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Evaluation of an in-house developed anaerobic culture medium from lentil

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As lentil contains considerable amount of nutrients and reducing substances like meat, the meat balls used in anaerobic medium like cooked meat broth(CMB) can be replaced by this easily preservable plulse grains. With this aim, an in-house develped medium containing 10% Indian red lentil suspension with methylene blue and its soft agar version adding 0.2% agar were evaluated. A 10^5 bacilli/ml. saline suspension of *Clostridium sporogenes,*ATCC11437, was prepared and 20 µl of the suspensions were simultaneously inoculated into 5 tubes of CMB and 5 each of test liquid media prepared from 5 different lots of lentil. After 48 hrs. incubation at 37^0C, the bacterial growths in different tubes were compared by counting stained smears prepared by spreading 20 µl of broths from each tube on 1 cm^2 area of slide. The bacterial numbers were about 4.5 times more in test media than in CMB. Ten twin swabs taken from the sites of isolation of *Cl. tetani* from OT floors are simultaneously grown in lentil methylene blue soft agar media and CMB. Growths were indicatred in test medium by reduction of dye from bottom within 24 hrs., much earlier than blackening of meat colur in CMB. So the innovative media can be tested for rapid detection of various anaerobes.

Key words : Anaerobiosis, culture medium, lentil, *Clostridium* sp,

INTRODUCTION

For primary isolation of obligate anaerobes in routine clinical bacteriology, prompt inoculation in reduced liquid medium is sometimes more rewarding and cost effective than cabinet or gas-flushing system (Watt *et al.,* 1974). Freshly steamed liquid media are temporarily anaerobic and soon become aerobic unless any of reducing agents like glucose (0.5-1%), ascorbic acid (0.1%), cysteine (0.1%), thioglycollate (0.1%) or particles of cooked meat are used (Collee and Marr, 1989). By incorporating 0.1-0.2% agar to liquid media the effectivenss of reducing agents can be increased further.

Robertson's cooked meat (RCM) broth and Thioglycollate broth (Ganguli *et al,* 1982) are two commonly used liquid media for anaerobic culture. In 1890, Smith first used fresh unheated animal tissue for cultivating anaerobic organisms. In 1916, Robertson used beef heart for preparing a cooked meat broth, named RCM broth. It was found suitable for growing anaerobes in air and also for preservation of stock cultures of aerobic organisms. Indian red lentil (*Lens esculenta;* Indian pulse, masoor) which is sometimes considered as meat equivalent food item, is tested here as substitute of meat balls used in RCM broth, not only for its nutritive value but also for containing many reducing substances (Gopalan *et al.,* 1996).

MATERIALS AND METHODS

Intitially 10 g red lentil was washed to remove any preservative and then 100 ml neutral P^H tap water was added to it. After sterilization by autoclaving, 01 ml of pre-sterilized aqueous solution of methylene blue (1 in 10000) was added and mixed thorougly. The media 10 ml, was aseptically poured into each sterile test tubes and cooled in vertical position. Five sets of media were prepared using five different lots

of lentil. Another set of soft agar medium was prepared by adding 0.2% agar-agar powder to lentile suspension, prior to sterilization. Tubes of RCM broths were prepared from RCM base (Hi-Media, India).

A saline suspension of reference strain *Clostridium sporogenes,* ATCC11437 was adjusted to 10^5 bacilli/ml. Into each tube 20 µl of prepared bacterial suspension was added to five RCM broths and five sets of Lentil-methylene blue suspension. Tubes were incubated at 37°C for 48 hrs. The bacterial growths were evaluated by counting 10 oil immersion fields from each of gram stained smear prepared by spreading 20 µl of culture suspensions from each tube and then uniformly spread on 1 cm^2 area of slide. Also ten twin swabs were taken from the site of isolation of *Clostridium tetani* in OT floors, prior to fumigation. Swabs were simultaneously tested in RCM broths and lentil methylene blue soft (LMS) agar media after dipping into upper 2/3rd depth of media. Tubes were incubated at 37°C and observed up to 2 weeks. In control sets, a sterile swab and a swab dipped in suspension of *Micrococcus* sp. were tested.

