SUITABILITY OF TENDER COCONUT WATER AGAR MEDIUM FOR GROWTH OF MICROORGANISMS AND ANTIBIOTIC SENSITIVITY TESTING

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Summary

Tender Coconut Water (TCW) is a natural sterile fluid of tissue origin containing many nutrients. TCW agar medium therefore incorporates all the nutrients present in TCW. This preliminary study evaluates the suitability of TCW agar for the growth of various pathogens and antibiotic sensitivity testing. Reference strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and standard isolate of *Vibrio cholerae01* (PSH 5) and *Klebsiella* (NK 1545) were simultaneously sub cultured in TCW agar and conventional media. Colony counts and colony forming units /ml were compared under identical test conditions. Antibiotic sensitivity testing was done simultaneously in TCW agar and Meuller Hinton Agar and the sizes of the zones of inhibition compared. Growths of *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella* were less in TCW agar but not statistically significant. *Staphylococcus aureus* and *Vibrio cholerae 01* failed to grow in TCW agar. Zones of inhibition were lesser in TCW agar than in conventional media. Keeping in mind the low cost and the easy availability of TCW, TCW agar can be considered to be an alternative to conventional media for the growth of some common organisms. However further study needs to be done with clinical isolates, clinical samples and other organisms. As the low pH of TCW is a limiting factor, study needs to be undertaken to see if further modifications can be done (eg. increasing the pH by addition of an alkali) so that TCW agar becomes a better medium to grow common organisms and have wider acceptability.

Key words: *Tender coconut water agar, microorganisms, antibiotic sensitivity testing*

Introduction

Microorganisms require the presence of adequate amount of nutrients and the absence of inhibitory substances for their optimum growth. They can be grown *in vitro*, in any culture medium that will provide the organisms with all the nutrients required for growth and energy turnover. For this, optimal pH, temperature and proper aeration are to be maintained[1] The composition of commonly used culture media may be altered within a range of tolerability if the basic requirements for optimal growth of a microorganism can be maintained. Tender coconut water (TCW) is a naturally obtained sterile fluid of tissue origin containing many nutrients.[2] It contains considerable amount of easily assimilable carbohydrates, many essential amino acids, minimum amount of fat, sufficient electrolytes and vitamins which can serve as the optimum recipe for the growth of many common bacteria and fungi.[3] The water of tender coconut contains reducing sugars (glucose and fructose), amino acids and sufficient ascorbic acid, in the early stages of maturity.[4] Coconut water tellurite egg medium has been used to culture *Corynaebacterium diphtheriae*[5] and Coconut water egg malachite green medium for isolation of *Mycobacterium tuberculosis* [6] TCW as a broth has proved to be an effective liquid medium that has supported the growth of many bacteria including anaerobes.[7] However, for the identification of organisms by Gram's staining and biochemical testing, it is necessary to obtain pure colonies of the organisms on a solid medium This is a preliminary study to evaluate the suitability of TCW agar medium for the growth of various pathogens and also if TCW agar medium can be used for antibiotic sensitivity testing.

Materials and methods:

Collection of tender coconut water and preparation of solid media

This study was undertaken in the Department of Microbiology, I.P.G.M.E.R., Kolkata, India in collaboration with the Department of Pharmacology, Medical College, Kolkata. Necessary clearance was obtained from the Independent Ethics Committee (I.P.G.M.E & R., Kolkata).

Tender coconuts of common Indian species (*Cocos nucifera*) were punctured aseptically and the water was collected in sterile conical flasks maintaining all sterile precautions and used when required. A small amount of the water was taken and its pH was measured using a Global Digital pH Meter (DPH 500). Biochemical analysis of the water was done to determine the levels of sugars (glucose), proteins, fat (triglycerides) and electrolytes (sodium, potassium, chloride, phosphate and calcium).

Two grams of agar-agar powder was dissolved in 20ml of distilled water and autoclaved at 121^0C for 15 minutes. 80ml of tender coconut water was poured aseptically into a sterile conical flask and placed in a 50^0C water bath for 10minutes. The autoclaved agar-agar was allowed to cool to 50^0C. Maintaining all sterility precautions the tender coconut water was added to the agar-agar with continuous shaking. Since some of the agar failed to dissolve completely, the media was then steamed until the entire agar was dissolved. The pH of the media thus prepared was measured. The media was then poured into sterile Petri dishes and left undisturbed until the medium had set. In this way a 2% TCW agar media was prepared. On attempting to streak these plates with an inoculating loop, it was found that the medium was too soft and was being cut through by the loop. In order to prepare a medium of suitable hardness, instead of 2 gm of agar-agar for 100ml media prepared, 2.5grams and 3grams of agar-agar was used respectively and the

whole process of media preparation was repeated as above. The 2.5% agar medium was also found to be soft, but the 3% agar medium was of a desired hardness. So, a 3% TCW agar medium was used as the standard solid medium for our study.

