

PROTEOLYTIC ACTIVITY OF *PSEUDOMONAS AERUGINOSA* ON NEOPEPTONE AGAR MEDIUM

CHENG-CHUANG TSENG

On the Neopeptone agar medium, *Pseudomonas aeruginosa* showed so characteristic pigment production and proteolytic activity that it was easily differentiated from other bacteria. Using these two properties on Neopeptone agar medium for isolation of *Ps. aeruginosa* from clinical specimens, we got the same isolation rate as that of using nalidixic acid cetrime agar medium. For biochemical identification of *Ps. aeruginosa*, Neopeptone agar medium seemed to be more characteristic than Kovacs oxidase, cytochrome oxidase, glucose oxidation and gelatin liquefaction tests and as accurate as gluconate oxidation and nalidixic acid cetrime agar medium. For isolation of achromogenic strains of *Ps. aeruginosa* and *Ps. pseudomallei*, the proteolysis on Neopeptone agar medium showed better result than the other methods. These two colourless strains can be differentiated each other by their ability of oxidizing gluconate into 2-ketogluconate. Although the use of Neopeptone agar medium for isolation of *Ps. aeruginosa* still requires some improvement, its simplicity in preparation, easiness in handling and cheap cost make the medium useful for clinical identification of *Ps. aeruginosa*.

Neopeptone agar medium can also be used for isolation and identification of *Serratia marcescens* by its reddish-purple pigment production and proteolytic activity, and of *Klebsiella* by its mucous-droplet colonies and white precipitate zones. Moreover, the medium serves to identify various pigment-producing *Staphylococci* and to show proteolytic activity of *Bacillus cereus* and *Proteus* species.

Since the wide use of antibiotics, the clinical incidence of *Pseudomonas* infection has gradually increased. Therefore, it becomes an important problem to develop simple and quick methods for identifying *Pseudomonas*—*Ps. aeruginosa*, *Ps. pseudomallei*, *Ps. maltophilia* and *Ps. stutzeri*⁽¹⁻²⁾—from clinical specimens.

The usual enterobacteria isolation media such as EMB and MaConkey are not satisfactory for the isolation of *Pseudomonas* because of possible overlooking of some strains and need of reidentification. Although the nalidixic acid cetrime (NAC) medium has shown

excellent results for the isolation of *Ps. aeruginosa*, it has some difficulty in differentiating the achromogenic strains of *Ps. aeruginosa* from other non-pigment-producing bacteria that can grow on this medium, and in isolating the other pathogenic *pseudomonas* strains such as *Ps. pseudomallei* and *Ps. maltophilia* that can not grow on the same medium⁽³⁾. Many important biochemical tests have been used for identification and classification of *Pseudomonas*^(1-2,4-7), however, it is still necessary to find a suitable differential medium for differentiate *Pseudomonas* from other related microorganisms.

Department of Microbiology, Taipei Medical College (Director: Dr. Yun-Chuan Tsan)
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Production of water-soluble pigments such as phenazine and pyoverdine⁽⁹⁾ by *Pseudomonas* is one of the important characteristics for identification of this bacterium. Although an excellent result of Sabouraud maltose agar medium for the pigment production of *Ps. aeruginosa* has been reported⁽⁸⁾, King A and B media are usually used for the test of pigment production. On another extracellular substance—protease—especially of the *Ps. aeruginosa* and *Ps. myogenes* sp. there have been many detailed studies⁽⁹⁻¹⁷⁾. Interested in the pigment production and proteolytic activity of *Pseudomonas*, the author studied various media and found that when cultured on the Neopeptone agar (NA) medium, *Ps. aeruginosa* produced both a water soluble yellow-green pigment and proteolytic enzyme. The present study is to investigate the possibility of using these characteristic pigmentation and proteolytic activity on the NA medium for the identification of *Ps. aeruginosa* and to compare its accuracy with other important biochemical tests.

MATERIALS AND METHODS

I. Organisms tested:

The suspected *Ps. aeruginosa* strains were freshly obtained from clinical materials of McCay Memorial Hospital. The other bacteria used in this experiment were the strains kept in our department. All the strains subjected to the tests were 18 hr fresh cultures on heart infusion agar slants (Difco).

II. Biochemical tests:

(1) Gluconate oxidation test: A large amount of freshly cultured strains were grown in the gluconate-HCl medium (Eiken) at 37°C for 6 hr. Then 3 ml of 1% molybdic acid solution (Eiken) and 0.2 ml of glacial acetic acid were added and the mixture was heated in boiling water for 5 min and then quickly cooled. Deep blue coloring indicated positive reaction.

