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REVIEW ARTICLE OPEN ACCESS

Challenges in Characterization of Coagulase Negative *Staphylococcus* by Conventional Methods and Comparison with Molecular Diagnostic Modalities

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Abstract

Coagulase-negative *Staphylococcus* (CoNS) has emerged as a significant primary pathogen, frequently determined in hospitalized individuals with immune deficiencies, and regularly with indwelling or implantable clinical devices. Most infections are nosocomial, and conservative infections can lead to a variety of chronic diseases, such as urinary tract infections (*S. saprophyticus*), infections brought on by medical devices, endocarditis, mediastinitis, meningitis, and bloodstream infections. Although many new species have been revealed in recent years, there is still a defect in clinical details for most of that genus. Additionally, interspecies variations that must be considered make certain species more pathogenic than others. Blood cultures and other samples are commonly contaminated because CoNS has the ability to colonize human skin. Determining cases where CoNS are causal agents rather than contaminants is hence the key diagnostic challenge. However, neither phenotypic nor genetic approaches have been successful in offering a satisfactory answer to this issue. MALDI-TOF MS (99.3% of strains properly identified) was the most effective method for speedy and precise CoNS differentiation. Vitek2 paired with partial tuf gene sequencing might be an alternative that yields 100% accurate strain identification when both techniques are used together. All of the staphylococcal subspecies under investigation, include *S.capitis* subspecies capitis as well as subspecies urealyticus, *S.cohnii* subspecies urealyticus, *S.saprophyticus* subspecies saprophyticus, and *Staphylococcus hominis* subspecies novobiosepticus and subspecies. As a result, staphylococci can be routinely identified by MALDI-TOF MS without incurring significant consumable expenses or requiring time-consuming DNA sequence analysis. The revolutionary high-quality RIDOM is superior to phenotypic techniques and the NCBI database. Staphylococci are easily identified using sequencing databases, even seldom isolated species and phenotypic differences. Another challenge facing CoNS is their comprehensive antimicrobial resistance profile is relevant to clinical practice, notably in medical facilities. Consequently, genuine infections brought on by CoNS require the usage of second-line antimicrobial medications the majority of the time.

Keywords: Coagulase-Negative Staphylococci, Hospital-acquired Infections, MALDI-TOF MS, Whole-Genome Sequencing

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INTRODUCTION

A broad category of Gram-positive cocci in clusters (GPC), known as Coagulase Negative Staphylococcus (CoNS), are related by the general lack of the pathogenic component, coagulase.[1] But in recent years, several CoNS species—including *Staphylococcus haemolyticus, Staphylococcus hominis,* and *Staphylococcus epidermidis* have become significant opportunistic infections, primarily infecting xenograft or opportunistic infections.[2] *Staphylococcus borealis*, which was first described in 2020, is the group's most recent species.[3] In routine clinical practice as opposed to possessing coagulases, CoNS are typically thought to be lower aggressive than *Staphylococcus aureus* (S.) and some other components of the *S. aureus* complex.[4-6] The published literature has a significant difference, with articles on *S. aureus* substantially outpacing those on CoNS. This mismatch highlights how little research has been done to evaluate his CoNS in previous years and decades. Foreign objects, such as catheters, are linked to numerous CoNS infections and encourage the production of biofilms, which increase CoNS pathogenicity.[7] The CoNS group includes *Staphylococcus lugdunensis*, which was initially identified in 1988.[8] Since then, interest in *S. lugdunensis* a growing factor in rheumatic fever. It exhibits clinical symptoms like *Staphylococcus aureus* and is reported to infect native valves more than other CoNS.[9] Numerous case studies have demonstrated that Huge septic embolisms and fast cardiac valve damage are two serious effects of *S. lugdunensis*.[10,11] The value of early surgical surgery is highlighted by several findings.[12] Additionally, *S. lugdunensis* is frequently susceptible to penicillin and other limited-spectrum antibiotics, according to recent research indicate that benzylpenicillin outperforms standard isoxazolyl penicillin or vancomycin treatments for susceptible isolates. Is possible alternative.[13,4] As a result, they are often classified as contaminants rather than infectious agents. It is not always easy to distinguish between contamination and infection, (shown in Figure 1) and the majority of prior attempts to pinpoint one or more distinctive markers have been fruitless. This review focuses on CoNS-associated infections, diagnostic challenges, and the growing clinical importance of current treatment options.

