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Morpho-Biochemical Aided Identification of Bacterial Isolates from Philippine Native Pig

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Abstract

The pure culture of the unknown bacteria isolated from Philippine native pigs was subjected to various morpho-biochemical tests/observations, and the Bergey's Manual of Determinative Bacteriology was used for its possible identification. The presumptive identification of isolates A4, A5 and A6 is *Pseudomonas* spp. that lack fluorescent yellow-green pigment. The identity of isolates A4, A5 and A6 was separated from *Vibrio* spp. and *Aeromonas* spp. based on the inability of the isolates to produce acid during glucose fermentation. Isolates D1, D11 and D14 are presumptively identified as *Serratia* spp. which is member of Family Enterobacteriaceae. The identity of isolates D1, D11 and D14 was further detached from other members of Family Enterobacteriaceae by using the results in lactose fermentation, indole test, urease, motility and H_2S production. The presumptive identification of isolate 9 is *Enterobacter* spp. which is a lactose-fermenter member of the Family Enterobacteriaceae. The isolate was identified from the rest of the Enterobacteriaceae members by having negative result on indole test and positive result on both Mehyl red and Voges-Proskaeur tests. For a more accurate identification of isolates, the use of MALDI-TOF MS and 16S rRNA sequencing are recommended.

Keywords: Philippine Native; Bacterial Isolates; Morpho-Biochemical

Introduction

Identification of unknown bacteria from various samples (e.g. blood, tissue, food and water, cosmetics) is one of the major responsibilities of the microbiologists. The process of identification produces benefits for many aspects of the research of microorganisms and helps physicians correctly treat patients. In addition, industrial organizations are constantly screening materials to isolate new antibiotic-producing microbes or microbes that will increase the yield of marketable products, such as vitamins, solvents and enzymes [1]. The science of classification is called taxonomy and deals with the separation of living organisms into interrelated groups. The science of taxonomy has grown from an artificial, imposed system of categorization based on gross physical

characteristics to a highly sophisticated study of genetic evolution [2]. Microorganisms have been classified and identified on the basis of a variety of characteristics including morphological, growth, tolerance, metabolic, biochemical, and genetic (Bergey and Holt, 1994). With the fundamental knowledge in isolation technique, growth characteristics of bacteria, staining methods, bacterial nutrition and biochemical activities, it becomes easier for identification of any unknown bacteria.

The establishment of the pig gastrointestinal microbiota is a large and successional process that is influenced by several factors. It starts immediately after birth, when environmental bacteria begin gut colonization. However, commercial weaning stresses the animal resulting in a disruption in the natural bacterial succession with both quantitative and qualitative changes. In consequence, the pig becomes more susceptible to overgrowth with potentially disease-causing pathogenic bacteria. After this alteration, the normal colonization continues and in the healthy adult pig becomes a stable and characteristic ecosystem with *Eubacterium*, *Clostridium* and bacteria belonging to the *Bacillus-Lactobacillus-Streptococcus* subdivision and the *Cytophaga-Flexibacter-Bacteroides* group as the main bacteria [3]. The general objective of this paper is to describe the morphological and biochemical characteristics of unknown bacteria which were isolated from Philippine native pigs. The specific objective is to identify the unknown bacteria using selected morpho-biochemical characteristics.

Materials and Methods

Identification of bacteria is a careful and systematic process that uses different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture or sample. Aseptic technique was put into practice all throughout the conduct of the activity. In characterization and identification, it is imperative that pure culture must be used in order to obtain consistent and reliable results. The pure cultures of the unknown bacteria were subjected to various morpho-biochemical tests/observations (Figure 1). The procedures in the laboratory manual MCB 101: Microbial Identification Techniques of the University of the Philippines Los Baños were followed strictly from the preparation of the various media/reagents until the observation of results. Reference bacteria (positive and negative control) in each test were used for comparison of results. Bergey's Manual of Determinative Bacteriology was used as a guide in the identification of the unknown bacteria.

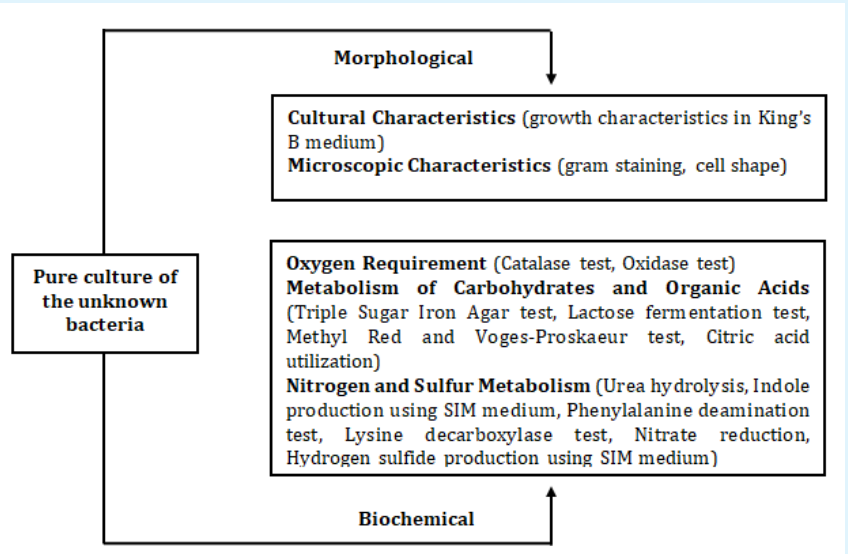

Figure 1: Morpho-biochemical observations/tests used in the characterization of the unknown isolates.

