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COMPARISON OF *MALASSEZIA* SPP. PROPORTIONS IN INFLAMMATORY AND NON-INFLAMMATORY FACIAL ACNE VULGARIS LESIONS

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ABSTRACT

Objective: The skin microbiome plays a role in the pathogenesis of acne vulgaris (AV). Among other species, *Malassezia* spp. can be found in the pilosebaceous follicle. However, its role on the pathogenesis of AV has not yet been described. The objective of this study was to identify the presence and the distribution of *Malassezia* spp. in facial AV lesions and also to compare the distribution of *Malassezia* spp. between inflammatory and non-inflammatory lesions.

Methods: One hundred and twenty subjects were allocated into two groups: inflammatory lesions and non-inflammatory lesions. Samples were taken from hair follicles and examined by microscopy using 20% potassium hydroxide and Parker ink and cultured for species identification.

Results: There was no difference in the spore load between the two groups (p=0.142). *Malassezia* spp. were isolated from 64.2% of subjects and consisted of *M. dermatis* (43%), *M. sympodialis* (18%), *M. slooffiae* (16%), *M. japonica* (5%), *M. furfur* (5%), *M. pachydermatis* (3%), and *M. restricta* (1%). There was a higher *Malassezia* spp. proportion in non-inflammatory lesions (p=0.019). The predominant species in inflammatory AV lesions was M. dermatis (45,8%), followed by M. sympodialis (17.1%), and M. slooffiae (11.4%).

Conclusion: *Malassezia* spp. were found in facial acne lesions. *M. dermatis* was the predominant species found in facial AV, followed by *M. sympodialis,* and *M. slooffiae.* A higher proportion of *Malassezia* spp. was found in non-inflammatory lesions.

Keywords: Acne lesion, *Malassezia spp*, Skin microbiome

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INTRODUCTION

Acne vulgaris (AV) is a disease of the pilosebaceous unit with multiple complex etiologies. This skin disorder affects 85-100% of adolescents, but can also afflict adults [1, 2]. AV is commonly thought of as a disease that heals spontaneously, which is why it is not given as much priority as other chronic diseases. However, its chronic nature, as well as the scars that form as a complication, have been acknowledged to have a negative effect on the quality of life of people with AV [3].

The microbiome is considered to be one of the key factors that aggravates inflammation, hence promoting the formation of inflammatory active lesions [4]. The microbiota found in the pilosebaceous follicles consists of *Propionibacterium acnes*, *Staphylococcus spp.*, and *Malassezia spp*. [5]. Changes in the balance between the relative proportions of each strain may induce the formation of AV lesions [2].

Malassezia is a normal flora on the human skin. This organism can be found variably in 75-98% of healthy individuals in various parts of the body. In certain conditions, *Malassezia* may become pathogenic and cause skin diseases such as pityriasis versicolor, and *Malassezia* folliculitis (MF). *Malassezia* is also known to play a part in seborrheic dermatitis, atopic dermatitis, psoriasis, and even systemic infections [6]. To date, there are only few studies regarding the existence and relationship between *Malassezia* and AV. Numata *et al.* and Akaza *et al.* reported a correlation between *Malassezia* colony density, in particular, *M. globosa* and *M. restricta*, with the number of inflammatory AV lesions [7, 8]. This result is thought to be due to *Malassezia* having higher lipase activity relative to *P. acnes*, which triggers an increase in free fatty acid and glycerol, which are chemotactic towards neutrophils, thus inducing inflammation in AV [8].

Follicle blockage and increased sebum production play a part in the pathogenesis of AV, as well as in MF, in which both entities can be found at the same time [9]. Kang *et al.* performed a study determining the distribution of *Malassezia* species and found that the coincidence of MF in patients with AV is 25% [10]. A study by Prindavile *et al.* shows that a history of pruritus and the use of antibiotics for *P. acnes* are markers for MF. A positive clinical response after the use of topical or systemic antifungals is also a diagnostic marker for MF [9]. Both *P. acnes* and *Malassezia* are known to have lipophilic properties and induce the release of pro-inflammatory cytokines through the activation of toll-like receptor 2 (TLR-2) in keratinocytes. This explains the similarities between clinical manifestations of AV and MF [8].