CoNS's Increasing Clinical Impact

CoNS are being utilized more frequently in therapeutically relevant samples like blood cultures or other samples that are largely sterile.[14] The use of implantable foreign bodies and advanced immunosuppressive or immunomodulatory treatment regimens in oncology are growing.

Neonatal sepsis is a leading cause of high death and disability ratio, and CoNS have reportedly been the leading neonatal intensive care unit's newborn sepsis-causing factor (NICU).[15] In 49 hospitals across the US, CoNS represented about 31% of all community-associated BSI cases over a 7-year period.[15] This result has since been verified in several additional cohorts from varied geographic backgrounds.[16] CoNS was shown to be the second very typical source of healthcare-associated infections (HAI), in an observational study that looked at the prevalence of hospital-acquired illnesses in German university hospitals.[17]

As a result of the formation of biofilms by certain CoNSs, bacteria can attach to surgical equipment and resist antibiotics.[18]

In addition, elderly, preterm infants, or immunocompromised patients, those with significant comorbidities, in particular, are more susceptible to CoNS-related BSI, inflammation of the skin and vulnerable tissues, and natural and prosthetic heart valve inflammation. Furthermore, Young, healthy people can potentially get CoNS-associated infections. For example, *S. saprophyticus*, particularly in younger women, causes urinary tract infections.[1]

The diagnostic difficulties of Coagulase negative Staphylococcus which differentiate among both infections and contamination

The studied organism, such as CoNS members, which may or may not be the cause of the patient's infection, provides the most diagnosis-related challenge. Various methods have been proposed to identify Staphylococcal species, as well as common methods of identification based on works by Kloos and Schleifer[19] and readily available tests for identification. The fact that phenotypic trait-based approaches depend on the manifestation of energy metabolism and/or physical features generally limits their applicability. Additionally, commercial systems frequently include two or several of his identification

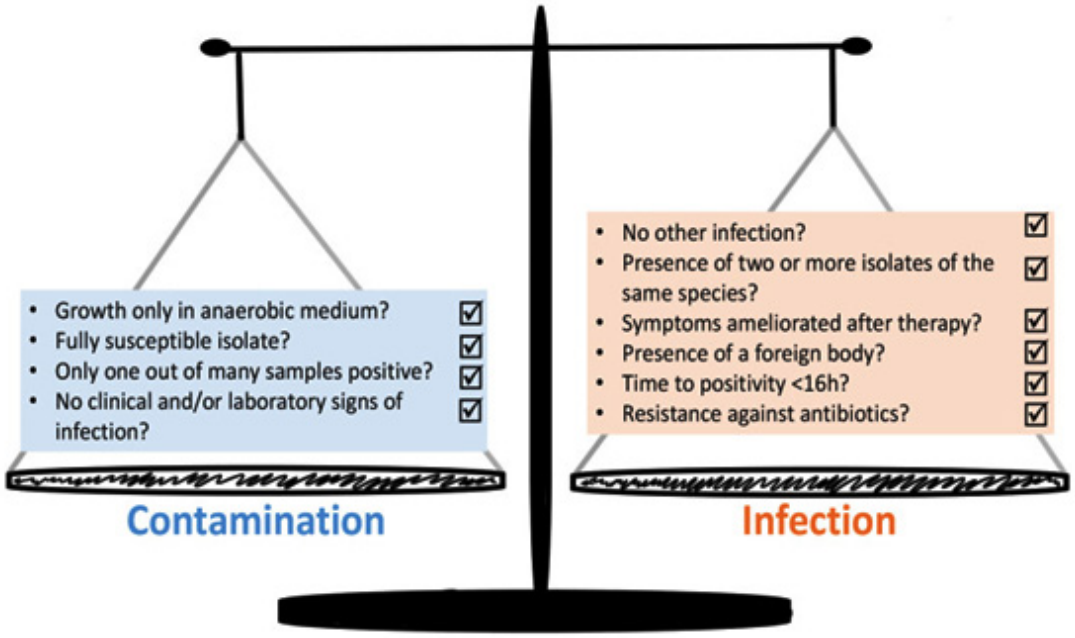

Figure. 1. When CoNS are discovered in a leading sterile material, a model for separating contamination from infection is used; BC stands for blood culture

