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A novel combinatorial approach for the Identification of *Cutibacterium namnetense* and *Cutibacterium modestum* from Facial Acne Samples

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Abstract

Background: *Cutibacterium* spp. is one of the most understudied bacteria and this is owed to its slow growing nature and its stringent requirement for anoxic conditions. To date, shortgun metagenomic sequencing and MALDI-TOF MS are widely used for species detection but, the latter is not able to distinguish *C. acnes* from *C. modestum* and *C. namnetense*. Our study has innovatively combined colony morphology, biochemical assays and16s rRNA gene sequencing to identify *C. acnes* as well as the underreported *C. namnetense* and C. *modestum* from facial clinical acne samples.

Methods: The clinical samples were obtained using a non-invasive method from acne patients at the Dermatology Clinic of Hospital Tuanku Jaafar, Seremban, Malaysia between January 2022 to December 2022. Colonies of *Cutibacterium* spp. were screened on BHI agar followed by subjecting them to the catalase and indole tests. The isolates were verified as *Cutibacterium* spp. using API20A and 16s rRNA Sanger gene sequencing.

Result: Out of 68 *Cutibacterium* spp. isolates, 3 were identified as *C. modestum* and 1 as *C. namnetense* while the rest were *C. acnes*. All isolates were present as raised, white colonies with 0.03 to 1mm in diameter on BHI agar. 89.71% of these isolates were indole producers. All isolates were identified as *C. acnes* in API20A but, the 16srRNA gene sequencing revealed 4 isolates as *C. modestum* and *C. namnetense*.

Conclusion: This study is the first to report the isolation of *C. namnetense* and *C. modestum* in clinical facial acne samples from Malaysia and across Asia, employing a modified combination of morphological, biochemical, and 16srRNA gene analyses. This methodical yet straightforward approach serves as a viable alternative in research settings lacking access to advanced techniques like MALDI-TOF and shotgun metagenomic sequencing. Moreover, this conventional isolation approach is valuable in assessing the sensitivity of the isolates to inhibitory agents apart from antibiotics, expanding researchers' abilities to develop potent antibacterial agents required for human health and wellbeing.

Introduction

Cutibacterium spp. are aerotolerant anaerobes and are comprised of *C. acnes, C. granulosum, C. avidum, C. modestum* and *C. namnetense* [1]. While *Cutibacterium acnes* is long known to cause acne vulgaris (AV), less is known of the role of *C. modestum* and *C. namnetense* in the pathophysiology of AV [1,2]. *Cutibacterium modestum* previously known as *Propionibacterium humerusii*, was first isolated from human humeral bone in 2011 [3]. They are also found in the pilosebaceous unit of the skin and have been isolated together with *C. acnes* and thus, the human skin is predicted to be the main reservoir for this bacterium. However, this is yet to be verified [1]. *Cutibacterium namnetense* was first identified in 2016 and was found in diverse locations of the human body including the skin and bones [1].

Unlike other skin bacteria, *Cutibacterium* spp. remains a challenge for identification using conventional techniques. This is because, they require anoxic conditions and at least 5 days or more to grow on a solid growth medium [2,4]. Presence of oxygen retards their growth even though they are aerotolerant cells. In addition, enriched media such as BHI, TSA, Blood agar, Brucella Blood Agar, SLM, Reinforced Clostridial Agar are needed for cultivation. Some of these media are not only expensive but also have a shorter shelf-life making the conventional isolation cost intensive. A similar study even used furazolidone in the growth medium to inhibit the growth of other skin bacteria to prevent them from deterring the detection of *C. acnes* in the clinical samples [5]. Altogether, these challenges have caused *Cutibacterium* spp. to be less commonly studied compared to other skin bacteria such as *Staphylococcus aureus* and *S. epidermidis* [2,6,7]. Due to this, MALDI-TOF is widely used as an alternative in the detection of *C. acnes* mainly in clinical samples. However, this robust system, is unable to discriminate *C. modestum* and *C. namnetense* leading to the misidentification of these species as *C. acnes* [1]. More recent studies have resorted the use of shortgun metagenomic sequencing to identify *C. modestum* and *C. namnetense* in clinical samples which is essential for rapid disease diagnosis [8-10]. However, this system cannot be used if progressive work is required to study these clinical isolates for their sensitivity towards potent antibacterial agents apart from antibiotics.