Results and Discussion

Provided in Table 1 is the morpho-biochemical characteristics of the unknown bacteria. The schemes used in the identification of the isolates are provided in Figures 2-4.

Tests	Isolates									Inference
	A4	A5	A6	D1	D9	D11	D14	Control (+)	Control (-)	
Gram Staining	--	--	--	--	--	--	--	*Bacillus polymya*	*Escheracoli*	Positive: Colonies stained violet/purple Negative: Colonies stained pink
Shape	Short rod	Short rod	Short rod	Short rod	Short rod	Short rod	Short rod	--	--	--
Gregersn	Thread of slime	Thread of slime	Thread of slime	Thread of slime	Thread of slime	Thread of slime	Thread of slime	*Bacillus polymya*	*Escherichia coli*	No slime/watery suspension: Gram positive, Thread of slime: Gram negative
Growth in King's B medium	--	--	--	--	--	--	--	*Pseudoas aeruginosa*	--	Positive: Formation of yellow green-fluorescent growth, Negative: Non-formation of yellow green-fluorescent growth
Motility (SIM)	Motile	Motile	Motile	Motile	Motile	Motile	Motile	*Proteus vulgaris*	*Staphylococcus aureus*	Motile: Diffusive zone of growth, Non-motile: Absence of diffusive zone of growth
Catalase	+	+	+	+	+	+	+	*E. coli*	*Lactobacillus plantarum*	Positive: Bubble formation, Negative: No bubble formation
Oxidase	+	+	+	--	--	--	--	*P.aeruginosa*	*E. coli*	Positive: Formation of blue/violet product, Negative: No change in color
Triple Sugar Iron Agar	KK	KK	KK	KA, G	K/A, G	A/A, G	K/A, G	K/K with H_2S: *P. aeruginosa*; A/A with H_2S: *P. vulgaris*; A/A: *E. coli*		A/A: Glucose and lactose and/or sucrose fermentation; with acid accumulation, K/A: No fermentation with acid production, K/K: No fermentation, H2S: Sulfur reduction, G: Gas production
Lactose Fermentation	--	--	--	--	+	--	--	*E. coli*	*P. vulgaris*	Positive: Red growth, lactose fermenter, Negative: White growth, non-lactose fermenter
Methyl Red	--	--	--	--	+	--	--	*E. coli*	*P. aeruginosa*	Positive: Formation of red color Negative: No color change
Voges-Proskauer	--	--	--	+	+	+	+	*B. polymya*	*P. aeruginosa*	Positive: Formation of pink/red color Negative: No color change
Citric Acid Utilization	+	+	+	+	+	+	+	*P. aeruginosa*	*E. coli*	Positive: Shift of the green color to Prussian blue color Negative: No color shift
Urea hydrolysis	--	--	+	--	+	--	--	*P. vulgaris*	*E. coli*	Positive: Formation of red/violet color Negative: Non-formation of red/violet color
Indole Production (SIM)	--	--	+	--	--	--	--	*P. vulgaris*	*S. aureus*	Positive: Development of pink/red color after addition of Kovac's reagent Negative: No pink/red color development
Phenylalanine Deamination	--	--	+	--	--	--	--	*P. vulgaris*	*E. coli*	Positive: Immediate appearance of intense green color Negative: Non-appearance of green color
Lysine Decarboxylase	+	+	--	+	+	+	+	*E. coli*	*P. vulgaris*	Positive: Formation of purple color Negative: No color change
Nitrate Reduction	--	--	+	+	+	+	+	*E. coli*	*Micrococcus luteus*	Positive: Formation of distinct red color Negative: Non-formation of red color
Hydrogen Sulfide Production (SIM)	--	--	--	--	--	--	--	*P. vulgaris*	*S. aureus*	Positive: Blackening of the medium Negative: No blackening of the medium

Table 1: Results of the morpho-biochemical tests for the characterization of the unknown isolates.