ideas with equivalent levels of security. With the development of molecular methods, it is now normal to practise microbiology to classify microbes using relative Genomic DNA analysis of genes for conserved macromolecules. The bigger rRNA molecules or their related genes, particularly 16S rRNA as well as a smaller extent 23S rRNA, are among the most beneficial and extensively researched classification marker components.[20] Although molecular analysis depending upon universal target sequence data has several benefits, including greater accuracy and quick turnaround times, There are many identical or not accurate sequence entries in the most recent databases, as well as other issues including irregular sequence endings and no longer relevant presence. There is no nomenclature or quality control.[20] When collecting CoNS from a patient's various blood cultures, precise CoNS identification is essential. Several antibiotics have been said to be resistant to many CoNS.[21-24] Analysis of bacterial protein profiles is possible with matrix-assisted laser adsorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). This is a newly invented method that has been proven to be effective and accurate.[25] The Staphylococci genus has around 40 known species and subspecies. Therefore, differentiating among staphylococcal species may have broad clinical and epidemiological significance.[7] Nevertheless, it is difficult to differentiate these organisms at the species level; particularly when traditional phenotypic techniques are used, certain strains can be mistaken for closely related species. Although not a single gene, DNA sequence analysis employing many nucleotide targets is an alternate method to identify Staphylococcal species (such as 16S rDNA).[26]

Due to matrix-assisted laser adsorption ionization time-of-flight mass spectrometry, it is now possible to immediately identify the protein composition of microbial cell lines (MALDI-TOF MS).[27] Comprehensive type and reference strain analysis as an integral part of the software that advertises its accuracy and reproducibility includes Waters MicrobeLynx and The BioTyper from Leipzig, Germany-based Bruker Daltonik GmbH (Waters Corp., Milford, MA, USA) Determination of inexpensive species of major groups of pathogenic bacteria.[7]

When CoNS are discovered in a leading sterile material, a model for separating

contamination from infection is used; BC stands for blood culture.

In order to avoid misidentifying CoNS as the source of infection, Beekmann et al. suggested that in fewer than five days, Coagulase Negative Staphylococcus should be detected in at least two different blood cultures. Alternatively, if clinical symptoms point to infection, a positive blood culture is sufficient.[28]

In a comparative study by Loonen et al., five of CoNS were compared. The identical set of CoNS strains received examination for transient 16S ribosomal RNA, partial 16S rRNA gene sequencing, partial tuf gene coding inside, MALDI-TOF MS, Vitek-2 (GRAM positive card REF-21342 from BioMérieux), ID 32 Staph strip, and transient 16S ribosomal RNA (MicroSeq from Applied Biosystems).[25]

Loonen, Anne JM found 142 distinct CoNS strains and others The National Institutes of Health (NIH), the American Type Culture Collection (ATCC), and Environmental Research provided 25 reference strains of isolates that were collected from clinical culture (n = 117). (RIVM; Bilthoven, The Netherlands). In this research, 25 reference strains were utilized. The 16S sequencing protocol failed to discriminate 9/25 reference strains in terms of species (36%). ID 32, Vitek2 Sequencing for staph and tuf misidentified 2/25 reference strains (8%). By using MALDI-TOF MS, all reference strains had been recognized. Others have looked into using MALDI-TOF MS to identify staphylococcal species straight from agar plates, with success rates as high from 74.2% to 99.3%. The statistics from this investigation are equivalent to those from Spanu et al.[7] Although Dubois et al.[29] discovered a 99.3% sensitivity, individuals during this investigation had just one strain of *S. hominis*, for unclear reasons, and it was unidentifiable by MALDI-TOF MS.[25]

Diagnostically Coagulase Negative Staphylococcal (CoNS) isolates were swiftly identified, according to Carbonelle et al.,[30] utilizing a set of peaks selected from each of the 23 reference strains that are clinically important species and subspecies of Micrococcaceae. With the help of this database, Carbonelle's group has successfully recognized the species in 97.4% of tested CoNS isolated using MALDI-TOF-MS analysis. They also showed how it might be used as a data structure.[31,7]

