

The Use of Modified Rimler-Shotts Agar as a Selective Medium for the Isolation of *Aeromonas* Species from Children Diarrhea in Mosul-Iraq

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ABSTRACT

Many selective simple and differential nutrient media were used for the isolation and identification of *Aeromonas* species. As for cultural characteristics, an examination of Modified Rimler Shotts was carried out and it was found that this medium was preferable for isolating *Aeromonas* species. This might be because this medium gave a kaleidoscopic colour variations for different *Aeromonas* species, besides some other positive discerning characteristics that this medium provides while other media block distinction. By accounting the recovery percentage compared to nutrient agar medium, it was noticed the *A. sobria* was the better growth on MRS medium followed by *A. hydrophila* and *A. caviae*.

Aeromonas

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Aeromonas

Aeromonas

A. hydrophila

MRS

A. sobria

. *A. caviae*

INTRODUCTION

Problems in isolation usually arise wherever quantitative recovery is needful, that is the account of the average number of emerging colonies after each process of dilution of

each isolate on tested media and on nutrient agar or in the case of availability in large number of contaminated microflora. Various media have been devised where several agents such as Ampicillin, Novobiocin, Sodium deoxycholate and Sodium lauryl sulphate besides Pril were used to augment selectivity (Joseph *et al.*, 1988). It has also been found that isolation from feces sample no single medium was generally accepted in spite of the effect of Ampicillin harbouring blood and the Cefulodin-Irgasan Novobiocin (CIN) medium which are commonly used on large scale (Travis and Washington, 1985). However, blood agar provides an elbow-room for direct oxidase test but unfortunately this agar is not highly selective because it dose not allow the growth of *Aeromonas* sensitive to the Ampicillin (Carnahan *et al.*, 1991).

Nazer research indicated that the employment of Alkaline Peptone Water (APW) at 37 °C for 3-6 hrs or 24 hrs. can help increase more isolations from fecal samples (Nazer *et al.*, 1985).

MATERIALS AND METHODS

The following simple, compound and selective nutrient media provided by Oxoid and BioMerieux Co. were used:

- Nutrient Agar (N.A.)
- Nutrient Broth (N.B.)
- MacConkey's Agar (M.A.)
- Cholera medium (T.C.B.S.)
- Modified Blood agar (M.B.A.) by applying 10 µg / cm³ from Ampicillin according to the method of (Koneman *et al.*, 1997).
- Alkaline Peptone Water (A.P.W.) prepared according to the method of (Koneman *et al.*, 1997).

In order to isolate and identify *Aeromonas* blood (Sheep blood) (5-7%) and Ampicillin (10) µg / cm³ containing blood agar as a selective medium was used for a preliminary isolation of fecal samples after having them cultured in APW which is suitable for encouraging the growth of such bacteria for 3-6 hrs.

- Pril Xylose Ampicillin Agar (P.X.A.) (Rogol *et al.*, 1979).
- Rimler-Shotts Agar (R.S.).

It is a selected media for *Aeromonas* prepared by mixing the following materials with the mentioned quantities:

L- lysine-hydrochloride	5.0 gm
L- ornithine-hydrochloride	6.5 gm
L- cystine-hydrochloride	0.3 gm
Maltose	3.5 gm
Sodium thiosulphate	6.8 gm
Bromothymol blue	0.03 gm
Ferric ammonium citrate	0.8 gm
Sodium deoxycholate	1.0 gm
Yeast extract	3.0 gm
Sodium chloride	5.0 gm
Agar	13.5 gm
Distilled water	1000 cm ³

pH was held at 7.0, then constituent parts were mixed by heat and the whole admixture was left to boil for one minute, put away and left to cool down to 45 °C, a 0.005 of Ampicillin was added (Shotts and Rimler, 1973).

- Modified Rimler-Shotts medium with fish extract:

The kind of fish used was *Chondrostoma regius* which belongs to F: Cyprinidae and the medium was finally prepared in tandem with Al-Jubori method (Al-Jubori, 1975).

