

Novel *Demodex* detection method involving non-invasive sebum collection and next-generation sequencing

Michał J. Kowalczyk¹, Natalia Derebecka², Ryszard Żaba¹, Joanna Wesoty², Piotr Pawlak³, Anna Szkaradkiewicz-Karpińska⁴, Amie Maher⁵, Kevin Kavanagh⁵

¹Department of Dermatology and Venereology, Poznan University of Medical Sciences, Poznan, Poland

²Laboratory of High Throughput Technologies, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland

³Department of Genetics and Animal Breeding, Faculty of Veterinary Medicine and Animal Sciences, Poznan University of Life Sciences, Poznan, Poland

⁴Department of Preclinical Conservative Dentistry and Preclinical Endodontics, Poznan University of Medical Sciences, Poznan, Poland

⁵Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland

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Abstract

Introduction: *Demodex* mites are common human ectoparasites found across a broad geographical range. They reside in pilosebaceous units of the skin and feed on sebum, epithelial and glandular cells. *D. folliculorum* is the more common mite, inhabiting the upper end of the pilosebaceous unit while *D. brevis* resides deeper in the skin and meibomian glands. Until now, *Demodex* mites have been obtained by various techniques such as skin scraping, cellophane tape, plucking eyelashes, and also by invasive biopsies.

Aim: To assess whether non-invasively collected sebum samples of patients suspected of rosacea or demodicosis are suitable for NGS DNA *Demodex* analysis.

Material and methods: Suspicion of seborrheic dermatitis or rosacea was the inclusion criterion. The study group consisted of 20 males, 1 female, age: 33–83, median: 58. Nasal dorsum was moisturized and an adhesive strip was applied. DNA was isolated from the sebum and sequenced with the use of MiSeq® Reagent Kit v2 and MiSeq® System.

Results: Out of 7 patients who were positive by microscopy, 6 were found positive by NGS. Additional 4 patients were found positive only by NGS, adding to a total of ten. The NGS approach showed superior sensitivity compared to light microscopy (63% and 44%, respectively). In 3 patients, both *Demodex* species were identified by NGS.

Conclusions: We believe to have proven that it is possible to study *Demodex* mites by NGS with sebum as the input sample. Furthermore, it is possible to identify and distinguish *Demodex folliculorum* from *D. brevis* in individual patients.

Key words: Next-Generation Sequencing, rosacea, demodicosis.

Introduction

The *Demodex* mite was first reported by Gustav Simon, a German dermatologist examining sebaceous follicle samples from an individual with acne lesions [1]. Simon first identified *Demodex folliculorum* in 1842, but it was not until 1963 that *Demodex brevis* was identified as a separate species [2].

Demodex mites are the most common human ectoparasites [3]. *Demodex* species can be found on all skin types across a broad geographical range [2]. *Demodex*

mites reside in the pilosebaceous units of the skin, with their heads directed at the bottom of the hair follicle, where most sebum can be found [4]. *Demodex* feed on epithelial and glandular cells as well as sebum typically secreted by active pilosebaceous units [5, 6].

Demodex folliculorum is a more common mite, possibly due to it inhabiting the upper end of the sebaceous unit and so making it easier to spread by direct contact [4]. This saprophytic mite colonizes the facial region commensally with *D. brevis* residing deep within the seba-

Address for correspondence: Michał J. Kowalczyk, Department of Dermatology and Venereology, Poznan University of Medical Sciences, 49 Przybyszewskiego St, 60-355 Poznan, Poland, phone: +48 531 224 221, e-mail: michalkowalczyk@ump.edu.pl

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ceous gland of the skin and the meibomian gland of the eye [7, 8].

It is believed that *Demodex* mites are first transmitted during nursing as *Demodex* mites have been isolated from the nipple [9]. As children mature, the sebaceous unit develops, creating a suitable habitat for *Demodex* colonization, which further improves as young adults produce sebum during puberty and change its composition [10]. *Demodex* densities increase with age, with almost 95% infestation found in individuals over the age of 71 [9].

As a result of *Demodex* feeding on sebum and epithelial cells, particularly around the nose, as it is rich in sebum, this may cause micro-abrasions to the skin surface [11]. This can commonly lead to epithelial hyperplasia on the face or even the eyelid margin of rosacea patients and blepharitis patients [11]. It has been suggested that the sebum composition rather than its volume is preferred by the mite and can determine *Demodex* densities [12]. Demodicosis is the infestation of *Demodex* mites on the face, whereby a minimum of 5 mites/cm² exist and induce symptoms such as redness of the skin (erythema), telangiectasia, itching, heat, and scaling [8].