RESULTS AND DISCUSSION

The number of gram positive becilli with or without spores noted in 10 oil immersion fields of each smear, are shown in Table 1.Result showed a parity of growths in media prepared from different lots of lentil and nearly 4.5 times higher growth in lentil based media than that of RCM broth. In LMS agar medium bacterial growths were indicated by bleaching of blue colour, which started from the bottom and spread upwards (Fig 1) within 24-48 hrs, while growths in RCM broths were indicated by blackening of meat balls, usually after 7 days. Growths were checked by examination of Gram stained smears. Bacilli were found as strongly Gram positive rods with drum-stick like rounded ends; some spores were unstained but most were stained deeply. Results in test medium totally corroborated with the results observed in conventional medium. Control swabs without bacteria showed no change of colour, while growth of *Micrococci* was indicated by fading of blue colour only on top.

RCM contains freshly minced bullock's heart, cooked in 1(N) NaOH and then dipped in peptone water. Here meat is main source of nutrients for bacterial growth containing about 21.4% protein, 3.6% fat and 1.1% minerals (Park, 2002). Because whole meat also contains certain reducing amino acids having –SH groups e.g. glutathione and cystine, it makes RCM broth suitable for growth of many anaerobes within meat pieces situated at the bottom of broth. To meet up small scale demand this medium is best and cost-effective for most centers of developing countries where costly equipments for anaerobiosis are not available. However, in remotest place of the country, RCM dehydrated base is not readily available in required small or large quantity. In-house preparation of RCM broth is used in such places, but its important ingredient beef heart is not available all the times, and can not be stored for a long time. The process of this media preparation is also cumbersome.

Red lentil (*Lens esculenta;* Indian pulse Masoor) can be better substitute for dehydrated meat balls of RCM base. For high protein content, red lentil is considered as meat equivalent non-vegetarian food for some Hindu widows. So after initial trials

Table 1 : Gram positive bacillus per 10 oil immersion fields from smears of different broth culture

Repitation	RCM broth	Lot 1(LIB)	Lot 2(LIB)	Lot 3(LIB)	Lot 4(LIB)	Lot 5(LIB)
1st	182	973	871	942	881	1015
2nd	206	885	892	983	916	891
3rd	193	1022	881	890	985	975
4th	187	867	1007	977	992	943
5th	215	896	958	994	973	937
Total	983	4643	4609	4786	4747	4761
Average	197	929	922	957	949	952

