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Article

Sequence-based identification of microbial contaminants in non-parenteral products

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Phenotypic profiles for microbial identification are unusual for rare, slow-growing and fastidious microorganisms. In the last decade, as a result of the widespread use of PCR and DNA sequencing, 16S rRNA sequencing has played a pivotal role in the accurate identification of microorganisms and the discovery of novel isolates in microbiology laboratories. The 16S rRNA region is universally distributed among microorganisms and is species-specific. Accordingly, the aim of our study was the genotypic identification of microorganisms isolated from non-parenteral pharmaceutical formulations. DNA was separated from five isolates obtained from the formulations. The target regions of the rRNA genes were amplified by PCR and sequenced using suitable primers. The sequence data were analyzed and aligned in the order of increasing genetic distance to relevant sequences against a library database to achieve an identity match. The DNA sequences of the phylogenetic tree results confirmed the identity of the isolates as *Bacillus tequilensis, B. subtilis, Staphylococcus haemolyticus and B. amyloliqueficians*. It can be concluded that 16S rRNA sequence-based identification reduces the time by circumventing biochemical tests and also increases specificity and accuracy.

Uniterms Non-parenteral products/microbiological contamination. Microbes/identification/non-parenteral products. Phylogenetic analysis/microbial identification. 16S rRNA/use/microbial identification.

Os perfis fenotípicos para identificação microbiana são incomuns para micro-organismos raros, de crescimento lento e exigentes. Na última década, em resultado do uso generalizado de PCR e sequenciação de DNA, a sequenciação do rRNA 16S tem desempenhado papel crucial na identificação precisa do micro-organismo e a descoberta de novos isolados em laboratórios de microbiologia. A região de rRNA 16S é universalmente distribuída entre micro-organismos e é espécie-específica. A genotipagem foi realizada sobre os organismos isolados a partir de formulações farmacêuticas não parenterais. O DNA foi separado dos cinco isolados obtidos a partir das formulações. As regiões alvo dos genes de rRNA foram amplificados por PCR e sequenciados utilizando os iniciadores adequados. Os dados dos sequência foram analisados e alinhados na ordem crescente de distância genética de sequências relevantes contra biblioteca de dados para obter a identidade. A sequência de DNA de árvores filogenéticas confirma a identidade dos isolados como *Bacillus-tequilensis*, *B. subtilis*, *Staphylococcus haemolyticus* e *B. amyloliqueficians*. Pode-se concluir identificação baseada na sequência do rRNA 16S reduz o tempo por evitar testes bioquímicos e também aumenta a especificidade e a precisão.

Unitermos: Produtos não-parenterais/contaminação microbiana. Micróbios/identificação/produtos não-parenterais. Análise filogenética/identificação microbiana. 16S rRNA/use/ identificação microbiana.

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INTRODUCTION

Microbiological contamination is a serious problem, because it not only results in the spoilage of medicines but also causes infections, and hence, several classical culture methods have evolved to determine the microbiological quality of non-parenteral pharmaceutical products (Jasson *et al*., 2010; Rosa, Medina, Vivar, 1995). These protocols are time-consuming and labor-intensive to ensure the microbiological quality of non-parenteral products. The inevitable time delay associated with incubation often determines that microbiological quality assurance data are only of retrospective value. Modern pharmaceutical production and economic pressures can no longer accommodate this delay (Newby, 2000). In the past decade, nucleic acid sequencing methods have undergone tremendous advances (such as whole-genome sequencing as well as the determination of 16S rRNA, 16S-23S rRNA, spacer and 23S rRNA sequences), which minimize the time needed for identification of microorganisms. 16S rRNA sequencing analysis is widely used, (Bansal, Meyer, 2002) and more useful in phylogenetic analysis compared to 16S-23S rRNA sequencing (Song *et al*., 2004) and also due to its rapidity, reliability, simplicity and reproducibility (Lane *et al*., 1985; Patel 2001; Easter 2003). This identification method not only confirms the microbial limits suggested in official pharmacopoeias but also demonstrates the presence of any other pathogens by virtue of its specificity (Rompre *et al.,* 2002; Gee *et al*. 2003; Rhoads *et al*., 2012). Recent studies and micro sequence packages indicate the cost of analysis is low (Cook *et al*., 2003; Hall *et al*., 2003; Woo *et al*., 2003). 16S rRNA sequencing is routinely used in the clinical laboratory but rarely used for microbial limits test for non-parenteral products. Hence, the aim of the present work was to evaluate 16S rRNA sequence-based identification of microbial contaminants in non-parenteral products.