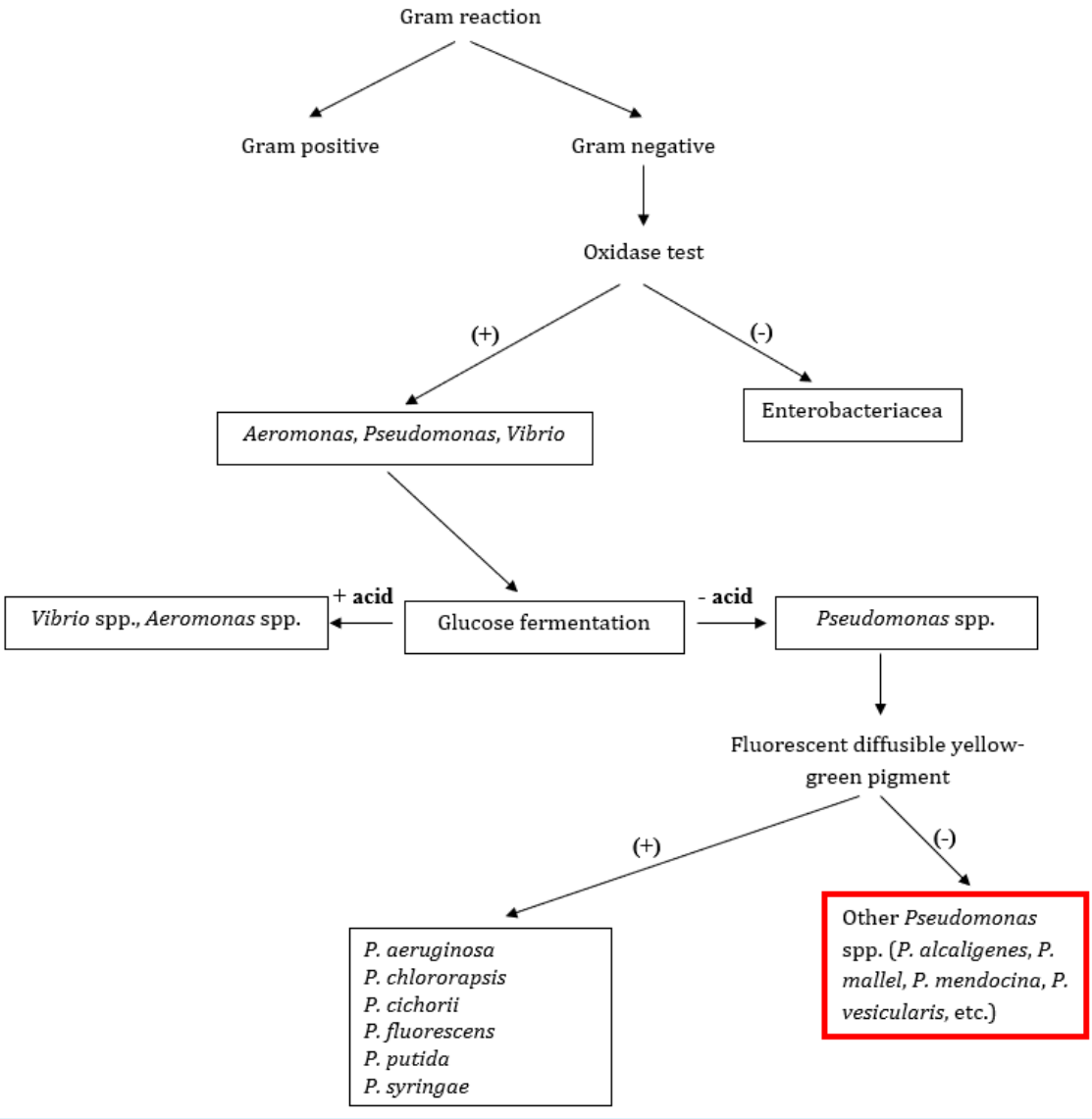

Figure 2: Identification flow chart for isolates A4, A5 and A6.