The following steps have been adopted:

a- Preparation of fish extract:

- 1-The fish entrails head and other parts were thoroughly washed.
- 2-These parts were all minced by a mincing machine. The minced meat was weighed and distilled water was added by ratio of 2 liters for each kilograms of meat. The distilled water contains 0.6% sodium carbonate, 7.5% chloroform.
- 3-The admixture was thoroughly shaken for quarter an hour.
- 4-The mixture was placed into an incubator at 37 °C for 48 hrs. While stirring it finely every hour so as to affect homogeneity and fish enzyme digestion.
- 5-After the pass of 48 hrs. the mixture was filtrated by a clean cloth of shash. Then, it was heated to up to 100 °C for half an hour. Another filtration was done by using a filtration paper type Wattman No. (1). This time the filtration took place while the solution was still hot and pH stood at 7.4-7.6.
- 6-The filter was sterilized by an autoclave 121 °C and under a pressure of 15 pound / inch² for 20 minutes so as to render it ready for use. The resulting extract has golden-yellow colour and the form of a concentrated liquid, so it may be used after dilution at the rate of 1 : 3 sterilized distilled water.

b- Preparation of *Aeromonas*-specific medium from the fish extract;

To prepare 100 cm³ of medium, the following components are being used:

Maltose	0.35 gm
Sodium thiosulphate	0.68 gm
Bromothymol blue	0.003 gm
Ferric ammonium citrate	0.08 gm
Sodium deoxycholate	0.1 gm
Sodium chloride	0.5 gm
Agar	2.5 gm
Fish extract	50 cm ³
Distilled water	50 cm ³

pH stands at 7.4. Then, the solution was mixed by heat and the whole admixture was left to boil for one minute, then left to cool down to 45 °C, then poured into petridishes.

Table (1) shows the kinds of media abstracted in *Aeromonas* isolation together with its various specifications:

Table1: The kinds of media for *Aeromonas* isolation and some important specifications

No.	Media	Abbreviation	Samples	Inhibitory agents	Differential agents	Incubation temperature / °C
1	MacConkey agar*	MA	Clinical	Bile salt	Lactose	37
2	Thiosulphate-Citrate-Bile salt Sucrose-agar*	TCBS	Clinical	Citrate Ox bile	Sucrose	37
3	Alkaline Pepton Water*	APW	Clinical	pH 8.6	-	35-37
4	Blood Ampicillin Agar*	BAA	Clinical	Ampicillin	Erythrocytes	37
5	Rimler-Shotts agar*	RS	Environmental Clinical	Citrate Ampicillin Deoxycholate	Lysine Ornithine Maltose	35-37
6	Pril-Xylose-Ampicillin agar*	PXA	Clinical	Pril Ampicillin	Xylose	37
7	Modified Rimler-Shotts agar (with Fish Extract)	MRS	Clinical	Citrate Deoxycholate	Maltose Fish extract	37

* (Jeppesen, 1995)

Culture Description:

The characteristics of bacterial cultures growing on Ampicillin containing blood agar used to isolate *Aeromonas* from fecal samples have been studied and the type of lysis was noted. Also, MacConkey's agar medium was inoculated so as to trace up the *Aeromonas* potentiality of fermenting Lactose. In order to confirm the characterization of *Aeromonas* cultures that have been purified on TCBS, RS and PXA media had been inoculated by streaking method. The plates were incubated at 37 °C for 18-24 hrs. and the shape and colour of the colonies were noted. It was found that these vary according to the components of media and the three species of colonies *A. hydrophila*, *A. sobria* and *A. caviae* were tested on MRS medium.

Qualitative estimation of growth on MRS:

MRS medium was inoculated by *Aeromonas* isolated from infant and children diarrhea, and the inoculation was performed by using streaking method so as to obtain single colonies that enable as to study their shapes and cultural behaviour. The plates were incubated at 37 °C for 18-24 hrs. (Moyer *et al.*, 1991).