In 1971 the skin surface biopsy was described to detect *D. folliculorum* presence on the skin. However, it was not until 1993 that the concept of assessing the *Demodex* density was first introduced [13–15]. This quickly led to the development and utilization of the standardized skin surface biopsy (SSSB) method on the stratum corneum,

immediately followed by a second deeper skin surface biopsy as outlined by Forton and De Maertelaer [15]. By combining these two consecutive samples' counts, an improved *Demodex* density could be found, proving this method as a useful diagnostic tool. The SSSB is a quick and reproducible procedure, tolerated well by patients as it is minimally invasive. It is also a cheap and straightforward diagnostic tool. The two consecutive SSSBs reached 89.3% sensitivity, compared to 55% with the use of just one biopsy [15].

A different modification of SSSB was proposed by Arasiewicz *et al.* In their approach, a hypoallergenic, sticking plaster is pressed onto the nose, chin, cheeks, and forehead. These are then removed after a night, followed by a microscopic analysis [16].

However, these microscopic methods present certain disadvantages. Performing a double SSSB is time-consuming, with sensitivities below 90%. The use of cyanoacrylic glue might also scare the patients. Thus, genetic approaches have been under examination. They give an opportunity to significantly increase sensitivity and provide additional information from the genetic material of the mite, the host, and the surrounding microbiota.

However, it has not been verified if non-invasively collected sebum can be successfully used by Next-Generation Sequencing (NGS) in *Demodex* analysis.

Aim

The purpose of this preliminary study was to assess whether non-invasively collected sebum samples of patients suspected of rosacea or demodicosis are suitable for NGS DNA *Demodex* analysis.

Material and methods

Patients

Suspicion of seborrheic dermatitis (criteria of the Polish Dermatological Society [17]) or rosacea (criteria of the National Rosacea Society [18]) manifesting themselves by facial skin inflammation was the inclusion criterion. Exclusion criteria involved age of under 18 and treatment within last 4 weeks. One person was free of symptoms and served as a negative control. The entire group consisted of 21 people: 20 males, 1 female, age range: 33–83, median age: 58. All patients gave written consent. The study was approved by the Bioethical Committee of the Poznan University of Medical Sciences.

Sebum collection

Sebum and epidermal debris were collected with the use of Deep Cleansing Face Pore Strips (Beauty Formulas, UK). Nasal dorsum was moisturized with PBS, and a strip was applied to it for 10 min (Figure 1). In most cases, the



Figure 1. Moisturized adhesive strip applied to nasal dorsum

strips were applied before the patients had their face cleaned or cream applied. Purposefully, the skin was not pinched to minimize the level of invasion, even though pinching could have yielded more sebum. Then, the strip was removed, put into a plastic zip bag, and immediately frozen at -20°C . The freezing step allowed the adhered sebum to harden and made it possible to be scratched off from the strip with a scalpel. The peeled debris were put into a 2.0 ml Eppendorf tube with lysis buffer.

DNA isolation

Due to minute amounts of input material and high fat content, DNA isolation proved to be challenging. Best results were achieved with the use of a NucleoSpin Food DNA isolation kit (Macherey-Nagel). The procedure was carried out according to the manual with slight modifications in that additional vortexing was applied during initial Proteinase K and lysis buffer incubation followed by centrifugation at 11000 xg. DNA was eluted in only 30 μl elution buffer (5 mM Tris/HCl, pH 8.5) to yield a higher concentration. The DNA concentration was measured with Qubit fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Isolated DNA was stored at -20°C prior to further processing.

Library preparation and validation

1.0 ng of each DNA sample was used as input for library preparation with the use of Nextera[®] XT DNA Sample Preparation Kit (Illumina), according to the user manual. Libraries were indexed with a dual-indexing strategy using Nextera[®] XT Index Kit (Illumina) that enabled multiplexing of the samples. The distribution of fragment length and concentration of final libraries was assessed by means of on-chip electrophoresis using Agilent High Sensitivity DNA Kit and Agilent Bioanalyzer 2100 System (Agilent). Each library was diluted to 2 nM concentration, an equimolar pool of all libraries was prepared, denatured, and diluted to the final 8 pM concentration [19].

NGS sequencing

The diluted pool of libraries was sequenced in paired-end, 2×150 cycles mode, with the use of MiSeq[®] Reagent Kit v2 (300 cycles) and MiSeq[®] System (Illumina), according to user manuals. 5% of PhiX Control (Illumina) was spiked in samples to obtain run quality metrics.

Bioinformatic analysis

The following analyses were performed using the Galaxy Web Portal at usegalaxy.org [20], apart from the NCBI BLASTn suite [21].