It has been established, particularly when conventional phenotypic markers were used, that unexplained disappearances frequently result from the variable behaviors of her CoNS populations obtained from clinical specimens. Dupont et al.[31] uploaded this on their website. Two independent information seeking the effectiveness of the current automated tests performed [Phoenix and Vitek 2 (bioMe'rieux, Marcy l'Etoile, France) (Becton-Dickinson Becton- Dickinson Biosciences, Franklin Lakes, NJ, USA) Systems] found that the proper detection rates for CoNS were only 90.5% and 87.5%, respectively. All 134 reported *S. aureus* isolates tested were successfully identified by Rajakaruna et al.[32] also using his MicrobeLynx software and MALDI-TOF MS. Compared to the 77.7% of his right recognition reported by Seng et al.,[33] MALDI-TOF recognized 99.1% of his CoNS isolates.[33]

The study of Becker, Karsten et al. showed, the 16S rRNA gene's 5' end (16S rDNA) has enough information to distinguish nearly all staphylococcal species, and microbial ribosomal differentiation (RIDOM). Established a new quality-controlled as part of the validation, performed using a database made up of 81 kinds and reference strains that included every genus and subtype of the genus Staphylococcus.[20]

Munoz-Gamito et al. in Barcelona studied the Higher accuracy of genotypic identification compared to phenotyping in the diagnosis of coagulase-negative staphylococcus infection in orthopedic surgery and found that among 255 samples All isolated CoNS strains were grouped in a surgical episode. Bacterial DNA was extracted using the QIAcube automated system from Qiagen in Hilden, Germany, and the rep-PCR method from DiversiLab System from bioMerieux in Marcy-l'Etoile, France, was performed in accordance with the manufacturer's directions. For each reaction in gene amplification, particular primers and conditions were required. The PCR products were separated, visualized, and the results were assessed using five different techniques: dendrogram analysis, similarity matrix analysis (similarity percentage among paired samples), visual inspection of the DNA band patterns in

the sample graphs (define differences between samples), graph analysis (overlapping graphs can reveal small differences), and analysis of the dispersion diagram, which offers a broad perspective of the grouping in clusters. 52 surgical events containing 255 CoNS strains were included. In 40 (77%) instances, genotyping was used to determine the infection.[34]

The isolated strains were identified and tested for antimicrobial susceptibility using the automated VITEK method (bioMerieux Marcy-l' Etoile, France). The term "phenotypically identical" was used to describe strains with similar variation in resistance as well as susceptibility to all of the antibiotics examined. Testing was repeated using the APIStaph disc-plate method (bioMerieux Marcy-l' Etoile, France) when the findings were deemed possibly false due to poor microbial growth or low discriminative power of the identification. 52 surgical events containing 255 CoNS strains were included. Phenotyping was used in 38 (73%) instances to identify the infection.

0.59 was the Kappa score. Phenotyping's sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 88%, 75%, 92%, and 64%, respectively, compared to rep-PCR. When using genotyping, 5/14 (36%) of the cases that were not deemed to be genuine infections by phenotyping were identified as infections. The kappa value was 0.68 in a subgroup of 203 strains from 41 surgical procedures involving orthopaedic implants. Sensitivity, Specificity, PPV, and NPV for phenotyping were 93%, 73%, 90%, and 80%, respectively. Once more, in 2/10 cases where CoNS was phenotypically determined to be non-infectious, infection was determined by genotyping.[34]

