1524.91**						
SD							**219.17**	**299.48**	**162.65**	**298.04**	**131.93**	**226.44**						
mode							**1552.95**	**1453.55**	**1470.96**	**1503.87**	**1488.09**	**1504.32**						

Appendix F. Atopic dermatitis corneocyte diameter (μm)

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
ADdog 1	32.67	38.41	39.20	42.14	44.71		42.42	43.84	38.86	41.55	44.47		36.44	44.40	38.04	40.23	37.23	
Labrador	35.61	36.67	43.16	36.06	41.22		41.99	42.90	46.83	42.61	41.60		28.35	42.90	31.90	43.46	43.74	
retriever	39.40	38.12	43.47	36.24	39.30		47.31	44.15	39.54	44.93	45.02		28.39	35.82	45.19	39.36	38.19	
1 year	40.39	34.75	46.06	33.65	38.54		41.40	43.73	38.86	26.16	34.10		32.93	35.31	35.50	12.02	42.62	
3 months	36.33	39.42	43.66	42.24	42.11		37.98	40.54	47.49	39.62	18.32		31.97	35.68	37.42	50.96	36.72	
FN	35.79	37.25	33.57	37.03	37.70		38.02	47.53	39.79	37.36	42.59		30.61	41.25	35.60	44.98	41.32	
	34.43	37.78	38.64	37.02	40.77		40.67	39.26	43.71	43.09	40.83		40.57	45.02	38.67	42.57	43.25	
	37.42	44.89	33.71	42.23	44.13		34.33	43.17	40.37	41.17	38.06		39.10	41.31	43.74	43.58	38.21	
	36.17	38.10	37.50	40.16	40.70		45.39	45.37	39.79	35.38	34.51		29.11	39.84	44.64	35.46	43.71	
	37.41	27.95	40.55	33.92	38.45		42.46	47.51	35.22	37.18	43.37		40.47	36.87	39.90	42.12	41.50	
Mean	**36.76**	**37.21**	**40.03**	**38.19**	**40.32**	**38.68**	**41.20**	**43.79**	**41.29**	**38.83**	**37.62**	**40.69**	**33.79**	**39.84**	**39.06**	**39.48**	**40.65**	**38.56**
SD	**2.30**	**4.44**	**4.49**	**2.88**	**2.04**	**3.54**	**3.75**	**2.80**	**3.97**	**5.08**	**8.18**	**5.14**	**4.95**	**3.71**	**4.36**	**10.45**	**2.79**	**6.17**
mode	36.76	37.25	40.03	37.03	40.32	38.43	41.20	43.73	39.79	38.83	38.06	41.47	32.45	39.84	38.36	41.18	40.98	39.61
ADdog 2	29.08	38.84	26.80	30.47	37.07		36.43	47.70	46.34	44.29	37.97		41.07	25.66	34.32	29.24	37.43	
Labrador	40.69	33.31	30.63	35.00	34.10		40.00	44.91	44.43	44.50	40.15		32.00	26.96	27.52	35.62	37.47	
cross	29.25	35.11	32.46	40.40	33.60		38.50	44.39	42.46	41.56	44.72		29.22	29.71	33.95	29.83	35.53	
5 years	32.65	37.17	31.32	30.59	34.78		44.58	45.33	43.94	37.59	41.83		30.38	33.29	30.66	29.00	34.65	
2 months	33.83	38.03	37.82	32.43	31.08		40.32	40.60	45.50	46.92	41.45		30.35	35.45	28.48	33.24	32.13	
M	30.84	37.73	24.70	34.94	34.96		40.80	42.71	42.29	39.19	40.72		38.26	31.36	32.79	30.04	36.40	
	32.46	41.49	30.72	30.84	36.55		42.78	40.77	46.79	41.49	42.67		31.63	36.45	28.15	34.18	36.97	
	29.48	28.39	26.90	24.06	34.00		43.20	43.21	43.74	40.04	38.89		32.69	24.68	31.23	33.95	37.99	
	30.84	34.59	35.40	36.63	36.18		37.27	41.08	41.53	39.06	41.89		26.37	32.41	34.96	21.13	41.18	
	33.97	33.20	32.78	29.92	40.75		43.23	44.86	45.95	44.64	38.47		34.40	32.74	31.05	28.18	37.32	
Mean	**32.31**	**35.44**	**31.41**	**32.75**	**35.11**	**33.37**	**40.71**	**43.10**	**44.07**	**41.66**	**41.20**	**42.27**	**32.64**	**31.07**	**31.31**	**30.44**	**36.70**	**32.43**
SD	**3.46**	**3.74**	**3.98**	**4.68**	**2.64**	**4.01**	**2.74**	**1.90**	**1.78**	**3.10**	**1.92**	**2.75**	**4.33**	**3.86**	**2.67**	**4.17**	**2.35**	**4.11**
mode	32.31	35.11	31.32	32.43	34.78	33.45	40.71	43.10	43.94	41.49	41.20	42.38	31.82	31.21	31.14	29.93	36.84	32.55
ADdog 3	34.61	38.75	44.52	40.05	41.39		39.79	49.98	32.95	38.52	42.73		40.61	46.24	43.75	44.00	40.25	
WHW	33.31	34.39	44.12	39.14	40.41		47.12	44.70	46.17	38.71	41.54		40.84	43.73	40.33	48.77	48.53	
1 year	35.30	36.97	44.94	38.39	41.60		42.40	43.82	45.30	47.42	42.17		46.42	49.30	43.40	45.00	46.76	
6 months	40.71	40.45	44.55	34.92	42.17		45.91	36.81	45.66	38.80	38.76		47.06	48.35	42.40	48.91	42.52	
M	33.40	36.40	40.99	34.58	33.88		41.43	33.57	42.23	36.64	42.72		48.44	35.56	40.36	40.07	47.81	
	33.48	45.98	44.96	32.97	42.61		37.82	44.80	44.70	41.10	43.95		39.03	45.07	42.35	42.31	41.64	
	34.08	43.16	43.76	42.75	41.86		38.26	38.95	46.88	43.82	43.39		47.09	44.94	39.14	41.79	45.43	
	38.08	36.60	39.63	35.01	44.13		42.80	44.21	43.58	42.39	38.42		47.15	35.06	38.43	48.36	43.81	
	37.04	39.06	49.78	43.08	44.96		44.61	48.18	32.86	39.78	34.35		49.91	35.69	45.86	41.79	49.92	
	34.76	41.59	39.64	37.38	43.52		42.17	39.15	44.68	40.38	41.24		44.49	37.96	53.10	39.75	42.40	
Mean	**35.47**	**39.40**	**43.59**	**37.58**	**41.68**	**39.59**	**42.23**	**41.57**	**43.58**	**41.11**	**40.72**	**41.79**	**45.10**	**42.19**	**42.91**	**44.07**	**44.91**	**43.84**
SD	**2.42**	**3.73**	**3.19**	**3.61**	**3.24**	**4.17**	**3.08**	**4.68**	**4.26**	**3.27**	**3.05**	**3.96**	**3.71**	**5.56**	**4.24**	**3.54**	**3.26**	**4.13**
mode	34.76	39.06	43.76	37.38	41.86	39.84	42.23	41.57	44.68	40.38	41.24	42.31	45.76	42.96	42.37	43.15	44.36	43.78

