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IN-VITRO ANTIMICROBIAL ACTIVITY OF *AEROMONAS* SPP ISOLATED FROM ESTUARY USING DIFFERENT SCREENING PROTOCOLS

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ABSTRACT

Screening is important in investigating antimicrobial activities of bacteria in a quest to discover new antimicrobials. Various protocols are readily available and employed by researchers worldwide studying microbiostatic and microbicidal activities of bacterial. This research therefore aims at evaluating available protocols and suggesting a standardized model. For the purpose of these research, antimicrobial ability of *Aeromonas* spp isolated from estuary was investigated using agar well diffusion method, deferred method and agar disc diffusion method respectively. Result obtained reveals that five isolates were positive to antimicrobial production among fifteen bacteria screened. Result was depended on the protocol utilized. Agar well diffusion produced a visible result when compared to others. Next to this was agar disc method. Deferred method involving both cross streaking and spot on lawn could not produce result as of swarming nature of the positive isolates tested. Based on our results, protocols utilized were grouped into preliminary screening test (PST) and confirmatory tests (CT) respectively. Preliminary screening protocol (PST) consists of deferred method – perpendicular cross streaking and spot-on-lawn while confirmatory protocols (CT) are agar well diffusion and agar disc diffusion methods. Preliminary protocols are not enough to conclude existence or non-existence of antimicrobial activity in bacteria. Conclusively, both preliminary and confirmatory protocols should be employed while screening bacteria for antimicrobials as suggested in our model.

INTRODUCTION: Increase in global antibiotic resistance of pathogenic bacteria, fungi and protozoa have geared up interest of researchers to investigate different sources for apparent antibiotics discovery. Emerging and re-emerging infections and diseases are continuously posing threat to human existence [1]. For the past decades, marine environment, soil and plants samples have been screened for potential antimicrobial compounds. Bacteria that have been identified as bioactive compounds producers include *Pseudomonas*, *Micrococcus* sp [2], *Vibrio ruber* [3, 4] *Lactobacillus acidophilus* [3, 5], *Bifidobacteria* [6], *Bacillus* [7], *Lactococcus* [8, 9, 10], *Staphylococcus*, *Actinomycetes* [11] and *Aeromonas hydrophila* [12]. Antimicrobials are products of microbial metabolism capable of inhibiting the growth of microorganisms. Antimicrobials have been reported in both Gram positive and negative bacteria [13]. Microbial inhibitory metabolites are either categorized as primary or secondary by-products [1]. According to Nanjwade *et al.,* 2010 [11], over 10,000

antibiotics have been discovered in the past five decades with more than 65% coming from actinomycetes. Bioactive metabolites from lactic acid bacteria are used in food preservation to elongate the shelf life of food products [3]. Identified metabolites capable of inhibiting the growth of other bacteria include organic acids, oxidizing agents like peroxide, siderophores and antimicrobial protein. Factors such as growth medium, incubation period, degree of alkalinity and acidity, temperature and protocols used for screening bacteria are capable of affecting results obtained. Only a careful selection of a mix of these factors can guarantee optimum results.

Research in life and natural sciences involve use of scientific protocols designed by various researchers for experiment purposes. Protocols can be modified to suit the purpose and aims of the researcher studies. Screenings for antimicrobial activity of bacteria have attracted different protocols. However, till date, no clear cut model has been developed. As a result of different existing protocols, a new model categorizing the existence method is hereby proposed. This research aim to investigate and compare antimicrobial activity screening of *Aeromonas* spp. isolated from estuary using different protocols

MATERIALS AND METHODS:

Isolation of Bacteria: Replicate marine water and sediment samples were collected from Melayu River, Johor Bahru, Malaysia in February, 2010. The water samples were processed via membrane filtration. The filters were then placed on modified Rimler Shott – mRS [14]. A standard microbiological method involving dilution and plate counts was carried out for sediment sample. 0.1ml of serially diluted sediment sample was plated on Marine agar (MA), Nutrient agar + 3% NaCl (NA+3%NaCl) and mRS respectively for isolation of presumptive *Aeromonas* spp. Discrete light green colonies with circular edge and dark centers on mRS agar were picked as presumptive *Aeronomas* spp. The isolates were Gram stained, phenotipically, morphologically and biochemically characterized using API kit 20NE (BioMerieux, France) and 0129/vibriostatic agent following manufacturers instruction to differentiate *Aeromonas spp* from *Vibrio spp.* Stock cultures of isolates were prepared and preserved.