(2) Starch hydrolysis: One percent of dissolved potato starch powder (Wako Chemicals) was added to the nutrient agar (Difco). Strains were placed on these plates by spot-inoculation and incubated at 37°C for 24-48 hr. If cloudy zones appeared around the colonies on the clear medium, and after flooding the plates with 95% ethanol the clear medium changed to white precipitate and the cloudy zones became clear, the test indicated positive starch hydrolysis.

(3) Tween 80 hydrolysis: One percent of Tween 80 and 0.01% of CaCl₂ was added to the nutrient agar (Difco). Strains were placed on these plates by spot-inoculation and incubated at 37°C for 48 hr. Cloudy zones around the colonies indicated positive reaction.

(4) Arginine hydrolysis: The medium consisted of peptone (Difco) 5 gm, yeast extract (Difco) 5 gm, K₂HPO₄ 2 gm, glucose 0.5 gm and arginine-HCl (Wako Chemicals) 3 gm in 1,000 ml of distilled water. After inoculation the medium (2 ml/tube) was kept in an incubator at 37°C for 24 hr and 0.25 ml of Nessler reagent⁽¹⁸⁾ was added to each tube. Appearance of brown color indicated a positive reaction.

(5) Protease production test: Three percent of skim milk (Difco) was added to the heart infusion agar (Difco). After autoclaving, the plates were inoculated with band inoculation and cultured for 24 hr. Appearance of clear zones around the colonies indicated production of protease by the tested strains.

(6) Other tests: Gelatin liquefaction⁽¹⁹⁾, Kovacs oxidase⁽²⁰⁾, cytochrome oxidase⁽⁷⁾, nitrate reduction⁽²¹⁾, dextrose oxidation⁽²²⁾ and carbohydrate fermentation⁽²³⁾ tests were also included in this experiment.

III. Culture media:

(1) Neopeptone agar medium (NA medium): The medium consisted of 2% of neopeptone (Difco), 2% of lactose (Difco) and 1.5% of agar (Difco). All

the ingredients were dissolved in distilled water, autoclaved and then poured into plates. This medium had adequate cloudy opaqueness. Strains were band-inoculated and incubated at 37°C for 24 hr for detection of their pigment production and proteolytic activity.

(2) Nalidixic acid cetrimide (NAC) agar medium (Eiken)⁽³⁾: After 24-hr cultivation, the growth and pigment production of the tested strains on this medium were examined.

IV. Comparison of NA and NAC media for isolation of *Ps. aeruginosa* from clinical specimens:

After 24-hr propagation on brain heart infusion broth (Difco), specimens were inoculated directly onto the NA and NAC media. The suspected colonies i.e. colonies showing both pigment production and proteolytic activity or only proteolytic activity on the NA medium, and those which produced pigment on the NAC medium were picked up and cultured on the usual agar slants. The purified strains were cultured again on the NA and NAC media for confirmation. Then the bio-

chemical tests listed in II. were carried out.

RESULTS AND DISCUSSION

Following the classification of Bailey and Scott⁽²⁾ and the reports of several other scholars^(1,4,5,24-27), out of 207 strains which showed positive proteolytic activity, 206 strains were identified as *Ps. aeruginosa* and one as *Ps. pseudomallei* (Table 1). Two strains were negative in proteolytic activity and did not produce brown pigment. They might be classified as *Ps. maltophilia*. Among the 206 strains of *Ps. aeruginosa*, 202 strains (98.1%) were chromogenic. Among the chromogenic strains, 197 produced water-soluble yellow-green pigment and 5 produced brown pigment. These proteolytic activity and pigment production on the NA medium may provide an auxiliary biochemical test for the identification of *Pseudomonas*, and this medium may also serve as a screening medium for selecting *Pseudomonas* strains from clinical routine cultures.

Table 1. Identification of *Pseudomonas* by cultural characteristics on Neopeptone agar medium and other biochemical tests. (209 strains)

Biochemical Characters Species	Number of strains tested	Kovacs oxidase test	Cytochrome oxidase test	Glucose oxidation test	Gelatin liquefaction	Glucose oxidation	Growth on NAC	NA medium	
								Pigmentation	Proteolytic activity
<i>Ps. aeruginosa</i>	202*	+(+)	+	+(+)	+(±)	+(+)	+(+)	+(+)	+
	4	+(+)	+	+(+)	+(±)	+(+)	+(+)	-(-)	+
<i>Ps. pseudomallei</i>	1	+(+)	+	-(-)	+(+)	+(+)	-(-)	-(-)	+
<i>Ps. maltophilia</i>	2	+(±)	+	-(-)	+(+)	-(-)	-(-)	-(+)	-

* : Out of 202 strains, 5 strains produced water soluble brown pigments.

() : Principal characteristics of pathogenic *Pseudomonas* as described in W. Robert Bailey & E. G. Scott's Diagnostic Microbiology, 3rd edition, p. 163, 1970.