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
ADdog 4	29.43	41.66	45.67	38.67	51.78		38.49	47.27	53.29	53.36	45.22		45.27	47.70	46.45	45.79	47.95	
JRT	31.86	44.97	42.12	38.40	38.68		37.17	48.32	44.38	44.39	43.23		39.48	50.64	52.06	51.52	40.81	
4 years	22.28	42.07	37.36	37.19	38.03		42.40	45.35	42.46	44.41	35.61		47.95	45.12	51.21	47.95	42.78	
FN	35.51	37.48	32.05	33.82	40.34		47.12	47.24	51.54	54.15	44.72		51.27	49.83	41.43	43.87	44.28	
	27.06	40.45	38.40	42.06	36.86		46.24	54.39	43.65	51.18	47.49		51.91	40.28	41.36	45.01	45.52	
	33.62	35.75	35.56	41.62	40.92		42.53	43.61	44.70	48.12	42.31		50.11	47.71	42.05	44.90	44.75	
	22.48	39.71	39.30	35.42	43.11		46.92	48.68	38.85	51.42	44.85		42.67	47.51	41.48	46.60	47.74	
	31.07	39.89	37.61	35.77	42.18		45.49	42.84	44.20	50.72	43.21		44.44	34.12	47.36	45.30	48.11	
	29.98	36.19	34.87	40.26	40.16		46.87	45.59	44.69	53.11	42.07		43.13	45.33	46.92	41.99	45.86	
	31.78	39.44	48.32	35.36	44.86		50.95	47.30	49.03	50.16	44.44		50.07	49.75	52.26	40.80	44.93	
Mean	**29.50**	**39.55**	**38.42**	**37.76**	**40.57**	**37.59**	**44.42**	**47.04**	**44.83**	**49.74**	**43.10**	**46.11**	**46.63**	**45.80**	**46.26**	**45.37**	**45.27**	**45.86**
SD	**4.40**	**2.89**	**4.68**	**2.98**	**2.54**	**5.72**	**4.24**	**3.41**	**3.64**	**3.47**	**3.25**	**4.28**	**4.23**	**5.09**	**4.52**	**3.00**	**2.34**	**3.84**
mode	**29.98**	**39.55**	**37.81**	**37.19**	**40.34**	**38.21**	**45.49**	**47.04**	**44.38**	**50.16**	**43.21**	**45.42**	**45.95**	**46.65**	**46.35**	**45.15**	**45.10**	**45.65**
ADdog 5	40.99	43.55	41.65	52.61	35.33		45.46	42.74	45.02	44.38	44.39		36.89	38.41	37.96	37.96	37.96	
WHW	43.46	48.85	50.39	40.04	34.69		45.48	46.35	47.90	38.90	39.67		41.83	36.94	44.93	44.93	44.93	
3 years	40.38	47.91	40.48	44.48	40.27		45.67	43.42	41.58	46.57	43.08		39.92	32.71	40.41	40.41	40.41	
M	41.47	47.94	48.66	45.72	33.46		46.97	44.73	45.20	47.03	41.79		36.05	42.43	39.21	39.21	39.21	
	38.40	42.37	37.75	45.63	35.83		33.72	44.39	48.36	41.17	50.76		41.66	31.43	42.71	42.71	42.71	
	39.68	40.82	39.51	39.63	33.92		46.66	49.47	47.97	40.87	45.17		46.39	39.88	39.68	39.68	39.68	
	38.07	44.27	47.48	50.19	35.11		40.69	41.11	50.89	43.03	49.32		43.66	32.72	42.16	42.16	42.16	
	45.79	47.93	44.62	40.24	33.24		48.31	43.53	45.93	57.74	47.00		43.42	41.99	40.05	40.05	40.05	
	40.80	39.18	43.80	51.43	41.67		46.63	47.00	43.94	48.11	47.43		40.28	35.87	35.92	35.92	35.92	
	46.58	47.16	43.24	48.63	47.55		54.06	35.80	47.68	50.64	42.50		40.56	42.80	45.56	45.56	45.56	
Mean	**41.56**	**45.16**	**43.99**	**45.11**	**37.30**	**42.65**	**45.36**	**43.98**	**46.60**	**46.04**	**45.21**	**45.33**	**41.07**	**37.52**	**40.86**	**40.86**	**40.86**	**40.23**
SD	**2.88**	**3.60**	**4.29**	**4.45**	**4.86**	**5.00**	**5.25**	**3.89**	**2.76**	**5.86**	**3.67**	**4.18**	**3.10**	**4.28**	**3.01**	**3.01**	**3.01**	**3.46**
mode	**40.80**	**45.16**	**43.80**	**45.11**	**35.11**	**42.02**	**45.67**	**43.98**	**46.60**	**46.04**	**45.17**	**45.47**	**40.81**	**37.23**	**40.23**	**40.23**	**40.23**	**40.16**

Appendix G. Atopic dogs corneocyte surface area (μm^2)

