SHORT COMMUNICATION

COMPARATIVE METHODS IN IDENTIFICATION OF BACTERIA FROM AGRICULTURAL WASTE USING BIOCHEMICAL TESTS AND 16S RRNA UARR SEQUENCING

M. AZLAN J.[1]*, NURZILLAH M.[2], NORLINDAWATI A.P.[2], M. NOOR I.[2], SITI HAJAR I.[1], MARLIAH A.[2] , NURHASLINA A.R.[3] AND ROHANA A.B.[1]

[1] Central Region Veterinary Laboratory, Jalan Nilai Banting, Bandar Baru Salak Tinggi, 43900 Sepang, Selangor, Malaysia
[2] Malaysia Veterinary Institute, KM13 Jalan Batu Pahat, Beg Berkunci 520, 86009 Kluang, Johor, Malaysia.
[3] Institute of Medical Science Technology (UniKL MESTECH), A1-1, Jalan TKS 1, Taman Kajang Sentral, 43000 Kajang, Selangor, Malaysia.
* Corresponding author: azlanjahaya@dvs.gov.my

ABSTRACT. Identification of microorganisms, including bacteria, are widely used especially in environmental studies, biotechnology, clinical microbiology, microbial forensics, and in research study. The conventional method of bacteria identification is based on phenotypic observation techniques by profiling an organism's metabolic attributes or some aspect of its chemical composition. Then, interpretation of test results involves substantial subjective judgement. Currently, general 16S rRNA sequencing and specific PCR play an important role in the accurate and faster identification of bacteria. The aim of this study is to compare the identification of the genus or species of bacteria from agricultural waste using conventional microbiology biochemical test and molecular techniques PCR 16S rRNA universal amplified ribosomal region (UARR) sequencing. A total of 72 agricultural waste samples and 2 ATCC culture as positive control were tested. Out of two ATCC bacteria and fifteen bacteria isolates identified by the biochemical test, twelve species (71%) of bacteria gave exactly the same bacteria genus as the 16S rRNA sequencing results. *Aeromonas hydrophilia, Alcaligenes faecalis* and *Acinetobacter calcoaceticus* was revealed as *Pseudomonas* sp. from the sequencing results. As for *Alcaligenes* sp., the results from the sequencing is *Stenotrophomonas maltophilia*. Previous reports also showed different results of the same isolate which were from similar classification, and closely related to each other. The limited number of biochemical tests available in a laboratory will contribute to misidentification of a proposed specie.

Keywords: bacteria identification, biochemical test, 16S rRNA, universal amplified ribosomal region, agricultural waste

METHOD AND RESULTS

There are 3 groups of agricultural waste samples: 24 samples from empty fruit bunches (EFB), 24 samples from rice straw (RS), and 24 samples from fruit waste (FW).

ATCC 25922 *E. coli* and ATCC 8090 *Citrobacter freundii* were used as positive control.

The samples were first being cultured on blood agar (BA) and MacConkey (MA). It was then incubated for 24 hours at 37 °C. The next day, the agar was examined to detect whether the plate had a mix or a pure culture. If there were a mix culture, the bacteria will be sub-cultured and incubated for 24 hours at 37 °C to obtain a pure colony. Biochemical tests were then carried out on the pure colonies. (Quinn P.J. *et al*., 2002)

The same bacteria isolates were extracted by using DNeasy Blood and Tissue Kit (QIAGEN), then continued with PCR 16S rRNA UARR sequencing using Toptaq Master Mix Kit (QIAGEN) and two universal primers, U1F: 5`-CTY AAA KRA ATT GRC GGR RRS SC-3` and U1R: 5`-CGG GCG GTG TGT RCA ARR SSC-3` (Rivas *et al*., 2004).

The band for the PCR product had a size of 435 bp and was sent to First BASE Laboratories Sdn Bhd for sequencing. The sequences obtained were compared against those held in data banks using the basic local alignment online search tools (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Results from agricultural wastes showed 15 pure isolates.

From biochemical tests, the bacteria species were identified as follows. EFB: *Klebsiella pneumoniae*, *Alcaligenes* sp. and *Bacillus* sp. FW: *Klebsiella pneumoniae*, *Alcaligenes faecalis*, *Acinetobacter calcoaceticus* and *Enterobacter cloacae*. RS: *Enterobacter aerogenes, Alcaligenes faecalis, Staphyloccoccus epidermidis, Bacillus* sp., *Escherichia coli, Aeromonas hydrophila, Klebsiella pneumonia* and *Enterobacter cloacae.* ATCC: *E. coli* and *Citrobacter freundii.*

From PCR 16S rRNA gene sequencing, the results were as follows. EFB: *Klebsiella pneumonia, Pseudomonas* sp., and *Bacillus* sp. FW: *Klebsiella pneumoniae, Pseudomonas* sp. and *Enterobacter cloacae.* RS: *Enterobacter aerogenes, Staphyloccoccus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Enterobacter cloacae*. ATCC: *E. coli* and *Citrobacter freundii.*