Screening for Inhibitory Activity:

Agar Well Diffusion Method: Presumptive *Aeromonas* isolates were screened for in vitro antimicrobial activity against selected Gram positive and negative bacteria using agar well diffusion method. This was carried out using agar well diffusion method [10]. Isolates were grown in 250ml Nutrient Broth + 3% NaCl, Lactose Broth + 3% NaCl, Marine Broth and Tryptone Soy Broth respectively. These were then incubated in a shaker incubator at 200rpm, 300°C for 18- 24hours. 50ml of the cultured broth were centrifuged at 4000 x g, 4°C for 20 minutes to obtain a cell free supernatant. 1.2ml of supernatant obtained was transferred into sterile eppendorf tube and centrifuged at 16000rpm for 10minutes.

The Cell-Free Supernatant was filtered using autoclaved 0.22µm membrane filter paper**.** These was then tested for antimicrobial activity using agar well diffusion method against the following bacteria: *Enterococcus faecalis*, *E aerogenes, Aeromonas hydrophila, Vibrio parahaemolyticus, Bacillus subtilis*, *B thurengensis* ATCC 10792, MRSA, *Salmonella typhimorium*, *S soneri* ATCC 29930, *S mutan* ATCC 25175,*Seratia marcescens*, *Staphylococcus aureus, E coli* ATCC 10536. 50µl of Cell-Free-Filterate of the producer bacteria was placed in each 6mm well bored with a sterile cork borer in Muller Hilton Agar (antimicrobial sensitivity agar) containing streaked indicator bacteria. Inoculated plates were incubated at 30°C for 24 hours [3, 8]. Isolates with clear zones of inhibition around the well were noted.

Heat Stability of Antimicrobial Extracts: The extracts were tested for heat stability at the following temperatures 27°C, 60°C, 100 C and 121°C for 15 minutes respectively. Extracts were heated at different temperatures and tested for antimicrobial activity thereafter.

Effect of Siderophores on Antimicrobial Activity: 60µl of 1% Ferric chloride ($FeCl_3$) was added to 20ml of TSB. 20µl of producer strains were added and incubated at 30°C, 200rpm for 18-24 hours. These were then centrifuged at 4°C for 20 minutes at 4000xg. Resultant cell free supernatants were tested for antimicrobial activity as described above.

Partial Purification: 40% and 60% ammonium sulphate were added to 5ml cell free supernatants and left overnight at 4°C for protein precipitation. 40l of each extracts was then tested against indicator bacteria using above antimicrobial activity method.

Agar Disc Diffusion Method: is quite similar to agar well except wells being replaced with sterile discs prepared from Whatman's filter paper [7].

Deferred Method: Bacteria was streaked on a straight line dividing the agar plate into equal half and incubated at 30°C for 18-24 hours**.** Indicator strains were then perpendicularly streaked across the producer strains and incubated at appropriate temperature and incubation period accordingly. Positive results are indicated by clear zone of inhibition close to producer strains [3, 4, 8, 11].

Antibiotic susceptibility of Indicator Bacteria: All the 14 indicator bacteria were tested against the following antibiotics for the respective susceptibility pattern: Neomycin 30μg, Novobiocin 30μg, Ampicillin 10μg, and Bacitracin 2IU, Vancomycin 5μg, Polymycin B 300IU, Ciprofloxacin 5μg, Erythromycin 15μg according to the method of [15] with slight modifications. Result was interpreted as follows inhibition zone $\geq$ 18mm-sensitive, inhibition zone 13-17mm - intermediate and inhibition zone < 13mm -resistance [15].