The distribution of *Malassezia* species in various skin diseases in different geographic locations varies considerably. Different areas of the body also show variable species distribution depending on geographic location. In tropical countries with high humidity, there is a higher growth of *Malassezia spp* [11]. Indonesia is a tropical country with a highly humid climate. There are limited studies regarding the existence of *Malassezia spp.* in AV lesions. To this end, there is a need to conduct a study to determine the abundance, as well as the distribution. of *Malassezia spp*. in AV lesions, particularly on the face, and the different proportions of *Malassezia spp.* in non-inflammatory and inflammatory AV lesions. This study is expected to provide results that could be used to emphasize the role of *Malassezia* in AV and provide guidance for treatment options.

MATERIALS AND METHODS

This study was a descriptive-analytical study with a cross-sectional design on AV patients in Jakarta. This study was approved by the Health Research Ethics Committee of our university. Clinical and microscopic examinations were performed in the Department of Dermatology-Venereology outpatient clinic in a tertiary hospital in Indonesia. Fungal cultures were performed in the Department of Clinical Microbiology of our university. The study was conducted from October to November 2018. Specimens were taken from four AV inflammatory or non-inflammatory lesions on each subjects' face. Study subjects were recruited based on the inclusion and exclusion criteria with informed consent. Inclusion criteria were an age of 15-49 y with AV and the presence of at least four inflammatory and four non-inflammatory lesions. Exclusion criteria were patients with prior topical retinoid acid or topical antifungal medication within

the past two weeks, systemic antibiotic or antifungal use within the past month, and corticosteroid use within the past six months. Determination of sample size was done with a non-paired numeric analytical equation using 80% power, which gave a proposed sample size of 120 subjects divided into two groups of 60: subjects with inflammatory lesions and those with non-inflammatory lesions.

The research record was compiled based on the clinical history and physical findings of subjects. Patient histories consisted of identity, sociodemographic data, prior medication use, and history of pruritus. The physical examination consisted of AV severity degree scoring based on Lehmann's criteria. Pictures of the subjects' faces were taken using a camera in five positions (anterior, right lateral, lateral oblique, also left lateral oblique, and left lateral).

Laboratory examination

The samples taken from each subject were the follicular contents of four non-inflammatory lesions (comedones), or four lesions from inflammatory lesions (papules, pustules, or nodes). The choice of lesion type taken was decided by randomization. Samples were taken by incision with a 26 ½-gauge needle and then extracted using a sterile comedo extractor. For each subject, one sample was mounted on a glass microscope slide for microscopic examination, while three other lesions were placed on sterile plates to be sent for culture on the same day within 2 h.

A 20% potassium hydroxide (KOH) solution and Parker ink were used to treat samples.

Each sample was given a drop, then gently heated, and then examined under a microscope using 400x magnification. Scores were given based on the existence and number of spores on a high-powered field according to Jacinto-Jamora *et al.*'s criteria, which are as follows: +1, one to two spores, no cluster; +2, small cluster of six or less spores or if dispersed, 12 spores; +3, large cluster of 7-12 spores or if dispersed, 20 spores; +4, a cluster of more than 12 spores or if dispersed, more than 20 spores [12].

Isolation and identification of *Malassezia* species

Two methods of identifying *Malassezia* species can be used: culture and biomolecular [13]. Identification through culture is the preferred choice for some research centers due to its low cost and simplicity. With regards to the culture medium, there are selective mediums such as Saboraud glucose agar supplemented with olive oil, Dixon agar, Lemming Notman agar, or chromogenic agar (CHROMagar *Malassezia*®, Paris, France) [14]. After culture in a primary medium, a further examination needs to be done to make use of the biochemical properties between species. Kaneko *et al.* reported an identifying method using CHROMagar *Malassezia*®, followed by culture on Saboraud agar and Tween 60-esculin, and the catalase reaction test. This yielded a sensitivity of 95-100% and specificity of 71-100% when compared to identification using the biomolecular method [15].