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
ADdog1	885.40	1138.03	1231.27	1186.24	1503.03		1199.23	1447.51	1272.99	1221.43	1382.97		1239.52	1350.71	1144.15	1162.45	1141.48	
Labrador	963.30	999.76	1144.00	1179.07	1322.72		1254.52	1478.95	1409.90	1417.50	1347.01		612.05	1407.43	799.28	1422.64	1366.53	
retriever	1061.72	1132.25	1510.80	976.85	973.66		1848.69	1441.96	950.14	1384.07	1370.03		923.83	773.59	1450.39	1125.86	1204.78	
1 year	953.74	1237.25	1384.68	1069.09	1116.51		1528.69	1195.95	822.51	563.34	826.82		949.32	1204.37	993.71	615.96	1014.47	
3 months	1078.46	927.24	1007.93	1026.66	1043.59		1081.88	1203.04	771.33	1085.58	446.60		726.73	1147.24	1109.22	1198.82	1082.50	
FN	1295.62	1034.03	1184.85	1067.50	1127.27		1089.28	1565.51	1264.80	885.19	1233.76		992.27	1148.68	806.48	1314.12	991.66	
	858.10	1045.18	1350.21	1134.44	1314.95		1061.06	988.78	1315.15	1520.26	1177.65		1328.10	1415.45	1264.80	1281.86	872.86	
	1050.56	879.42	1415.16	768.05	1338.46		636.92	1715.10	1320.70	1363.86	1129.56		1062.77	1417.71	1277.95	1366.74	1049.61	
	1176.48	962.70	1070.09	1116.31	1063.12		1460.46	1686.33	1115.59	715.43	929.38		613.26	917.67	1361.80	1047.76	1348.24	
	1315.35	1078.86	1277.29	1387.27	1313.35		1292.75	1666.19	943.56	1075.51	1444.32		1204.37	136.78	1187.31	1561.16	839.77	
Mean	1063.87	1032.97	1260.56	1080.58	1179.29	1133.56	1247.35	1437.98	1101.52	1110.08	1100.57	1210.99	965.22	1063.21	1138.99	1214.99	1085.60	1099.52
SD	158.63	108.77	169.43	165.67	142.80	169.71	323.74	255.95	237.27	335.17	317.79	303.05	254.25	413.20	232.39	272.78	188.08	273.18
mode	1061.72	1032.97	1260.56	1069.09	1127.27	1116.41	1247.35	1441.96	1101.52	1085.58	1129.56	1244.14	949.32	1147.24	1138.99	1214.99	1049.61	1145.70
ADdog2	786.38	976.85	832.20	841.37	1006.13		1014.09	1533.47	1909.30	1435.67	1309.08		850.53	703.50	953.14	781.60	1083.04	
Labrador	918.47	1380.50	745.74	920.86	913.09		1276.70	1518.11	1629.77	1526.14	1336.24		634.16	783.19	715.65	686.56	1064.31	
cross	919.47	1004.54	737.96	878.62	803.31		1410.20	1322.24	1327.67	1417.73	1639.17		805.11	694.93	879.02	741.35	995.38	
5 years	1014.30	1352.01	747.93	851.33	729.60		1336.04	1551.64	1292.16	1282.97	1283.17		729.40	899.54	666.44	549.29	1041.00	
2 months	873.05	1009.92	896.75	922.06	1114.12		1446.96	1177.47	1535.13	1614.11	1427.12		753.11	868.28	977.25	814.47	822.24	
M	744.94	983.82	878.62	1217.92	915.08		1378.64	1351.27	1347.30	1321.63	1236.58		873.25	831.80	707.88	593.32	1010.72	
	783.59	1146.40	681.38	749.12	976.05		1534.28	1155.94	1523.02	1254.55	1222.17		823.63	920.07	807.30	693.93	1444.45	
	798.73	1099.58	639.14	738.35	876.43		1493.35	1637.71	1585.48	1494.81	1345.84		656.28	672.22	875.84	706.09	1017.29	
	892.37	1073.48	597.11	964.89	1031.44		970.01	1099.11	1452.19	1254.55	1364.22		894.76	816.06	830.61	583.96	970.87	
	838.18	1007.33	710.87	914.49	1072.48		1218.20	1710.41	1437.36	1347.74	1111.86		780.05	824.23	707.66	585.15	1135.04	
Mean	856.95	1117.51	737.28	906.41	936.84	910.17	1307.85	1391.32	1458.90	1390.47	1329.60	1387.97	780.05	812.26	796.41	661.57	1055.70	825.10
SD	82.07	150.66	99.18	140.43	125.39	164.11	191.70	222.68	118.92	129.96	146.58	180.37	87.72	84.51	103.63	88.41	168.28	165.97
mode	856.95	1073.48	737.28	906.41	915.08	904.92	1336.04	1351.27	1452.19	1347.74	1329.60	1357.75	780.05	816.06	796.41	661.57	1017.29	815.27
ADdog3	1038.41	1297.42	1502.83	1343.24	1159.56		1132.65	1715.81	804.11	1292.43	1224.89		1464.17	1710.03	1356.79	1506.61	1521.36	
WHW	1179.07	1085.63	1587.70	1145.00	1261.75		1589.69	1373.72	1776.57	1716.41	1366.75		1737.33	1533.71	1097.18	1642.09	1602.84	
1 year	1115.12	1023.07	1452.82	1336.66	1569.37		1521.56	1464.17	1366.67	1957.28	1419.35		1562.40	1659.03	1681.74	1514.58	1576.94	
6 months	1215.33	1050.96	1590.29	919.67	1410.38		1517.17	1245.02	1621.97	1636.91	1393.65		1761.83	1429.11	1585.11	1778.77	1445.65	
M	943.77	1006.33	1436.48	1171.30	816.06		1342.44	1023.87	1578.73	855.71	1324.31		1587.90	1493.46	1516.18	1346.43	1548.05	
	927.44	1431.10	1517.17	823.83	1156.76		1239.64	891.57	1538.69	1378.11	1169.51		1509.40	1453.22	1168.91	1609.22	1277.89	
	1039.21	1241.43	1331.88	1110.53	1254.98		1511.19	1726.17	1514.38	1741.71	1074.47		1865.63	1102.76	937.80	1372.33	1665.80	
	1470.55	1281.68	1591.88	1228.48	1339.25		1176.88	1338.86	1659.42	1221.51	1412.17		1689.91	1012.11	1633.33	1526.73	1530.12	
	1193.61	1380.50	1680.74	1357.98	1718.20		1710.03	1335.07	1162.93	1441.66	1199.79		1779.16	1172.69	1344.43	1331.68	1555.23	
	1112.92	1024.26	1497.65	1156.96	1081.05		1250.79	1607.23	1648.87	1250.00	1465.77		1605.63	1142.41	1768.61	1024.07	1067.70	
Mean	1123.54	1182.24	1518.94	1159.37	1276.74	1252.17	1399.20	1333.94	1543.14	1466.59	1313.97	1398.96	1656.34	1333.17	1414.81	1460.86	1474.47	1476.10
SD	157.11	161.83	98.99	177.09	254.22	222.61	195.93	261.45	179.08	334.75	134.30	248.13	130.09	227.49	290.97	221.23	188.27	227.48
mode	1115.12	1085.63	1517.17	1156.96	1261.75	1234.96	1399.20	1335.07	1543.14	1441.66	1324.31	1390.16	1656.34	1333.17	1414.81	1460.66	1530.12	1524.05

