Clinical, microscopic and microbial characterization of exfoliative superficial pyoderm-associated epidermal collarettes in dogs

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Background – The microscopic and microbial features of the spreading epidermal collarettes of canine exfoliative superficial pyodermas are poorly characterized.

Objectives – To characterize the clinical, cytological, microbial and histopathological features of epidermal collarettes in five dogs.

Results – Cytology from the margins of collarettes identified neutrophils, extracellular and intracellular cocci within neutrophils but no acantholytic keratinocytes. Phenotypic and genotypic analyses identified all bacterial isolates from the centre and margin of five epidermal collarettes as Staphylococcus pseudointermedius. PCRs of collarette-associated Staphylococcus strains did not amplify genes encoding for the known exfoliative toxins expA and expB, whereas the predicted siet and speta amplification products were detected in all isolates.

Microscopically, epidermal collarettes consisted of interfollicular, epidermal spongiotic pustules. Advancing edges of lesions consisted of peripheral intracorneal clefts in the deep stratum disjunctum above an intact stratum compactum; they contained lytic neutrophil debris, bacterial cocci and fluid, but no acantholytic keratinocytes. This intracorneal location of bacteria was confirmed using Gram stains and fluorescent in situ hybridization with eubacterial- and Staphylococcus-specific probes. The indirect immunofluorescence staining patterns of desmoglein-1, desmocollin-1, claudin-1, E-cadherin and corneodesmosin were discontinuous and patchy in areas of spongiotic pustules, whereas only that of corneodesmosin was weaker and patchy in advancing collarette edges.

Conclusion – Epidermal collarettes represent unique clinical and histological lesions of exfoliative superficial pyoderma that are distinct from those of impetigo and superficial bacterial folliculitis. The characterization of possible causative staphylococcal exfoliatin proteases and the role of corneodesmosin in collarette pathogenesis deserve further investigation.

Introduction

Staphylococcus (S.) aureus is an important pathogen of humans that causes – in addition to skin diseases of other phenotypes – two blistering dermatoses: bullous impetigo (BI) and the staphylococcal scalded skin syndrome (SSSS).1 Both conditions share a common pathogenesis that involves the proteolytic cleavage of the extracellular segment of human desmoglein 1 (DSG1) by S. aureus exfoliatin toxins A, B or D (ETA, ETB, ETD); these entities differ mainly in the site of toxin secretion and extent of skin damage.2-4 In BI, a common skin infection of children, the local production of exfoliatin toxin produces small vesicles that enlarge rapidly into superficial flaccid bullae filled with a cloudy fluid and surrounded by an erythematosus rim. These bullae rupture easily, leaving shiny erosions with scale-crusts.1 The SSSS is a generalized exfoliative dermatosis that most commonly affects newborns, young children or sometimes adults with immunosuppression or renal failure.1 In this syndrome, an extracutaneous infection (of the pharynx, umbilicus, nose, ear or conjunctiva) with exfoliatin-producing staphylococci results in high levels of these toxins in the circulation, and this leads to sloughing of the epidermis that exfoliates in large sheets overlying widespread erosions.1 Histologically, both diseases share a similar intraepidermal cleavage beneath or within the stratum granulosum, with the difference being that the remainder of epidermis in SSSS appears normal, whereas in BI blisters are filled with neutrophils and inflammation is present in the dermis.5

Accepted 11 May 2016
This article is based on a Supporting original Study presented at the 8th World Congress of Veterinary Dermatology held May 2016 in Bordeaux, France.
Sources of Funding: This study was self-funded.
Conflict of Interest: No conflicts of interest have been declared.
In dogs, there is increasing evidence suggesting that some lesions of superficial bacterial skin infections are caused by S. pseudintermedius producing exfoliative toxins. Although a first attempt failed to detect in vitro exfoliative toxin production from staphylococci historically classified as S. intermedeus in dogs, a 30 kDa S. intermedeus exfoliatin toxin (SIET) has been isolated. Subcutaneous injections of purified SIET into the skin of two dogs reportedly induced lesions with a positive Nikolsky sign and secondary crusting after 40 h, but the level of epidermal cleavage was not described or histologically confirmed. In contrast to these findings, recombinant SIET has been shown not to cause intraepidermal splitting when injected in canine skin; SIET also was found to be unable to digest canine DSG1 and DSG3. At this time, whether or not SIET is relevant to the pathogenesis of canine exfoliative superficial pyoderma (ESP) remains unknown.

A novel exfoliative toxin (EXI with “I” for “intermedeus”) was identified after screening 43 strains of S. pseudintermedius isolated from dogs with uncharacterized pyoderma using PCR with degenerate primers for consensus sequences of the exfoliative toxins of S. aureus (ETA, ETB, ETD) and S. hyicus (SHETB). Thereafter, EXI-negative S. pseudintermedius strains were isolated from a pustule of a miniature dachshund diagnosed with BI, and were found to have a gene encoding another protein that resembled EXI. The S. pseudintermedius exfoliative toxin EXI was then renamed EXPA (exfoliatin of S. pseudintermedius type A) and the novel protein was named EXPB (exfoliatin of S. pseudintermedius type B). Results from this study also showed that both EXPA and EXPB could digest canine DSG1 and cause subcorneal splits in the epidermis when injected in mice, characteristics that are likely relevant to the pathogenesis of intraepidermal splitting in canine impetigo.

Whereas the clinical and histological characteristics of impetigo and superficial bacterial folliculitis have long been described in dogs, those of exfoliative forms of superficial staphylococcal infections have not been studied in depth. The clinical lesions of epidermal collarettes – expanding coalescing erythematous rings with peripheral peeling – were first described as a lesional stage of superficial bacterial folliculitis. The term “superficial spreading pyoderma” was used to describe a condition in a border collie with chronic, recurrent epidermal collarettes on the ventral abdomen with microscopic examination revealing superficial epidermal spongiotic pustules with lifting of the stratum corneum. It was later proposed that this condition be renamed “exfoliative superficial pyoderma” to better account for the exfoliative superficial feature of this entity and to differentiate it from a worsening bacterial folliculitis that would also qualify for the same “superficial spreading pyoderma” denomination. However, despite proposed changes in disease terminology, the characteristics of epidermal collarettes remain poorly characterized and it is still classified under the umbrella term of “superficial bacterial folliculitis.”