MATERIAL AND METHODS

(1) Sequencing Kit: ABI 3100 (Applied Biosystems) with Big Dye Terminator Kit v.3.1 (Applied Biosystems) (2) Universal primers: 27F (5′GAGTTTGATCATGGCTCAG3′), 1492R (5′GGTTACCTTGTTACGACTT3′) [for PCR] and 518F (5’CCAGCAGCCGCGGTAATACG3’) and 800R (5’TACCAGGGTATCTAATCC3’) [for 16S rRNA sequencing] were purchased from Macrogen. (3) Sequencer: ABI PRISM 3730XL Analyzer (96 capillary type) (3) PCR machine: MJ Research PTC-225 Peltier Thermal Cycler. All chemicals were purchased from Hi-media &Sigma Aldrich Mumbai. The non-parenteral products (syrup and tablets) were purchased from retail medical shops in TamilNadu.

Preparation of the sample

For isolation of specific organism present in the non parenteral products (Syrup, Tablet) six different media were used. All media including mannitol salt agar, MacConkey agar, xylose lysine deoxycholate agar, cetrimide agar, Columbia agar and Sabouraud dextrose agar were prepared according to the directions given on the label of the container and sterilized in an autoclave (USP-35, 2012; IP, 2007). From syrup, microbial growth was observed on Sabouraud dextrose agar, and from tablets, microbial growth was observed on mannitol salt agar, Maconkey salt agar, xylose lysine deoxycholate agar and Sabouraud dextrose agar (Senthilraj, Prasad, Janakiraman, 2014). The microbial growth from syrup was coded as SYRSI2SAB and that from tablets as TABSIV1MAN, TABSIV2MAC, TABSIV3XYL and TABSIV4SAB, respectively. The isolated cultures were allowed to grow overnight in nutrient broth at 37°C for isolation of DNA.

DNA Isolation

The isolate was centrifuged at 5000 rpm, mixed with 10 µl Tris–EDTA buffer (pH 8.0), and centrifuged again until a pellet was obtained. The Tris–EDTA buffer and lysozyme were added to the microbial pellet and then incubated at 37 °C for 30 minutes. The cells were lysed by the addition of 3 µL of 10% sodium dodecyl sulfate and 3 µL of proteinase K solution followed by a 15-minute incubation at 37 °C. Subsequently, 1 µL of 5 M sodium chloride and 80 µL of cetyltrimethylammonium bromide were added. The water phase was extracted with chloroform:isoamyl alcohol (24:1) (780 µL), and the mixture was centrifuged at 10,000 rpm. Isopropanol (0.6 mL) was added to the supernatant and the mixture was again centrifuged at 10,000 rpm for 5 min, after which the supernatant was removed. The ethanol washed, air-dried pellets were suspended in Tris–EDTA buffer and stored at 4 °C until they were used. (Nishiguchi *et al*., 2002; Vural, Ozgun, 2011). DNA was extracted using the QIAamp Tissue Kit (Loeffler *et al*., 1996).

16S gene Amplification

PCR for the amplification of the 16S rRNA gene was carried out using universal primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) as forward and

TABLE I - Percentage similarity of tested strains against representative species in BLAST search

S. No.	Test strains	Representative species	Percentage similarity (BLAST)
1.	SYRSI2SAB	*Bacillus tequilensis* (NR 140919)	99%
2.	TABSIV1MAN	*Bacillus amyloliquefaciens* (NR 116022)	99%
3.	TABSIV2MAC	*Bacilus subtilis* (NR 112629)	99%
4.	TABSIV3XYL	*Sthaphylicoccus haemolyticus* (NR 036955)	99%
5.	TABSVI4SAB	*Bacillus amyloliquefaciens* (NR 112685)	99%

1492R (5′-GGTTACCTTGTTACGACTT-3′) as reverse primer. (Lane, 1991; Turner *et al*., 1999). Approximately 10 to 100 ng of template were added to a reaction mix containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 µL of each deoxynucleoside triphosphate, 0.4 µM of each primer, and 2.5 U Taq DNA polymerase to complete a final volume of 50 µL. The PCR conditions were: initial denaturation of 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C and 1 min at 72 °C, and a final extension at 72°C for 3 min. The reaction product was visualized on a 1% agarose gel under UV light after ethidium bromide staining (Silva *et al*., 2013; Devereux, Wilkinson, 2004).