	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
ADdog 4	644.13	1327.30	1717.00	1116.51	1584.71		1389.26	1835.75	1434.49	1691.50	1729.75		2064.67	2196.95	1921.02	1680.94	1838.54	
JRT	791.36	1426.52	1421.14	1326.11	1156.36		1430.30	1921.02	1321.92	2178.83	1596.47		1476.33	2415.72	1692.70	1183.45	1353.60	
4 years	593.52	1259.56	954.73	1143.41	1316.54		1721.98	1714.41	1829.37	1583.32	1246.01		1780.76	1733.94	1407.79	1291.04	1587.50	
FN	661.86	1104.95	1377.11	1103.96	1414.17		1733.34	1686.72	1491.67	1468.96	1766.61		1713.82	1984.97	1262.35	1907.87	1710.83	
	653.49	1111.53	1000.16	1343.84	1528.33		1843.32	2041.76	1641.29	2089.57	1503.03		2000.71	1337.86	1613.00	1529.32	1690.31	
	710.67	1101.77	1141.81	1172.10	1165.52		1563.00	1562.40	1837.34	1740.51	1451.03		1592.68	1509.60	1162.93	1816.02	1367.55	
	587.34	1112.92	1231.67	995.97	1301.40		1184.05	1527.33	1591.68	1755.26	1340.25		1619.98	946.16	1770.40	1373.72	1733.14	
	961.31	795.15	1366.47	1061.92	1210.95		1732.15	1516.37	1307.78	1565.19	1656.44		1467.36	1777.37	1714.21	1162.33	1680.94	
	755.90	1144.40	1176.88	1174.09	1154.56		1645.48	1782.35	1521.36	1688.71	1473.54		1389.26	1593.28	1587.50	1835.75	1533.91	
	635.56	980.63	1283.27	1068.50	1219.71		1729.36	1759.24	1652.25	1882.57	1485.09		1779.56	1706.05	1913.65	1567.38	1615.00	
Mean	**699.51**	**1115.27**	**1219.25**	**1154.43**	**1274.17**	**1112.18**	**1597.22**	**1723.51**	**1577.18**	**1772.55**	**1502.05**	**1636.83**	**1688.51**	**1667.44**	**1569.39**	**1518.54**	**1585.86**	**1631.91**
SD	**112.93**	**173.01**	**167.20**	**117.14**	**129.27**	**257.84**	**204.30**	**178.12**	**190.20**	**239.01**	**157.39**	**207.55**	**224.54**	**408.53**	**245.69**	**286.16**	**142.48**	**276.75**
mode	**661.86**	**1111.53**	**1219.25**	**1143.41**	**1219.71**	**1143.91**	**1645.48**	**1714.41**	**1577.18**	**1740.51**	**1485.09**	**1648.87**	**1619.98**	**1667.44**	**1587.50**	**1518.54**	**1587.50**	**1650.46**
ADdog 5	1262.15	1427.32	1148.59	1982.18	1370.93		1559.21	1548.05	1782.15	1518.37	1578.14		995.58	1141.02	1061.52	960.31	1083.04	
WHW	1567.38	1464.17	1537.49	1626.55	1075.47		1847.30	1574.35	1044.79	1450.23	1574.75		1118.31	1172.50	984.62	1226.09	1064.31	
3 years	1372.13	2030.40	1316.14	1452.82	1103.76		1775.98	1532.51	1853.68	1610.01	1530.92		1255.38	845.95	1216.13	1402.81	995.38	
M	1440.27	1587.50	1940.54	1003.54	845.35		1699.07	1673.17	1800.88	1405.20	1807.26		922.85	936.60	1365.95	1057.74	1041.00	
	1314.35	1373.32	1237.84	1406.00	1054.75		2068.65	1250.00	1459.39	1841.13	1699.07		1188.23	996.37	853.92	1319.93	822.24	
	1244.02	1416.16	1183.65	1816.02	1102.17		1602.48	1717.60	1461.39	1243.42	1507.81		1742.90	953.74	1070.69	838.58	1010.72	
	1404.60	1439.87	1385.08	1601.65	998.96		1742.90	1310.17	1522.95	1906.08	1509.00		934.61	1378.90	1360.17	1144.20	1444.45	
	1424.33	1517.57	1706.64	1713.02	1242.83		1565.19	1424.53	1315.55	1526.93	1690.31		1225.89	1340.25	1183.45	1233.06	1017.29	
	1730.95	1433.89	1309.77	1143.21	1073.08		1699.47	1337.26	1406.40	1494.26	1662.21		1215.93	1165.32	1199.59	1359.78	970.87	
	1538.89	1286.26	1492.67	1576.54	1088.22		1326.30	1376.31	1242.43	1551.64	1352.80		1214.53	1373.92	692.74	1274.90	1136.04	
Mean	**1429.91**	**1505.46**	**1456.65**	**1482.13**	**1084.95**	**1396.22**	**1708.66**	**1466.21**	**1456.38**	**1558.77**	**1592.68**	**1563.59**	**1181.22**	**1129.28**	**1103.03**	**1208.34**	**1055.70**	**1130.15**
SD	**149.66**	**214.20**	**243.24**	**264.44**	**104.84**	**259.56**	**197.94**	**165.76**	**254.58**	**207.07**	**135.84**	**203.98**	**233.71**	**204.85**	**226.19**	**173.94**	**168.28**	**195.66**
mode	**1424.33**	**1439.87**	**1385.08**	**1482.13**	**1073.08**	**1405.30**	**1708.66**	**1424.53**	**1456.38**	**1526.93**	**1574.75**	**1549.85**	**1188.23**	**1129.28**	**1103.03**	**1226.09**	**1017.29**	**1138.03**