In order to test the hypothesis that ESP-associated epidermal collarettes represent a unique clinical phenotype, potentially due to superficial epidermal splitting by protease(s) that cleave superficially expressed keratinocyte adhesion molecule(s), our objectives were to characterize the clinical, cytological, microbial and histopathological features of epidermal collarettes that occur in canine ESP. Furthermore, the expression patterns of the principal superficial epidermal desmosomal and non-desmosomal adhesion proteins were evaluated in biopsies collected at the advancing edges of epidermal collarettes to detect any evidence of suspected toxin-related adhesion protein digestion.

**Material and methods**

**Study participants**

Dogs of any breed, body weight and sex with ESP-associated epidermal collarettes were selected for the study. Dogs with different stages (early, expanding) of epidermal collarettes were screened and dogs were selected if they had at least one actively expanding epidermal collarette, which developed within the preceding 2 days with an erythematous rim. Dogs with the more widespread phenotype of ESP, which is characterized by regional and/or generalized erythema with scaling composed of large sheets of stratum corneum, were excluded. To be selected, dogs were not being currently treated with antibacterial shampoos, systemic and/or topical antibacterial agents. Withdrawal times from anti-inflammatory medications were 4 weeks for topical (skin and ear) and oral glucocorticoids and 8 weeks for injectable glucocorticoids and oral ciclosporin. The study was preapproved by the Institutional Animal Care and Use Committee, and all owners were provided a written informed consent.

**Skin cytology, bacterial cultures and susceptibility testing**

Two samples were obtained using a nonsterile cotton-tipped applicator from the leading edge (margin) and the centre of expanding epidermal collarettes. All slides were heat-fixed and stained with Diff–Quick (Fisher Scientific; Pittsburgh, PA, USA) and Gram (Becton–Dickinson and Company; Sparks, MD, USA) stains. Each slide was evaluated in its entirety, by the same investigator (FB), using a subjective, semiquantitative method at high power (×100). The presence of neutrophilic granulocytes, lymphocytes, nonlymphocyte mononuclear cells (Langerhans cells, macrophages), eosinophils, cocci bacteria (free or intracytoplastic in neutrophils), other bacteria and acantholytic keratinocytes was graded 0–4+. The Gram-stained slides were evaluated for the presence and types of bacteria, and these were graded as above. Sterile culture swabs were taken from sites different from those of cytology at the leading edge and the centre of expanding epidermal collarettes. The swabs were inoculated onto Columbia agar with 5% sheep blood (Fisher Scientific) using the four quadrants technique and incubated at 35°C for 18–24 h. All isolates were identified phenotypically, biochemically and through PCR amplification of hspa80 and nuc gene, as described previously.

Antimicrobial susceptibility by the broth microdilution method was performed using an automated system (Sensititre, Trek Diagnostic Systems; Cleveland, OH, USA) according to the Clinical and Laboratory Standards Institute guidelines for minimum inhibitory concentration testing. Metillin resistance was confirmed by PCR amplification of mecA, the gene conferring metillin-resistance. All S. pseudintermedius isolates were stored frozen (−80°C) in tryptic soy broth containing 15% sterile glycerol until further testing was performed.

**Genotypic relatedness of isolated staphylococcal strains from epidermal collarettes**

Preparation of bacterial genomic DNA, digestion by Smal and pulse field gel electrophoresis (PFGE) were performed according to methods modified from the Centers for Disease Control (Atlanta, GA, USA) methods. Similarity coefficients were calculated and dendrograms constructed using the Dice coefficient and unweighted pair median method.

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group method with arithmetic means, respectively, with an optimization value of 1.0% and a position tolerance of 0.65%. Isolates were considered to be of similar genotypes if PFGE band patterns were identical. Genotypes with grouping above 80% on the dendrogram (BioNumerics 6.0, Applied Maths Inc.; Austin, TX, USA) were considered members of the same genotype group or cluster of similarity.21

**Detection of S. pseudintermedius exfoliative toxin genes**

The presence of *S. pseudintermedius* exfoliative toxin genes *expa*, *expb* and *siet* in the isolated staphylococcal strains was determined by PCR using previously described primers.8,9 In addition, bacterial strains were analysed for the presence of a novel suspected *S. pseudintermedius* exfoliative toxin SPETA using the published sequence (Table S1 in Supporting information).22 Ten frozen *S. pseudintermedius* isolates collected from pustules of dogs with superficial bacterial folliculitis served as controls, as previously suggested.10 Frozen isolates were revived from the glycerol stock, thawed and inoculated into Columbia agar with 5% sheep blood agar and incubated at 35°C for 18–24 h. Template DNA was prepared by a simple and rapid boiling procedure.23 The PCR assays were performed as described previously,31 annealing temperatures were chosen depending on the primer set used (Table S1 in Supporting information). All PCR products were resolved by electrophoresis through a 1.2% (w/v) agarose gel and visualized by the application of the SYBR safe DNA gel stain (Fischer Scientific). The PCR products of predicted size from positive isolates were purified using QIAquick PCR Purification Kit (Qiagen Inc.; Valencia, CA, USA) and sequenced. The sequences were aligned using the ABI Sequencing analysis software, with contiguous sequences matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) and positively identified if there was ≥98% sequence similarity with a reference sequence.

