

Identification of *Demodex Hominis* using Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

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Abstract: This research explored the utilization of Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for developmental stage differentiation in *Demodex hominis* and the creation of a reliable spectral reference database. VITEK® Mass Spectrometry (MS) was employed to capture the spectral profiles, with SARAMIS™ super spectra tool establishing the reference database. Validation experiments and blinded assessments demonstrated that species-level and developmental stage identification accuracy was confirmed through SARAMIS™ software evaluation. Consistent and reproducible MS profiles were obtained in 28 specimens. A spectrum database, including 4 super spectra, revealed 15 discriminating mass peaks. Peaks 4304.7 and 8609.8 were specific peaks for *Demodex hominis*. Peaks 3346.8 and 5236.6 are shown in adult *Demodex brevis* spectra. Similarly, peaks 3788.2 and 7673.3 emerged in the larva *Demodex brevis*. Peaks 4109.6, 5383.2, and 7518.3 presented in adult *Demodex folliculorum* spectra. Peak 4696.8 appeared only in larva *Demodex folliculorum*. Validation experiments revealed successful developmental stage determination in 44 out of 48 target *Demodex* samples during blinded testing. This research demonstrates the diagnostic potential of MALDI-TOF-MS-based proteomic analysis for precise differentiation of *Demodex hominis* subspecies and their maturation phases. A preliminary spectral reference database has been compiled, documenting protein profiles of two clinically significant *Demodex hominis* variants identified in China.

Keywords: *Demodex Hominis*; Mass Spectrometry; Mites; Eyelash; Matrix-assisted Laser Desorption/Ionization.

1. Introduction

Demodex represents a genus of microscopic parasitic mites inhabiting mammalian skin structures, particularly hair follicles and sebaceous glands. Taxonomically classified within Animalia kingdom, Arthropoda phylum, Arachnida class, and Acari subclass, these organisms demonstrate host-specific adaptations. Current research has identified over 140 distinct species and subspecies, with nomenclature typically reflecting their host organisms. In human hosts, two primary variants exist - *Demodex folliculorum* and *Demodex brevis* - though scientific discussions propose their potential reclassification as *Demodex hominis*, colloquially termed eyelash mites. These ectoparasites have been implicated in multiple dermatological and ocular conditions including inflammatory skin disorders, facial erythema conditions, persistent eyelid inflammation, and sebaceous gland abnormalities. [1-4].

In clinical practice, taxonomic categorization of *Demodex hominis* has principally depended on anatomical features, with particular emphasis on body-tail proportion [5] (Figure 1). However, these arachnids exhibit high susceptibility to environmental conditions across different life cycles, demonstrating marked adaptability and morphological diversity in their physical traits. The simultaneous presence of multiple *Demodex* species within a single host organism further introduces complexities and ambiguities in conventional morphological assessment. Recent advancements in molecular biology, particularly the emerging DNA barcoding methodology, now enable sophisticated genomic differentiation, species verification, and evolutionary analysis of these mites [6]. While molecular

approaches demonstrate precision across all mite life stages, their implementation requires substantial temporal investment, specialized labor, and significant resource allocation compared to traditional methods. Over the past decade, Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) has emerged as a reliable analytical tool for microbial identification across scientific investigations and clinical applications. This proteomic methodology has evolved into a cost-efficient solution that enables efficient, precise, and consistent characterization of clinically significant microorganisms including bacterial strains, fungal species, and yeast cultures, requiring minimal sample preparation time. [7, 8] The technique's application spectrum extends beyond microbial analysis, having been successfully implemented in arthropod vector identification (fleas, ticks)[9, 10] and insect species differentiation (*Drosophila*[11] and mosquitoes[12]). These advancements position MALDI-TOF-MS as a valuable taxonomic tool for elucidating intra-species variations and interspecies connections among phylogenetically proximate organisms. Nevertheless, research exploring its potential for *Demodex Hominis* identification remains scarce.

Therefore, this investigation sought to examine the utilization of MALDI-TOF-MS technology in characterizing *Demodex hominis* across various life cycle phases while developing a standardized protocol for acquiring consistent spectral data, thereby contributing to the construction of a *Demodex hominis* reference database.