Malassezia samples were inoculated into CHROMagar *Malassezia*®, Dextrose Saboraud agar, and Tween 60-esculin agar. Samples in CHROMagar *Malassezia*® were incubated at 32 °C for 2 d. Then, the grown colony was spread on CHROMagar *Malassezia*® using a streak technique and incubated again at 32 °C for 4 d. The grown isolate was taken with a probe and then inoculated on Saboraud agar and Tween 60-esculin agar and then incubated on 32 °C for 5-7 d before observation. Dextrose Saboraud agar was used to determine the fungal isolate's dependence on lipids for growth, while Tween 60-esculin agar was used to determine the fungal isolate's ability to hydrolyze esculin and utilize Tween 60. The catalase test was also done on the fungal isolates using 3% hydrogen peroxide.

The agar plates were observed for the formation of precipitate and fungal colony morphology and color on the 4th and 7th day in CHROMagar *Malassezia*® medium. The size of the colony was measured based on a single isolated colony, categorized into small (<1 mm), medium (1-2 mm), or large (2-5 mm). Fungal growth in ASD was observed until the 14th day. A positive result was noted if there was fungal colony growth with a shiny cream color. Growth in Tween 60-esculin agar was determined by whether there was fungal colony growth and whether there was blackening of the medium. Morphological characteristics of the fungal colony on CHROMagar *Malassezia*® and the result of biochemical examination on each *Malassezia* species was based on the revised criteria by Kaneko *et al.* [15]. The identification criteria based on morphological characteristics of the fungal colonies on CHROMagar *Malassezia*® and result of biochemical examination on each *Malassezia* species can be seen in table 1. Data were analyzed using chi-square tests to test differences in the distribution of *Malassezia spp.* between the two groups. Statistical significance was defined by $p<0.05$.

RESULTS

Demographic characteristics of subjects

The demographic characteristics of research subjects based on the two groups is shown in table 2. There was no difference in characteristics between the two groups for all variables analyzed ($p>0.05$).

Proportion of spore existence from microscopic examination of facial AV lesions

There was no significant statistical difference in the proportion of samples with spores nor the number of spores based on microscopic examination on both groups (table 3).

Distribution of *Malassezia* species

There were 120 subjects who underwent cultures, of which 77 (64.2%) had positive culture *Malassezia spp.* Culture results. Fig. 1 shows the proportion of *Malassezia* species successfully identified.

Six samples were identified containing two species of *Malassezia*, one with *M. dermatis* and *M. furfur*, one with *M. dermatis* and *M. sympodialis*, and one with *M. slooffiae* and *M. sympodialis*. We also found two samples with *M. dermatis* and *M. japonica*, and one with *M. dermatis* and *M. pachydermatis*. There were 2 isolates that we could not identify by physiological or biochemical characteristics. The first isolate had a negative catalase reaction, but growth was seen without the formation of black areas on the Tween 60-esculin agar. The second isolate also had a negative catalase reaction, but a precipitate formed on CHROMagar *Malassezia*®. We concluded that both isolates could not be identified.

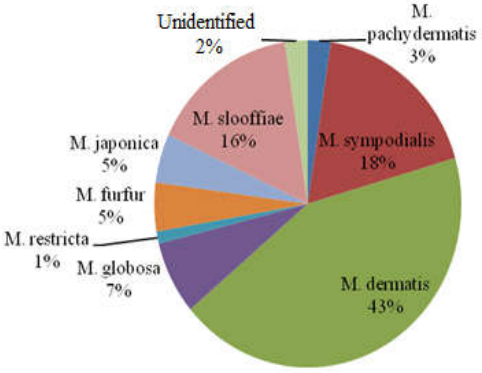

Fig. 1: Proportion of *Malassezia* species based from culture and biochemical examination of facial acne lesion

Proportion of *Malassezia spp.* from culture examination in facial AV lesions

In this study, positive cultures were found in 75% of samples taken from non-inflammatory lesions, while only 53% of samples from inflammatory lesions were positive. The difference in proportions was significantly different ($p=0.013$) (table 4).

Malassezia species tended to be less common in inflammatory lesions taken from subjects with mild AV (43.6%), while in

inflammatory lesions taken from subjects with moderate to severe AV, a larger proportion of isolated *Malassezia* species were found (71.4%). The relationship between culture and AV severity and the morphology of lesions can be seen in table 5.