16S rRNA gene sequencing and phylogenetic analysis

The amplified PCR products of microbial gene fragments were purified and sequenced at MACROGEN sequencing company, Seoul, Korea using the automated sequencer ABI 3100 with Big Dye Terminator Kit v. 3.1. Primers 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3') were used for sequencing (Ghyselinck *et al*., 2013). The sequences thus obtained were compared with the NCBI database through BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In this comparison, sequences of type strains most closely related to the sequences of the isolates were searched. The sequences were aligned with Clustal W, and a phylogenetic tree was constructed from the evolutionary distances by the neighbor-joining method with the software MEGA (Nikunjkumar, 2012; Tamura *et al*., 2011).

RESULTS AND DISCUSSION

Isolate-1 from syrup (SYRSI2SAB) had a nucleotide sequence of 1425 bp (Fig. 1). NCBI-BLAST search results showed the highest sequence similarity with *Bacillus tequilensis* belonging to the family *Bacillaceae*, and the accession number was NR 104919.

Isolates-2, -3, -4 and -5 from tablets (TABSIV1MAN, TABSIV2MAC, TABSIV3XYL and TABSIV4SAB) had nucleotide sequences of 1600, 1882, 1590 and 1550 bp and are shown in Figs. 3, 5, 7 and 9. The NCBI-BLAST search results for isolates-2, -3, -4 and-5 had highest sequence similarity with the following bacteria, whose accession numbers are given in parentheses: *B. amyloliqueficians* (NR116022), *B. subtilis* (NR112629), *Staphylococcus haemolyticus* (NR036955) and *B. amyloliqueficians* (NR104919). Thus, the BLAST search of Genbank for all isolates provided the percentage similarity between the microorganism tested and those detected in Genbank as shown in Table I.

Furthermore, the neighbor-joining tree based on 16S rRNA gene sequences was constructed to show the relationship between isolate-1 and 12 representative species of the family *Bacillaceae* (Fig. 2). This result also confirmed the result of NCBI-BLAST for isolate-1. Likewise, for isolates-2, -3, -4 and -5, the neighbor-joining trees based on 16S rRNA gene sequences were constructed to show the relationship between the isolates and their representative species of the family *Bacillaceae*, and *Staphylococcaceae,* as shown in Figs. 4, 6, 8 and 10. Thus, the above results showed that contaminants of non-parenteral pharmaceutical products belonged to *Bacillus* and *Staphylococcus* species and the names of the contaminants are given in Table II.

CONCLUSION

As discussed earlier, the universal primers 27F and 1492R produced well-amplified 16S r RNA PCR products for all isolates. The nucleotide sequence obtained for all isolates was approximately 1500 bp by using the universal sequencing primers 518F and 800R (Ghyselinck *et al*., 2013) which are sufficient for NCBI-BLAST searches and phylogenetic analysis for the identification of unknown microorganisms.

The time for microbial identification was reduced by molecular-based PCR techniques when compared to conventional methods of detection. But PCR detects only the fixed target microorganism and it will not detect

AACTGGGGAACTTGAGTGCAGAGTAGGAGAA
AGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGA
CTCTCTGGTCTGTAACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTAAG
TGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACG
GTCGCAAGACTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCCTCTGACAATCCTAGAGATAGGAC
GTCCCCTTCGGGGGGCAGAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
CTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGAC
CTGGGCTACACACGTGCTACAATGGACAGAAC
AAAGGGCAGCGAAACCGCGAGGTTAAGCCAA
TCCCACAAATCTGTTCTCAGTTCGGATCGCAGT
CTGCAACTCGACTGCGTGAAGCTGGAATCGCT
AGTAATCGCGGATCAGCATGCACTGGTGTTCC
TCCACATCTCTACGCATTTCACCGCTACACGTG
GAATTCCACTCTCCTCTTCTGCACTCAAGTTCC
CCAGTTTCCAATGACCCTCCCCGGTTGAGCCGG
GGGCTTTCACATCAGACTTAAGAAACCGCCTG
CGAGCCCTTTACGCCCAATAATTCCGGACAAC
GCTTGCCACCTACGTATTACCGCGGCTGCTGGC
ACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACC
GTCAAGGTACCGCCCTATTCGAACGGTACTTGT
TCTTCCCTAACAACAGAGCTTTACGATCCGAA
AACCTTCATCACTCACGCGGCGTTGCTCCGTCA
GACTTTCGTCCATTGCGGAAGATTCCCTACTGC
TGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT
CCCAGTGTGGCCGATCACCCTCTCAGGTCGGCT
ACGCATCGTTGCCTTGGTGAGCCATTACCTCAC
CAACTAGCTAATGCGCCGCGGGTCCATCTGTA
AGTGGTAGCCGAAGCCACCTTTTATGTTTGAAC
CATGCGGTTCAAACAACCATCCGGTATTAGCC
CCGGTTTCCCGGAGTTATCCCAGTCTTACAGGC
AGGTTACCCACGTGTTACTCACCCGTCCGCCGC
TAACATCAGGGAGCAAGCTCCCATCTGTCCGC
TCGACTTGCATGTATTAGGCACGCCGCCAGCG
TT.