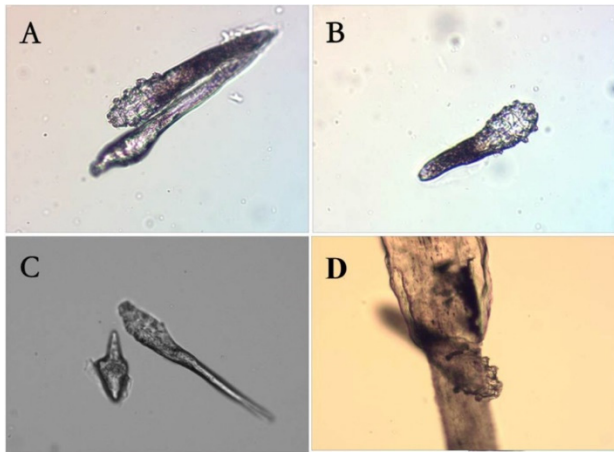


Figure 1. Microscopic features of different development stage demodex hominis. These *Demodex* are divided into head-neck and body-tail parts, with 8 short legs attached to the former body segment. The body-to-tail ratio is 1:2 to 1:4 for *Demodex folliculorum* but close to 1:1 for *Demodex brevis*. **A.** Adult and larva *Demodex folliculorum*. **B.** Adult *Demodex brevis*. **C.** Larva and ovum *Demodex folliculorum*. **D.** Larva *Demodex brevis*.

2. Materials and Methods

(1) Demodex collection

The collection of eyelash specimens for *Demodex* analysis followed established protocols from our prior investigations [13, 14]. Sterilized forceps were employed to carefully extract three lashes from a healthy donor's upper eyelids, which were subsequently transferred onto microscope slides. Each follicular unit underwent examination with sterile distilled water applied to its root portion for parasite detection. The study cohort comprised 240 specimens across developmental stages (**Figure 1**), consisting of 140 *D. folliculorum* (15 ovum, 50 larval forms, 75 mature adults) and 100 *D. brevis* (5 eggs, 30 nymphs, 65 adult specimens). Collected parasites underwent brief aqueous preservation prior to species differentiation through morphological characterization [15] with taxonomic verification conducted by certified medical entomology specialists.

(2) Sample preparation for MALDI-TOF-MS

For MALDI-TOF-MS sample preparation, fresh specimens underwent cleansing with deionized water. Five *Demodex* specimens (including eggs, larvae, or adults) per sample were homogenized using a tissue homogenizer (OSE-Y20, TIANGEN BIOTECH, Beijing) for 5-minute mechanical disruption. Following centrifugation at 12,000 rpm for 30 minutes, 1 µL aliquots of supernatant were combined with equivalent volumes of CHCA matrix solution (bioMérieux, France) and deposited onto MALDI-TOF-MS FlexiMass-DS target slides (Cat. #: 149 TO-430) in triplicate, with three replicate spots per sample being allowed to air-dry. *Escherichia coli* ATCC 8739 strain was utilized as the calibration standard during spectral acquisition.

(3) MALDI-TOF-MS analysis

Protein mass spectral data were obtained through MALDI-TOF Vitek Mass Spectrometry (VITEK® MS RUO, bioMérieux, Marcy l'Etoile, France) covering the 2–20 kDa molecular weight range. Measurements were performed using a Vitek MS system equipped with a 337 nm nitrogen laser operated in linear positive-ion mode, employing delayed extraction under an accelerating voltage of 58 kV. Spectral acquisition involved automatic recording of 500 cumulative laser pulses per sample (100 pulse cycles repeated five times) in positive ionization configuration. Data processing utilized

Saramis premium software (SARAMIS™ version 4.0.0.14) with spectral refinement including mass range restriction (2–20 kDa) and elimination of low-intensity signals (<5% relative intensity). The super spectrum algorithm in SARAMIS™ facilitated mass binning and average mass computation. Analytical retention criteria required spectra exhibiting over 60% dendrogram similarity with 100–300 detectable peaks. Spectral data demonstrating >80% reproducibility were incorporated into SARAMIS™ super spectral databases for subsequent evaluation.