**Biopsy samples for histopathology, fluorescent in situ hybridization (FISH) and immunomapping**

All dogs were sedated with medetomidine (Domitor, Zoetis; Florham Park, NJ, USA) intravenously. An injection of 1 mL of lidocaine hydrochloride 2% (Hospira Inc., Lake Forest, IL, USA) was administered subcutaneously to provide additional local anaesthesia. One 8 mm skin biopsy sample was taken from the leading edge of epidermal collarettes and bisected: one half was placed in 10% neutral buffered formalin for paraffin embedding and routine histopathology, mal collarettes and bisected: one half was placed in 10% neutral buffered formalin for paraffin embedding and routine histopathology, the other half was stored at −80°C for subsequent subtyping and genotyping. For PFGE, the DNA from the frozen isolates was isolated using a modified method of phenol/chloroform extraction and was digested with a restriction enzyme selected to create fragments of approximately 500–2000 bp. Digests were mixed with electrophoresis loading buffer and resolved by electrophoresis through a 1% (w/v) agarose gel, and visualized by the application of the SYBR safe DNA gel stain (Fischer Scientific). The PFGE products of predicted size from positive isolates were purified using QIAquick PCR Purification Kit (Qiagen Inc.; Valencia, CA, USA) and sequenced. The sequences were aligned using the ABI Sequencing analysis software, with contiguous sequences matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) and positively identified if there was ≥98% sequence similarity with a reference sequence.

**Results**

**Study subjects and skin cytology**

Five dogs of different breed, age and weight met the inclusion criteria (Table 1). All dogs presented with widespread expanding epidermal collarettes (Figure 1), ranging in size from 1.5 to 4 cm and which were distributed on the lateral thorax, axilla, ventral abdomen and inguinal areas. In three dogs (cases 1, 2 and 5), additional lesions of early epidermal collarettes were present (Figure 1b). All collarettes sampled had an active erythematous rim with a peripheral scaling/crusting (Figure 1). Focal, follicle-centred pustules, pustules and crusts, representing typical clinical lesions of superficial bacterial folliculitis, were not observed in any dogs. Detailed cytology results are presented in Table 1. Neutrophils, as well as extracellular and intracellular cocci within neutrophils, were identified from marginal cytology samples in all dogs. Acantholytic keratinocytes were not seen, but occasional non-acantholytic keratinocytes were found in marginal samples of dogs 2 and 3. Gram stains of cytology swab samples taken from the margin of epidermal collarettes revealed Gram-positive cocci in all dogs.

**Bacterial culture, susceptibility testing and genotypic relatedness**

Phenotypic, biochemical and genotypic analysis identified all bacterial isolates from the centre and margin of epidermal collarettes as *S. pseudintermedius*. The degree of bacterial growth between marginal and centre samples differed in three dogs (cases 1, 4 and 5), being more abundant when sampled at the margins of epidermal collarette (Table S2 in Supporting information). There were no differences in the antimicrobial susceptibility profiles between bacterial isolates collected from the margin or the centre of an epidermal collarette from the same dog (Table S2 in Supporting information). Meticillin resistance was
### Table 1. Signalment, skin cytology results and sampling sites (number of biopsies) for adhesion molecule immunomapping of five dogs with epidermal collarettes.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Breed, age, sex</th>
<th>Skin cytology</th>
<th>Immunomapping of adhesion molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diff-Quick stain</td>
<td>Gram stain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Margin</td>
<td>Centre</td>
</tr>
<tr>
<td>Case 1</td>
<td>Dachshund, 1.5 years, mc</td>
<td>2+ neutrophils, 1+ cocci (intra- and extracellular)</td>
<td>None</td>
</tr>
<tr>
<td>Case 2</td>
<td>Hound, 10 years, mc</td>
<td>2+ neutrophils, 2+ cocci (intra- and extracellular), 1+ non-acantholytic keratinocytes</td>
<td>None</td>
</tr>
<tr>
<td>Case 3</td>
<td>American Staffordshire terrier, 5 years, mc</td>
<td>1+ neutrophils, 1+ cocci (intra- and extracellular), 1+ non-acantholytic keratinocytes</td>
<td>1+ non-acantholytic keratinocytes</td>
</tr>
<tr>
<td>Case 4</td>
<td>Golden retriever, 5 years, mc</td>
<td>1+ neutrophils, 1+ intracellular cocci</td>
<td>None</td>
</tr>
<tr>
<td>Case 5</td>
<td>Irish setter, 9 years, mc</td>
<td>1+ neutrophils, 1+ cocci (intra and extracellular)</td>
<td>None</td>
</tr>
</tbody>
</table>

mc, male castrated.

The Diff-Quik® cytology findings using subjective semiquantitative method (0–4+) and presence of bacteria on Gram stains from margin and centre of epidermal collarettes are described.

determined in isolates from two dogs and the presence of mecA gene was confirmed in these two isolates (cases 1 and 5). Marginal and centre-collected ESP bacterial isolates from three dogs (cases 1, 4 and 5) were defined as multidrug resistant according to definitions proposed for *S. aureus*. Six bacterial isolates collected at the margin and centre of epidermal collarettes from three dogs (cases 2, 3 and 5) were available for PFGE analysis; the remaining four bacterial isolates (cases 1 and 4) were not available for testing. According to PFGE analysis, marginal and centre-collected ESP bacterial isolates from the same dog had identical band patterns suggesting that they were identical isolates. Furthermore, isolates from cases 2 and 3 belonged to the same genotype (Figure S1 in Supporting information).

Detection of *S. pseudintermedius* exfoliative toxins

Six bacterial isolates from three dogs (case 2, 3 and 5) were available for PCR analysis. The analysis of these isolates showed an absence of exfoliative toxin genes expa and expb, whereas amplicons corresponding to the predicted sizes of siet and speta products were present in all isolates. These exfoliative toxin genes were also detectable in the ten control *Staphylococcus* isolates from superficial bacterial folliculitis: expa (3/10; 30%), expb (1/10; 10%), siet (8/10; 80%) and speta (9/10; 90%). Sequencing of the amplicons from epidermal collarette and control isolates of exfoliative toxin genes expa, expb, siet and speta confirmed the 100% sequence identity when compared with the corresponding published gene sequence.