Pigment production and proteolytic activity of various pathogenic or non-pathogenic bacteria on the NA medium are shown in Table 2. *Serratia marcescens*, *Bacillus cereus*, coagulase-negative *Staphylococci* and *Proteus* sp. showed proteolytic activity of different

strength accompanied with their specific pigment e.g. reddish purple (*Serratia marcescens*), yellow or white (*Staphylococci*). These two properties of *Ps. aeruginosa* on the NA medium were more characteristic than other microorganisms. Although some other bacteria

can produce protease or pigment too, it is easy to differentiate *Ps. aeruginosa* from others by the shape of colonies and its characteristic water-soluble green-pigment.

The results of using NA medium for isolation of *Ps. aeruginosa* from the clinical specimens in comparison with NAC medium are shown in Table 3. From the total of 370 clinical specimens, 57 strains of *Ps. aeruginosa* were isolated

on both NA and NAC media. 11 strains of suspected *Ps. pseudomallei* which only showed proteolytic activity on NA medium were also isolated. Other bacteria found on the NA medium were: 6 strains of *Serratia marcescens* which produced reddish-purple pigment and proteolytic activity, 14 strains of *proteus* sp. which showed weak proteolytic activity, yellow or white colonies of *Staphylococci*, and *Klebsiella* with

Table 2. Proteolytic activity and pigment production of various pathogenic or non-pathogenic bacteria on Neopeptone agar medium

Species	No. of strains tested	Response on NA medium	
		No. of proteolytic strains (%)	Pigmentation
<i>Serratia marcescens</i>	39	39 (100)	Reddish-purple
Coagulase-negative <i>Staphylococci</i>	387	56 (14.5)	Yellow or white
Coagulase-positive <i>Staphylococci</i>	133	0	Yellow or white
<i>Bacillus cereus</i>	1	1	None
<i>Proteus</i> sp.	14	14 (100)	None
<i>Salmonella</i>	51	0	None
<i>Shigella</i>	310	0	None
<i>Klebsiella</i>	94*	0	None
<i>Escherichia coli</i>	30	0	None
<i>Corynebacterium xerosis</i>	1	0	None
<i>Candida albicans</i>	1	0	None

* : Cloudy zone formation.

Table 3. Comparison of Neopeptone agar medium with nalidixic acid cetrimide agar medium for isolation of *Pseudomonas* from clinical specimens

Kind of specimens	Specimen	NA medium			NAC medium (Isolated no.)
		Pigmentation	Proteolytic activity	Isolated no.	
Sputum	89	+	+	33	33
		—	+	6	0
Pus	64	+	+	10	10
Urine	107	+	+	8	8
		—	+	2	2
Throat swab	47	+	+	4	0
		—	+	1	1
Stool	20	+	+	3	3
Others	43	+	+	1	0
		—	+		
Total no.	370			68	57

white precipitate and mucous-droplet growth.

A special medium was used for the successful isolation of *Ps. pseudomallei* from clinical specimens⁽²⁸⁾. Thomason *et al.*⁽²⁹⁾ reported that they identified this bacterium on the slide by fluorescence antibody technique from the mixed culture of many microorganisms. In the present experiment NA medium showed an excellent result for the isolation of *Ps. pseudomallei*. As shown in Table 4, out of 11 strains of suspected *Ps. pseudomallei*, 7 (*Ps.* -11, 19, 40, 88, 93, 94 and 97) had the same properties as those described by Bailey and Scott⁽²⁾ i.e. positive reactions to the tests of Kovacs oxidase, dextrose oxidation,

gelatin liquefaction and carbohydrates fermentation (glucose, sucrose, maltose and mannitol in nutrient broth), no growth on the NAC medium; no oxidation of gluconate to 2-keto-gluconate; and no pigment production. They also showed the following properties as described by Yabuuchi⁽⁴⁾: no growth on 3% NaCl nutrient agar medium and positive reaction to the tests of starch hydrolysis, Tween 80 hydrolysis and arginine dihydrolation. *Ps.* -128 strain had properties partly similar to *Ps. aeruginosa* and partly similar to *Ps. pseudomallei*. Other 3 strains *Ps.* -67, 75 and 107 had rather confused biochemical characteristics and could not be identified.