Quantitative estimation of growth on MRS medium:

Genus *Aeromonas* of several species are used to estimate the quantitative effect of growth on MRS medium which has been already prepared: verile bacteria in a suspension of Phosphate Buffer Saline was prepared in such a way that it gave an approximate number of (10^3 - 10^4) living cell per each cm^3 . Several dilutions were also made on the suspension and the number of living cells was counted using the standard method of plate count (Cruickshank *et al.*, 1975).

The medium was inoculated by high dilutions of the suspension and the nutrient agar was also inoculated using the same dilutions for comparison. The plates were then

incubated at 37 °C for 18-24 hrs. Later, the number of growing colonies on both media was registered and the recovery percentage on the tested medium was taken to have it compared with the nutrient agar (Moyer *et al.*, 1991).

RESULTS

Aeromonas colonies, as a result, appeared on the blood agar and they were green in colour surrounded by a complete blood lysis in the case of *A. hydrophila* and *A. sobria* while the blood was not of the same shape in the case of *A. caviae*. On the TCBS medium, the three species appeared yellow in colour because its potentiality to ferment sucrose that exists in the medium. Similar yellow colonies of the same species emerged on RS medium since they have the ability to ferment maltose that exists in the medium, fig. (1). On the other hand, the three species had pinky colour on PXA medium since they do not ferment xylose that exists in the medium.

It is significant to note that the colonies emerging from the three kinds have shown variance in colour while growing on MRS agar in spite of it having a maltose content. In this respect, the *A. hydrophila* was green, *A. sobria* greenish-yellow to green while the *A. caviae* colonies had a yellow colour after 24 hrs. of incubation, see fig. (2), (3) and (4). The colonies of all three species were turned into green after 48 hrs. being placed in a fridge, the strains of these bacteria had the power to persist in slow growth at fridge temperature ranging between (4-10) °C.

This genus is also characterized by the centralization of pigment in the centre of the colonies emerging from the three species. The centre usually turns to dark-green while the periphery fades to light-green. Therefore, these colonies are termed Bulls-eye colonies.

The preference for growth of the three species on MRS medium in comparison with their growth on NA medium was also scrutinized through using quantitative estimation, the recovery percentage was usually coupled to this effect too. Fig. (5) shows that the growth of *A. sobria* colonies was better than that of the other colonies as it gave a higher recovery percentage on MRS medium that ranges between 85.3% to 100%. *A. hydrophila*, on the other hand, gave a lesser recovery percentage on the same medium which ranges between 79.4% to 84.8%. Fig. (1) also shows the *A. caviae* was the least in growth for its recovery percentage stood at 59.0% to 60.5%.