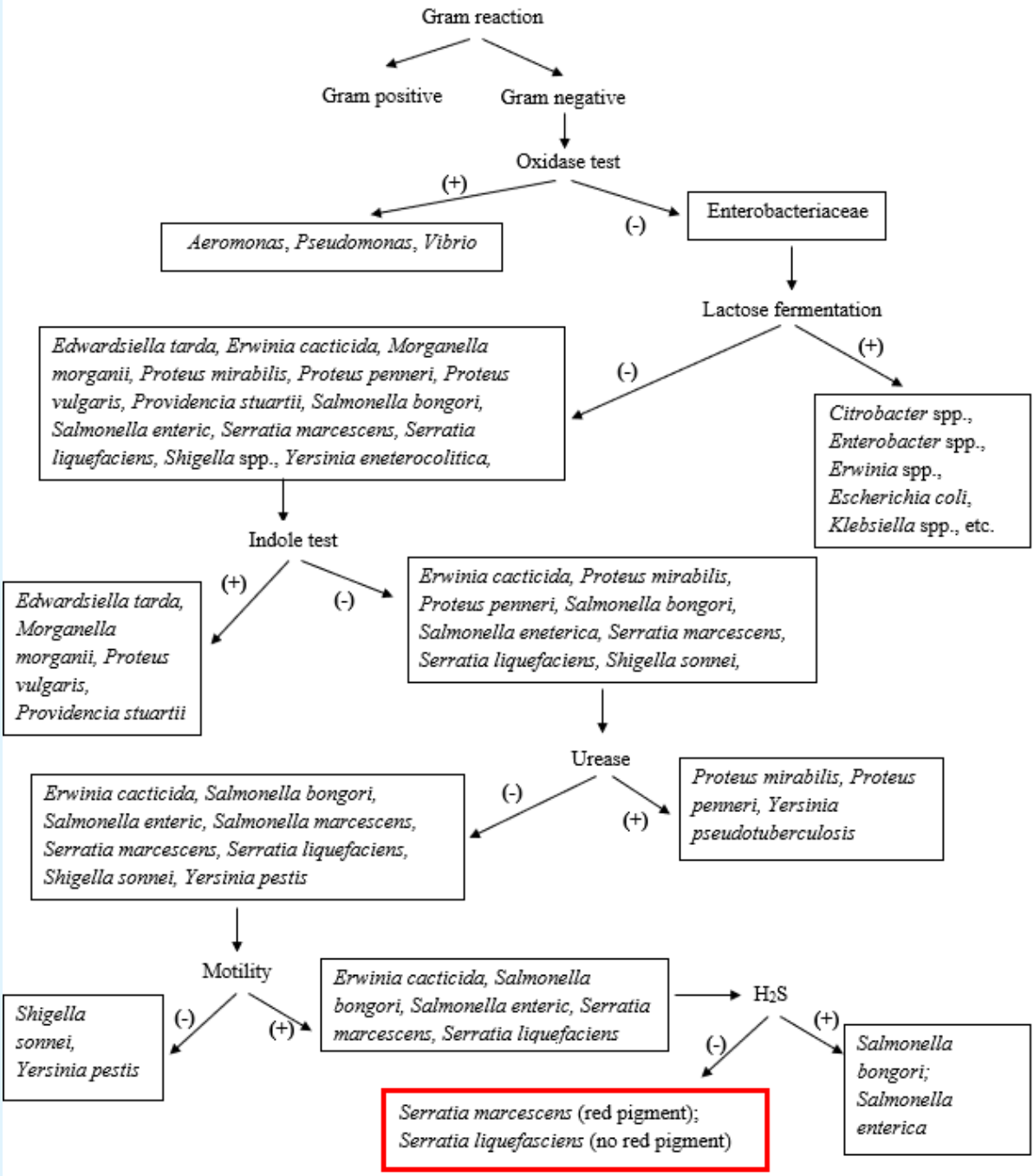

Figure 3: Identification flow chart for isolates D1, D11 and D14.

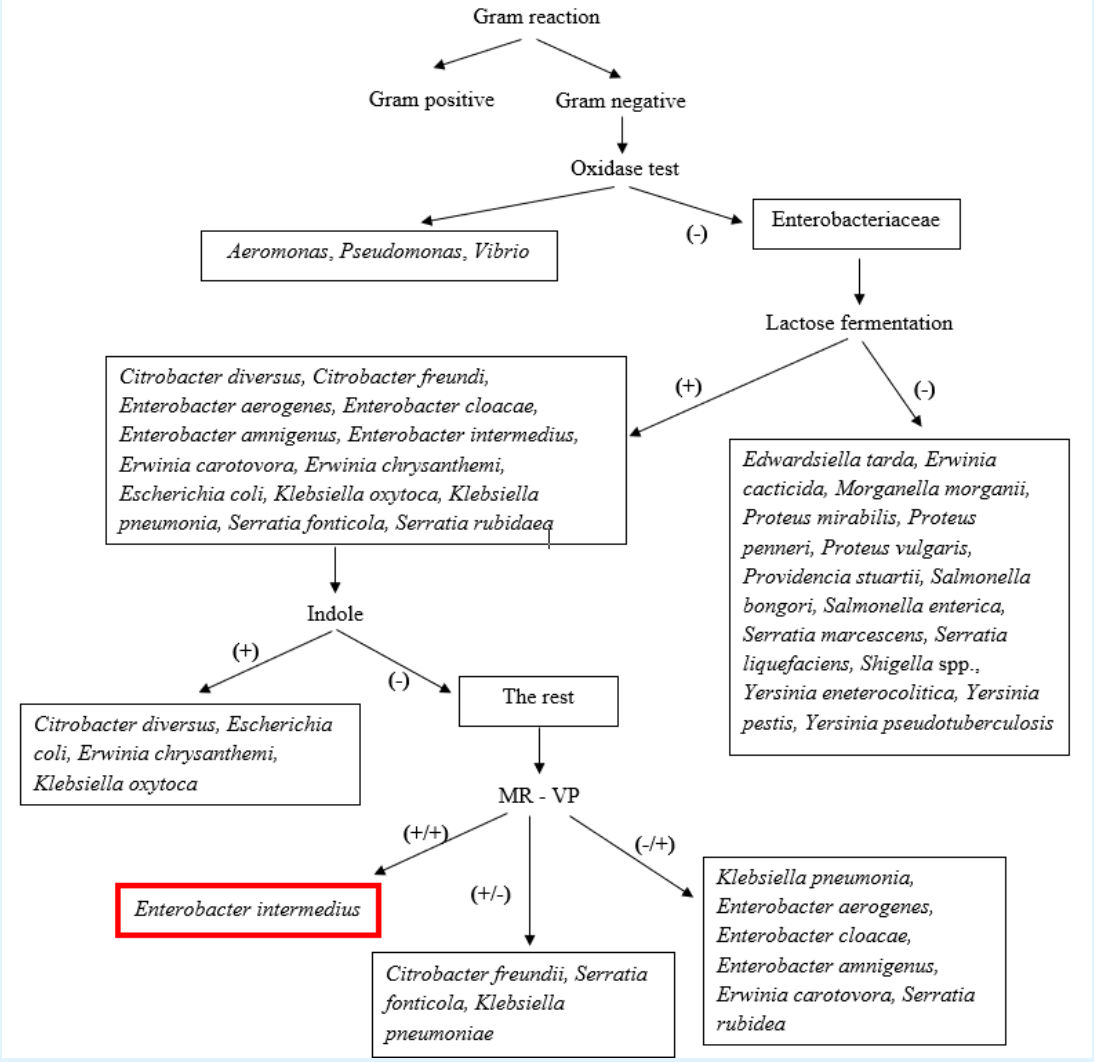

Figure 4: Identification flow chart for isolate D9.