FIGURE 1 - The 16S rRNA sequence generated for isolate-1 had 1425 bases.

CTTAAGTCTGATGTGAAAGCCCCCGGCTCAAC
CGGGGAGGGTCATTGGAAACTGGGGAACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACAC
CAGTGGCGAAGGCGACTCTCTGGTCTGTAACT
GACGCTGAGGAGCGAAAGCGTGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTAAGTGTTAGGGGGTTTCCG
CCCCTTAGTGCTGCAGCTAACGCATTAAGCACT
CCGCCTGGGGAGTACGGTCGCAAGACTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGACA
ATCCTAGAGATAGGACGTCCCCTTCGGGGGCA
GAGTGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGATCTTAGTTGCCAGCATT
CAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAAT
CATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGACAGAACAAAGGGCAGCGAAA
CCGCGAGGTTAAGCCAATCCCACAAATCTGTT
CTCAGTTCGGATCGCAGTCTGCAACTCGACTGC
GTGAAGCTGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTTTGT
AACACCCGAAGTCGGTGAGGTAACCTTTTAGG
AGCCAGCCGCCGAAGGTGGGACAGATGATTGG
GGGGCCACTGGTGTTCCTCCACATCTCTACGCA
TTTCACCGCTACACGTGGAATTCCACTCTCCTC
TTCTGCACTCAAGTTCCCCAGTTTCCAATGACC
CTCCCCGGTTGAGCCGGGGGCTTTCACATCAG
ACTTAAGAAACCGCCTGCGAGCCCTTTACGCC
CAATAATTCCGGACAACGCTTGCCACCTACGT
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGT
GGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCC
CTATTTGAACGGCACTTGTTCTTCCCTAACAAC
AGAGCTTTACGATCCGAAAACCTTCATCACTC
ACGCGGCGTTGCTCCGTCAGACTTTCGTCCATT
GCGGAAGATTCCCTACTGCTGCCTCCCGTAGG
AGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCC
GATCACCCTCTCAGGTCGGCTACGCATCGTCGC
CTTGGTGAGCCGTTACCTCACCAACTAGCTAAT
GCGCCGCGGGTCCATCTGTAAGTGGTAGCCGA
AGCCACCTTTTATGTCTGAACCATGCGGTTCAA
ACAACCATCCGGTATTAGCCCCGGTTTCCCGG
AGTTATCCCAGTCTTACAGGCAGGTTACCCAC
GTGTTACTCACCCGTCCGCCGCTAACATCAGG
GAGCAAGCTCCCATCTGTCCGCTCGACTTGCAT
GTATTAGGCACGCCGCCAGCG

FIGURE 3 - The 16S rRNA sequence generated for isolate-2 had 1600 bases.

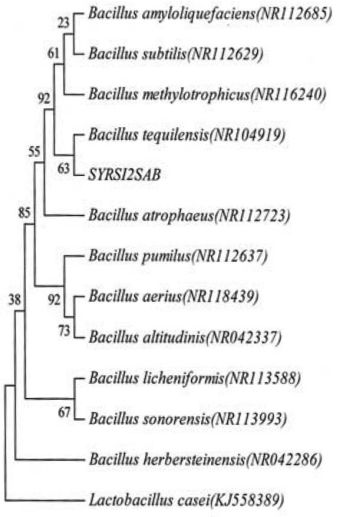

FIGURE 2 - Neighbor-joining tree based on 16S rRNA (1425) sequences showing the relationship between unknown isolate SYRSI1SAB and other closely related species of the genus *Bacillus.*

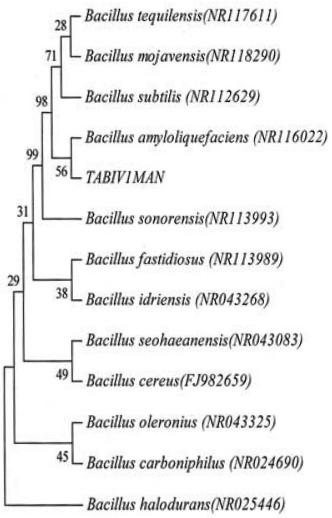

FIGURE 4 - Neighbor-joining tree based on 16S rRNA (1600) sequences showing the relationship between unknown isolate TABSIV1MAN and other closely related species of the genus *Bacillus*.