Whole-Genome Sequencing (WGS) and Phylogenetic Analysis

To isolate genomic DNA for PacBio sequence analysis from blood cultures, use the Master Pure Gram-positive DNA extraction kit (Lucigen). Add RNase A (10 mg ml-1; Qiagen) for genotyping. Isolate genomic DNA from blood cultures in accordance with Chachaty and Saulnier. It now includes Lucigen. The Wizard G-DNA kit was used to isolate the genomic DNA from four microbial isolates (Promega). The Illumina Genome Analyzer II and Illumina MiSeq were used for WGS, respectively, for the four commensal strains and the blood culture strains. At the Norwegian Sequencing Center (NSC) in Oslo, Norway, the strain 51-48T was further sequenced using a PacBio RS II instrument (Pacific Biosciences). Shovill 0.8.0 (https://github.com/tseemann/shovill), a software program, was used to merge Illumina data. Utilizing HGAP version 3, PacBio Long Reads produced and put together consensus sequences (SMRT Analysis Software, Pacific Biosciences, version 2.3.0). Cavanagh et al. used Pilon version 1.23 to test this assembly. BWA-MEM was used to carry out PacBio assembly and Illumina sequence mapping (version 0.715-r1140). The resulting draught genome sequence was saved as BioProject PRJNA638539 in GenBank.[35]

The Role of Coagulase-Negative Staphylococci Biofilms on Late-Onset Sepsis: Current Challenges and Emerging Diagnostics and Therapies. In this study, a growing number of molecular techniques rely on the identification of nucleic acids (DNA or RNA). These either rely on methods for amplification (PCR, qPCR), probe hybridization (fluorescence in situ hybridization), or sequencing. (whole generation sequencing).[36,37] The use of amplification-based techniques in the clinical setting has been encouraged by the development of automated systems, as well as the simplification of nucleic acids amplification processes and tools. Therefore, it is not surprising that the majority of techniques used in neonatal research rely on nucleic acid amplification, followed by hybridization- and sequencing-based techniques, which are typically more time- and money-intensive, respectively.[37]

Kits like GeneXpert, FilmArray, Verigene, PNA-FISH, and QuickFISH, which can produce results in as little as 3 h, are among the common molecular techniques presently used for LOS diagnosis.[38] There are kits specifically designed to detect *Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus saprophyticus,* and *Staphylococcus capitis*, which are among the most frequently isolated in the skin microbiome and significant in the context of LOS. Although these kits enable the detection of CoNS, due to the relevance of CoNS in the development of LOS, there are kits that.[39-41] Although quick, these methods do not represent a significant advantage because they do

not eliminate the need for earlier cultures because they depend on positive blood cultures. In this way, new developments in molecular techniques have made it possible to amplify nucleic acids directly from biological samples.[42] The kits SeptiFast from Roche and SepsiTest from Molzym are two examples. Both require 1 to 1.5 mL of whole blood and have turnaround times of 6 hours and 10 hours,[43] respectively, to be able to identify 25 to more than 300 pathogens simultaneously. One hundred litres of blood can be used with SeptiFast, but the sensitivity decreases to 80%.[44]

However, the possibility of false-positive findings from PCR/qPCR-based methods and the reported discrepancies between blood culture and PCR/qPCR results have prompted concerns about the effectiveness of molecular methods. Additionally, the quantity, integrity, and purity of the template (nucleic acids) and the initial sample quality rely on the principles underlying the methods used to isolate the nucleic acids, which in turn depends on the sensitivity of these techniques.[45] Furthermore, contamination from other organisms, the laboratory environment, modest levels of bacteremia, or both can have an impact on the molecular assay's quality. Because of this, molecular methods currently have a bright future as tests that supplement but are not substitutes for others.[37]

Conventional method

As an alternative to conventional identification techniques, numerous commercial staphylococci quick identification technologies have been developed in recent years. However, these diagnostic techniques usually produce erroneous results because of problems with cost and incubation time.[34] Numerous of these kits also have are less specialized because all known CNS species exist in them (clinical, veterinary & nutritional isolates). The current study compared four methods for identifying CNS, including a standard protocol,[34] its conventional API-Staph technology, and two methods modified from the standard procedure in our lab to create novel detection methods using both convenience, accuracy, and relatively inexpensive, especially to locations with limited resources.[34]

Characterization of CNS - Isolates from clinical samples were grown on blood agar and stained with gram stain to establish their purity, lifespan, and specific characterisation. The ability to ferment and oxidise glycogen, the bacitracin-resistant (0.04 U) feature, the coagulase and