(4) Species identification

Validation was confirmed through blinded validation trials employing newly collected fresh *Demodex* samples from both species. Three to five immature or mature specimens per species underwent comparative analysis against the spectral reference library incorporated into the SARAMIS™ software platform. All specimens received randomized codes prior to analysis initiation. Following SARAMIS™ Premium protocol standards, the acceptance threshold was established at 75% confidence level for biomarker correspondence with reference spectral profiles.

3. Results

(1) MALDI-TOF-MS spectrum analysis and database assembly

A total of 48 flea specimens were subjected to MALDI-TOF/MS premium analysis. Spectral evaluation through SARIMAS software indicated that homogenized supernatant samples produced stable, repeatable profiles characterized by prominent peaks between 3–12 kDa (**Figure 2**), with each spectrum displaying 198–411 distinct peaks. Cross-group spectral analysis confirmed consistent reproducibility across all tested tick species, including both *Demodex folliculorum* and *Demodex brevis* samples. A reference database was subsequently constructed, incorporating 28 larval and adult specimens representing two species. Mass spectral data from 14 specimens per species were aggregated to create composite profiles, yielding a dendrogram with clearly differentiated phylogenetic branches (**Figure 3**). Four representative super spectra were additionally produced, encompassing both developmental stages (adult and larva) of *Demodex folliculorum* and *Demodex brevis*.

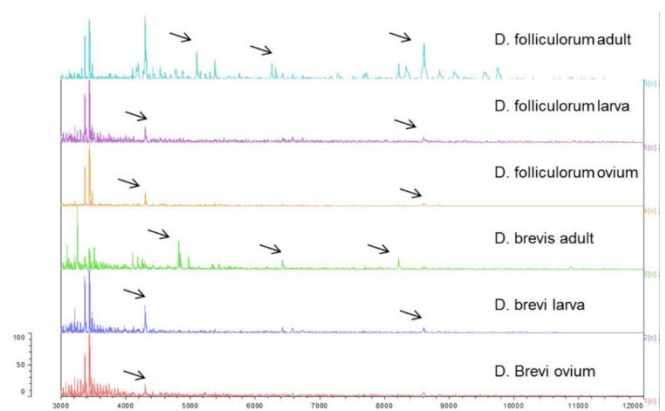


Figure 2. Vitek Mass Spectrometry (VITEK® MS) spectra in the range of 3–12 kDa of different stages for *Demodex hominis*. Four super spectra were created for the larva and adult stages. Most specific peaks are indicated by arrows.

(Abbreviations: *D. folliculorum*=*Demodex folliculorum*; *D. brevis* =*Demodex brevis*)

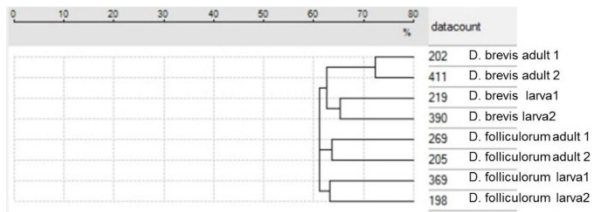


Figure 3. Dendrogram of Vitek Mass Spectrometry (VITEK® MS) spectra in the range of 3–12 k Da from two individual species of *Demodex hominis*.
(Abbreviations: *D. folliculorum*=*Demodex folliculorum*; *D. brevis* =*Demodex brevis*)

(2) Study validation using a blind test.
The reliability of the *Demodex hominis* database was

subsequently verified using blinded testing protocols. Specimen analysis utilizing VITEK® MS technology demonstrated highly consistent results across the database entries, achieving species-level differentiation with confidence thresholds between 90% and 99.9% spectral similarity. Fifteen specimens representing various life stages (adults and larvae) underwent blinded analysis against the reference database, with all samples achieving precise taxonomic classification exceeding 90% confidence thresholds (refer to **Tables 1** and **2**). Developmental stage-specific proteomic signatures were successfully captured (**Figure 2**), confirming the database's efficacy when utilizing supernatant-derived spectral data for both database construction and subsequent blinded verification processes.