Thus, this study was aimed to devise a cost-effective systematic approach to identify *Cutibacterium* spp. from clinical acne samples using a combination of colony characteristics, biochemical tests and 16s rRNA gene Sanger sequencing enabling researchers to further investigate this understudied bacterial species.

Methods

Ethical Clearance

Ethical approval was acquired from the National Medical Research Register, Malaysia (NMRR-21-1891-61558) and the Research & ethical Committee of INTI International University (INTI/UEC/2018/001) before the commencement of the study. The Patient Information Sheet (PIS) or the informed consent form as approved by the Medical Research Ethics Committee (MREC) was signed by the patients who took part in this study. Two language versions of the PIS were available: English and Bahasa Malaysia. The Case Report Form was used to record patients brief demographic details along with the sample number.

Sample Collection

138 acne samples were obtained from acne patients at the Dermatology Clinic of Hospital Tuanku Jaafar Seremban (HTJS) between January 2022 to December 2022. Patients who were pregnant, below 18 years old, receiving topical antibiotic therapy, or had used antibiotics in the past were excluded from this study. A panel of dermatologists at HTJS evaluated the severity of the acne (mild, moderate, and severe) using the Comprehensive Acne Severity Scale (CASS) before sample collection. Samples were taken from the patient's forehead, cheeks, jaw, and areas of papules, pustules, and nodules. The skin area above the acne lesions was gently swabbed with an alcohol pad (70%) followed by swabbing with the Amies transport media swab (Microscience) with charcoal. The swabs were transported to the Molecular Biology Laboratory 1 of INTI International University within 24-48 h upon collection for processing.

Colony Screening on BHI Agar

The Amies swabs were streaked on BHI agar (Oxoid) and incubated for five days at 37 °C in anoxic conditions (Gaspak EZ, BD). Pure cultures were obtained by subculturing single colonies with morphology typical of the positive control *Cutibacterium acnes* ATCC 11827 (raised, smooth, pearly-white, 0.03 mm to 1 mm diameter) on BHI agar under the same growth conditions. The pure cultures were subjected to indole and catalase tests as well as gram staining prior to identifying with Biomerieux's API20A [11–13].

Molecular Analyses

DNA Extraction

Single colonies were cultivated in BHI broth (Oxoid) and grown in anoxic conditions. After centrifuging the bacterial culture for 5 min at 4000 rpm, the pellet was resuspended in 200 µL of cell lysis buffer (Tween20, TritonX, 0.5M EDTA pH 8.0, 1M Tris-HCL, pH). The cell lysate was vortexed vigorously for 2-3 min and placed

in a dry heat block at 95 ℃ for 15 min [14]. The cell lysate was re-centrifuged for 5 min at 4000 rpm, and the supernatant containing the DNA was electrophoresed in a 1% agarose gel that had been prestained with ViSafe Red Gel dye (Vivantis).

Amplification of 16S rRNA gene and Phylogenetic Analysis

The DNA of the isolates and *C. acnes* ATCC 11827 were amplified using universal primers; forward primer, 50-AGAGTTTGATCCTGGCTCA-30 (27), and reverse primer, 50- AAGGAGGTGATCCAGCCGCA-30 (1525) corresponding to bases 27 and 1525 of the 16s rRNA gene [15]. The PCR mixture was done using the GoTaq Green Master Mix, 2X, Promega in a total volume of 25 µL adapted from the study by Alnabati et al (2021) [15]. The thermocyling conditions were applied as described by Alnabati et al (2021) [15]. The PCR products were sent to Apical Scientific Sdn Bhd to determine the sequence. The data obtained was analysed using Bioedit.Ink and MEGA11 (version 11) and compared with the sequences in the NCBI database. The confirmed isolates of *Cutibacterium* spp., were aligned with several reference sequences that served as ingroup and outgroup species using the ClustalW method in MEGA11. The reference taxa were chosen based on the study by Dekio et al (2019) [16]. The Bayesian information criterion (BIC), the HKY model was chosen to generate the tree. The reliability of the internal branches was assessed using standard bootstrap (SH-aLRT), aBayes test and the ultrafast boot-strap (UFBoot) tests for 1000 replicates respectively. The phylogenetic tree obtained through IQ-Tree was reconstructed in FigTree V1.4.4 and MEGA11.