Histopathology

A total of nine skin lesions (six expanding and three early collarettes) from five dogs were evaluated in 45 histological sections. At the outer leading edge of epidermal collarettes, large laminar epidermal clefts (eight of nine; 89%) occurred in the deep stratum disjunctum, just above the stratum compactum (Figures 2 and 3). The roof of clefts had a normal basket-weave pattern of cornification (eight of eight; 100%) that centrally often appeared thinner than normal due to the release of individualized corneocytes from its inner surface. At the cleft base, a thin layer of stratum disjunctum was present or, more often, the exposed surface of the stratum compactum was observed, which was mostly intact. Near the leading cleft margin, the stratum compactum was disrupted multifocally by discrete small areas of superficial corneocyte swelling and pallor (six of eight; 75%), whereas occasional full-thickness loss of stratum compactum was observed for only a few corneocytes. In more central areas, along the cleft base, very small parakeratotic foci (seven of eight; 88%) and/or mild lamination and thickening of the stratum compactum were seen.

The epidermis underlying the leading edge of clefts was minimally altered, and only mild epidermal hyperplasia and occasional individual lymphocytic exocytosis occurred (Figure 2a). The epidermis just behind the peripheral cleft margin contained multifocal, discrete, epidermal spongiosic foci with neutrophil exocytosis (five of eight; 63%) that progressed to spongiosic pustules (Figure 2b,c). Above these spongiosic foci, the stratum compactum disintegrated into individual corneocytes, and neutrophils transmigrated to the cleft lumen and spilled onto the cleft floor along with fluid (Figure 2c). More centrally, small crusts formed on the cleft floor from resolving neutrophilic spongiosic pustules (seven of eight; 88%), often still under an intact cleft roof formed by the upper stratum disjunctum layers. Interestingly, acantholytic keratinocytes were not observed (zero of eight; 0%) and acantholytic epidermal pustules were not a feature in any skin sections.

The lumen of stratum corneum clefts at the expanding collarette margin contained either only bacteria and fluid, or lytic neutrophil debris and fluid with bacteria (Figure 3a,b). Cocci were present individually and/or in clusters in clefts (seven of eight; 88%) (Figure 3b), either free...
or closely apposed to the cleft base, cleft roof or individualized corneocytes. Gram stains and FISH (Figure 4), the latter performed with both eubacterial and *Staphylococcus* probes, confirmed *Staphylococci* in the leading cleft. Gram-positive cocci were also observed in spongiotic foci and focal crusts (six of eight; 75%) and sometimes closely apposed to the cleft base or roof, more centrally. A few clefts had no visible content at their margin, perhaps lost from aging of lesions or from tissue processing.

In the dermis, superficial perivascular dermatitis (nine of nine; 100%) was generally moderate centrally, mild or absent under the most peripheral epidermal cleft and included lymphocytes, plasma cells, neutrophils and eosinophils. Neutrophils were most numerous under neutrophilic spongiotic foci in the epidermis. Neutrophils were not observed in the epidermis below the leading edge of the epidermal cleft (eight of eight; 100%) and generally did not extend in the dermis beyond the margin of the epidermal cleft. A mild eosinophilic dermal infiltrate was common (nine of nine; 100%) and mild exocytosis and mild eosinophilic micropustules were occasional in the epidermis of more central collarette areas of biopsies. Dermal oedema, fibrin exudation and haemorrhage were most often mild or absent in the superficial dermis. Hair follicles were generally not affected and extension of epidermal clefts into hair follicle infundibula (two of nine; 22%) was uncommon and mild. Neutrophilic folliculitis (zero of nine; 0%) was not observed. A single follicle in the central collarette area had mild luminal eosinophilic folliculitis (one of nine; 11%). Biopsy sections contained few hair follicles and typically occasional mildly atrophic follicles, lacking a hair shaft, were present with a few anagen and/or telogen follicles.

Figure 1. Canine epidermal collarettes – clinical lesions. (a,c,d) Annular to polycyclic epidermal collarettes with erythema, lifting and peeling of stratum corneum, and partial alopecia confined to the centre of the lesions (a,c – Case 3; d – Case 1). (b) Early epidermal collarettes were characterized by central flattened crust with erythematous leading edge and lifting of peripheral stratum corneum (arrowheads) (Case 3).
Immunomapping of adhesion molecules

Immunomapping was performed on three biopsies collected from early collarettes and seven biopsies from larger expanding lesions with staining of normal skin from each dog serving as a control. The expression patterns of DSG1, DSC1, CLDN1, CDH1 and CDSN were assessed for their continuity on the entire epidermis of each section with detailed examination of advancing intracorneal cleavage areas. The staining patterns of DSG1, DSC1, CLDN1, CDH1 and CDSN in control canine skin samples (normal skin) were identical to previously reported patterns for normal canine skin (data not shown). The skin tissues samples from the early and expanding epidermal collarettes contained normal (i.e. basket-weave) and stratum corneum separation sites, as they were taken at the margin of the lesions. The immunostainings for DSG1, DSC1, CLDN1, CDH1 and CDSN were discontinuous and patchy in areas of spongiotic pustules, whereas only that of CDSN was weaker and patchy in advancing collarette edges (Figure 5).

Discussion

Two often overlapping phenotypes of ESP are proposed to exist in dogs: the first, which is more common, is characterized by skin lesions of rapidly expanding epidermal...
collarettes that can coalesce to cover large areas with a polycyclic pattern, whereas the second type consists of an acute onset of regional or generalized erythema with overlying scaling composed of large sheets of stratum corneum.\textsuperscript{11,14} The present study establishes the clinical, cytological, microbial and histopathological features of ESP-associated epidermal collarettes in five dogs. In these dogs, epidermal collarettes featured an erythematous leading edge where the stratum corneum lifted and peeled in a round, oval to polycyclic pattern with partial alopecia confined to the centre of the lesions. Hyperpigmentation, presumed post inflammatory, in the centre of epidermal collarettes is sometimes a sequel of aging lesions, but it was not seen in this study due to their acute onset, which was a criterion for inclusion. Although concurrent signs of bacterial folliculitis were not detected in any dogs, ESP-associated epidermal collarettes anecdotally have been mentioned to co-exist in some dogs with superficial bacterial folliculitis.\textsuperscript{13}