Supporting figures on gram staining and selected biochemical tests are provided in Figures 5-18.

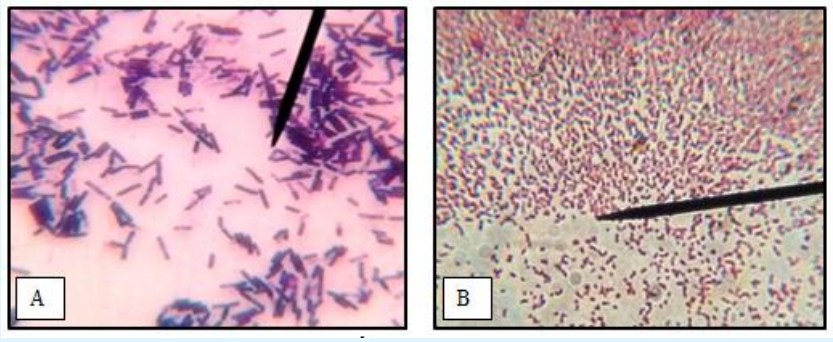

Figure 5: Gram staining. (A) Bacillus polymyxa, gram positive bacterium; (B) Isolate A5, gram negative bacterium.

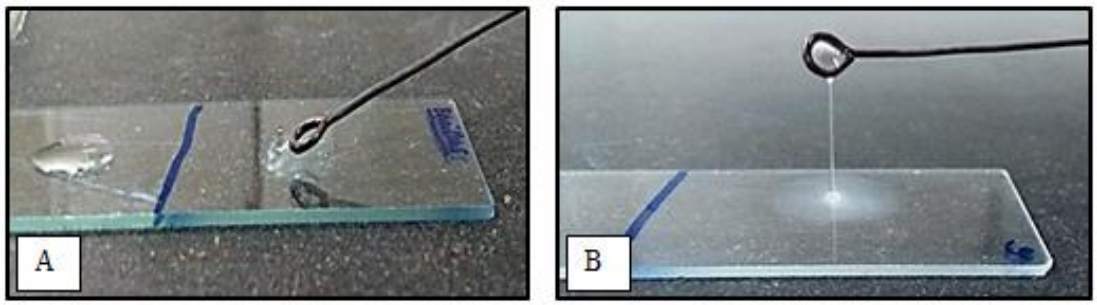

Figure 6: Gregersen. (A) B. polymyxa, negative result; (B) Isolate A6, positive result as characterized by thread of slime.

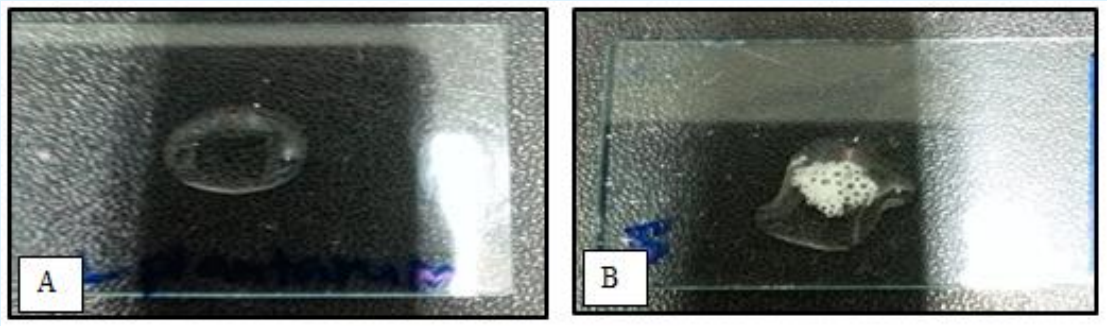

Figure 7: Catalase test. (A) Lactobacillus plantarum, negative result; (B) Isolate A5, positive result with noticeable formation of bubbles.

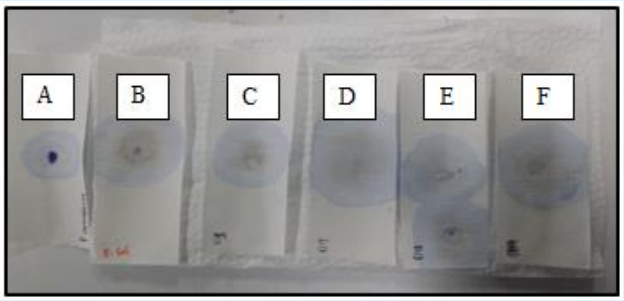

Figure 8: Oxidase test. (A) Pseudomonas aeruginosa, positive result as indicated by blue color; (B) Escherichia coli, negative result; (C-F), Isolates D1, D9, D11 and D14, negative results.

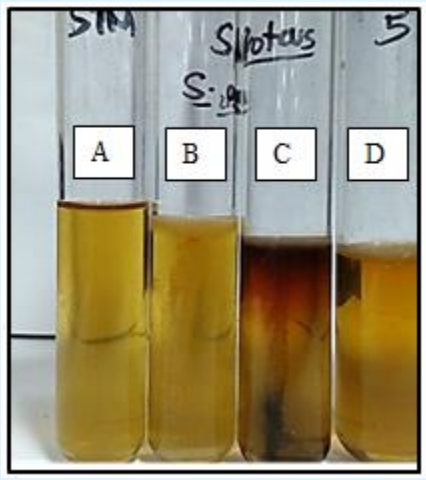

Figure 9: SIM medium: (A) Uninoculated; (B) Staphylococcus aureus, negative result on sulfide and indole production and non-motile; (C) Proteus vulgaris, positive result on sulfide (blackening) and indole (red color in the surface) production and motile (diffuse growth); (D) Isolate A5, negative result on sulfide and indole production and motile.