Results

Colony Identification on BHI Agar

A total of 138 facial acne swabs were obtained from the acne patients who participated in this study from January 2022 to December 2022. 68 *Cutibacterium* spp. isolates were obtained with 94% (n=64) of these isolates were identified as *C. acnes* while, 4% (n=3) isolates were identified as *C. modestum*. 2% (n=1) of the isolate was identified as *C. namnetense*. The facial acne swabs streaked on BHI agar produced colonies with heterogenous appearance. However, the colonies of *Cutibacterium* spp. were distinct from the other colonies that were mostly pale yellow, convex with irregular morphologies and a diameter of more than 1mm (Figure 1). All *Cutibacterium* spp. isolates were observed as raised, pearly-white and smooth colonies ranging from 0.03 to 1.00 mm in diameter within 5 days of incubation in anoxic conditions. Interestingly, no distinct differences were observed between the *C. acnes*, *C. namnetense* and *C. modestum* colonies (Table 1). This colonies were subcultured on BHI agar to obtain pure cultures and were further identified using gram staining and other biochemical tests as well as 16s rRNA gene sequencing.

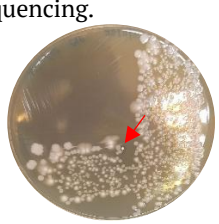


Figure 1: The raised, white colony of *C. acnes* (arrow) in acne sample N22/118 following growth on BHI agar. Colonies of Cutibacterium spp. were distinguishable from the rest of mixed culture population due to their colony characteristics.

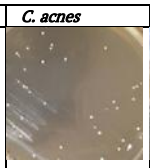
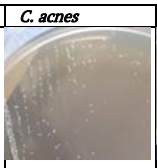
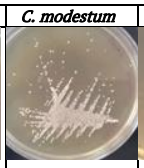
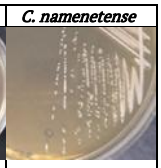
Species	*C. acnes*	*C. acnes*	*C. modestum*	*C. namenetense*
Colony appearance on BHI Agar				
Isolate Number	ATCC11827 (positive control)	J22/04 (5)	M22/57(4)	J22/78(5)

Table 1: Colony appearance of *Cutibacterium* spp. on BHI agar. All colonies were observed as pearly-white, raised and with smooth consistency. No variations were detected in colony morphology between the isolates of *Cutibacterium* spp.

Biochemical Identification of *Cutibacterium* spp.

All of the *Cutibacterium* spp. colonies identified on BHI agar were Gram positive pleomorphic rods with either single or pair wise arrangements (Table 2). All isolates were also catalase-producers.

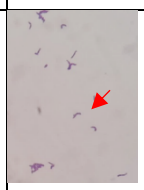
Types of Test	Gram Staining	Catalase	Indole
			
Observation description	Gram positive pleomorphic rod isolate O22/107(5) when viewed under brightfield microscope at 100x magnification (oil immersion). Cells were either in single or pairwise arrangements (arrow)	Catalase positive J22/04(5) with bubble formation (arrow) upon the addition of 3% H_2O_2 (v/v)	The variations in indole reaction among the *Cutibacterium* isolates. A: Indole-negative isolate J22/67(5) with the absence of the red ring, (B) Indole-positive isolate J22/68(5) – presence of red ring.

Table 2: Summary of gram staining results and biochemical tests. All isolates were observed as catalase producing pleomorphic rods. 7 isolates tested negative for indole production while, all other isolates were indole producers.