The phenotypic and genotypic analysis of bacterial cultures from the leading edge and centre of ESP-associated epidermal collarettes identified \textit{S. pseudintermedius} as the main causative agent of epidermal collarettes in dogs, which is similar to previous results.\textsuperscript{13,27} The identical antibiotic susceptibility pattern and PFGE results of bacterial isolates collected from leading edges and centres of epidermal collarettes suggested that each lesion was caused by a single strain of \textit{S. pseudintermedius}. The sampling method used in this study yielded higher bacterial growth on blood agar when sterile swabs were rolled gently over erosions underneath the stratum corneum of clefts at the leading edge than from sampling the centre of collarettes. This finding corresponds to the detection of a high number of bacteria in the superficial stratum corneum at the leading edge of skin sections using cytology, Gram stains and FISH. The appropriateness of sampling methods for ESP-associated epidermal collarettes has received only limited scrutiny. One study recommended no surface disinfection before bacterial isolation and rolling a sterile swab three to four times across the epidermal collarettes, which resulted in four negative bacterial cultures from 22 dogs with epidermal collarettes.\textsuperscript{27} In contrast, the results of our study, which included the identification of bacteria in skin sections using routine and specialized histological techniques, support a sampling method underneath the scales/crusts at their leading edge for future studies on epidermal collarettes. Furthermore, given the findings reported in an abstract that different staphylococcal strains may be associated with superficial pyoderma lesions in the same dog,\textsuperscript{28} an investigation using PFGE analysis is necessary to compare the genetic relatedness of isolates across multiple epidermal collarettes in order to understand if all epidermal collarettes on the same animal are caused by the same bacterial strain. This information about clonal relationship may have a crucial impact for therapeutic effectiveness, because any possible variability in the staphylococcal strains between epidermal collarettes

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Exfoliative superficial pyoderma (ESP) in a dog. Photomicrographs of Case 2 (a) and Case 3 (b). (a) A peripheral intracorneal cleft in the deep stratum disjunctum contains bacteria (box insert, arrowheads) floating in fluid and lytic neutrophilic debris. (b) Some peripheral clefts contained bacteria (arrow) and only a scant amount of fluid. Below some clefts, thinning and discontinuity of the stratum compactum (arrowheads) is associated with swelling, pallor and separation of corneocytes (between arrow heads). Haematoxylin and eosin. 60×.}
\end{figure}

\begin{table}
\centering
\caption{Clinical and Histological Features of ESP-Associated Epidermal Collarettes in Dogs}
\begin{tabular}{|l|l|}
\hline
Feature & Description \\
\hline
Clinical & Acute onset of regional or generalized erythema with overlying scaling
\begin{itemize}
\item Stratum corneum lifts and peels in a round, oval to polycyclic pattern
\item Partial alopecia confined to the centre of the lesions
\end{itemize}
\hline
Cytological & Erythematous leading edge with bacteria floating in fluid and lytic neutrophilic debris
\hline
Microbial & \begin{itemize}
\item \textit{S. pseudintermedius} identified
\item Identical antibiotic susceptibility pattern
\item Identical PFGE results
\end{itemize}
\hline
Histopathological & \begin{itemize}
\item Thinning and discontinuity of stratum compactum
\item Swelling, pallor and separation of corneocytes
\end{itemize}
\hline
\end{tabular}
\end{table}
potentially could result in variable antibiotic susceptibility patterns.

It has been hypothesized that ESP-associated epidermal collarettes could be caused by exfoliative toxins of *S. pseudintermedius* similar to those of *S. hyicus* in piglets. In our study, PCR results for collarette-associated *Staphylococcus* strains showed a lack of amplification of exfoliative toxin genes *expa* and *expb*, whereas amplicons corresponding to predicted sizes of *siet* and *speta* products were present in all isolates. Our results

are consistent with a previous study that reported only one isolate of EXPB-producing S. pseudintermedius among 13 isolates from dogs with scales/epidermal collarettes. In the present study, the prevalence of expa and expb amplicons in control strains from bacterial folliculitis ranged from 10 to 30%, as described previously. Although several collarette- and superficial folliculitis-associated Staphylococcus strains were positive for the proposed exfoliative toxins SIET and SPETA in our study, their role in the pathogenesis of epidermal collarettes remains unknown. The putative toxin SIET shares no homology in amino acid sequence with other known exfoliative toxins and it does not contain the typical catalytic triad, the active site of serine exfoliatin proteases. Further investigation revealed a lack of epidermal changes after intradermal injection of recombinant SIET into canine skin, emphasizing the controversy existing on the true role of SIET in canine pyoderma. A novel toxin, designated SPETA, was discovered after whole-genome sequencing of S. pseudintermedius ED99; an exfoliative role for this toxin was presumed based on the high amino acid similarity to the exfoliatin SHETA from S. hyicus. Because the functionality testing of recombinant SPETA has not been reported and as acantholytic keratinocytes were not seen in any skin sections in our study, future studies are warranted to determine if SPETA is a truly exfoliative toxin.

Microscopic examination of the leading edge of epidermal collarette-associated ESP skin lesions identified early epidermal separation in the deep stratum disjunctum, a few corneocyte layers above the stratum compactum, and it was similar to images reported previously. Epidermal clefts appeared to expand through minimally altered epidermis, consistent with the content of the cleft itself causing the peripheral extension of epidermal separation. Gram-positive cocci, lytic neutrophils and small amounts of fluid were all found in the leading cleft; any combination of these could promote epidermal separation. Although historically regarded as nonmotile organism, S. aureus aggregates have recently been shown to have active spreading behaviour under certain conditions, which resembles the so called bacterial gliding motility. This may explain cleft extension in several skin sections where only clusters of cocci bacteria without neutrophils were revealed.