Table 4. Biochemical characteristics of suspected *Ps. pseudomallei* which showed proteolytic activity but no pigmentation on the Neopeptone agar medium

Biochemical Characteristics	Tested strains 11, 19, 40, 88 93, 94, 97	107	67, 75	128	<i>Ps. aeruginosa</i> (control strain)
Kovacs oxidase	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+
Gluconate oxidation	—	—	—	+	+
Dextrose oxidation	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+
Tween 80 hydrolysis	+	—	+	+	+
Growth on: NAC medium	—	—	—	—	+
3% NaCl medium	—	—	+	+	+
7.5% NaCl medium	—	—	+	—	—
Starch hydrolysis	+	—	—	—	—
Arginine dihydrolation	+	—	—	—	+
Nitrate reduction	—	—	—	—	+
Neopeptone agar medium:					
pigment production	—	—	—	—	+
proteolytic activity	+	+	+	+	+
Protease production	+	+	+	+	+
Carbohydrates in nutrient broth					
Dextrose	+	+	+	+	+
Lactose	—	+	+	+	—
Sucrose	+	+	—	+	—
Maltose	+	+	+	+	—
Mannitol	+	+	—	+	—

The media now generally used for biochemical identification and isolation of *Ps. aeruginosa* are more or less unsatisfactory. For example, in the Kovacs and cytochrome oxidase tests, not only *Ps. aeruginosa* but also other bacteria such as *Alcaligenes faecalis*, *Aeromonas* species and *Flavobacterium* species can react positively. Gluconate oxidation test offers more accurate result for identifying *Ps. aeruginosa* but the procedure is rather complicated⁽⁶⁾. NAC medium is excellent for biochemical identification and clinical isolation of *Ps. aeruginosa*⁽³⁾, but since this medium has no inhibitive effect on the growth of some organisms such as *Klebsiella aerobactor* species, they can not be differentiated from the achromogenic strains of *Ps. aeruginosa*. Especially because the growth of both *Ps. pseudomallei* and *Ps. maltophilia* is inhibited on this medium, they will be missed when using NAC medium for isolation of *Pseudomonas*.

When NA medium was used as a differential medium for *Pseudomonas*, though its surface could often be covered by *Proteus* species, the result for isolation of *Ps. aeruginosa* was very good as compared with NAC medium or other biochemical tests, and it was also suitable for the isolation of *Ps. pseudomallei*. Out of 263 strains (206 in Table 1 and 57 in Table 3) of *Ps. aeruginosa* isolated from clinical specimens, 100% were proteolytic activity positive and 98.5% were pigment-producing strains on the NA medium. Compared with other biochemical characteristics especially with gluconate oxidation and growth on NAC medium, no doubt the NA medium was better than NAC medium or other biochemical tests for the isolation and identification of *Ps. aeruginosa*, especially for isolation of its achromogenic strains and *Ps. pseudomallei*.

NA medium also can be used for the isolation or biochemical identification of *Serratia marcescens* which produce

purple-red pigment and proteolysis, yellow and white pigment-producing *Staphylococci*, and *Klebsiella* which grow mucous-droplet colonies and produce white precipitate zones.

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綠膿桿菌在新蛋白胨 (Neopeptone) 瓊脂培養基之 蛋白胨分解活性之研究

曾 金 章

綠膿桿菌 (*Ps. aeruginosa*) 生長在新蛋白胨瓊脂培養基 (Neopeptone agar medium), 會產生色素及分解蛋白胨。因其性質相當特異, 故使綠膿桿菌和其他細菌的分離更為容易。若應用綠膿桿菌在新蛋白胨瓊脂培養基之色素產生及蛋白胨分解活性之二大特點, 於臨床檢體之直接分離時, 綠膿桿菌之分離率 and 選擇性培養基 nalidixic acid cetrimide 瓊脂培養基的分離率得到相同的結果。檢討新蛋白胨瓊脂培養基之應用於綠膿桿菌之鑑定, 似乎較 kovacs 氧化酶, 細胞色素氧化酶, 葡萄糖氧化及明膠液化等試驗更有特異性, 而和 gluconate 氧化試驗及選擇性 nalidixic acid cetrimide 瓊脂培養基之分離同樣地具有準確性及特異性。新蛋白胨瓊脂培養基除應用於色素產生之綠膿桿菌之外, 更可應用在無色素產生性之綠膿桿菌及 *Ps. pseudomallei* 之分離

與鑑定, 因兩者在新蛋白胨瓊脂培養基都具有蛋白胨分解活性的能力, 故應用本培養基於此兩種細菌之鑑定較其他之生化方法優越, 而兩者之間可由 gluconate 之氧化能力而區別之。雖然新蛋白胨瓊脂培養基之使用於分離培養仍須加以改善, 但取其製作簡單, 操作方便及成本低廉等等優點, 對綠膿桿菌之分離及鑑定得有所裨益。

新蛋白胨瓊脂培養基尚可應用於在本培養基可以產生紫紅色素及蛋白胨分解活性陽性之靈菌 (*Serratia marcescens*) 的分離及鑑定, 同時亦可用於可產生白色沉澱環及露滴樣生長的肺炎桿菌 (*Klebsiella*) 的分離。並且還可當為葡萄球菌 (*Staphylococci*) 之色素產生用培養基及變形桿菌 (*Proteus*) 和 *Bacillus cereus* 的蛋白胨分解用培養基。