89.71% (N=61) of the 68 *Cutibacterium* spp. isolates and *C. acnes* ATCC11827 were indole positive while the rest were indole-negative. Out of the 3 isolates of *C.*

modestum, 2 were negative for indole production while, the *C. namnetense* were indole positive. All 68 isolates were identified as *C. acnes* with the percentage of identity of more 99% using the API20A kit.

16srRNA Gene Sequencing

The PCR products for the 16srRNA gene in all isolates were separated by electrophoresis in a 1.5 % agarose gel and were observed at 1500 bp in size (Figure 2).

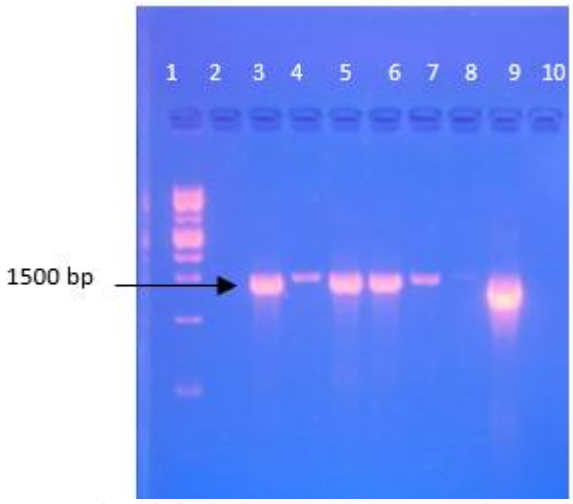


Figure 2: The gel electrophoresis of 16s rRNA amplicons. The PCR products were amplified at 1500 bp (arrow). Lane 1: VC 1kb DNA ladder; Lane 2: Empty; Lanes 3-9: 16s rRNA gene of M22/55(5), M22/56(4), M22/57(4), M22/58(4), M22/59(5), M22/60(5) and J22/61(5); Lane 10: Negative control.

The partial sequencing of the 16srRNA gene which were analyzed using Bioedit.Ink followed by BLAST revealed more than 99% of nucleotide similarities with the *Cutibacterium* sequences deposited in the GenBank database. The phylogenetic analysis using MEGA11, FigTree and NCBI database revealed that the some of the isolates of *C. acnes* along with the positive control *C. acnes* ATCC11827 were clustered with reference strain LC7522328.1 *C. acnes* ATCC6919 and *C. acnes* elangotum NR145912.1 (Figure 3). *C. namnetense* (Isolate J22/78 (5)) were clustered with strains KM507346.1 *C. namnetense* whereas, *C. modestum* isolates M22/57(4) and J22/67(5) were clustered with strains LC466959.1 (*C. modestum*), LC637867.1 (*C. modestum*) and LR118646.1 (*P. humerusii* – now, known as *C. modestum*) (Figure 3). The support values at the main branches showed more than 91% of confidence level for SH-aLRT (Figure 3). Although the UFBoot analysis showed a varying degree of confidence ranging between 85%-100%, the additional bootstrapping support of 0.91 to 1 for the Bayesian analysis (aBayes test) indicates the overall reliability of the phylogenetic tree. Accession numbers NR115826.1 and NR114803.1 were used as the outgroup (Figure 3).

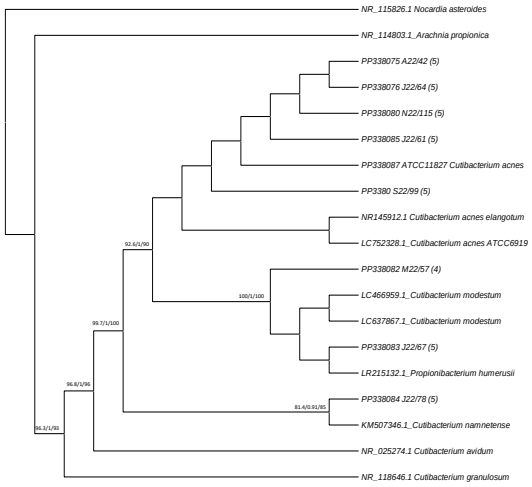


Figure 3: Maximum likelihood tree generated using IQ-Tree, FigTree v1.4.4 and MEGAX from the 16s rRNA gene sequences of the isolates of *Cutibacterium* spp. including *C. acnes* ATCC11827 and the reference sequences from NCBI. Highlighted in green boxes are isolates identified as *C. modestum* and *C. namnetense*. The branch support values (in blue) are in the sequence of SH-aLRT, aBayes and UFBoot with an accepted confidence level of 85%, 1 and 95% respectively.