Case	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
Cat 1	38.92	38.32	40.47	39.39	39.93		15.83	16.37	21.71	21.71	18.75							
DSH	39.37	39.85	45.34	42.65	43.99		16.96	18.14	15.74	15.74	15.76							
11 years	35.99	44.83	39.24	42.03	40.63		17.54	16.11	18.18	18.18	11.66							
MN	37.81	40.14	35.41	37.77	39.59		20.13	23.78	19.42	19.42	17.28							
	44.79	39.83	46.50	43.16	44.83		19.01	16.49	21.54	21.54	17.28							
	44.13	37.27	42.21	39.74	40.97		24.13	20.61	16.94	16.94	10.89							
	42.32	38.14	47.76	42.95	45.35		18.71	18.31	15.58	15.58	22.02							
	41.58	45.87	48.89	48.38	48.64		20.40	15.31	25.51	25.51	16.86							
	43.57	39.36	42.95	41.15	42.05		16.28	22.46	23.40	23.40	17.38							
	40.85	43.24	43.69	43.57	43.73		24.46	11.23	19.20	19.20	11.11							
Mean	40.93	40.69	43.06	41.88	42.47	42.12	19.39	17.88	19.72	19.72	15.50	17.76						
SD	2.89	2.94	3.87	2.46	3.02	4.38	2.99	3.68	3.30	3.30	3.83	3.54						
mode	49.89	39.89	43.00	41.95	42.26	41.53	18.38	17.18	19.31	19.31	16.38	17.33						
Cat 2	34.23	37.46	38.44	36.49	40.56													
DSH	36.28	42.21	31.83	37.32	37.79													
10years	41.51	32.38	36.37	33.73	37.88													
MN	44.33	43.60	38.53	47.19	39.84													
	31.14	32.05	32.82	38.79	42.90													
	47.81	32.90	38.45	35.76	34.29													
	40.10	39.36	38.57	39.49	43.81													
	37.39	41.35	39.24	33.68	40.12													
	42.70	36.73	35.63	42.93	37.53													
	40.68	36.03	35.49	36.83	33.48													
Mean	39.62	37.40	36.53	38.12	38.82	38.10												
SD	4.95	4.18	2.59	4.23	3.33	3.93												
mode	39.36	37.06	36.45	36.91	38.34	37.82												
Cat 3	47.18	40.11	43.51	50.46	50.00								50.10	50.08	58.85	49.26	42.15	
DLH	37.61	40.98	42.03	39.51	54.25								52.81	51.52	57.11	56.28	48.59	
5 years	36.26	47.12	43.61	40.44	51.66								52.79	62.19	58.02	46.54	48.03	
MN	33.23	44.50	37.69	49.44	39.05								50.48	45.53	53.26	54.82	46.95	
	37.66	42.06	43.25	50.31	46.84								60.33	64.11	54.32	53.32	49.40	
	41.16	52.43	43.59	44.38	41.42								52.85	55.02	61.28	55.94	49.12	
	37.38	40.67	32.72	46.76	49.68								49.95	50.30	55.14	56.66	49.88	
	47.30	45.94	38.44	45.23	46.31								55.52	50.56	54.87	48.46	48.47	
	37.10	40.03	42.70	42.90	43.44								50.09	54.60	54.96	52.10	48.49	
	43.58	38.81	48.43	42.07	47.94								45.37	51.47	53.55	61.50	47.18	
Mean	40.14	43.25	41.70	45.05	45.06	43.04							52.03	52.81	55.83	53.96	48.47	52.41
SD	4.39	4.24	4.08	3.99	5.49	4.70							3.86	4.55	2.56	4.52	0.99	4.42
mode	37.63	41.52	42.36	44.71	45.68	43.08							52.03	51.52	54.96	53.96	48.47	52.44
Cat 4																		
DSH	20.02	37.74	35.47	46.05	43.60		14.95	15.31	17.80	11.82	12.58		31.86	37.30	43.90	43.67	50.89	
9 years	39.10	38.28	29.94	34.80	32.14		16.71	12.34	14.05	22.62	19.45		39.92	47.03	52.46	48.50	40.84	
FN	31.33	37.65	38.40	38.11	35.95		15.76	18.59	11.20	19.57	18.31		45.22	45.89	44.23	43.97	48.47	
	35.90	32.85	35.53	34.11	38.91		15.05	12.24	15.07	16.56	14.98		35.83	39.96	46.32	38.86	50.23	
	34.97	31.02	39.04	34.79	38.53		15.14	13.02	17.02	19.12	17.05		41.04	37.85	48.20	40.15	41.54	
	41.81	44.23	37.64	36.51	38.25		16.55	17.32	14.13	12.36	15.21		36.83	38.09	43.47	48.19	56.77	
	44.17	42.96	43.67	33.51	33.41		13.93	13.28	21.48	9.93	20.28		31.27	37.22	54.57	39.26	54.70	
	37.83	21.29	35.68	36.19	35.61		13.89	9.58	15.84	12.89	11.49		37.42	35.98	50.19	42.82	45.09	
	40.53	45.70	33.19	38.36	37.56		13.54	20.49	14.76	16.15	10.44		36.14	46.52	43.69	47.85	47.17	
Mean	41.43	39.21	35.18	34.73	39.67		21.50	19.37	14.15	14.10	15.98		41.44	39.76	39.91	43.08	40.48	
SD	37.01	37.09	36.47	36.81	37.86	37.05	15.71	15.14	15.27	15.92	15.91	15.49	37.49	41.25	47.00	43.07	47.25	43.16
mode	6.77	7.23	3.74	3.72	2.98	5.00	2.31	3.65	2.79	4.03	3.35	3.13	3.96	4.86	4.75	3.43	5.92	5.75
	37.42	37.70	35.61	35.49	38.06	37.64	15.14	13.28	14.76	15.92	15.91	15.10	37.42	39.76	46.32	43.07	47.17	43.13
Cat 5																		
DSH							16.59	12.85	15.29	17.37	15.60							
9.7 years							12.66	25.00	13.85	20.38	17.92							
FN							11.94	22.83	11.29	15.27	15.65							
							12.44	19.00	18.89	17.52	16.57							
							12.52	21.00	15.99	19.91	16.16							
							16.57	17.05	15.06	16.93	14.57							
							10.44	22.55	14.50	19.58	14.04							
							18.95	21.32	18.59	19.72	17.64							
							13.68	20.19	15.52	18.64	16.47							
Mean							13.93	22.37	13.26	14.76	24.57							
SD							13.97	20.42	15.22	17.83	18.94	16.84						
mode							2.61	3.42	2.29	1.85	2.94	3.40						
							13.17	20.71	15.14	17.44	16.31	16.57						