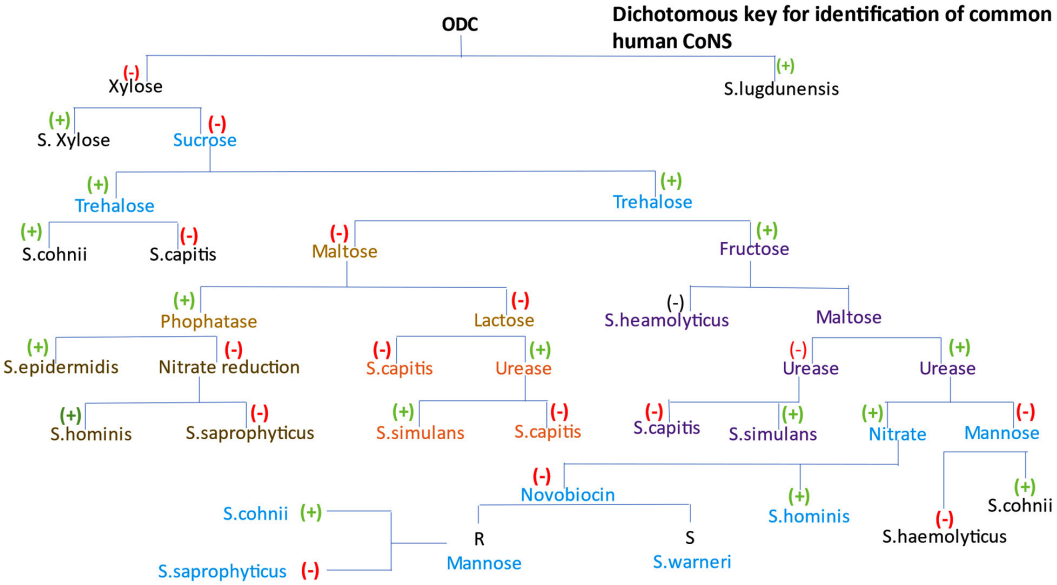

Figure. 2. Dichotomous key for identification of common human CONS

Table. Summary of the test used in the first step of the simplified method for the identification of human *Staphylococcus* species

Species	Coagulase	D-Xylose	Sucrose	D-Trehalose	Maltose	Manitol	Anaerobic growth thioglycolate	Hemolysis
S. aureus[a]	+	-	+	+	+	+	+	+
S. schleiferi subsp. Coagulans[a]	+	-	+,-	-	-	+,-	+	+
S. schleiferi subsp. schleiferi[b]	-	-	-	+,-	-	-	+	+
S. epidermidis[a]	-	-	+	-	+	-	+	+,-
S. haemolyticus[b]	-	-	+	+	+	+,-	+	+
S. saprophyticus[b]	-	-	+	+	+	+,-	+	-
S. warneri[b]	-	-	+	+	+	+,-	+	+,-
S. hominis subsp. hominis[a]	-	-	+	+,-	+	-	-	-
S. hominis subsp. novobiosepticus[b]	-	-	+	-	+	-	-	-
S. simulans[a]	-	-	+	+,-	+	+	+	+,-
S. lugdunensis[b]	-	-	+	+	+	-	+	+
S. capitis subsp. capitis[a]	-	-	+	-	-	+	+	+,-
S. capitis subsp. capitis subsp. urealyticus[a]	-	-	+	-	+	+	+	+,-
S. cohnii subsp. cohnii[b]	-	-	-	+	+,-	+,-	+	+,-
S. cohnii subsp. urealyticum[b]	-	-	-	+	+	+	+	+,-
S. xylosus[a]	-	+,±	+	+	+	+	+,-	-
S. caprae[b]	-	-	-	+	+,-	+,-	+	+,-

+ : positive reaction ; - : negative reaction ; +,- : positive , negative ; a : species identified in a single step; b : species identified in two steps.

peroxidase assays on the isolates, and these characteristics were verified, shown since there isn't any microbicidal region or the beginnings an inhibitory region with a maximum diameter of 9 mm, and sensitivity to furazolidone (100 g), shown by inhibition zones measuring 15 to 35 mm diameter, were used to differentiate Staphylococcus species from Micrococcus species.[35]

The CNS was located using one of the four techniques listed below. *S. xylosus* American Type Culture Collection 29979, *S. xylosus* American Type Culture Collection 12228, *S. simulans* American Type Culture Collection 27851, *S. warneri* American Type Culture Collection 10209, *S. saprophyticus* American Type Culture Collection 15305, S. epidermidis American Type Culture Collection -12228, all of which are recognized CNS strains internationally, served as the controls.