Discussion

While many studies of similar nature have used invasive methods to obtain cultures of *C. acnes,* our study has shown that even the isolation of *C. namnetense* and *C. modestum* as well as *C. acnes* is possible using a non-invasive approach [15-17]. A similar finding was reported in a previous study in Malaysia but no studies have been documented in Malaysia or in Asia on the use of non-invasive approach to isolate *C. namnetense* and *C. modestum* from clinical acne samples [17]. The isolation of *Cutibacterium* spp. colonies on BHI agar was crucial in this study as it led to successful identification of *Cutibacterium* spp. thereafter. This was done by thorough screening of colonies that showed similar morphology with the positive control *C. acnes* ATCC11827. These colonies always appeared raised and pearly-white in colour with a diameter ranging from 0.03 to 1.00 mm making them easily distinct from other colonies which were significantly larger, with a convex elevation and irregular morphologies. No distinct differences were observed between the colonies of *C. acnes, C. modestum* and *C. namnetense* making it relatively easy to identify these bacteria if this method is reproduced for future work using clinical and non-clinical samples. Such detailed descriptions on the colony morphology of *C. acnes, C. modestum* and *C. namnetense* on BHI agar to our best knowledge, is the first. *C. acnes* is generally grown in media specific for anaerobic or fastidious bacteria such as Schaedler agar, chocolate agar, Brucella blood agar, Wilkins-Chalgren

agar, reinforced clostridial medium, blood agar as well as brain heart infusion agar [18-21]. In our study, the brain heart infusion agar proved to be a cost- effective medium with an extended shelf life enabling the growth of not only *C. acnes* but also *C. namnetense* and *C. modestum.* Although the number of *Cutibacterium* spp. colonies were significantly lower than the larger colonies which are either *Staphylococcus* or *Corynebacterium*, the other common bacteria in acne samples, the stark differences in the elevation, the colony colour and consistency eased the screening process [22]. In fact, the colonies screened to be similar or with the same colony morphology with the positive control were all identified as *Cutibacterium* spp. This is noteworthy as the identification of colony morphology is the pivotal step in the identification of the *Cutibacterium* spp. We have also shown that the isolation of *Cutibacterium* spp. from clinical isolates can be done without the incorporation of furazolidone in the growth medium in contrast with the study by Sheffer-Levi et al. (2020) [5]. However, the bacterial cultures must be incubated at 37°C in an anoxic condition for 5 to 7 days [15]. *Cutibacterium* spp. including the positive control were initially attempted to be grown using Candle jars as they are aerotolerant anaerobes but, no growth was visible even after 7 days (data not included). Hence, future isolation of *Cutibacterium* sp must be done in anoxic conditions using anaerobic growth systems such as GasPak (BD BBL) and Anaerogen (Merck, Millipore) [4].

The indole production using the Kovac's indole reagent showed mixed results. While most of the previous studies have shown indole production as a main biochemical property for the isolation of *Cutibacterium* spp. from clinical or non-clinical samples, we report that a small percentage of the isolates of *C. acnes* as well as *C. modestum* and *C. namnetense* were indole-negative [23-25] . Puhvel (1968) has pointed out that the differences in the production of indole is mainly due to the differences in the strains of *C. acnes* [26]. Thus, the variation in indole production must be considered when *Cutibacterium* spp. including *C. modestum* and *C. namnetense* are being identified to prevent the indole-negative strains from being disregarded for further analysis.