Figure 10: TSIA. (A) Isolate D1, no fermentation; (B) Uninoculated; (C) P. aeruginosa, no fermentation but with H2S production; (D) P. vulgaris, with glucose and lactose or sucrose fermentation and H2S production; (E) E. coli, with glucose and lactose or sucrose fermentation with acid accumulation.

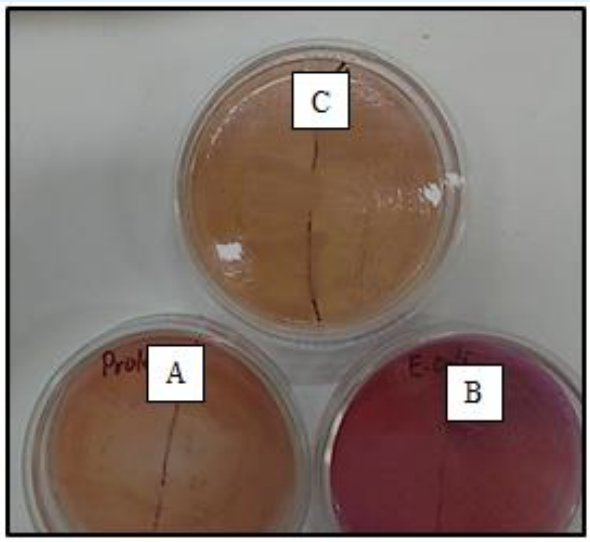

Figure 11: McConkey agar plates. (A) P. vulgaris, non-lactose fermenter (white/colorless growth); (B). E. coli, lactose-fermenter (pinkish growth); (C) Isolate A4, non-lactose fermenter.

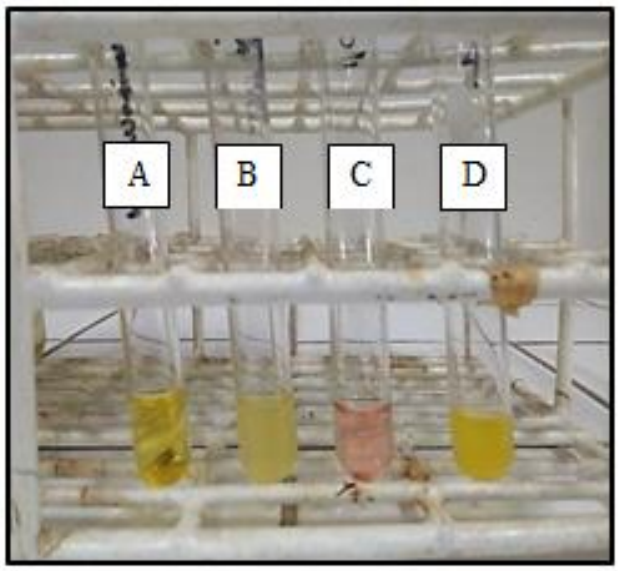

Figure 12: MR test. (A) Uninoculated tube; (B) P. aeruginosa, negative result; (C) E. coli, positive result as indicated by the production of red color; (D) Isolate A4, negative result.

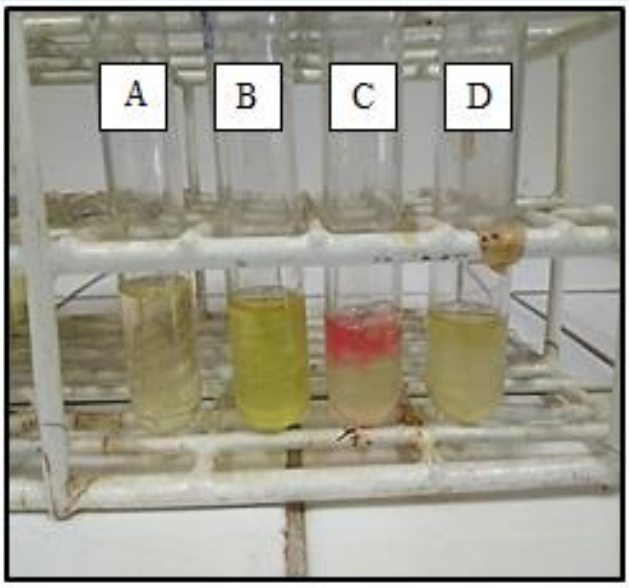

Figure 13: VP test. (A) Uninoculated tube; (B) P. aeruginosa, negative result; (C) B. polymyxa, positive result as indicated by the presence of red color in the surface; (D) Isolate A4, negative result.