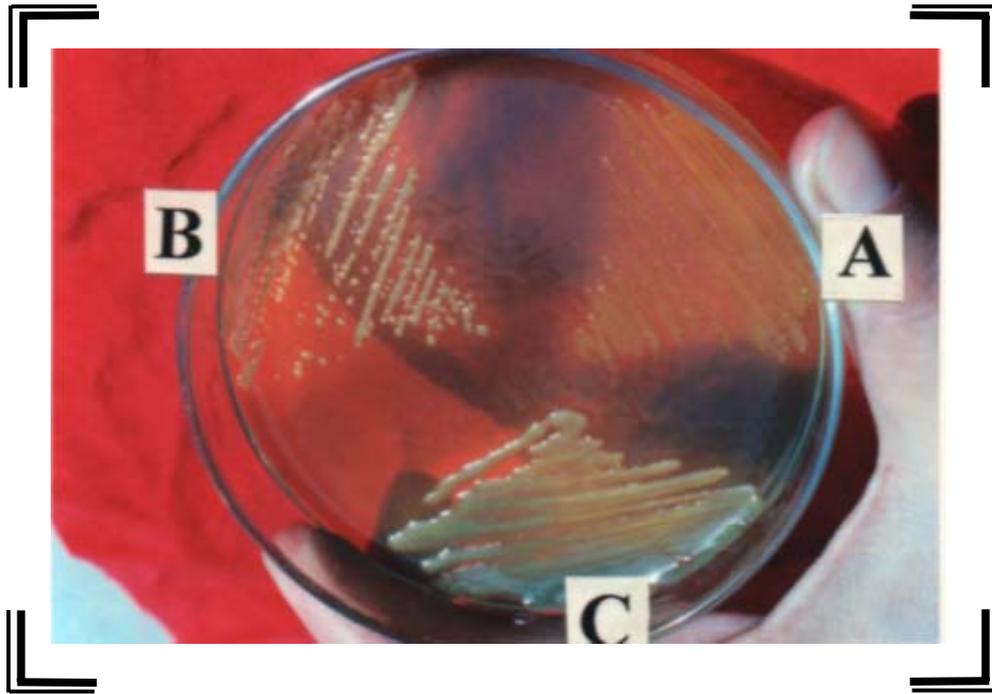


Fig 1: The culture of *Aeromonas* species on Rimler-Shotts agar medium. (A) *A. sobria* (B) *A. hydrophila* (C) *A. caviae*.

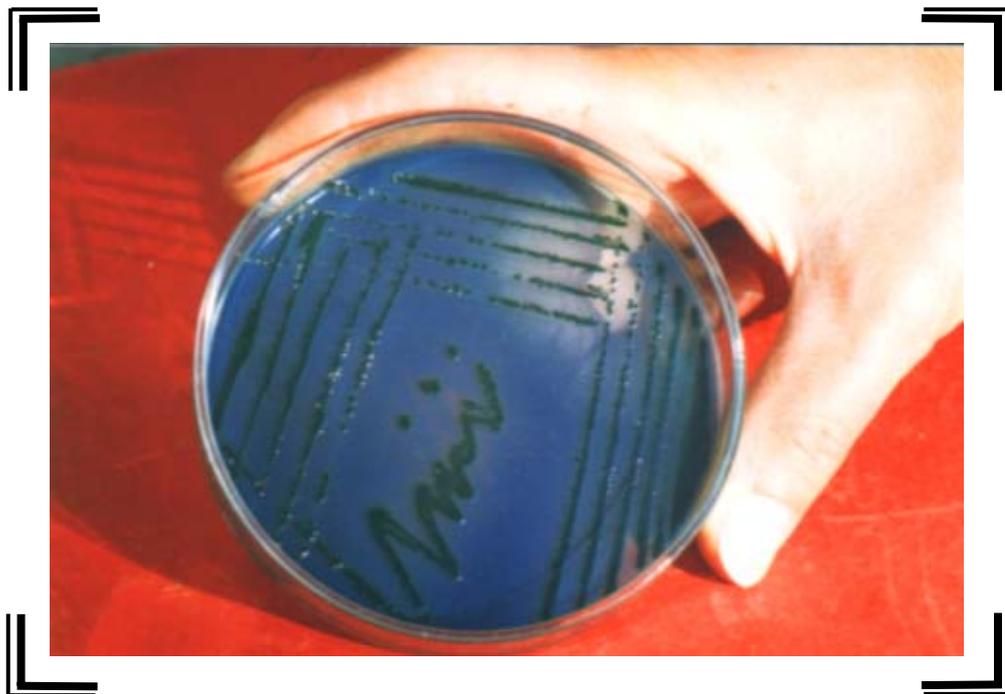


Fig 2: The culture of *Aeromonas* type *A. hydrophila* on Modified Rimler-Shotts agar medium.