API20A was used in this study as only *C. acnes* was anticipated to be isolated from the clinical acne samples due to the limited studies reported on *C. namnetense* and *C. modestum* during the research period. However, similar studies in the future should use API rapid 32A to enable rapid discrimination of *C. acnes* from *C. modestum*, *C. namnetense* and possibly other species of *Cutibacterium* [23]. The 16S rRNA gene is widely used for the identification of bacteria as the presence of hypervariable regions provide significant sequences to distinguish bacterial species [27]. The stretch of the conserved regions which flank the hypervariable regions enables the design of universal primers for the identification of bacterial species [27]. The primers used in this study were forward primer, 5'-AGAGTTTGATCCTGGCTCA-3'(27), and reverse primer, 5'-AAGGAGGTGATCCAGCCGCA-3'(1525) used by Alnabati et al (2021) was able to distinguish *C. acnes* and the closely related *C. modestum* and *C. namnetense* and this was not reported elsewhere at the time of writing [15]. This is noteworthy, as it shows that a relatively simple and cost effective 16srRNA gene sequencing is discriminative enough to differentiate species of *Cutibacterium* unlike the limitations reported by Ruffier d'Epenoux et al. (2020) which showed the misidentification of *C. namnetense* as *C. acnes* when MALDI-TOF was used (Ruffier d'Epenoux et al., 2020) [28]. Similar limitation was also reported by Goldenberger et al (2021) when MALDI-TOF is widely used in clinical settings in the detection of *C. acnes* from clinical samples [7].

The phylogenetic analysis was also consensus with the study by Dekio et al (2021) as the isolates obtained in this study were closely clustered with *C. acnes, C. namenetense, C. modestum* and *C. avidum* [1]. In addition, all *C. acnes* isolates including *C. acnes* ATCC11827 were grouped with the reference strain LC752328.1 *C. acnes* ATCC6919 while, some isolates of *C. acnes* were grouped closer to NR145912.1 *C. acnes* subs *elangotum*. In general, the maximum-likelihood tree indicated reasonably accepted relationships between the isolates and the reference strains. This is evidenced by the support values at the main branches showing more than 97% of confidence level for SH-aLRT [29]. Although the UFBoot analysis showed a varying degree of confidence ranging between 67%-100%, the additional bootstraping support of 0.7 to 1 for the Bayesian analysis (aBayes test) indicates a reliable phylogenetic tree. SH-alRT, UFBoot and Bayesian analysis are bootstrapping methods incorporated in IQ-Tree. IQ-Tree is a fast and efficient in constructing phylogenetic tree [31]. It is also widely used for its rapid analysis. The inclusion of 3 branch support analyses in IQ-Tree which are the standard bootstrap (SH-aLRT), aBayes test and the ultrafast boot-strap (UFBoot) in a single run makes it a robust system [30-32].

The isolation and identification of *Cutibacterium* species succeeded with the screening of colony morphology on a cost-effective brain heart infusion agar. The morphological identification proved to be a crucial step in isolating *Cutibacterium* species, complemented by 16srRNA gene analysis that overcame limitations observed with the MALDI-TOF

system. While shotgun metagenomic analysis is currently favored for its rapid and accurate identification of *Cutibacterium* species, our methodical conventional approach offers a viable alternative, particularly in regions lacking access to advanced analytical methods. In conclusion, our study presents an accessible method for the isolation and identification of *Cutibacterium* species from clinical samples and possibly even from non-clinical samples advancing research efforts in various global settings. The systematic and cost-effective approach which starts with the colony morphology screening on BHI agar, biochemical tests, API 20A and 16s rRNA gene sequencing gave a 100% success rate in the identification of *C. acnes* isolates from facial acne clinical samples and is expected to do the same when this methodical approach is replicated using other clinical samples for *Cutibacterium* spp. isolation.

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Author Contributions

Lalita Ambigai Sivasamugham: Planned and designed the study, experimental conduct, data analysis, manuscript generation
Geetha Subramaniam: Planned the research, supervised the study, reviewed the data, manuscript revision
Wong Ling Shing1: Reviewed the data, supervised the study, manuscript revision
Preamala Gunabalasingam: Sample collection
Nithiya Visayaragawan: Sample collection
Nurfara Ain Ramli: Sample collection
Gan Li-Lian: Clinical advisor
Ravindran Vythilingam: Sample collection
Anshoo Agarwal: Advisor

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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