KIB = lentil infusion broth

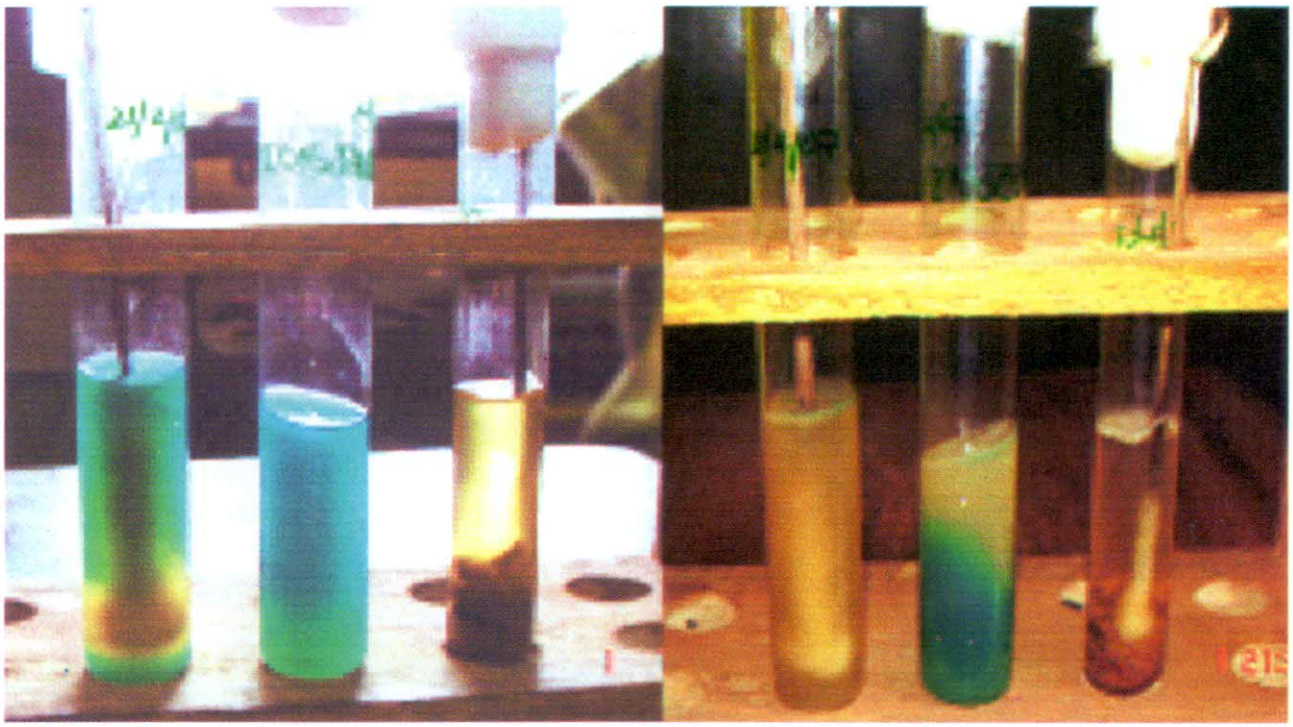

Fig. 1 : **A,** (Lt. to Rt.) Early groth after 18 hrs.: *Cl. tetani* in LIA; negative control *Micrococcus* in LIA; RCM broth with stock culture of Cl. tetani. **B,** Growth after 48 hrs.: *Cl. tetani* in LIA; *Micrococcus sp.*in LIA, *Cl. tetani* in RCM.

with different pulse items lentil is selected for preparing RCM substitute medium. By composition (Gopalan *et al,* 1996) 100 g dry weight of Indian red lentil contains about 25.1g protein, 59g sugar and 0.7 g fat, sufficient vitamins and minerals, Due to high reducing components (Anomymous, 2004) like cystine, methionine, starch, amylose, ascorbic acid and mineral ions, it may be used as meat substitute reducing substance. The presence of superoxide dismutase in lentil (Federico *et al.,* 1985) may also favor bacterial growth in appropriate conditions. Lentil was tested as basis in in-house developed media and RCM broth for growth of anaerobes. Results showed superiority of the test medium by appearance of early growth than that of RCM broth, as well as by higher rate of growth.

Due to growth of anaerobic organisms in the depth of medium, excess methylene blue is reduced by locally diffused hydrogen acceptors as product of their substrate phosphorylation. Growth of aerobic bacteria can also be indicated by dye reduction starting from upper part of medium, for their metabolic products of oxidative phosphorylation. This property of the dye has also been used for testing contamination of milk by Methylene blue test (Senior, 1989). By use of 0.2% agar, not only convection currents were prevented but also spread of motile bacteria and retardation of diffusion of air into media were ensured. Probably for that reason stock LMS agar can also be used for anaerobic culture without prior heating before inoculation.

In many centers routine monitoring of sterility for OT environments is done by inoculating OT swabs in RCM broths to note growth of *Clostridium* sp. It takes longer time to indicate growth of Gram positive anaerobic spore bearers by change of meat colour, which not only delays early interventions but also prolongs post-fumigation OT closure.

So the in-house developed LMS medium is not only cheaper but also can be used for rapid detection of *Clostridium sp.* if not all anaerobes. The medium can

also be tested for maintaining anaerobic stock cultures. Lentil infusion broth may be used for enrichment of anaerobes from clinical materials. Drug sensitivity test by broth dilution method can be carried out using this broth after appropriate standardization. Further studies are to be done with this innovative medium.

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