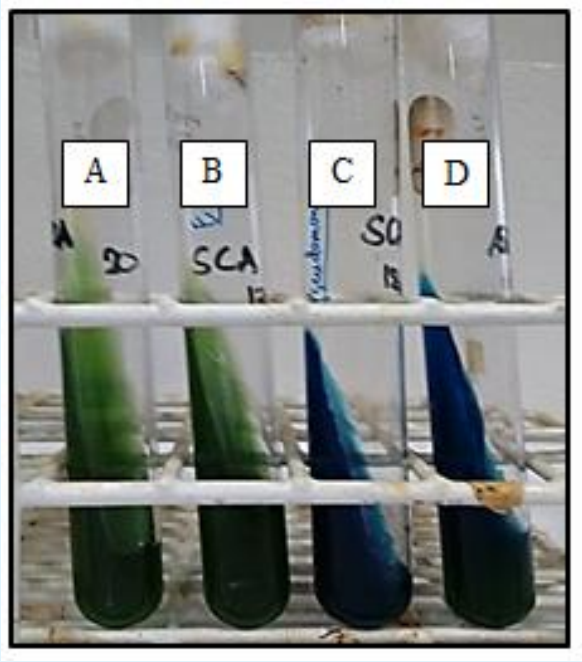

Figure 14: Citric acid utilization. (A) Uninoculated tube; (B) E. coli, negative result; (C) P. aeruginosa, positive result as indicated by the change of color from green to Prussian blue; (D) Isolate A6, positive result.

Figure 15: Urea hydrolysis. (A) Uninoculated tube; (B) P. vulgaris, positive result as indicated by the formation of red color; (C) E. coli, negative result; (D) Isolate A4, negative result; (E) Isolate A5, negative result; (F) Isolate A6, positive result.

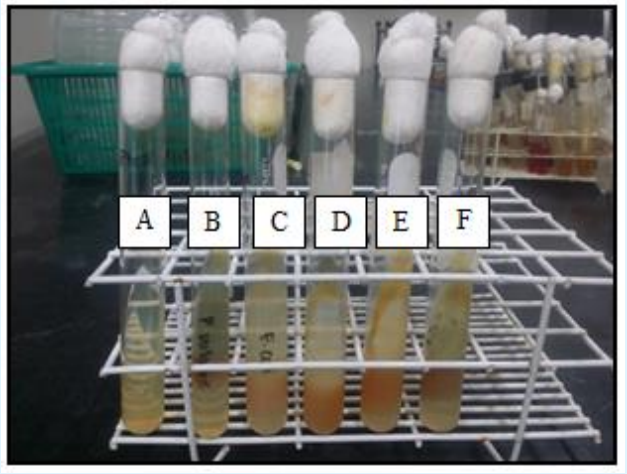

Figure 16: Phenylalanine deamination test. (A) Uninoculated tube; (B) P. vulgaris, positive result as indicated by the appearance of green color; (C) E. coli, negative result; (D) Isolate A4, negative result; (E) Isolate A5, negative result; (E) Isolate A6, positive result.

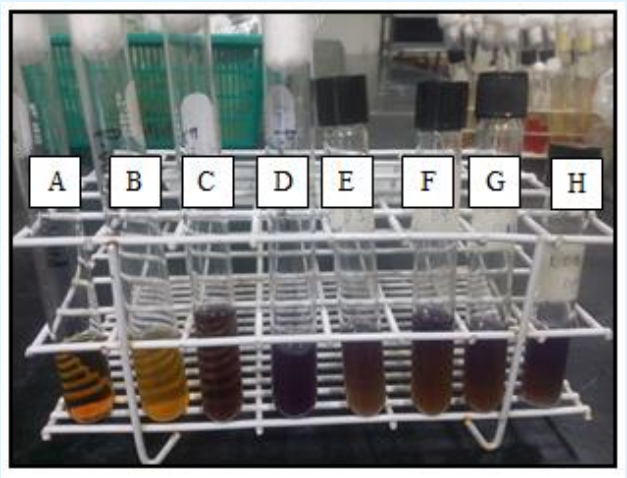

Figure 17: Lysine decarboxylase test. (A) Uninoculated tube; (B) P. vulgaris, negative result; (C) E. coli, positive result as indicated by appearance of purple color; (D) P. aeruginosa, positive result; (E) Isolate D1, positive result; (F) Isolate D9, positive result; (G) Isolate D11, positive result; (H) Isolate D14, positive result.

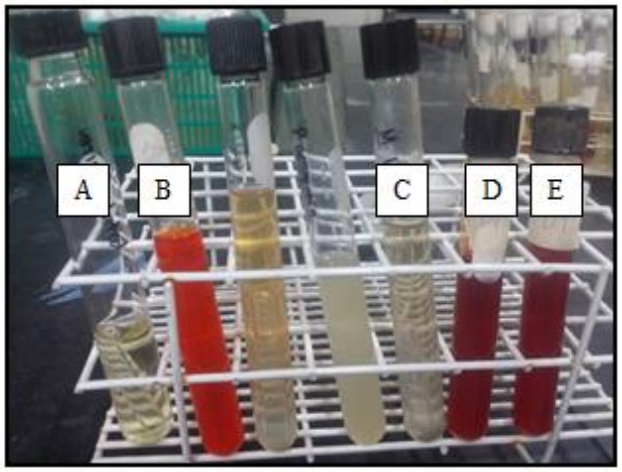

Figure 18: Nitrate reduction test. (A) Uninoculated tube; (B) E. coli, positive result as indicated by appearance of distinct red color; (C) Micrococcus luteus, negative result; (D) Isolate D1, positive result; (E) isolate D11, positive result.