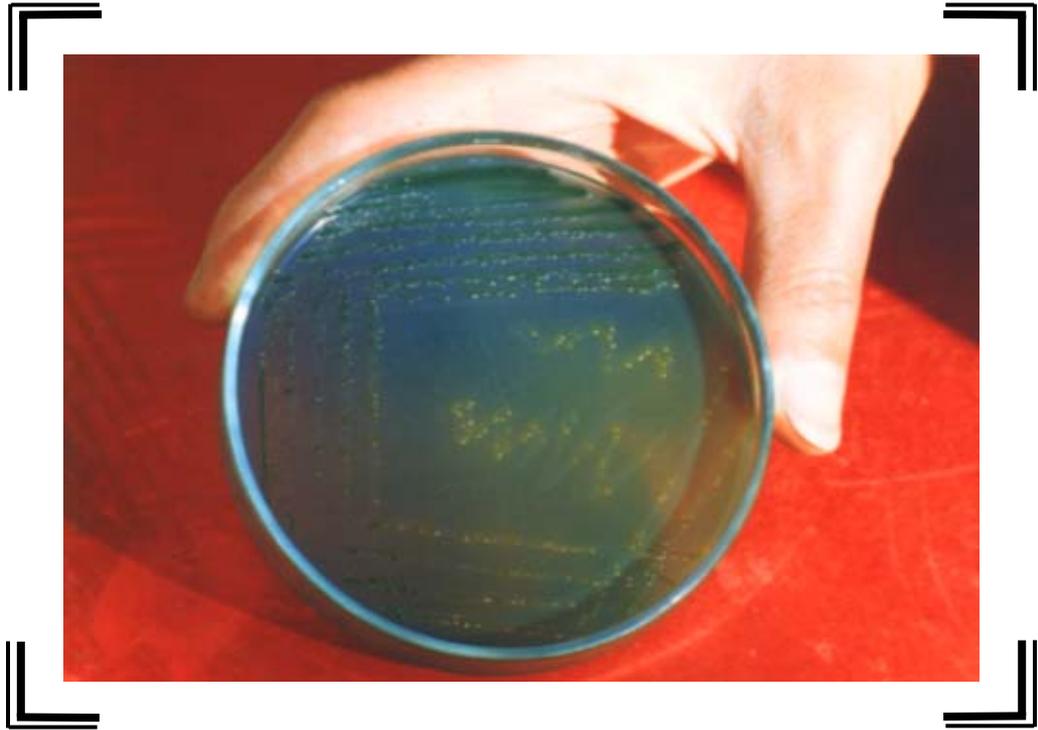


Fig 3: The culture of *Aeromonas* type *A. sobria* on Modified Rimler-Shotts agar medium.