Table 1: Morphological characteristics of colonies on CHROMagar *Malassezia*® and results of biochemical examination on *Malassezia spp*

Species	Growth on ASD	Colony characteristics on CHROMagar *Malassezia*®			Colony characteristics on tween 60-esculin		Catalase reaction
		Size	Color/morphology	Precipitate formation	Growth	Black zone formation	
M. pachydermatis	(+)	large	faint pink/smooth surface	(+)	(+)	(+)	(+)
M. sympodialis	(-)	large	faint pink/smooth surface	(+)	(+)	(+)	(+)
M. globosa	(-)	small	purple/smooth surface	(+)	(-)	(-)	(+)
M. dermatis	(-)	large	faint pink to purple/smooth surface	(+)	(+)	(-)	(+)
M. furfur	(-)	large	faint pink/wrinkled surface	(-)	(+)	(+)	(+)
M. slooffiae	(-)	small	faint pink/smooth surface	(-)	(+)	(-)	(+)
M. obtuse	(-)	medium	faint pink/irregular surface	(-)	(-)	(+)	(+)
M. restricta	(-)	small	faint pink/smooth surface	(-)	(-)	(-)	(-)
M. japonica	(-)	large	pink faint pink/smooth surface	(-)	(+)	(+)	(+)

Table 2: Clinical characteristics of subjects classified by AV lesion type

Parameter		Total		Groups				p-value
				Non-inflammatory lesions		Inflammatory lesions		
		N	%	N	%	N	%	
Age (years)		22.7	(±6.192)	21	(15-40)	21	(15-36)	
Sex	Male	46	38.3	22	47.8	24	52.2	0.707
	Female	74	61.7	38	51.4	36	48.6	
Education	Low (did not graduate primary school)	1	0.84	1	100	0	0	0.355
	Middle (high school)	78	65	36	46.2	42	53.8	
	High (diploma, university)	41	34.16	23	56.1	18	43.9	
Marital status	Single	90	73.33	45	51.5	43	48.9	0.673
	Married	30	25	14	46.7	16	53.3	
AV severity	Mild	86	71.7	47	54.7	39	45.3	
	Moderate-to-severe	34	28.3	13	38.2	21	61.8	0.105
Pruritus	No	74	61.7	40	54.1	34	45.9	
	Yes	46	38.3	20	43.5	26	56.5	0.26
Prior medication	Yes	81	67.5	39	48.1	42	51.9	
	No	39	32.5	21	53.8	18	46.2	0.559

Table 3: Proportion of spore existence based on microscopic examination facial lesions of AV patients

Lesion morphology	Microscopic examination								p-value
	No spores found		Spores found		+1		+2		
	N	%	N	%	N	%	N	%	
Non-inflammatory (n=60)	31	51.7	29	48.3	22	36.7	7	11.7	0.142
Inflammatory (n=60)	23	38.3	37	61.7	35	58.3	2	3.4	
						0.047		0.297	
					OR 2.1(1-4.5)		OR 0.38 (0.07-2.02)		

Notes: +1: 1-2 spores non clustered; +2: 3-12 spores, or 6 clustered spores.

Table 4: Proportion of *Malassezia spp.* based on the culture of inflammatory and non-inflammatory facial AV lesions

Lesions morphology	Culture results				
	No growth		Growth		p-value
	N	%	N	%	
Non-inflammatory (n=60)	15	25	45	75	0.013
Inflammatory (n=60)	28	46.7	32	53.3	

Table 5: The proportion of *Malassezia spp.* based on the culture of inflammatory and non-inflammatory facial AV lesions on the face categorized by severity

AV severity degree	Lesion morphology	Culture			
		No growth		Growth	
		N	%	N	%
Mild (n= 86)	Non-inflammatory (n= 47)	10	21.3	37	78.7
	Inflammatory (n=39)	22	56.4	17	43.6
Moderate-to-severe (n=34)	Non-inflammatory (n=13)	5	38.5	8	61.5
	Inflammatory (n=21)	6	28.6	15	71.4

DISCUSSION

The role of *Malassezia* species in AV is not precisely known. To date, there is only one study (Song *et al.*) that aimed to identify and determine the relationship between species of *Malassezia* in AV patients. The limitations of this study were that the extraction of samples was done with a smear technique which did not involve the contents of the pilosebaceous follicle and there was no comparison between inflammatory and non-inflammatory AV lesions [16].