Elimination of human reads

Since the data from two runs in MiSeq[®] System paired-end (PE) sequencing are stored as individual

files, a list of data-set pairs was created. These pairs were then mapped against the *Homo sapiens* hg38 reference genome to eliminate human DNA reads using Bowtie2 v.2.3.4 with default settings [22, 23]. Read pairs that did not align concordantly to the human genome were processed further.

Mapping against *Demodex* mitochondria

Since the full genomic sequences of both *Demodex folliculorum* and *brevis* are not yet available, the unaligned reads were mapped against their known mitochondrial genomes (NC_026102.1 and NC_026101.1, respectively [24]) using Bowtie2. Mapped results were then filtered and converted into FASTA format with BAM Tools (Galaxy Version 2.4.0) [25].

The FASTA files were used to perform NCBI BLASTn searches as the final check of the results ('Somewhat similar sequences' mode, 'Nucleotide collection (nr/nt)' database, 'Low complexity regions' unchecked) [21]. Reads were considered positive if the hit with the lowest e-value was referring to the appropriate *Demodex* species. The BLASTn confirmation step was omitted for samples with more than 100 PE reads mapped.

Results

The results of the DNA isolation are provided in Table 1. Unfortunately, there was not enough facial material yielded from some patients, resulting in unsuccessful DNA isolation. A total of 5/21 (23.8%) isolation attempts failed. All of these happened in the first half of the collected samples. During this time we established two additional sample entry criteria: 1) the sample must be collected before any dermatological treatment starts, 2) frozen sebum must be visible on a strip before scratching it with a scalpel.

Run quality

Overall quality of the sequencing run was high (cluster PF = 91%, > Q30 = 95%; error rate = 0.6) and over 22 million passing filter sample reads were generated (0.7–2.2 million per sample).

Elimination of human reads

Firstly, both the default and sensitive-local options in Bowtie2 were run to compare the two modes for the best results. Initial analysis showed that the surplus hits generated by the *--sensitive-local* option are in fact human reads that were not removed in the primary elimination of human reads or hits, yielding no results under BLASTn. It was therefore decided to use the default options of Bowtie2.

As expected, most of the DNA was indeed human. The summary of human DNA elimination is provided in Table 1.

Table 1. Results

Patient*/sex	Age	DNA concentration [ng/μl]	Microscopic analysis	Paired-end reads total	Non-human PE reads	<i>D. folliculorum</i> mapped PE reads	BLASTn <i>D. folliculorum</i> confirmed PE reads	<i>D. brevis</i> mapped PE reads	BLASTn <i>D. brevis</i> confirmed PE reads
1/M	58	6.30	Negative	638554	72019 (11.28%)	0	–	0	–
4/M	61	75.0	Negative	605509	70936 (11.72%)	0	–	0	–
6/F	61	0.42	Negative	519298	81469 (15.69%)	2	2	4	4
7/M	65	0.15	Negative	963428	114277 (11.86%)	31	31	0	–
8/M	40	33.3	Negative	672734	79421 (11.81%)	0	–	0	–
11/M	33	0.45	Positive	728772	84344 (11.57%)	0	–	0	–
12/M	66	0.16	Positive	970263	443816 (45.74%)	59 + 1 SE	59 + 1 SE	11 + 1 SE**	11
13/M	55	0.14	Positive	764057	144228 (18.88%)	260 + 2 SE	Not assessed	1**	0
14/M	41	1.05	Positive	690920	110420 (15.98%)	1 + 1 SE	1 + 1 SE	0	–
15/M	34	0.24	Negative	671291	121220 (18.06%)	0	–	0	–
16/M	58	0.22	Negative	627240	73727 (11.75%)	14 + 1 SE	14 + 1 SE	0	–
17/M	54	0.25	Positive	638441	72116 (11.30%)	100 + 1 SE	Not assessed	0	–
18/M	73	0.78	Negative	615519	241721 (39.27%)	4	4	0	–
19/M	37	1.93	Negative	671320	77000 (11.47%)	0	–	0	–
20/M	83	0.41	Positive	1132927	216371 (19.10%)	25	25	1	1
21/M	76	0.20	Positive	342636	253606 (74.02%)	1	1	0	–
N studied			16			10/16	10/16	4/16	3/16
Min.	33	0.14		342636	70936 (11.28%)				
Max	83	75		1132927	443816 (74.02%)				
Mean	55.9	7.56		703307	141043 (21.2%)				
Median	58	0.42		671306	97382 (13.8%)				

*Failed isolation patients are excluded, **false positive hit. PE – paired-end read, SE – single-end read.

Demodex detection

No statistically significant correlation was observed between the initial DNA concentration and the number of paired-end reads yielded (Spearman $r_s = -0.28$, $p = 0.29$).