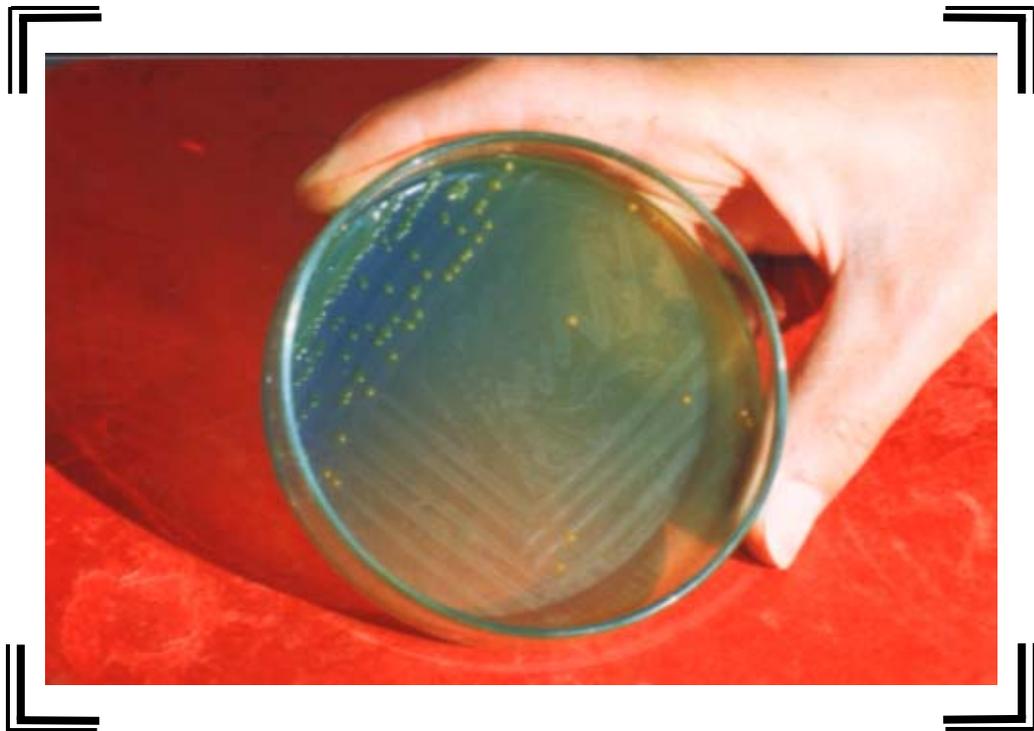


Fig 4: The culture of *Aeromonas* type *A. caviae* on Modified Rimler-Shotts agar medium.

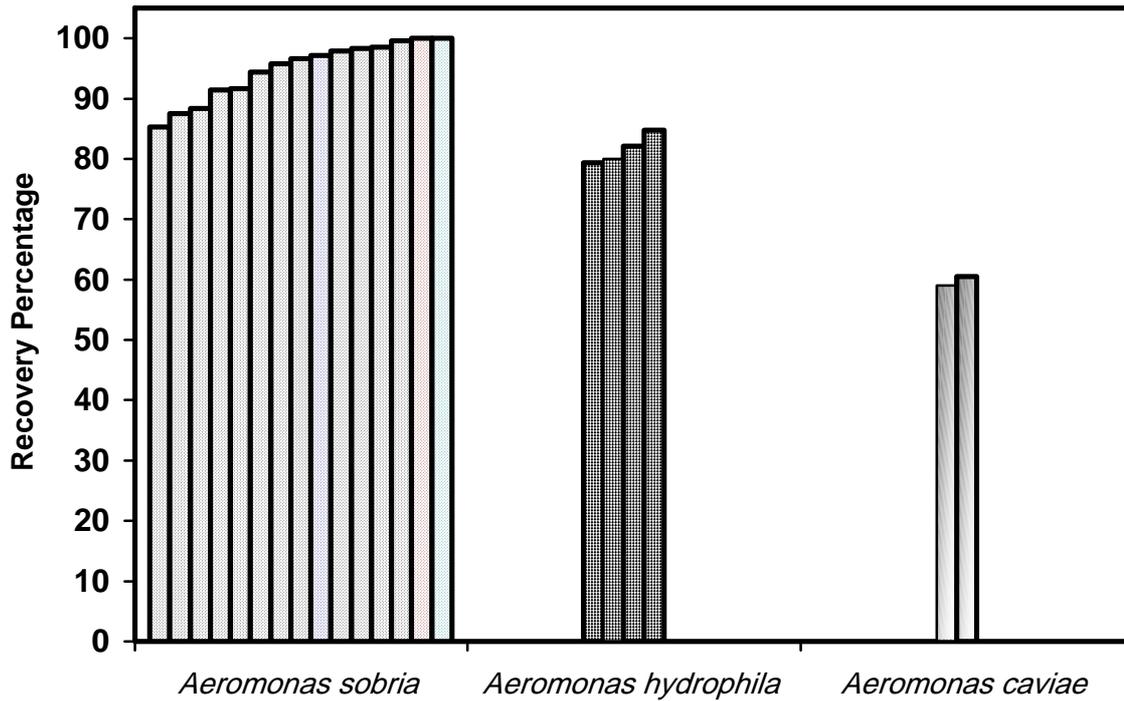


Fig 5: Recovery percentage to three species of *Aeromonas* on MRS medium comparative with NA medium

DISCUSSION

APW is being used as one of the enrichment medium that encourages the growth of *Aeromonas* from fecal samples at a range of pH of (8.6-9.8) which greatly helps the growth of such bacteria even if found in small numbers in fecal samples (Moyer *et al.*, 1991).