Kloos and Schleifer's and Bannerman's reference methods to determine the utilization of the carbohydrates the nitrate reduction action as well as hemolysin generation, urease being present and ODC, the sugars xylose, arabinose, sucrose, trehalose, maltose, mannitol, lactose, xylitol, ribose, fructose, and mannose, and other factors, This approach includes a number of many after being of incubated for 24 hrs, 48 hrs, and 72 hrs at 37°C in an incubator, the test results were obtained.

Using a homogenous suspension of bacteria with such a McFarland viscosity of 0.5, the API Staph system (bioMerieux) combines 20 biochemical tests with a portable test battery. After incubation for 24 hours at 37°C and adding the NIT (NIT1 and NIT2), PAL (ZYM A and ZYM B), as well as VP (VP1 and VP2) components provided in the kit, the reaction was evaluated and the microorganisms analyzed catalog. Identification depends on a 7-digit number system that provides a percentage identification (%ID), allowing values above 80%.

Modified Methods

The disc approach and the basic method were both used as modified identification methods in our lab. Two steps make up the streamlined process. In the initial step, the anaerobic growth of thioglycolate was evaluated, together with Sucrose, Xylose, Maltose, Mannitol, Trehalose and Hemolysin Synthesis. (Table).

A novobiocin resistance test, hemolysin formation, urease and ornithine decarboxylase test, and fermented maltose, lactose, arabinose, sucrose, mannitol, and trehalose were all part of the disc method. For only the carbohydrate fermentation experiment, discs (HiMedia) made specifically, a sugar was poured in for each vials with 2.5 millilitres of Violet medium-based broth. According to Kloos and Schleifer's instructions, bacterial suspensions were inoculated after 24, 48, and 72 hours of incubation at 37°C, and measurements for the two techniques were taken. CNS species are shown in Figure 2.

Statistical analysis

The tests' sensitivity and specificity were evaluated to identify the level of convergence between the Specified methods and the methods used to identify CoNS (simplified method, disc technique, and API Staph).

Sensitivity

The ratio of CoNS strains, when tested using the reference technique, screened positive for a particular species and when tested using the method under study, were determined as *S. epidermidis, S. haemolyticus, S. saprophyticus, S. hominis, S. warneri, S. capitis, S. simulans, S. cohnii, S. xylosus* and *S. saccharolyticus* species (simplified method, Disk or API Staph).

Applicability

The percentage that unfavourable Coagulase Negative Staphylococcus strains using test method and also negative using the analysed method for the same species (simple method, Disk or API Staph).[35]

CONCLUSION

So let's review some Coagulase Negative Staphylococcus, which serve as significant skin microbes that are getting increasingly important, particularly in infections linked to the healthcare system. Even more CoNS species will emerge as a result of the widespread regular microbiological testing diagnoses using MALDI-TOF MS and

the growing viability regarding whole-genome sequence analysis, as well as the improvement of our understanding of their resistance as well as pathogenicity profiles. As there is the fundamental problem in standard diagnostics still is to properly assign the responsible function of CoNS acquired predominantly sterile materials because there is nothing unique or combination screening method or combination a large sufficient specificity and sensitivity to enable a meaningful prognosis. Future studies seeking to make use of both contemporary pathogen detection techniques and host response evaluation could offer information on the therapeutic value of such a combined approach. Molecular biology technology makes it possible to classify different types of microorganisms by comparing their relative DNA sequences to the genes found in conserved macromolecules. The bigger rRNA molecules and their related genes, particularly 16S and 23S rRNAs to a lesser amount, are currently the most helpful and actively studied taxonomic marker molecules. However, examination of rDNA sequences does not always support classifications depending on traditional phylogenic techniques. While phylogenetic analyses of universal targets used in the molecular analysis have several advantages, such as increased accuracy and speed, It is crucial to be aware of the numerous restrictions placed on the current databases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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