Microorganisms tested:

Reference strains of *Escherichia coli* (ATCC 25922)
Reference strains of *Pseudomonas aeruginosa* (ATCC 27853)
Reference strains of *Staphylococcus aureus* (ATCC 25923)
Standard isolate of *Vibrio cholerae 01*(PSH 5),
Standard isolate of *Klebsiella* (NK 1545).
All the above strains were obtained from the National Institute of Cholera and Enteric Diseases (NICED), Beliaghata, Kolkata.

Methodology:

Standard inoculum, (broth culture) of each of the organisms tested was prepared by matching turbidity with McFarland 0.5 standard. For each of the test organisms, three types of media were taken and labeled properly: (i) TCW agar plate, (ii) MacConkey agar plate and (iii) blood agar plate. In addition a Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plate was used for *Vibrio cholerae.* A loopful of inoculum (standard loop supplied by HIMEDIA, having an internal diameter of 2.2mm and a loop capacity of 0.005ml) from each of the broth cultures of Reference strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), standard isolate of *Vibrio cholerae 01*(PSH 5) and standard isolate of *Klebsiella* (NK 1545) was taken and subcultured on the correspondingly labeled plates of the TCW agar, Mac Conkey agar and Blood agar. In addition a loopful of broth culture of the Standard isolate of *Vibrio cholerae 01*(PSH 5) was also subcultured onto a TCBS agar plate. All the plates were incubated aerobically at 37^0C overnight and examined on the next day for growth. The growth on the TCW agar plate was compared with that on the conventional media for each organism and the number of colonies was counted and the colony forming units /ml were determined using standard loop technique.

Antibiotic Sensitivity testing:

From stock cultures of *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) nutrient agar plates were prepared. From each nutrient agar plate, using a sterile wire 3-5 isolated colonies were touched and emulsified in 3ml of nutrient broth. The broths were incubated aerobically at 37^0C for 1 hour. At the end of 1 hour each broth culture was observed and the turbidity matched with McFarland 0.5 standard. Thus a standard broth suspension of each organism was prepared. Thereafter antibiotic sensitivity testing was done for each of the organisms on Mueller Hinton agar plate and TCW agar plate in parallel by the modified Kirby-Bauer technique[8] using appropriate antimicrobial discs. The following day, zones of inhibition produced in the two types of media for each organism were measured and compared.
The antimicrobial discs used in the technique were supplied by HIMEDIA

Discs for *Escherichia coli* (ATCC 25922) and their respective disc potency

Amikacin	(AK)	30µg	Chloramphenicol	(CH)	30µg
Gentamicin	(GM)	10µg	Ceftrioxone	(RP)	30µg
Norfloxacin	(Nx)	10µg	Ciprofloxacin	(RC)	5µg
Nitrofurantoin	(FD)	300µg			

Discs for *Pseudomonas aeruginosa* (ATCC 27853) and their respective disc potency:

Amikacin	(AK)	30µg	Ceftrioxone	(RP)	30µg
Gentamycin	(GM)	10µg	Cefotaxime	(CF)	30µg
Piperacillin	(PC)	100µg	Cefoperazone- Sulbactum	(CM)	105µg
Ceftazidime	(FG)	30µg			

Results

Table 1
pH and Chemical Composition of Coconut Water

pH	Glucose (mg/dl)	Reducing sugar (mg/dl)	Protein (mg/dl)	Triglyceride (mg/dl)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Phosphate (mg/dl)	Calcium (mg/dl)
4.75	742.9	1846	81	0	5.1	37.97	19.5	15.56	28.13

The pH of TCW is acidic and chemical composition of TCW reveals it has considerable amounts of glucose, reducing sugars, some proteins, no triglycerides and sufficient amounts of minerals

Table 2
Comparative growth of Reference strains of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus, Vibrio cholerae 01*(PSH 5) and *Klebsiella* (NK 1545) in TCW agar, MacConkey agar and blood agar and TCBS agar (for only *Vibrio cholerae*) [n-6]

Colony count	TCW agar		MacConkey agar		Blood agar		p-value
	mean	SD	mean	SD	mean	SD	
Eschericia coli(ATCC 25922)	1428.33	6.17	1432.67	5.89	1436.67	6.15	0.089
Pseudomonas aeruginosa (ATCC 27853)	1306.33	8.41	1310.67	9.93	1320.17	9.58	0.060
Klebsiella (NK 1545)	1239.17	9.17	1241.83	10.71	1256.87	16.69	0.454
Colony forming units/ml X 10^5							
Eschericia coli(ATCC 25922)	**2.857**	0.118	**2.865**	0.124	**2.873**	0.123	0.454
Pseudomonas aeruginosa (ATCC 27853)	**2.613**	0.017	**2.621**	0.02	**2.640**	0.019	0.056
Klebsiella (NK 1545)	**2.478**	0.09	**2.484**	0.09	**2.514**	0.07	0.048

p- value <0.05 was considered to be significant.