Microscopic spongiotic pustules were located from centrally to the leading cleft edge, and these were therefore unlikely to directly induce epidermal separation and cleft extension; however, they could contribute to the extension of epidermal clefts by supplying fluid and/or neutrophils to the leading cleft edge. Interestingly, presumed older epidermal clefts usually were observed at the interface of the stratum disjunctum and the stratum compactum, a slightly deeper epidermal position than early clefts at the very margin of lesions. Additionally, thinning of the cleft roof was observed. Both of these observations were attributed to lesion aging and keratinocyte desquamation from the exposed inner surfaces of the cleft. The common occurrence of individualized corneocytes in the cleft lumen supports this possibility. Individualized corneocytes were also observed at foci of disintegrating stratum compactum, just above spongiotic pustules, which suggests a possible role for neutrophil enzymes, and/or fluid mediators in corneocyte separation.

**Figure 5.** Immunostaining of frozen skin sections for corneodesmosin (CDSN). (a) The expression of CDSN can be seen as a thick line (arrowheads) in the lower stratum corneum of positive control unaffected skin sections (Case 3). (b) Negative control immunostaining reveals blue DAPI positive-stained paired coccoid bacteria within the cleft of stratum corneum (Case 5). (c,d) The immunostaining of epidermal collarettes for CDSN is discontinuous in areas of spongiotic pustules, whereas in leading collarette edges CDSN staining was weaker and patchy (bottom centre). In contrast, a normal CDSN immunostaining pattern was found beyond the collarette’s leading edge (arrowheads) (Case 5). (e,f) Abrupt interruption of normal CDSN immunostaining (arrowheads) was found in the leading collarette edge (Case 3). Immunofluorescence for CDSN with Alexa 488 secondary antibody (green). DAPI-stained nuclei are blue. a,c,e: 10×, b,d,f: 20×.

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Neither keratinocyte acantholysis nor the formation of acantholytic pustules were observed in ESP-associated epidermal collarettes, despite the close proximity of cocci to breaks in the stratum compactum over spongiotic pustules and exposed stratum granulosum keratinocytes. Therefore, based on morphology, S. pseudintermedius involved in epidermal collarettes should not be expected to produce DSG1-digesting exfoliative toxins thought to induce keratinocyte acantholysis and pustules in other forms of superficial pyoderma such as impetigo. In the current study, dogs lacked clinical evidence of bacterial folliculitis. Histologically, epidermal clefts did not involve hair follicles significantly and neutrophilic luminal folliculitis was not observed. Therefore, ESP lesions can occur in the absence of bacterial folliculitis and folliculitis is unlikely to be a key mechanism underlying ESP epidermal collarette lesion induction or spread, an association that had been suggested – but was not proven – in previous reports. In our study, mild follicular atrophy/inactivity with hair shaft shedding might be the mechanism for alopecia described for some ESP lesions; however, changes were mild in this study and biopsies did not include the centres of lesions and therefore may not fully include typical follicular changes. Another explanation for alopecia may include the digestive activity of bacterial and/or neutrophilic proteases targeting the adhesion molecules between hair shafts and hair follicles. Indeed, the loss of CDSN normally present in the hair follicle inner root sheath leads to alopecia in mice. Indirect immunofluorescence revealed only anomalies in CDSN staining at the leading edge of epidermal collarettes in our dogs. Interestingly, an extracellular S. aureus enzyme, commonly referred to as the V8 protease, impairs the epidermal barrier permeability and causes stratum corneum structural disturbance in mice. Analysis of the stratum corneum by transmission electron microscopy revealed that the V8 protease induced the loss of corneodesmosome integrity and resulted in disturbance of corneocyte cohesion. As S. aureus V8 protease shows a sequence similarity to that of other “classic” exfoliative toxins, the epidermal barrier disruption after V8 protease application to the skin could be due to its enzymatic cleavage of corneodesmosomes in stratum corneum. The exfoliative toxin EXPMA from S. pseudintermedius shares homology (33% amino acid identity) to the staphylococcal V8 protease, however, recent whole-genome sequencing of a single strain of S. pseudintermedius ED99 isolated from superficial bacterial pyoderma lesions (likely to be pustules) did not reveal the presence of a V8 protease as in S. aureus. Further studies involving whole-genome sequencing of collagen-associated Staphylococcus strains and sequence alignment to previously published exfoliative toxins, including V8 protease, are needed to reveal new proteases that might be involved in the pathogenesis of epidermal collarettes in dogs.

In conclusion, the results of this study indicate that epidermal collarettes represent unique clinical and histological lesions of ESP that are distinct from those of impetigo and superficial bacterial folliculitis. Strains of S. pseudintermedius – presumably secreting yet unknown toxin(s) – with or without lytic neutrophils spread in fluid and disrupt corneodesmosomes in the deep stratum disjunctum, forming an epidermal cleft where the intact stratum corneum roof maintains hydration at the lesion margin. Desquamation of the cleft base, superficial injury to the stratum compactum and bacterial mediators induce the formation of peripheral neutrophilic spongiotic pustules that rupture through the stratum compactum to the cleft lumen and provide more fluid and neutrophils to the cleft, thereby enhancing the bacterial mediated extension of the cleft in a cyclical process. Centrally within the collarettes, rupture and opening of the epidermal cleft, along with epidermal barrier reparative responses, and upregulation of innate defence mechanisms, promote the drying and resolution of skin lesions. The mechanism underlying exfoliation and spreading of epidermal collarettes does not appear to be associated with EXPA, EXPB, SIET and SPETA exfoliative toxins of S. pseudintermedius; another mechanism that involves CDSN proteolysis and corneodesmosome separation is suspected to contribute to the exfoliation.

Acknowledgements
The authors acknowledge the help of Stanley Dunston, Lisa Mamo, Kathleen Hover, Megan Jacob and Kevin Anderson regarding bacterial storage, immunofluorescence staining, pulse field gel electrophoresis, polymerase chain reactions and sequencing.

References
Épidermiques collarettes de chiens

Résumé

Contexte – Les critères microcopiques et microbiens des collarettes épidermiques extensives des pyodermites superficielles exfoliatives canines sont faiblement caractérisés.

Objectifs – Caractériser les critères cliniques, cytologiques, microbiens et histopathologiques des collarettes épidermiques pour cinq chiens.