Out of two ATCC bacteria and fifteen bacteria isolates identified by biochemical tests, twelve species (71%) of bacteria were identified with exactly the same bacteria genus as the 16S rRNA sequencing results. *Aeromonas hydrophilia, Alcaligenes faecalis* and *Acinetobacter calcoaceticus* were identified *Pseudomonas* sp. by sequencing. As for *Alcaligenes* sp., the sequencing result is *Stenotrophomonas maltophilia.*

DISCUSSION

The conventional biochemical identification methods have been widely used in laboratories. However, because of the limited capability and not enough of biochemical tests available, identification of a proposed specie can be doubtful and imprecise. This problem may lead to mistakes and may result in the discovery and re-analysis of the same bacteria species by different investigators who might give the same group name but slightly different morphological, cultural, and phenotypic criteria. (Michael *et al*., 2002). The conventional approach is very time consuming, making important cases of bacteria identification at risk when involving lives. Besides that, the difficulty and misinterpretation of bacteria species

might happen if there is mixture of cultures and defects in the media.

According to Hugh and Ryschenkow (1961), *Stenotrophomonas malthophilia* had been misidentified as *Bordetella bronchiseptica* and *Alcaligenes faecalis*. The first detected *S. maltophilia* specie was initially described as *Pseudomonas maltophilia* in 1981, later reclassified as *Xanthomonas maltophilia* and finally classified as *Stenotrophomonas maltophilia* (Martina *et al.,* 2011).

Aeromonas hydrophilia, Alcaligenes faecalis and *Acinetobacter calcoaceticus* share some of biochemical characteristics when compared to the *Pseudomonas* sp. There are only few characteristics that differentiate them. The similar characteristics that could lead to misidentification of *Pseudomonas* and other species are motility positive characteristics, oxidase positive, indole negative, citrate positive and nitrate positive. Identification of *Pseudomonas* is difficult because of the lack of biochemical reaction pattern for each specie. Each specie is dependent on unstable pigment production and unstable pathogenicity (Liu, 1961). The genus *Achromobacter*, *Agrobacterium* or *Pseudomonas* appear to be acceptable as species of *Alcaligenes* (Margaret *et al.,* 1974). Strains of *Pseudomonas* have been reported as *Alcaligenes*, *Flavobacterium*, or *Mima-Herellea* (Vera, 1968).

Improvement of *Pseudomonas* identification is based on observation of characteristics such as flagellation, oxidase activity, arginine dihydrolase activity, storage of intracellular fat, growth at 4 °C and at 41 °C, denitrification and gelatin hydrolysis (Vera, 1968).

Currently, 16S rRNA sequencing has become an alternative method in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections. PCR 16S rRNA UARR sequencing has provided a method of advance phylogenetic classification of microorganisms allowing more rapid and reliable identification.

REFERENCES

1. Hugh R. and Ewdokia Ryschenkow (1961). *Pseudomonas malthophilia*, an *Alcaligens*-like specie. *Journal of General Microbiology,* **26**: 123-132
2. Liu P.V. (1961). Identification of pathogenic *Pseudomonads* by extracellular antigens. *Journal of Bacteriology*. **81**: 28
3. Margaret S., Hendrie A.J. and J.M. Shewan (1974). Emended descriptions of the genus *Alcaligenes* and of *Alcaligenes faecalis* and proposal that the generic name *Achromobacter* be rejected: status of the named species of *Alcaligenes* and *Achromobacter, International Journal of Systematic Bacteriology* **24(4)**: 534-550
4. Martina A., Jorg O., Stephan B., Josef W., Reinhard F and Thomas S. (2011) Genotyping of environmental and clinical *Stenotrophomonas maltophilia* isolates and their pathogenic potential, *PlOS ONE* **6(11)**: e27615
5. Michael J. and Sharon, L.A. (2002) Bacterial identification for publication: when is enough enough? *Journal of Clinical Microbiology,* **40**(6): 1887-1891.
6. Quinn P.J., Markey B.K., Carter M.E., Donnelly W.J. and Leonard F.C. (2002). Veterinary microbiology and microbial disease. Blackwell Publishing. p23-25
7. Rivas R., Velazquez E., Zurdo-Pineiro J.L., Mateos P.F. and Molina E.M. (2004). Identification of microorganisms by PCR amplification and sequencing of a universal amplified ribosomal region present in both prokaryotes and eukaryotes. *Journal of Microbiological Methods*, **56**: 413-426
8. Vera L. Sutter (1968). Identification of *Pseudomonas* species isolated from hospital environment and human sources. *Applied Microbiology,* **16(10):** 1532-1538