Differential and selective media to isolate genus *Aeromonas* from other bacteria have also been employed. Since MacConkey's agar medium was, according to Jeppesen, (Jeppesen, 1995) not suitable to select *Aeromonas* since this genus includes lactose unfermenting strains besides fermenting strains of the same sugar in addition to agar TCBS that is used to isolate *Vibrio* and to a lesser value for isolating *Aeromonas* which appear yellow for both genera with untransparent centre and transparent peripheries that came as a result of sucrose contained in the medium. Therefore, the use of these media to isolate *Aeromonas* was being adopted and in certain cases even this use has been replaced by selective media as sheep blood (5-7)%, Ampicillin (10) $\mu\text{g}/\text{cm}^3$ containing agar (Janda and Abbott, 1999). The *A. hydrophila* and *A. sobria* have shown a blood lysis β -haemolysin while *A. caviae* did not show this phenomenon. The existence of Ampicillin in the medium also limits the growth of numerous bacteria that are responsive to its effect.

RS medium was, on the other hand, considered a differential and selective medium at the same time since it helps in isolating the *Aeromonas* (Atlas, 1997) maltose sugar that is included in the medium was usually used as a source for carbon and amino acids such as lysine and ornithin that help in revealing the carboxyl group. As for cysteine amino acid, it was used in discovering the emitting of hydrogen sulphate with the addition

of some selective agents such as Ampicillin, Bile salts and Bromothymol blue pigment as a guide. This is because *Aeromonas* colonies usually appear yellow on this medium for it was fermented with maltose. The same medium was being used to segregate *Aeromonas* from other bacterial species such as *Citrobacter* and *Proteus* which grow on this medium and give yellow colonies with a black centre. As for other species that do not ferment maltose such as *Salmonella* and *Edwardsiella*, green colonies with black centre appear while *Pseudomonas*, *E. coli*, *Klebsiella*, *Shigella* and *Plesiomonas*, they usually appear greenish-yellow to green.

PXA medium was used for *Aeromonas* since it harbours an ampicillin content besides pril which restrict the growth of *Aeromonas* (Joseph and Carnahan, 1994), on this medium *Aeromonas* colonies with pinkish bright colour emerged because they were not fermented to xylose.

In this study MRS as a selective medium to isolate *Aeromonas*, it has also been used to distinguish the species contingent on this genus. The three species have shown variance in colour, green in *A. hydrophila*, green-yellowish to green in *A. sobria* and yellow in *A. caviae*. It also emerged that while the three species ferment maltose, the variance was attributed to the nature of these isolates and their physiological needs besides the difference in the metabolism of the fish extract from amino acids content of the protein. Moreover, the species, *Aeromonas* differ in its use of amino acids such as Lysine, Ornithine, Arginine, Prolin, Serin, Cysteine, Alanine and Histidine, on the other hand, the variance in time taken for sugar fermentation in the medium besides the shift in colonies of all strains into green after 48 hrs. placed in a fridge, this persistence and change in colour were possibly due to the lysis of certain amino acids in the fish extract leading to a higher pH of the medium resulting from alkaline increase, this in turn affects the light colour of bromothymol blue pigment that is blue and is used as a queue. *Aeromonas* is also distinguished by its capability of persistence in growth in fridge temperature (4-10) °C (Beuchat, 1991) which hightens the probability of continuity of these bacteria with the same metabolic effectiveness. This, of course brings in the formal changes in the shape of these colonies such as the centralization of the pigment which is commonly termed Bulls-eye phenomenon.

The preference of growth for the three species has also been investigated. Fig. (5) showed that *A. sobria* taking into account the rate of recovery percentage in comparison to nutrient agar medium-was found to be the highest in growth rate, followed by *A. hydrophila* and *A. caviae*. The difference, here, was normally attributed to the fact that *A. sobria* was the most actively metabolic for components taken from fish extract and its amino acids in particular (Altwegg *et al.*, 1987).

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