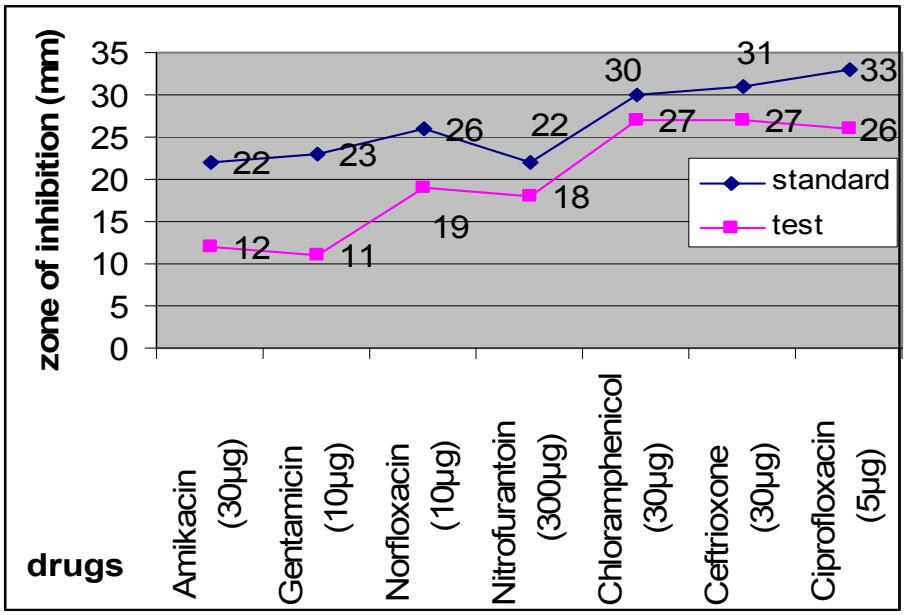
No growth was seen in TCW agar with *Staphylococcus aureus* (ATCC 25923) and *Vibrio cholerae 01* (PSH 5), though growth was observed in both Mac Conkey agar & Blood agar along with TCBS agar (*Vibrio cholerae*). The results of the growth were not shown this table.

The growths of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella* (NK 1545) were less on TCW agar media than that on the blood agar and the MacConkey agar plates but this was not statistically significant. *Staphylococcus aureus* (ATCC 25923) failed to grow on the TCW agar media, but growth was apparent in the blood agar and the Mac Conkey agar plates. Standard strain of *Vibrio cholerae 01*(PSH 5) showed no growth on TCW agar media but revealed growth on blood agar, Mac Conkey agar and TCBS agar plates.

On comparison of the size of the zones of inhibition around the antimicrobial discs on performing the antibiotic sensitivity test by the Modified Kirby Bauer method for the Reference strains of *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), it was seen that the zone sizes around the discs tested in the TCW agar media were less than that in the Meuller Hinton agar

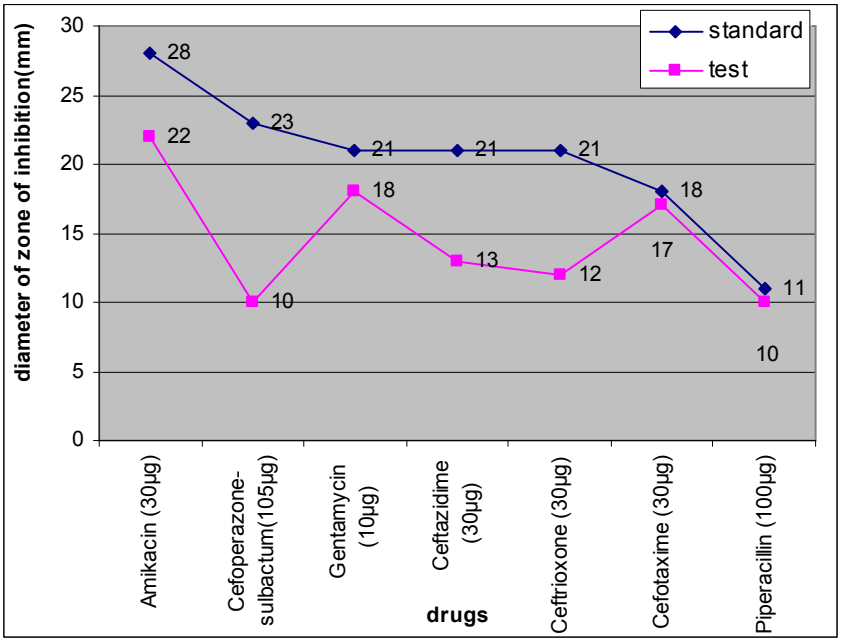
A comparative line diagram was drawn for zones of inhibition by different drugs in two media with two different bacterial growths. **Fig. 1 shows comparison in *Escherichia coli* (ATCC 25922) and the Fig.2 shows that of in *Pseudomonas aeruginosa* (ATCC 27853).** Both the figures reflect a unique characteristic that inhibition by Gentamicin(10µg) is always on the reverse direction compared to the series of values we get in the standard medium. Further in-depth study with more number of samples would help in explaining the issue. From this study its evident that sensitivity by Gentamicin(10µg) is not standardized in this medium.

