# Salivary Allergens of *Ctenocephalides felis*: Collection, Purification, and Evaluation by Intradermal Skin Testing in Dogs*

**Glenn R. Frank, DVM, PhD**
**Shirley W. Hunter, PhD**
**Lynda J. Wallenfels, BS**
*Heska Corporation*
*Fort Collins, Colorado*

**Kenneth W. Kwochka, DVM, Diplomate ACVD**
*Department of Veterinary Clinical Sciences*
*College of Veterinary Medicine*
*The Ohio State University*
*Columbus, Ohio*

## INTRODUCTION

Flea allergy dermatitis is the most common dermatologic disease of dogs. Management of allergic dogs consists of symptomatic treatment and flea control. Specific immunotherapy will rely on identification of allergens responsible for initiating the allergic response. It was hypothesized that the major allergens involved in this disease could be found in the saliva of fleas, and that a method to collect saliva would enable the identification of the specific allergens. Intradermal skin testing (IDST) of sensitized dogs could then be used to evaluate the collection methodologies and purified antigens separated from flea saliva.

## MATERIALS AND METHODS

A saliva collection apparatus was adapted from an existing artificial flea feeding system (Flea Data, Inc., Freeville, NY). The apparatus consisted of a flea housing with mesh on two sides that was contacted with a glass moisture chamber containing damp blotting pads (Figure 1). The flea housing was maintained at ambient temperatures, while the moisture chamber was maintained at 37°C. The flea housing and moisture chamber were separated by two membranes. The saliva collection membrane consisted of a Durapore™ (Millipore) membrane situated adjacent to the blotting pads in the moisture chamber. The membrane separating fleas from the saliva collection membrane consisted of stretched Parafilm™ (American National Can Company) and was used to prevent contamination of the saliva collection membrane with nonsaliva flea-related contaminants. Freshly emerged *Ctenocephalides felis* were placed in the housing and allowed to probe through the Parafilm™ membrane and deposit salivary products onto the saliva collection membrane for 24 to 72 hours. Salivary products were eluted from the Durapore™ membrane using acetoni-

**The pathophysiology of flea bite allergy in the dog and cat involves IgE-mediated hypersensitivity to components of flea saliva injected at the time the flea bites.**

*Abstracted from the Proceedings of the Third World Congress of Veterinary Dermatology, 1996.

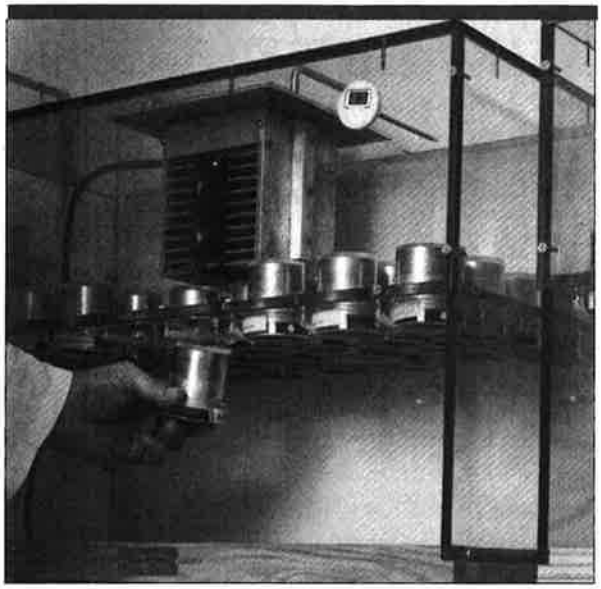

*Figure 1. Artificial flea feeding system.*

trile and trifluoroacetic acid and dried under vacuum. Dried whole saliva was dissolved in 0.1% trifluoroacetic acid and separated into fractions using C4 reverse phase high performance liquid chromatography (HPLC) and an increasing acetonitrile gradient. Whole saliva and subsequent fractions were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by IDST in artificially and naturally sensitized dogs. IDST consisted of 50 µl injections of whole or fractionated flea saliva in saline, saline (negative control), and histamine (positive control). Injections were in the lateral thorax of dogs. Selected injection sites were biopsied at 15 minutes and 24 hours, fixed, embedded in paraffin, stained, and the histology evaluated. Purified proteins from reactive fractions were subjected to N-terminal amino acid sequencing. Degenerate oligonucleotide primers were constructed, using the amino acid sequence data, and the genes encoding these proteins were cloned and expressed. The recombinant flea salivary allergens were used for IDST to confirm the biological activity of the expressed proteins.