Résultats – La cytologie des marges des collarettes a identifié des neutrophiles, des cocci intracellulaires et extracellulaires mais pas de kératinocytes acantholytiques. Les analyses phénotypiques et génotypiques ont identifié toutes les souches bactériennes du centre et des marges de cinq collarettes épidermiques en tant que *Staphylococcus pseudintermedius*. Les PCR des souches de *Staphylococcus* associées aux collarettes n’ont pas permis l’amplification les gènes codant pour les toxines exfoliatives connues expA et expB, tandis que les produits d’amplification attendus, siet et speta, ont été détectés dans tous les isolats. Microcopiquement, les collarettes épidermiques consistaient en des pustules interfolliculaires, épidermiques, spongiosiques. Les données avancées des lésions consistaient en un clivage intracornéen périphérique que dans le stratum disjunctum profond sous un stratum compactum intact; ils contiennent des débris de neutrophiles lytiques, des bactéries de type cocci et des fluides mais pas de kératinocytes acantholytiques. Cette localisation intra cornéenne des bactériennes a été confirmée par coloration de Gram et hybridation fluorescente in situ avec des sondes spécifiques d’ubactérie et *Staphylococcus*. L’immunofluorescence indirecte des patrons de coloration de desmogérieine-1, desmocolline-1, Claudine-1, E-cadherine et cornéodésminose étaient discontinus et en patch dans les zones de pustules spongiosiques, tandis que les cornéodésminoses étaient plus faibles et en patch en périphérie des collarettes évolutées.

Conclusions – Les collarettes épidermiques représentent les lésions cliniques et histologiques uniques de pyodermites superficielles exfoliatives qui sont distinctes de l’impétigo et des pyodermites bactériennes superficielles. La caractérisation des protéines exfoliatives staphylococciques en cause et le rôle des cornéodésminoses dans la pathogenie des collarettes nécessite d’autres investigations.
Resumen

Introducción – Las características microscópicas y microbianas de los collaretes epidérmicos expansivos de las piodermas exfoliativas superficiales caninas están pobamente caracterizados.

Objetivos – Caracterizar las características clínicas, citológicas, microbianas e histopatológicas de collaretes epidérmicos en cinco perros.

Resultados – Citología de los márgenes de collaretes identificaron neutrófilos, cocos extracelulares e intra-
celulares dentro de los neutrófilos, pero no se observaron queratinocitos acantolíticos. Los análisis
fenotípico y genotípico identificaron todas las cepas bacterianas del centro y el margen de cinco collaretes
epidérmicos como Staphylococcus pseudintermedius. La PCR de cepas de Staphylococcus asociadas con
collaretes no amplificaron genes que codifican para las toxinas exfoliativa conocidas expA y expB, mientras
que los esperados amplicones siet y speta fueron detectados en todos los aislados. Microscópicamente,
los collaretes epidérmicos consistieron en pústulas interfoliculares epidérmicas espongiformes. Los bordes
externos de las lesiones consistían en hendiduras intracorneales periféricas en el estrato profundo disjunto
por encima de un estrato compacto intacto; contenían restos lípticos de neutrófilos, cocos bacterianos y
líquido, pero no había queratinocitos acantolíticos. Esta ubicación intracornal de bacterias se confirmó
usando tinciones de Gram y la hibridación in situ fluorescente con sondas específicas ebubacterianas y de
Staphylococcus. Los patrones de tinción de inmunofluorescencia indirecta para desmogleína-1, desmocó-
liga-1, claudina-1, E-cadherina y corneodesmosina fueron discontinuos y multifocales en áreas de pústulas
espongiformes, mientras que sólo la corneodesmosina fue más débil y multifocal en los bordes de los colla-
retes.

Conclusion – los collaretes epidérmicos representan lesiones clínicas e histológicas únicas de piodermas
superficiales exfoliativas que son distintos de los de impétigo y folliculitis bacteriana superficial. La caracte-
rización de posibles proteasas exfoliativas estafilocócicas causales y el papel de corneodesmosina en la
patogénesis de los collaretes merecen investigación adicional.

Zusammenfassung

Hintergrund – Die mikroskopischen und mikrobiellen Charakteristika von sich ausbreitenden epidermalen
Colaretten der exfoliativen oberflächlichen Pyodermie des Hundes sind nur ungenau beschrieben.

Ziele – Eine Charakterisierung der klinischen, zytologischen, mikrobiellen und histopathologischen Merk-
male von epidermalen Colaretten bei fünf Hunden.

Ergebnisse – Die Zytologie von den Rändern der Colaretten zeigte Neutrophile, extrazelluläre und intrazellu-
läre Kokken in den Neutrophilen, aber keine akantholytischen Keratinozyten. Mittels phänotypischer und
genotypischer Analyse wurden alle bakteriellen Isolate aus dem Zentrum und dem Rand von fünf epider-
malen Colaretten als Staphylococcus pseudintermedius identifiziert. Mittels PCR konnten die bekannten
Gene der exfoliativen Toxine expA und expB nicht aus den Staphylococcus Stämmen der Colaretten ampli-
fiziert werden, während die prognostizierten siet und speta Amplifikationsprodukte in allen Isolaten gefun-
den wurden. Mikroskopisch bestanden die epidermalen Colaretten aus interfollikulären, epidermalen
spongösen Pusteln. Die fortgesetzten Ränder der Veränderungen bestanden aus peripheren intracorne-
aalen Spalten im tiefen Stratum disjunctum über einem intakten Stratum compactum; sie enthielten lyti-
sches neutrophiles Debris, bakterielle Kokken und Flüssigkeit, aber keine akantholytischen Keratinozyten.
Diese intracorneale Lokalisation der Bakterien wurde durch eine Gramfärbung sowie durch Fluoreszenz
in situ Hybridisierung mit ebubakteriellen und Staphylococcus-spezifischen Proben bestätigt. Das Färbever-
halten von Desmoglein-1, Desmocollin-1, Claudin-1, E-Cadherin und Corneodesmosomen mittels indirekter
Immunfluoreszenz war diskontinuierlich und fleckenhaft in den Gebieten mit spongösen Pusteln,
 während nur jene der Corneodesmosomen im Bereich der sich ausbreitenden Colarettenränder schwächer
und fleckhafter war.