All of the unknown isolates are characterized as gram negative and short rods (Figures 5-6). In Bergey's Manual of Determinative Bacteriology, gram negative and rod shape bacteria belong to either Group 4 or Group 5. Members of Group 4 are gram negative, aerobic/microaerophilic rods and cocci Figure 7) which are represented by *Acinetobacter*, *Pseudomonas*, *Beijerinckia*, *Acetobacter* and others. Meanwhile, members of Group 5 are described as facultatively anaerobic gram negative rods which are represented by Family Enterobacteriaceace and Vibrionaceae. The presumptive identification of isolates A4, A5 and A6 is *Pseudomonas* spp. that lack fluorescent yellow-green pigment. The identity of isolates A4, A5 and A6 was separated from *Vibrio* spp. and *Aeromonas* spp. based on the inability of the isolates to produce acid during glucose fermentation (Figure 2). This inability of acid production was revealed in Methyl Red (MR) test (Table 2; Figure 12). When isolates A4, A5 and A6 were grown in King's B medium, no fluorescent yellow-green pigment was observed. In addition, isolates A4, A5 and A6 and *Pseudomonas* spp. share the same biochemical characteristics such as non-lactose/glucose fermentation (Figure 11), inability to produce acetoin from glucose utilization (Figure 13), ability to utilize citrate as carbon source (Figure 10) and inability to reduce sulfur to hydrogen sulfide (Figure 9) [4]. The biochemical results of A4 and A5 were the same, indicating the possibility that they belong to the same species. The possible candidate species for isolates A4 and A5 are *P. arvilla*, *P. salopia* or *P. desmolytica*. Meanwhile, some of the biochemical results (e.g. urea hydrolysis, indole production, nitrate reduction) of A6 were different from A4 and A5 (Figures 9, 15 and 16) suggesting that it belongs to different species of *Pseudomonas*. The possible candidate species for isolate A6 are *P. mephitica*, *P. putrefaciens* or *P. cohaerens* [5]. Isolates D1, D11 and D14 are presumptively identified as *Serratia* spp. which is a member of Family Enterobacteriaceae. Family Enterobacteriaceae was separated from other gram negative rods (*Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp.) through negative result in oxidase test (Figure 3; Figure 8). The identity of isolates D1, D11 and D14 was further detached from other members of Family Enterobacteriaceae by using the results in lactose fermentation (Figure 10), indole test, urease, motility, H_2S production and nitrate reduction test. As shown in Figure 3, the three isolates had no capability to ferment lactose, failure to produce indole and to catalyze the hydrolysis of urea, motile, inability to reduce sulfur to hydrogen sulfide and ability to reduce nitrate (Figure 18). Possible candidate species based on Figure 3 are *S. marcescens* and *S. liquefaciens*, however, the identity of isolates D1, D11 and D14 is more towards *S. liquiefaciens* due to the absence of red pigment when grown in solid media. *Serratia* spp. are also characterized by the presence of the enzyme lysine decarboxylase which converts lysine to amine and also the ability to utilize citrate as carbon source [6]. These biochemical traits are also present in isolates D1, D11 and D14 (Figure 17).

The presumptive identification of isolate D9 is *Enterobacter* spp. which is a lactose-fermenter member of the Family Enterobacteriaceae. The isolate was identified from the rest of the Enterobacteriaceae members by having negative result on indole test and positive result on both MR and Voges-Proskauer (VP) tests (Figure 4). A typical *Enterobacter* spp. has positive result on catalase test, citrate test and urea hydrolysis test, while negative results on indole test and hydrogen sulfide test [7]. These biochemical results are also the same with isolate 9. In Figure 4, isolate D9 was specifically identified as *E. intermedius*. According to Jensen, et al. [8], the major bacterial groups isolated from the pig intestine are *Streptococcus*, *Lactobacillus*, *Prevotella*, *Selenomona*, *Mitsuokella*, *Megasphera*, *Clostridia*, *Eubacteria*, *Acidodaminococci* and the Enterobacteria. In the study of Elazhary, et al. [9], Enterobacteriaceae was isolated in the colon and cecum of 8 weeks, 10 weeks and 12 weeks pigs, while *Pseudomonas* spp, was only isolated in the 12 week-old pigs. In intensively and extensively fed pigs, Enterobacteriaceae and *Pseudomonas* spp. were also isolated in the intestine [10].

For a more reliable and faster identification of the isolates, MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry) is recommended. It is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules and large organic molecules. A portion of colony of the microbe in question is placed onto the sample target and overlaid with matrix. The mass spectra generated are analyzed by dedicated software and compared with stored profiles. Species diagnosis by this procedure is much faster, more accurate and cheaper than other procedures based on immunological or biochemical tests [11]. Genotypic approach is also highly recommended for the identification of the bacterial isolates. The use of broad-range 16S rRNA gene PCR as a tool for identification of bacteria is possible because the 16S rRNA gene is present in all bacteria. The 16S rRNA gene is consist of highly conserved nucleotide sequences, interspersed with variable regions that are genus-or species-specific. PCR primers targeting the conserved region of rRNA amplify variable sequences of the rRNA gene [12]. The bacterial isolates can be identified by nucleotide sequence analysis of the PCR product followed by comparison of this sequence with known sequences stored in a database [13].

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