TABLE 1: INDICATOR STAINS

Gram positive	Gram negative
E. faecalis	*E. coli ATCC 10536.*
MRSA	*V. parahaemolyticus ATCC 17802*
S. aureus	*S. sonnei ATCC 29930*
S. mutan ATCC 25175	*A. hydrophila*
B. subtilis	*P. mirabilis ATCC 12453*
B. thuringensis ATCC 10792	*E. aerogenes ATCC 10792*
	S. typhi
	S. marcescens

TABLE 2: ANTIBIOTIC SUSCEPTIBILITY OF INDICATOR STRAINS

Bacteria	Antibiotics susceptibility (mm)							
	N	NB	PB	VA	BC	AMP	CIP	E
E. faecalis	-	14	15	-	-	-	15	18
MRSA	-	14	15	-	-	-	15	18
S. aureus	-	-	-	-	-	-	-	19
S. mutan **ATCC 25175**	21	16	15	-	-	-	-	22
Bacillus subtilis	17	-	-	-	-	-	15	-
B. thuringensis **ATCC 10792**	19	-	-	-	-	-	-	-
V. parahaemolyticus **ATCC 17802**	20	15	-	-	-	-	15	20
E. coli **ATCC 10536**.	20	16	-	-	-	-	-	20
S. sonnei **ATCC 29930**	20	16	-	-	-	-	16	20
A. hydrophila	17	14	-	-	-	-	14	16
P. mirabilis **ATCC 12453**	19	14	14	-	-	-	-	18
E. aerogenes **ATCC 10792**	20	16	-	-	-	-	18	18
S. typhi	18	-	-	-	-	-	18	-
S. marcescens	-	-	14	-	-	-	16	22

N=Neomycin, NB = Novobiocin PB = Polymycin B, VA = Vancomycin, BC = Bacitracin, AMP = Ampicillin, CIP= Ciprofloxacin, E= Erythromycin, - = resistance

TABLE 3: CRITICAL ANALYSIS OF MERIT AND SHORTCOMINGS OF EACH PROTOCOL

Protocol	Merit	Shortcomings
Agar well diffusion	- Suitable for screening all bacteria. - Can be used for multiple producer bacteria screening. - Quantity of antimicrobial extracts used can be determined - Uses standard medium (Muller Hilton Agar). - Generally accepted for antimicrobial susceptibility testing including conventional antibiotic test. - Result can be read easily and compared	- Can only be tested against one indicator bacterium. - Time consuming (drilling well) - Consumes material like medium and plates if used for many isolates.

Deferred Methods – perpendicular cross streaking	- Fast. - Many producer bacteria can be tested. - Reduces materials - Any suitable or selective medium can be used to grow indicator bacteria. - Similar to cross streaking except the replacement spot with line - Plates can be labeled easily. - Positive result can also be easily observed and recorded.	- Not suitable for screening swarming bacteria. - Pathogens can grow across each other thereby affecting results. - Volume and concentration of antimicrobial cannot be determined.
- spot-on-lawn	- Can be used to search more than one producer bacteria suitable - Selective media can be used. -Possibility of cross-contamination is avoided. - less time and materials consuming	- No standard medium - Not suitable for swarming bacteria except extract. - Involves initial growth of bacteria in both media in order to extract antimicrobial.
Agar disc diffusion	- Used for screening more than one producer bacterium. - Similar to agar well except it uses disc instead of well. - Results can easily be compared - Less materials are used - Volume and concentration of antimicrobial are quantified	- Not suitable for swarming bacteria except extracting first. - Involves initial growth of production bacteria in broths media in order to extract antimicrobial