Table 1. Validation of VITEK® MS of 15 *Demodex hominis* in different stages.

Species stage	Number	Correspondence of MALDI-TOF to morphological identification (%)	Confidence rang
<i>Demodex brevis</i> adult	15	13/15 (86.67)	75-99.9
<i>Demodex brevis</i> larva	10	10/10 (100.00)	90-99.9
<i>Demodex folliculorum</i> adult	13	12/13 (92.31)	70-99.9
<i>Demodex folliculorum</i> larva	10	9/10 (90.00)	70-99.9

Abbreviations: VITEK® MS, Vitek Mass Spectrometry. MALDI-TOF, Matrix-assisted laser desorption/ionization time of flight.

Table 2. Comparison of MALDI-TOF *Demodex hominis* in different stages, ranging from 3-20 k Da.

Mass m/z (Da)	<i>Demodex brevis</i> adult	<i>Demodex brevis</i> larva	<i>Demodex folliculorum</i> adult	<i>Demodex folliculorum</i> larva
3187.7	yes	yes	no	no
3278.6	no	no	yes	yes
3346.8	yes	no	no	no
3788.2	no	yes	no	no
4109.6	no	no	yes	no
4304.7	yes	yes	yes	yes
4696.8	no	no	no	yes
5236.6	yes	no	no	no
5383.2	no	no	yes	no
6426.2	no	no	yes	yes
6581.8	yes	yes	no	no
7518.3	no	no	yes	no
7673.3	no	yes	no	no
8609.8	yes	yes	yes	yes
8865.1	no	no	yes	yes

Abbreviations: MALDI-TOF, Matrix-assisted laser desorption/ionization time of flight.

4. Discussion

Demodex mites have been identified as carriers of multiple human pathogens, contributing to their global public health importance [1, 16]. This investigation confirmed that protein extracts from specimens prove suitable for distinguishing arthropod species through MALDI-TOF-MS analysis, provided reference spectra for the target species exist in the database. The research demonstrated that whole-body homogenates of *Demodex* specimens yield consistent spectral patterns for reliable species differentiation using this technique. These outcomes align with prior arthropod identification studies involving *Drosophila*[11], ticks[17, 18], *Culicoides*[19], and mosquito species[20, 21], where either complete specimens or specific body segments were processed for protein analysis. Our experimental data revealed that spectral patterns obtained from fresh intact

specimens through MALDI-TOF-MS exhibited high reproducibility. This consistency matches earlier findings regarding the reliability of whole-body protein profiles in arthropod identification methodologies. Previous research indicated that newly collected specimens exhibited 70% greater mean data quantities compared to those preserved long-term in ethanol solutions.[22]

In this investigation, MALDI-TOF-MS technology was employed for *Demodex hominis* characterization, successfully developing China's inaugural spectral reference database for this species. This innovative database enables economical and efficient differentiation of *Demodex* variants and developmental phases through distinctive spectral patterns, offering enhanced specificity relative to genetic identification methods[6]. Validation confirmed that the VITEK® MS platform generated superior spectral profiles using CHCA matrix preparations. Mass spectrometry demonstrated exceptional discriminatory capacity for *Demodex* specimens, achieving reliable identification

outcomes across the studied biological samples.

5. Conclusion

In conclusion, this investigation demonstrates that MALDI-TOF-MS serves as an effective and reliable technique for rapid detection of *Demodex* parasites. Additional research is needed to examine spectral profiles across different mite species. A foundational spectral database containing two human *Demodex* species has been successfully compiled, with plans for continuous expansion through incorporation of newly collected specimens. Compared to traditional morphological analysis and molecular techniques, MALDI-TOF-MS offers simplified operation and cost-efficiency after initial equipment acquisition, eliminating dependence on expensive reagents. This methodology shows promise as a standardized identification protocol for *Demodex* and may develop into a cost-effective diagnostic solution for mite-related analyses.

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