Out of the 7 patients that were previously confirmed positive by microscopic analysis, 6 were also found positive by NGS. One patient (no. 11) was found negative by NGS and positive by microscopy. On the other hand, additional 4 patients were found positive by NGS, being negative by microscopy, adding to a total of ten. Thus, the NGS approach showed superior sensitivity compared to light microscopy (63% and 44%, respectively).

Interestingly, in 3 patients, both *Demodex* species were detected by NGS. One of these patients was negative in light microscopy. It is worth mentioning that the BLASTn analysis was unambiguous in all 3 cases. This step seems crucial, however, since it pointed to 2 false positive hits. One of these was a SE read with no influence on the final results since other PE reads were present. The other was a single PE read for that patient

(no. 13); thus, the status was changed from positive to negative only as far as *Demodex brevis* was concerned since this patient was strongly positive for *Demodex folliculorum*.

Discussion

Genomic, transcriptomic, and microbiotic analyses of human *Demodex* species are a challenge. The mites cannot be cultured *in vitro* but only kept alive for a few days [26, 27]. Several genetic analyses of *Demodex* mite have been carried out. For instance, Thoemmes *et al.* carried out a phylogenetic analysis on the 16S rRNA and the 18S rRNA genes to determine possible genetic diversities and the lineage history of the *Demodex* mite evolution [2]. A genetic variance between the two *Demodex* species has been highlighted as mites located at the eyelashes have a different CO1 mitochondrial gene compared to mites inhabiting the skin [28].

Until now, *Demodex* mites have been obtained from sebaceous glands of individuals by various techniques such as scraping the skin gently, cellophane tape, pluck-

ing in the case of eyelashes and eyebrows, and also by invasive methods, i.e. a biopsy [2]. Nevertheless, the mites are microscopic, thus difficult to handle, while the amount of the sample for any DNA or RNA analysis is far from desirable. It is the methodology of extraction that must be resolved to successfully diagnose patients with high sensitivity, not only qualitatively but quantitatively, allowing thorough scientific research to be done. Our extraction and DNA isolation methodology was acceptable as far as this research was concerned but obviously requires more insight and optimization. Perhaps, not pinching the skin to minimize the invasion level was a mistake since a visible sebum sample at this early stage was important for later DNA isolation success.

The authors are aware that at the moment the usage of NGS in the diagnostics of individual patients is not likely due to the overall cost of the procedure per patient. The procedure might be used in such a way in the future as the prices per sample decrease.

For the time being, genetic diagnostics of *Demodex* parasites can be aided by PCR and second-generation sequencing that are far cheaper per patient yet less informative. Worth mentioning is the ongoing research for the most suitable barcoding regions [29, 30].

However, we believe to have proven that it is now possible to study the *Demodex* genomics in individual people. Coupled with the microbiota analysis of both the outside and inside of mites inhabiting the skin, it opens new research possibilities of that pathogen.

Furthermore, we have been able to successfully identify and distinguish *Demodex folliculorum* from *D. brevis* in individual patients. Both human *Demodex* mites coexist commensally and share common characteristics but also have structural differences and habitual preferences. *D. brevis* is the smaller of the two species and is solitarily confined deep within the glands and ducts but is located in more widespread areas of the face such as the ear canals [9]. The two species reside in different ecological niches, thus might require different treatment approaches. The authors are also aware that relying on just one mitochondrial sequence per species as reference points is likely not the ideal situation as far as specificity is concerned. Our conclusion that the two *Demodex* species can be specifically distinguished from one another and from other mites is based on the assumption that any yet to be discovered mitochondrial diversity would not overlap. That is likely the case, but the possibility of a much smaller genetic distance is not entirely excluded. More sequences need to be deposited so that researchers can assign species to their sequences with more certainty. Worth mentioning is the outstanding research on the transcriptome of *Demodex* mites recently made by Hu *et al.* It paves the way for understanding the differences between the two human invading species and developing reliable treatment strategies [27, 31].

The causality of *Demodex* spp. in the etiopathogenesis of rosacea is a subject of an ongoing scientific debate. One of the most common hypotheses on the matter suggests it may act like a syringe by bringing bacteria and antigens deeper into the skin than normally present. This can be especially seen when mites die deep in pilosebaceous units releasing contents of their intestines. This hypothesis is supported by studies pointing to certain bacteria, such as *Bacillus oleronius*, whose proteins have been shown to elevate inflammatory response *in vitro* [3, 7, 11, 12, 32–34]. These studies show that further analyses on the host-mite-bacteria interactions in the etiopathogenesis of rosacea are necessary. NGS enables researchers to look for genetic traits of these bacteria and even study the entire microbiota inside or outside mites, both *in vitro* and in patients.

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Conflict of interest

The authors declare no conflict of interest.

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