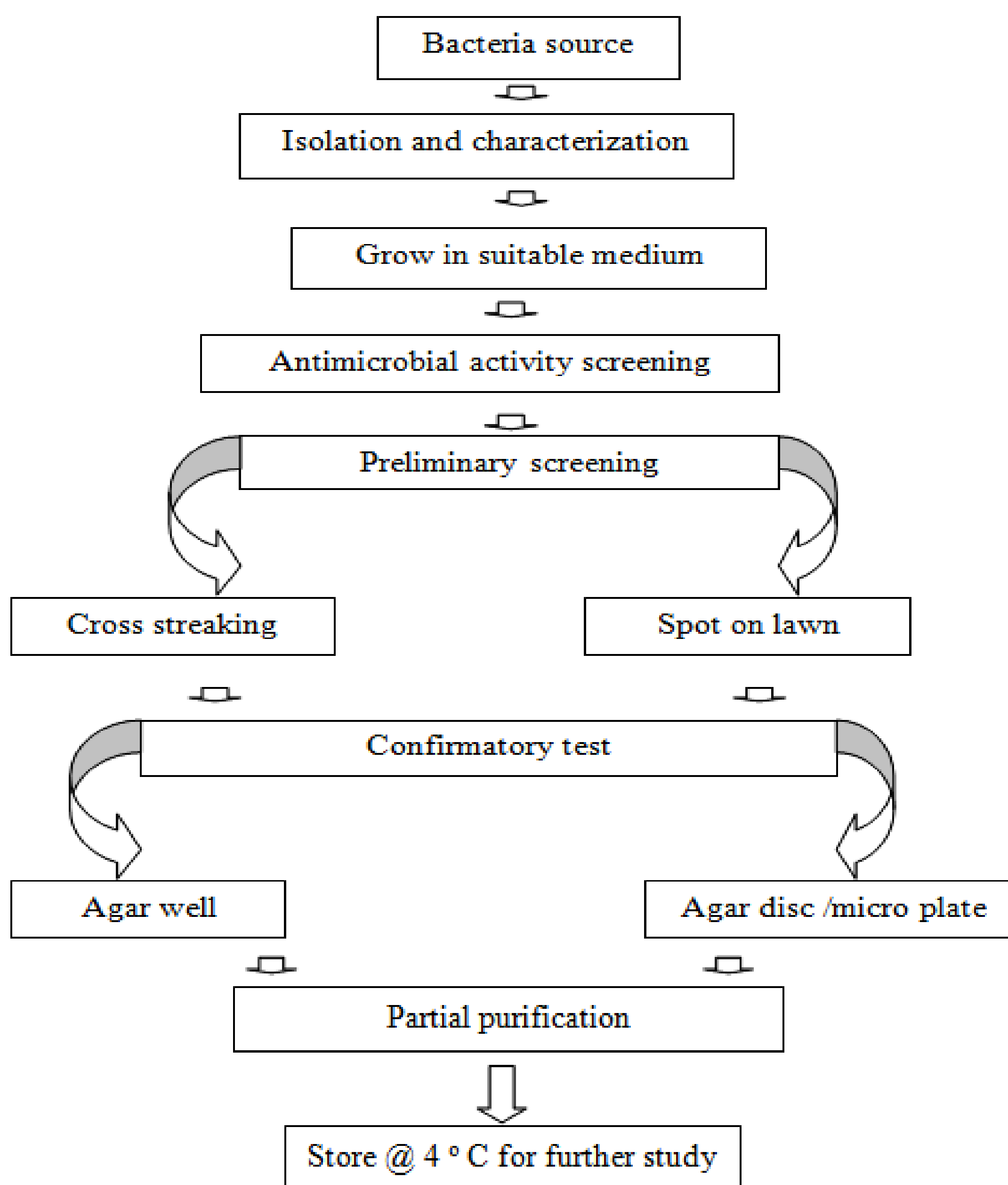

FIGURE 1: SUGGESTED ANTIMICROBIAL ACTIVITY SCREENING MODEL

RESULTS AND DISCUSSION: A total of 15 isolates were isolated from estuary samples. Cell and Colony morphologies for each isolates were noted. The isolates were catalase and oxidase positive, Gram negative, resistant to 0129/Vibriostatic agent and were able to grow on NA +3.5 %NaCl.