## RESULTS

Flea saliva was evaluated for protein content. Approximately 13 ng of protein per flea was collected during a 72-hour period. Reverse phase HPLC separation of saliva resulted in about 15 fractions. SDS-PAGE of whole saliva and fractions demonstrated predominant proteins ranging in molecular mass from approximately 6 to 44 kilodaltons.

The initial characterization of allergens in flea saliva required the availability of flea bite hypersensitive dogs that were not concurrently hypersensitive to other allergens. Laboratory-reared beagles were chosen, and they were sensitized to flea bites by low level intermittent exposure to fleas by previously described methods.[1] The sensitization procedure involves allowing 25 fleas, contained within a feeding chamber, to feed on dogs for 15 minutes once a week. Using this procedure, approximately 60% of beagles become hypersensitive to flea bites over 20 weeks, with 80% being IDST positive at 40 weeks (see p 24). IDST of these dogs with 1 to 2 µg of whole flea saliva or with 50 to 500 ng of protein fractions derived from whole flea saliva by HPLC, resulted in positive intradermal skin test reactions comparable to the histamine positive control. Naive, nonsensitized beagles exhibited negative skin tests, demonstrating the specificity of the reaction in the hypersensitive animals. Skin punch biopsies of the reactive injection sites demonstrated a subacute superficial dermatitis with perivascular and periadnexal infiltration of mastocytes and eosinophils consistent with an allergic Type I hypersensitivity reaction.

**Identification and characterization of the specific antigens involved in the induction of flea bite allergy is the critical step for development of *in vitro* diagnostics and *in vivo* therapeutics for this disease.**

Because IgE is a major component of Type I hypersensitivity responses, the serum from these hypersensitive dogs provides a valuable reagent to identify and further characterize the specific allergens in flea saliva. Protein fractions prepared from flea saliva were evaluated by enzyme-linked immunosorbent assay (ELISA) for reactivity with antibodies in the serum of flea-allergic dogs. To conduct these tests, the purified allergens are adsorbed to wells of a 96-well plastic plate, and sera from sensitized dogs were allowed to react with the allergens. The allergen-reactive IgE contained within the serum binds to the allergen and is identified

using either monoclonal antibodies reactive with canine IgE or with the high-affinity Fc epsilon receptor alpha chain (FcεRIα) as previously described (see p 10). These ELISA techniques allow the identification of specific flea salivary allergens in the laboratory without having to continuously expose allergic animals to extraneous allergens by IDST. In addition, sera from clinically diagnosed flea bite allergic dogs, provided to us by practicing veterinarians, can be evaluated to assist in validating the importance of each allergen. This system allows for the rapid identification of important allergens, provides a means to monitor the purity of allergens separated from complex mixtures, and provides a way to determine if recombinant allergens cloned from the flea salivary gland cDNA library are likewise bound by IgE from flea allergic dogs.

**Whole flea extracts contain less than 0.5% flea saliva.**

Sera from flea bite hypersensitive dogs can also be used to identify protein allergens by immunoblot analysis. This procedure involves the separation of flea salivary allergens according to size and stabilizing their positions in a gel matrix using SDS-PAGE. After separation, the proteins are transferred to a nitrocellulose membrane and allowed to react with the serum from flea sensitized dogs. Again, the presence of IgE reactive with the flea allergens was detected using either monoclonal antibodies specific for IgE or the very specific high-affinity Fc epsilon receptor alpha chain (FcεRIα). This system shares many of the same features as the ELISA but, in addition, allows visualization of the protein and estimation of its molecular weight. The immunoblot analysis can simplify the quality control of protein purification and aids in the identification of proteins suitable for amino acid sequencing or for the production of allergen-reactive antibodies. These latter techniques and reagents are then used in the molecular cloning of the relevant allergens.

This combination of flea biology expertise, the ability to collect purified flea saliva, and the availability of a colony of laboratory beagles sensitized to flea bites, combined with expertise in classical protein biochemistry and development of the novel high-affinity Fc epsilon receptor alpha chain (FcεRIα) technology for measurement of allergen-specific IgE, has allowed Heska's scientists to detect, characterize, and then clone and express a number of important allergens present in flea saliva. The availability of recombinant flea allergens has led to the study of the immunopathology of flea bite hypersensitivity, the development of highly specific *in vitro* allergy diagnostics, and implementation of controlled immunotherapy studies using well-defined antigens.

## REFERENCE

1. Halliwell REW, Longino SJ: IgE and IgG antibodies to flea antigen in differing dog populations. *Vet Immunol Immunopathol* 8:215, 1985.