Schlussfolgerung – Epidermale Colaretten repräsentieren einzigartige klinische und histologische
Veränderungen der exfoliativen superfiziellem Pyodermie, die sich deutlich von Impetigo und superfiziel-
er Bakterieller Follikulitis unterscheiden. Die Beschreibung von möglichen Staphylokokken Exfoliatin Pro-
teasen als Verursacher und die Rolle von Corneodesmosin bei der Pathogenese der Colaretten bedürfen
einer weiteren Untersuchung.

要約

背景 – イヌの表皮剥落性表在性皰皮症の拡大性表皮小環は類微鏡学的、微生物学的特微がほとんど明らかに
されていない。

目的 – 5症例のイヌにおける表皮小環の臨床学的、細胞診・微生物学的および病理組織学的特微を明らかにするこ
とである。

結果 – 表皮小環の辺縁からの細胞診で好中球と、好中球の細胞質および細胞内桿菌が認められたが、嫌酸性
性ケラチノサイトは認められなかった。表現型および遺伝型解析では、5例の表皮小環の中央および辺縁から分離さ
れたすべての桿菌がStaphylococcus pseudintermediusと特定された。表皮小環感性Staphylococcus菌株
のPCRでは表皮剥落毒素のexpAやexpBとして知られた遺伝子は增幅されなかったが、すべての分離菌において、

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表皮かいは毛包間、表皮総合状態物で
構成されている。癌病の非辺縁の尖端付近の上部、剥離層下部の位置に未梢性の角層内裂隙から構成され
ていた。裂隔には分解した中性球を含み、液体が含まれていたが、粒状体クラチノサートは含まれていなかっ
た。この細胞のくぼみの周辺はゲルマ染色および真正細菌特異的ブロッサムあるいはStaphylococcus特異的ブロッサムを
用いた免疫蛻光in situ hybridizationで確認された。デスモゲニン-1、デスモコリン-1、クラウディン-1、E-カドー
およびコルネオデスモシンの間接免疫蛻光染色パターンは表皮総合状態において不連続で斑点に認められた一
方、コルネオデスモシンのみが表皮かいの辺縁においてより強く斑点に認められた。

結論 — 表皮かいは表皮剥離病表在性黒色病のジニーな臨床および病理学的な変化を示しており、黒色病や表在
性細菌性毛包炎とは異なる特徴を示す。潜在的な原因となり得るアピソウ球菌のexfolatinプロテアーゼの特徴および
表皮かいの病因およびコルネオデスモシンの役割にさらなる調査が必要とされる。

摘要

背景 — 大鰭型または皮膚病病の表皮かい形態層、特に表皮かいと微細構造が特徴である。

目的 — 描述5犬の表皮かい形態層の臨床表現、細胞学、細胞生物学と組織病理学的特徴

結果 — 表皮かい形態層の細胞学的観察、表现で中性粒細胞、中性粒細胞外と細胞内芽胞、および細胞内松脂細胞
自ら表皮かい形態層の尖端で、その周囲層内裂隙に、特に表皮かいの尖端層には、細胞内芽胞を含む中性粒細胞の
形態、細胞内松脂細胞の見られることが観察された。表皮かいの細胞内芽胞を含む中性粒細胞外皮膚病細胞、細胞内芽胞は、細胞
内で、細胞内芽胞が見られることが観察された。表皮かいの細胞内芽胞を含む中性粒細胞外皮膚病、細胞内芽胞の
形態、細胞内松脂細胞の見られることが観察された。

総括 — 表皮かい形態層は、頸片状脱皮症の脱皮障害物、表皮かい形態層、特に表皮かいと細胞学的特徴を示す。

Resumo

Contexto — As características microscópicas e microbiológicas dos colarinhos epidérmicos em expansão,
as piodermias superficiais exfoliativas caninas, são pouco elucidadas.

Objetivos — Caracterizar as particularidades clínicas, citológicas, microbiológicas e histopatológicas
do boxe, em cinco caes.

Resultados — Nos cortes das margens dos colarinhos epidérmicos, foram identificados neutrofilos,
cocos extracelulares e intracelulares, dentro de neutrofilos, mas não foram observados queratinócitos
clonados. Provas fenotípicas e genotípicas identificaram todos os isolados bacterianos do centro e mar
gem de cinco colarinhos epidérmicos como Staphylococcus pseintermedius. A PCR de cepas de Staphyloc
occus associadas a colarinhos não amplificou os genes codificadores das toxinas exfoliativas conhecidas
exA e exB, enquanto que os produtos de amplificação preditos siet e sjeta foram detectados
em todos os isolados. Microscopicamente, os colarinhos epidérmicos consistiram-se em pápulas epidérmicas
esponjogênicas e interfoliáceas. As bordas em expansão das lesões consistiram em fissuras
periféricas intracéreos no estrato disjunctum profundo, sobre um estrato compactum intacto; nelas, esta
vam contidos debrís líticos de neutrofilos, bactérias cocóides e fluidos, mas não queratinócitos acantolí
icos. Esta localização intracelular das bactérias foi confirmada utilizando coloração de Gram e hibridização
in situ fluorescente com clones eucariontes e clones específicas para Staphylococcus. Os padrões de
depósito para desmogleina-1, desmocolina-1, claudina-1, E- caderina e caronezmosina, na imunofluo
tencência indireta, foram contínuas e irregulares em áreas de pápulas esponjogênicas, ao passo que,
apenas da caronezmosina foi mais fraco e irregular nas bordas dos colarinhos em expansão.

Conclusões — Colarinhos epidérmicos são lesões clínicas e histológicas singulares de piodermite superfi
cial exfoliativa que se diferem daquelas de impetigo e foliculite bacteriana superficial. A caracterização de
proteases exfoliativas estafilocócicas potencialmente causadoras e o papel da caronezmosina na patogênese
do colarinhos merecem maior investigação.

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