GGGAGGGAGGTGACCGGATTATTGGGCGTAAGGGCT
CCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCC
GGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAAC
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTA
GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT
GGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGA
GGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACG
CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGA
CTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACAATCCTA
GAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTT
AGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACAGAACAAAGGGCAGCGAAACCGCGA
GGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGA
TCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCG
CTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACG
AGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTT
TAGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGG
GGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGT
GCGCTGAACCCCCCCCCTTTCTTATATAAATCCTTTCG
CTTCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGA
GTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACG
CATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTC
TGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCG
GTTGAGCCGGGGGGCTTTCACATCAGACTTAAGAAAC
CGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAA
CGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACG
TAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGG
TGCCGCCCTATTTGAACGGCACTTGTTCTTCCCTAAC
AACAGAGCTTTACGATCCGAAAACCTTCATCACTCAC
GCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAA
GATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCG
TGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGT
CGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCA
CCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTG
GTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGT
TCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGGA
GTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTA
CTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCC
CATCTGTCCGCTCGACTTGCATGTATTAGGCACGCCG
CCAGCGTTCGTCCTGAGCGGGGCCAAAAAACCAAAA
AAAAAAAAAAAAAAACCTTTAACCCT

FIGURE 5 - The 16S rRNA sequence generated for isolate-3 had 1822 bases.

TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGAAAACTTGAGTGCAGA
AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAA
TGCGCAGAGATATGGAGGAACACCAGTGGCGAAG
GCGACTTTCTGGTCTGTAACTGACGCTGATGTGCG
AAAGCGTGGGGATCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT
AGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
GTTGAAACTCAAAGGAATTGACGGGGACCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAAATCTTGACATCCTTTGAC
AACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACA
AAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGG
CACTCTAAGTTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGATTTGGGCTACACACGTGCTACAATGGACAATA
CAAAGGGCAGCGAAACCGCGAGGTCAAGCAAATC
CCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGC
AACTCGACTACATGAAGCTGGAATCGCTAGTAATC
GTAGATCAGCATGCTACGGTGAATACGTTCCCGGG
TCTTGTACACACCGCCCGTCACACCACGAGAGTTT
GTAACACCCGAAGCCGGTGGAGTAACCATTTGGA
GCTAGCCGTCGAAGGTGGGACAAATCCACTGGTG
TTCCTCCATATCTCTGCGCATTTCACCGCTACACAT
GGAATTCCACTTTCCTCTTCTGCACTCAAGTTTTCC
AGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGC
TTTCACATCAGACTTAAAAAACCGCCTACGCGCGC
TTTACGCCCAATAATTCCGGATAACGCTTGCCACC
TACGTATTACCGCGGCTGCTGGCACGTAGTTAGCC
GTGGCTTTCTGATTAGGTACCGTCAAGACGTGCAT
AGTTACTTACACGTATGTTCTTCCCTAATAACAGA
GTTTTACGATCCGAAGACCTTCATCACTCACGCGG
CGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAGA
TTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCG
TGTCTCAGTTCCAGTGTGGCCGATCACCCTCTCAG
GTCGGCTACGTATCGTCGCCTTGGTAAGCCGTTAC
CTTACCAACTAGCTAATACGGCGCGGGTCCATCTA
TAAGTGATAGCAAAACCATCTTTCACTATCGAACC
ATGCGGTTCGAAATATTATCCGGTATTAGCTCCGG
TTTCCCGAAGTTATCCCAGTCTTATAGGTAGGTTA
CCCACGTGTTACTCACCCGTCCGCCGCTAACGTCA
AAGGAGCAAGCTCCTTGTCTGTTCGCTCGACTTGC
ATGTATTAGGCACGCCGCCAGCGT

FIGURE 7 - The 16S rRNA sequence generated for isolate-4 had 1590 bases.