Cat 6																		
DSH	40.91	44.41	40.94	40.75	44.13		15.66	17.46	20.28	13.01	15.85							
7.3 years	40.35	40.55	33.90	45.14	39.42		18.17	13.85	5.86	15.53	18.05							
MN	37.04	37.44	40.24	42.96	42.60		14.70	14.14	19.14	15.86	18.46							
	38.55	49.81	43.25	40.10	40.89		14.03	11.51	9.99	17.28	14.72							
	46.53	36.26	45.43	38.85	40.08		16.91	14.87	12.18	13.34	19.56							
	37.55	43.90	46.68	43.22	36.98		19.94	15.76	14.01	9.77	18.32							
	42.89	37.15	44.54	49.48	45.31		15.40	14.98	19.87	19.00	14.84							
	38.78	43.34	41.39	37.65	46.59		10.60	12.52	16.85	10.33	18.65							
	44.68	43.19	47.08	43.45	40.47		13.15	15.23	9.59	9.40	14.52							
Mean	40.94	38.69	47.33	46.31	45.38		14.38	11.62	10.27	15.57	13.86							
SD	40.82	41.49	43.18	42.79	42.16	42.09	15.29	14.19	13.80	13.92	16.75	14.79						
mode	3.10	4.22	4.22	3.60	3.11	3.63	2.63	1.88	5.02	3.30	2.16	3.27						
	40.57	41.02	43.21	42.87	41.53	42.60	14.89	14.17	12.99	13.63	16.30	14.90						
Cat 7																		
DSH													42.22	37.49	47.46	46.59	39.57	
7.2 years													33.17	44.59	30.64	46.60	31.34	
MN													36.66	43.92	41.60	41.28	37.93	
													41.08	35.35	47.57	39.58	41.03	
													33.37	33.87	44.89	38.43	42.47	
													34.53	43.30	45.96	36.69	40.26	
													39.37	38.75	40.33	40.77	34.26	
													38.13	37.35	41.38	39.86	20.45	
Mean													39.74	41.07	42.94	43.64	46.38	
SD													39.59	36.89	39.25	46.64	38.66	
mode													37.78	39.19	43.10	42.41	38.19	40.13
													3.21	3.67	3.16	3.70	5.10	4.29
Cat 8													37.95	38.12	42.27	41.03	38.42	39.80
DSH													51.83	46.49	52.92	48.28	51.43	
13.4 years													55.53	50.37	47.94	49.14	53.03	
MN													54.89	57.37	56.33	55.78	59.81	
													56.36	42.62	55.24	52.09	53.30	
													52.65	62.68	52.36	53.22	59.00	
													53.95	57.61	51.59	50.03	53.16	
													47.30	55.83	51.54	55.60	49.61	
													55.42	51.27	53.80	57.58	51.28	
Mean													58.25	51.13	45.34	59.31	51.76	
SD													54.39	54.74	47.88	44.95	52.76	
mode													54.03	53.03	51.18	52.66	53.51	52.89
													2.98	5.87	3.14	4.54	3.31	4.07
													54.21	52.15	51.56	52.67	52.89	52.97
Cat 9																		
DSH							10.82	17.37	17.90	11.85	11.38							
1.9 years							8.62	15.23	10.90	14.41	13.12							
MN							15.18	15.74	12.91	13.37	17.90							
							10.17	11.89	15.73	6.33	21.58							
							13.59	15.22	15.85	9.01	17.78							
							14.12	12.76	20.91	15.32	14.23							
							15.14	11.04	9.75	11.74	17.38							
							14.20	11.99	14.23	8.45	16.21							
							7.91	8.96	9.60	16.00	15.82							
Mean							16.59	9.80	9.59	8.88	14.60							
SD							12.61	12.88	14.36	11.53	16.00	13.50						
mode							3.03	2.80	3.85	3.27	2.88	3.43						
							13.10	12.37	14.30	11.63	15.91	14.16						
Cat 10																		
DSH													54.96	54.46	53.14	47.34	59.52	
16 years													46.58	49.97	52.33	48.41	50.90	
MN													64.09	51.67	68.86	49.89	56.07	
													49.31	52.26	54.38	51.88	51.85	
													51.97	52.91	55.11	50.50	52.18	
													62.03	50.32	56.19	49.66	55.16	
													55.52	49.22	60.51	52.41	51.45	
													50.28	58.22	50.63	59.41	55.98	
													59.23	53.15	50.82	55.60	52.93	
Mean													50.99	48.34	58.09	52.41	54.33	
SD													54.39	51.85	55.07	51.75	55.94	53.80
mode													5.68	2.45	3.36	3.56	5.76	4.53
													53.18	51.76	54.57	51.12	54.70	52.66

Appendix 1. Normal cats corneocyte surface area (μm^2)