Figure-1
Line diagram showing zone of inhibition for *Escherichia coli* (ATCC 25922)



Standard: Mueller Hinton agar plate, Test: TCW agar plate

Figure-2:
Line diagram showing zone of inhibition for *Pseudomonas aeruginosa* (ATCC 27853)



Standard: Mueller Hinton agar plate, Test: TCW agar plate

Discussion

Although future trends in clinical microbiology continue to point in the direction of rapid, non-growth dependant methods for detecting the presence of infectious agents, the isolation and the identification of viable pathogens is still the 'gold standard' for diagnosis of infectious diseases.[1] A pure culture of a clone of identical cells has been necessary for performing biochemical differentiation tests and susceptibility studies. In the laboratory, nutrients are incorporated into culture media on or in which bacteria are grown. If the culture medium meets a bacterial cell's growth requirements and if placed in optimal environment, then that cell will multiply to sufficient numbers to allow visualization by the unaided eye. The water of tender coconut, which is technically the liquid endosperm, is a highly nutritious fluid containing a number of nutrients. [2]

Preparation of TCW agar medium results in a solid medium incorporating all the essential nutrients present in TCW.[3] On attempting to prepare a solid medium by mixing agar powder with TCW, it was seen that the 2% and 2.5% TCW agar media prepared were too soft and were being cut through with the inoculating loop, while the 3% TCW agar medium was of the desired hardness. This is perhaps due to the fact that agar is hydrolyzed to products that do not solidify properly on cooling if it is heated at a low pH [9] and the pH of TCW is approximately 5. So, by increasing the concentration of agar a desirable hardness of the solid media was obtained. A limitation was that it was not possible to add any good indicator to TCW agar medium, as TCW and therefore the TCW agar medium, is highly acidic.

Most of the indicators used for conventional identification of organisms are active in the acidic range of the pH and therefore, the detection of organisms grown in TCW agar medium using such indicators is not appropriate.

The growth of Reference strains of *Escherichia coli* (ATCC 25922) and Pseudomonas *aeruginosa* (ATCC 27853) and the standard strain of *Klebsiella* (NK 1545) occurred in the TCW agar media, but was less than that in the conventional media, perhaps because of (i) the limiting factor of the low pH, though some organisms regulate their internal pH over a wide range of external pH[10] (ii) since a higher concentration of agar was used, a greater amount of the inhibitory substances present as impurities in agar were available[9] These factors inhibited the growth of Reference strain of *Staphylococcus aureus* (ATCC 25923) completely. *Vibrio cholerae* failed to grow on TCW agar media because the pH of the media was approximately 5 and *Vibrio cholerae* has a low tolerance to acid. Growth is rapid in the pH range of 7.4-9.6 while the limits for growth are approximate pH 6.8 and 10.2. [11] The organism is rapidly killed by acid.

On comparison of the size of the zones of inhibition around the antimicrobial discs on performing the antibiotic sensitivity test by the Modified Kirby Bauer method for the Reference strains of *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), it was seen that the zone sizes around the discs tested in the TCW agar media were less than that in the Meuller Hinton agar. TCW has an acidic pH of ~5 which decreases the activity of aminogycosides and macrolide antibiotics and a change in the pH interferes with the activity of a number of other antibiotics as well[12]. Moreover, TCW being a natural product, the pH and the composition of the nutrients vary from one tender coconut to another and also with the different stages of maturity of the coconut. Also, TCW contains a number of undefined components, some of which could be inhibitory to the growth of some organisms. Therefore, with such variations and limitations, it is difficult to standardize the antibiotic sensitivity testing using TCW agar medium.

Thus we observed that TCW agar medium supported the growth of some of the organisms tested. Keeping in mind the easy availability of tender coconut in our country and also its low cost this TCW agar medium can prove to be an effective alternative to conventional media for the growth of some common organisms. However this is a preliminary study, and further study needs to be done with other organisms. Also study has to be undertaken with clinical isolates and with clinical samples like pus and urine. We are forced to think if further modifications can be done in this medium (eg. increasing the pH by addition of an alkali) so that this TCW agar medium can become a better medium to support the growth of common organisms and have a wider acceptability.

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