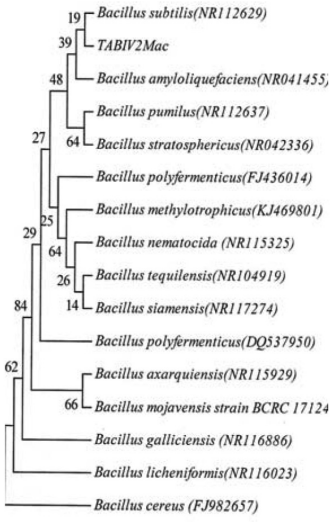

FIGURE 6 - Neighbor-joining tree based on 16S rRNA (1822) sequences showing the relationship between unknown isolate TABSIV2MAC and other closely related species of the genus *Bacillus*.

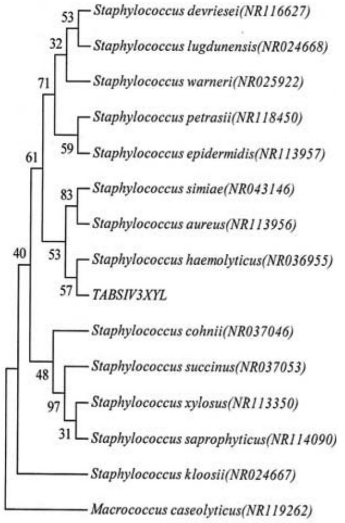

FIGURE 8 - Neighbor-joining tree based on 16S rRNA (1590) sequences showing the relationship between unknown isolate TABSIV3XYL and other closely related species of the genus *Staphylococcus.*

CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGG
GAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAA
GAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGAC
TCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCC
GCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCG
CCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CTTGACATCCTCTGACAATCCTAGAGATAGGACGTCC
CCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTC
AGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGGCAG
AACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATC
CCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAA
CTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
ACCCGAAGTCGGTGCCACTGGTGTTCCTCCACATCTC
TACGCATTTCACCGCTACACGTGGAATTCCACTCTCC
TCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTC
CCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGA
AACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGA
CAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGC
ACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCA
AGGTGCCGCCCTATTTGAACGGCACTTGTTCTTCCCT
AACAACAGAGCTTTACGATCCGAAAACCTTCATCACT
CACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCG
GAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGG
CCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCA
GGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACC
TCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAA
GTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGC
GGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCC
GGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTG
TTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGC
TCCCATCTGTCCGCTCGACTTGCATGTATTAGGCACG
CCGCCAGCG

FIGURE 9 - The 16S rRNA sequence generated for isolate-5 had 1550 bases.

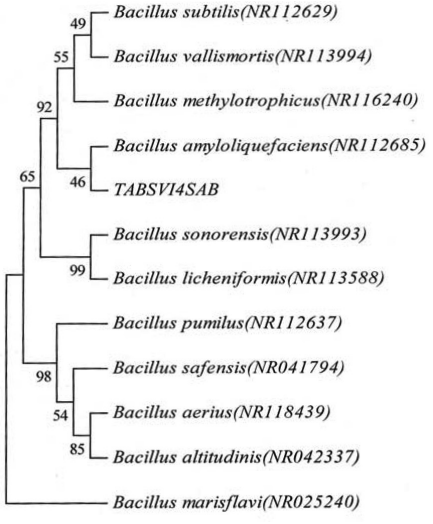

FIGURE 10 - Neighbor-joining tree based on 16S rRNA (1550) sequences showing the relationship between unknown isolate TABSIV4SAB) and other closely related species of the genus *Bacillus*.

TABLE II - Microbial isolates from non-parenterals identified by 16S rRNA sequence

Isolates	Fprmulation	Microbial isolate (contaminant) identified by 16S rRNA sequence
Isolates-1	Syrup	*Bacillus tequilensis*
Isolates-2	Tablets	*Bacillus amyloliquefaciens*
Isolates-3	Tablets	*Bacilus subtilis*
Isolates-4	Tablets	*Sthaphylococcus haemolyticus*
Isolates-5	Tablets	*Bacillus amyloliquefaciens*

other microorganisms of interest (Jimenez, Ignar, 2000). Although it takes 6 to 7 hours more than PCR methods, 16S rRNA sequence-based analysis gives very accurate information about all microbial contaminants and also confirms the presence of any other pathogens by virtue of its specificity (Rompre *et al*., 2002; Gee *et al*., 2003; Rhoads *et al*., 2012). As discussed earlier about sequence analysis, this study also confirmed 16S rRNA as the most ideal and suitable method for the identification of microbial contaminants in non-parenteral products. Hence, microbial quality control for non-parenteral products by 16S rRNA sequence-based identification can be employed on a routine basis as a pharmacopoeial protocol to ensure simplicity, reliability and rapidity.

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