Case	Ear						Thorax						Groin					
	1	2	3	4	5	Total	1	2	3	4	5	Total	1	2	3	4	5	Total
Cat 1	1345.23	1005.34	1020.06	1238.24	1314.75		250.04	180.31	290.28	207.20	149.03							
DSH	1072.68	1315.55	1140.22	1423.53	1354.90		243.86	180.90	310.61	135.68	177.12							
11 years	1274.90	970.27	1086.65	1681.72	1024.07		194.45	147.83	318.38	129.70	215.57							
MN	960.43	1375.91	1049.97	1362.14	1493.06		232.51	232.71	242.07	160.38	243.07							
	895.96	1442.85	1157.15	1464.57	1444.25		339.89	220.35	202.62	212.18	242.27							
	1062.92	1044.69	1428.71	1163.93	1485.89		298.25	227.53	253.19	189.87	218.57							
	760.68	1286.25	1213.94	1239.44	1100.17		310.01	140.26	252.23	187.68	174.53							
	724.02	1217.52	1258.75	1148.79	954.13		250.24	254.22	226.93	213.78	208.60							
	1006.93	1355.79	1135.04	1216.13	1545.86		238.09	217.86	316.38	196.64	245.25							
	1052.95	1076.68	1343.44	1272.91	1096.59		269.37	223.94	294.67	249.04	244.06							
Mean	1017.67	1209.12	1183.39	1311.74	1281.46	1200.68	262.67	202.60	271.64	188.20	211.61	227.34						
SD	196.74	171.38	129.59	142.05	218.66	197.07	42.02	38.08	40.49	37.00	34.39	50.13						
mode	1012.30	1213.32	1148.69	1256.18	1298.10	1215.04	250.14	210.28	267.41	186.93	213.59	226.73						
Cat 2	834.79	1149.59	1269.05	824.23	1020.88													
DSH	991.99	974.26	974.06	867.67	1218.92													
10years	942.58	1058.56	856.31	1112.53	1102.17													
MN	1229.68	933.21	596.71	1185.55	1258.37													
	984.92	1607.03	875.44	1064.51	1451.03													
	1024.86	1143.01	1556.62	757.09	1122.89													
	1306.19	1461.78	533.02	1065.04	1350.41													
	943.77	1279.09	1157.95	1231.67	1205.57													
	1060.92	1119.50	1423.13	1251.99	1384.68													
	1146.40	1088.62	1016.30	1467.36	1146.79													
Mean	1069.80	1184.45	1043.28	1113.28	1248.98	1121.13												
SD	129.10	224.72	296.05	210.38	122.63	211.12												
mode	1024.86	1119.50	974.06	1112.53	1218.92	1116.02												
Cat 3	1308.37	1479.72	1153.35	1168.91	1261.55								1943.73	1983.58	2379.86	1870.81	1771.99	
DLH	1105.55	1635.72	1340.25	1396.63	1499.64								1988.16	2042.15	2862.40	1921.82	1546.46	
5 years	1414.57	1281.08	1464.57	1361.37	1566.18								2345.19	1904.25	2589.05	2175.24	1404.01	
MN	1331.09	2040.16	1202.98	1006.74	1312.16								2061.68	1983.76	2164.68	2090.97	1633.13	
	1125.08	1678.55	1242.23	1712.62	1623.76								1648.47	2148.15	2092.38	1784.94	1797.52	
	1445.25	1119.50	1186.64	1577.54	1823.59								1885.36	2232.82	1690.34	2342.80	1808.05	
	985.91	1053.15	1486.69	1620.97	2138.38								2235.61	2082.00	2005.69	2040.96	1556.22	
	1467.86	1170.90	1646.08	1510.00	1630.83								2132.21	2283.63	1465.77	1778.17	1784.34	
	1100.97	1590.09	1020.06	1498.44	1210.55								1938.35	2079.21	1889.14	2083.79	2010.58	
	1072.08	1195.01	1107.60	1335.47	1174.09								2168.87	2212.90	1765.21	1980.89	1648.27	
Mean	1229.81	1418.24	1309.68	1446.31	1553.25	1379.85							2044.77	2103.23	2080.69	2022.30	1680.96	1986.75
SD	188.69	332.37	192.65	207.14	305.95	257.57							205.86	130.24	423.21	181.32	177.64	276.04
mode	1125.08	1281.08	1242.23	1446.31	1553.25	1337.88							2044.77	2082.00	2005.69	2022.30	1548.27	1955.87
Cat 4	1305.19	1042.99	1149.36	1463.18	1125.68		206.01	147.83	151.30	143.65	208.50		1125.48	1871.61	1473.94	1377.11	2006.09	
DSH	1267.13	1197.80	1307.19	1016.49	1360.37		186.48	189.63	168.75	169.75	120.94		960.43	1076.58	1597.88	1428.11	1870.61	
9 years	955.73	1110.14	1198.99	965.61	930.43		192.66	207.20	144.84	235.69	136.28		1048.17	1279.29	1399.62	1491.97	1789.33	
FN	1218.12	1331.48	956.72	772.43	733.58		162.18	233.50	233.62	190.27	107.19		1008.73	1028.85	1628.55	1135.83	1760.84	
	1130.26	1139.02	1082.24	977.05	869.46		146.04	190.87	232.42	167.76	210.99		1274.11	1250.40	1663.81	1275.10	1653.05	
	944.37	1108.14	1114.92	1092.60	1052.16		179.31	128.90	172.14	136.97	206.51		1054.75	1420.34	1086.03	1282.27	2031.59	
	1129.26	1090.41	865.11	1222.70	966.49		163.17	161.88	189.87	171.94	152.41		1045.78	1420.14	1344.83	1050.96	2086.78	
	1252.99	1090.01	1092.60	1267.33	761.08		140.06	225.14	190.27	205.61	106.79		1228.98	1253.38	1685.52	1606.43	1673.57	
	1139.62	951.54	1016.69	1196.02	1371.93		214.18	207.20	148.43	155.90	223.74		1060.56	1172.10	1722.38	1318.53	1564.99	
	1056.34	1141.22	965.49	1126.86	1060.33		147.63	189.67	180.71	223.74	203.42		1237.94	1102.96	1496.45	1458.40	1346.43	
Mean	1121.60	1128.86	1065.57	1072.90	1011.76	1093.85	178.41	193.79	177.89	184.27	163.15	177.86	1103.03	1222.70	1513.67	1338.72	1753.24	1404.73
SD	117.89	100.79	136.10	153.49	230.32	158.50	24.93	32.02	21.35	32.58	47.95	32.78	111.22	140.85	206.20	176.30	229.92	295.85
mode	1129.26	1110.14	1085.57	1072.90	968.49	1100.47	163.17	193.79	177.89	171.94	152.41	181.01	1050.56	1222.70	1513.67	1318.53	1753.24	1361.77
Cat 5							291.28	163.57	153.01	195.85	178.31							
DSH							97.82	195.05	231.11	203.02	226.33							
9.7 years							185.29	128.11	139.46	209.79	123.13							
FN							136.08	113.76	228.72	207.60	240.48							
							217.66	151.22	186.09	141.86	210.79							
							202.02	189.08	193.46	173.13	165.76							
							305.63	148.23	100.21	163.37	282.99							
							205.01	251.04	199.44	248.25	154.61							
							149.03	210.39	180.71	227.73	216.57							
							173.93	176.92	192.68	330.53	234.90							
Mean							185.86	173.64	183.43	211.70	203.95	192.19						
SD							56.88	43.06	41.24	55.35	45.98	49.24						
mode							165.29	173.64	186.09	207.60	210.79	193.06						