Results obtained from this agar well diffusion screening revealed that isolates Sg-20, Sg-19, Sg-17, Sw-16 and Sg-13 showed antimicrobial activity against the following indicator bacteria: MRSA, *S. soneri, S. typhimurium, V. parahaemolyticus, A. hydrophila, E. faecalis, B subtilis* on different medium. Sg-20 in LB broth showed the highest zone of inhibition against MRSA, *S. soneri, S. typhimurium, V. parahaemolyticus* and *A. hydrophila,*. However, no inhibition was observed when Sg-20 was grown in TSB, MB and NB respectively against the pathogens except *E faecalis.* It also show inhibition against *B subtilis* when grown in Nutrient broth supplemented with 3 % NaCl.

Sw-20 in LB did not show any inhibition against the four bacteria when incubated at 25-27°C for 24 hours. Isolate Sg-20 showed a broad spectrum activity. The extracted antimicrobial was found to be inactivated when heated. Cell-free filtrate was heated at the following temperature 27 ^{o} C, 60 ^{o} C, 100 ^{o} C and 120 ^{o} C respectively for 15 minutes each. The filtrate was stable at 60°C and inhibited tested indicator strains. No inhibition was observed at 100 ^{o} C and 120 ^{o} C respectively. It was also observed that the isolate did not exhibit antimicrobial property when grown for 48-72hours.

Siderophores are low molecular weight chelating agents required by bacteria in scavenging iron in growing environment. Iron is needed for DNA synthesis. Results obtained showed that no inhibition was observed against tested strains indicating antimicrobial activity of producer bacteria was not as a result of siderophores production by bacteria as also observed by [1].

All isolates showing inhibitory abilities did not give any positive result using cross streak method of screening. This was due to the fact that they spread over the plates after 18-24 hours of incubation. As a result of this, it was difficult to streak the plates perpendicularly with indicator bacteria. Similar thing was also observed with spot on lawn method. As seen in **table 3**, each of the protocol has its own merit and demerit. Swarming bacteria cannot be screened for antimicrobial activity using both spot on lawn and cross streak methods respectively.

Proven, accepted and reproducible laboratory procedures are of immense importance in life and natural sciences research. Search for new and effective antimicrobials that can be used in the fight against antibiotic resistance of bacteria have attracted different protocols.

Factors responsible for this includes sources of bacteria, cultural characteristics of bacteria and availability of appropriate materials growth medium. Marine bacteria require different environmental and laboratory growth conditions in order to produce needed metabolites likewise soil bacteria and environmental bacteria. Protocols needed for their studies may therefore differ. However, uniformity can still be achieved in this area by using more simplified and combination of existing protocols It is therefore of great importance if these protocols are grouped into preliminary screening and confirmatory test.

Preliminary screening is required to test possibility of inhibition of the growth of indicator bacteria by the suspected antimicrobial producers. Confirmatory test however confirms this initial claim of antimicrobial activity. Initial screening will help save time, energy and research materials. It also gives a green light to proceed with the investigation. However, due to media and cultural conditions, some antibiotic producers may not exhibit this during preliminary screening. It is therefore important to ensure appropriate medium at optimum growth conditions are provided.

As seen in **figure 1**, the suggested model consists of two stages involving preliminary screening and confirmatory test respectively. Stage 1: In this stage, cross streaking method or spot on lawn is used to screen the isolates for potential antimicrobial abilities. Stage 2: A more detailed test is needed to establish the result obtained in preliminary screening hence confirmatory test is to be carried out as described in proposed model. Any of agar well diffusion, agar disc diffusion and micro plate could be used.

This is required because some bacteria that are initially positive can lose their inhibitory abilities. All screening tests should be classified as either preliminary/primary screening lest or confirmatory test (CT). Screening protocols that does not involve extraction of the antimicrobial activity constituent should be used as preliminary/ primary screening test. Protocols such as deferred method, spot-on-lawn are suitable for the first test.

On the other hand, confirmatory screening test involves extraction of the active constituent from cell-free supernatant and then testing it on the same indicator organism. Agar well diffusion and Agar disc diffusion methods are best suitable in this category.

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