In our study, despite the severity of the disease, spores were found in 61.7% of inflammatory lesions and 48.3% of non-inflammatory lesions in AV patients and this was not a significant difference (p=0.142). However, we also found in that non-inflammatory lesions there was a trend towards a higher number of spores from each sample compared to those of inflammatory lesions (11.7% and 3.3%, respectively). This study also showed that in inflammatory lesions, only 38.3% of patients had spores and most had a score of+1 (58.3%). This finding is similar to Jacinta-Jamora *et al.*'s study in that papulopustular lesions or nodes of MF contained lesser or no *Malassezia* spores, while molluscoid comedo lesions contained more spores, a pathognomonic manifestation of MF [12].

M. dermatis was identified in 36 samples and was predominant in both inflammatory (45.8%) and non-inflammatory lesions (41.7%). This finding differs from a Korean study by Song *et al.* that reported *M. restricta* as the most common species identified in inflammatory AV lesions [12]. In studies by Akaza *et al.* and Numata *et al.*, the identification of *Malassezia* species was done using polymerase chain reaction (PCR) with primers for *M. globosa* and *M. restricta* only, resulting in an inability to identify other *Malassezia* species, including *M. dermatis* [7,8]. In an Indonesian study of seborrheic dermatitis patients, Zoulba *et al.* reported that *M. dermatis* is one of the most common species identified in seborrheic dermatitis of the scalp (23.2%), followed by *M. globose* [17].

M. sympodialis was identified from 15 samples. This species was also identified in the Korean study [16] and was almost always reported on various studies of *Malassezia spp.* in differing proportions [18–20].

M. slooffiae was identified in 13 samples. This species is a normal flora that is found on the face and is reported to have a specific reservoir in the external auditory canal in humans [21] This species was not identified in the Japanese and Korean studies; however in Bosnia and Herzegovina, this species was found to be predominant on the scalp [22]. Studies on seborrheic dermatitis in Serbia identified *M. slooffiae* as the predominant species in seborrheic dermatitis lesions of the scalp [19].

The detection of two *Malassezia* species in a single AV lesion had not been reported previously. Although Akaza *et al.* reported similar findings, the study was on MF, in which most MF lesions had more than two species per sample [23].

Results based on lesion morphology are shown in table 4. There were positive results in 75% of cultures of samples taken from non-inflammatory lesions, and 53.3% of samples from inflammatory lesions. Statistical analysis showed a significant difference (p=0.013). This result had not been previously reported and is a novel finding.

The relationship between the existence of *Malassezia spp* and AV is still inconclusive. Song *et al.* showed a lower rate of *Malassezia spp.* Found in AV (50%) compared to non-AV patients (71.6%) using the skin smear method. This result was thought to be caused by the excess growth of *P. acnes* [16]. We found that the number of spores in inflammatory AV lesions tended to be fewer than that of non-inflammatory AV lesions.

The identification of *Malassezia* species in non-inflammatory AV lesions may be linked to the suggested role of *Malassezia spp.* in AV lesion formation through lipase activity and activation of TLR-2, which in turn trigger the release of pro-inflammatory cytokines [24]. This process is also linked to inflammation in AV that is not only induced after the formation of microcomedones, but is also present before lesions form. A study by Jeremy *et al.* reported an increase in the number of inflammatory cells and pro-inflammatory cytokines surrounding normal pilosebaceous follicles of AV patients [25].

The results of this study can also be attributed to the fact that most of our subjects had mild AV. *Malassezia* species tended to be less common from inflammatory lesions from subjects with mild AV (43.6%); however, in inflammatory lesions from subjects with moderate-to-severe AV, the proportion of *Malassezia* species isolates was higher (71.4%). In this study, we did not use a standard *Malassezia* species strain (reference strain) as a positive control, which would have otherwise strengthened our results. This was due to its limited availability in Indonesia.

CONCLUSION

Malassezia spp. were found in AV lesions on the face. *Malassezia* species predominantly found in AV lesions on the face in this study were, in order of abundance, *M. dermatis, M. sympodialis,* and *M. slooffiae.* Other species included *M. globosa, M. japonica, M. furfur*, and *M. restricta*. There was a difference in the proportion of *Malassezia* species between inflammatory and non-inflammatory AV lesions with a higher abundance found in non-inflammatory lesions.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

All authors have none to declare.

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