Cat 6	1026.66	1173.69	1367.85	1235.25	1058.13	
DSH	1224.65	1726.17	1354.00	1213.34	1547.85	
7.3 years	962.50	1422.14	1655.84	1531.32	1225.45	
MN	1071.68	1551.44	1728.56	1537.89	1151.58	
	1314.55	1633.52	1774.78	1659.23	1087.02	
	1367.35	1281.28	1489.09	1340.25	1341.45	
	868.07	1271.32	1352.21	1416.56	1174.49	
	894.76	1354.20	1220.31	1590.09	986.01	
	1481.11	1023.27	924.45	1380.50	1327.50	
	1402.21	1133.25	1044.59	1401.02	1456.21	
Mean	1161.38	1357.03	1391.12	1430.53	1236.57	1315.32
SD	224.34	226.80	281.11	147.76	180.27	231.35
mode	1116.63	1317.74	1360.68	1408.79	1204.97	1340.85

Cat 6	130.50	127.71	208.80	112.77	136.28	
DSH	201.03	157.40	144.25	185.29	230.12	
7.3 years	151.22	246.45	139.86	159.39	125.12	
MN	227.92	106.19	150.22	209.40	112.37	
	149.83	137.67	171.54	210.76	199.83	
	130.30	135.49	157.99	96.23	153.81	
	148.63	179.71	139.46	86.97	134.08	
	148.23	96.03	116.15	143.25	189.47	
	167.76	168.56	170.74	153.21	196.05	
	246.06	159.79	103.80	119.74	210.79	
Mean	174.44	154.14	143.78	152.46	177.96	158.82
SD	40.68	44.33	22.74	46.61	52.51	42.26
mode	151.22	154.14	143.78	152.46	177.96	150.72

Cat 7	1529.52	1295.22	1150.38	1319.73	1137.03	
DSH	924.25	1197.60	922.26	1512.89	1001.92	
7.2 years	1285.66	1268.92	1118.90	1365.15	1102.96	
MN	1190.83	936.80	1159.65	1045.38	1148.09	
	1363.16	1086.03	1413.17	1085.62	1204.97	
	1447.64	1430.70	1536.30	1311.76	1311.96	
	815.07	1311.96	1454.61	1350.41	1232.47	
	1154.17	1169.31	1205.06	1441.26	1417.55	
	931.82	1007.53	975.05	1262.35	1146.00	
Mean	1223.10	1127.47	928.43	1570.17	1694.69	
SD	1148.41	1170.70	1199.26	1327.66	1257.95	1227.36
mode	215.11	153.42	234.60	176.57	196.67	195.21
	1154.17	1169.31	1159.55	1327.68	1204.97	1214.04
Cat 8	2327.06	1730.35	1819.41	2023.03	1948.71	
DSH	2425.48	2524.90	1848.71	2506.37	1972.22	
13.4 years	2345.39	1521.95	1850.97	2305.54	2308.73	
MN	2170.26	2346.56	2026.81	2087.18	2190.78	
	2141.57	1994.54	2261.44	2544.42	1932.58	
	2322.28	2128.62	1902.69	2181.82	2118.46	
	2456.56	1486.29	2183.41	1681.14	2163.13	
	2475.09	2261.11	2174.45	2021.63	2136.19	
	2229.43	2204.73	1800.48	1969.63	1927.78	
Mean	1584.71	1566.96	2147.35	1872.18	1743.50	
SD	2238.97	2003.97	2021.59	2130.68	2053.71	2077.76
mode	273.52	387.64	181.27	285.27	172.52	269.70
	2236.97	2003.97	2021.50	2087.18	2053.71	2132.41

Cat 9	149.43	230.32	131.69	79.30	139.46	
DSH	157.79	111.37	97.23	99.62	157.59	
1.9 years	73.72	221.35	168.95	165.95	139.07	
MN	139.07	138.07	102.01	208.60	214.97	
	73.92	164.57	164.77	122.13	141.26	
	175.72	101.01	127.11	51.60	229.52	
	102.21	171.54	143.85	129.30	94.44	
	102.41	110.87	78.49	132.29	124.72	
	100.02	83.46	173.13	157.99	97.23	
	160.58	137.07	111.57	170.11	105.40	
Mean	120.60	137.60	129.79	138.51	144.89	135.46
SD	38.45	42.82	34.52	46.30	46.77	42.33
mode	102.41	137.07	127.11	132.29	139.07	134.68

Cat 10	2176.43	1691.50	2077.22	1713.22	3364.27	
DSH	2380.45	1878.98	2139.38	1922.41	2506.37	
16 years	2104.51	2279.04	2368.50	2004.90	2126.63	
MN	2268.88	2355.15	2320.29	2005.89	2498.40	
	2316.30	2001.51	2152.53	2393.01	1925.00	
	2172.85	2173.65	2266.29	2018.05	2408.35	
	2042.55	1955.29	2193.57	2110.89	2013.66	
	2267.39	2236.21	2080.21	2380.26	1920.82	
	2127.43	1757.65	2218.08	2263.09	2177.49	
	2168.67	2250.55	1844.51	2133.40	2023.63	
Mean	2205.50	2098.67	2175.93	2139.09	2177.81	2163.95
SD	109.27	306.30	153.73	173.76	236.42	257.58
mode	2172.85	2098.67	2175.93	2110.89	2126.63	2160.60

Appendix J. Scripts used in image analysis studies.

Script 1. 400 x 400 pixel box.

```
macro 'box 400 x 400';
{Produces a centrally located box 400 x 400 pixels. Copies selection to new window.}

var
  w,h:integer;
begin
  GetPicSize(w,h);
  MakeRoi((w-400)/2,(h-400)/2, 400, 400);
copy;
paste;
end;
```

Script 2. 200 x 200 pixel box.

```
macro 'box 200 x 200';
{Creates a 200x200 selection centred on the image. Copies selection to new window.}
var
  w,h:integer;
begin
  GetPicSize(w,h);
  MakeRoi((w-200)/2,(h-200)/2, 200, 200);
copy;
paste;
end;
```

Script 3. Count bacteria

```
.macro 'count bacteria';
{Opens user define ROI. Copies selection to new window.
Opens threshold plugin. Find objects gives count. Opens object classification.
Open dialog box with result. Closes all windows}

begin
GetUserRoi('Outline the area to add to the object set', true);
copy;
close;
paste;
PlugIn('C:\\Program Files\\UTHSCSA\\ImageTool\\Plug-Ins\\bilevel');
FindObjects;
NextWindow;
PlugIn('C:\\Program Files\\UTHSCSA\\ImageTool\\Plug-Ins\\objclass.dll');
ShowMessage('count is: ', GetResults('count', 0));
CloseAll;
end;
```

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