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Demonstration of lepra bacilli in clinical materials of human leprosy cases with special references to cultural prospect

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Abstract
Background: Apart from clinical examination and laboratory examination to demonstrate Lepra Bacilli from slit skin smear examination of clinically suspected patients with microbiological grading of cases & an attempt is taken to develop an artificial culture media for growth of Lepra Bacilli by inclusion of their requirement present in natural habitat.
Methods: This is a prospective study involving 100 clinically suspected Leprosy patients who were attended OPD in the Department of Dermatology in a Tertiary care Hospital, Kolkata, India from April 2013 to October 2014. Slit skin smear examination and microbiological grading of the cases were done followed by in-vitro culture from multi-bacillary cases were done in Dept. of Microbiology of said hospital. Statistical analysis was done by using pie charts and bar charts.
Results: The study reveals 58% of pauci-bacillary cases and 42% of multi-bacillary cases. Pauci-bacillary cases are more in number in newly diagnosed cases as well as old treated cases. Incidence and prevalence of leprosy are more in males than in females. Pauci-bacillary cases (59%) are more in male and female both than multi-bacillary cases (41%). Problem is more in rural area than urban. In my study, among 80 confirmed leprosy cases 2 pauci-bacillary cases were considered as relapsed cases as both of them were found to be persistently slit skin smear positive.
In both cases no decline in morphological index were detected.
Conclusions: Leprosy is associated with lower socioeconomic status predominantly in rural areas, close contact with an index case especially of lepromatous leprosy, lack of BCG vaccination and male preponderance. So, socioeconomic improvements in nutrition, housing and sanitation are key factors in altering immune susceptibility of leprosy transmission. An attempt was taken regarding in-vitro cultivation and considering growth characteristics in natural habitat, at the end of my work I have isolated a Corynebacterium species (Corynebacterium ureicelerivorans) in my developed cultural media.

Keywords: lepra bacilli, pauci-bacillary, multi-bacillary

Introduction
Leprosy is a chronic, non-fatal, immunological disease and the causative agent Mycobacterium Leprae was the first recognized acid-fast bacterial pathogen of human disease. This disease has the maximum social stigma attached with it due to physical deformity, reduced productivity and social isolation. Today, with early diagnosis and prompt Multidrug Therapy deformities and other visible manifestations can largely be prevented. In India, the National Leprosy Eradication Program was launched in 1983. Later WHO in 1991 adopted a resolution, calling for elimination of leprosy as a public health problem by the year 2000AD reducing prevalence less than 1 case per 10,000 population. In this study microbiological grading of cases done to assess the present scenario of NLEP in a Tertiary Care Hospital.
Till date, M. Leprae has not satisfied Koch's postulate as it has not so far been possible to cultivate it in-vitro culture media. It still remains one of the least understood human Pathogen. There have been several reports of cultivation, but None has been confirmed. Thus as one of many trials, an attempt has been taken in this study to develop an artificial culture medium for in-vitro isolation of Lepra Bacilli from clinical materials.

Methods
This is a prospective study involving 100 clinically suspected Leprosy patients who were attended OPD in the Department of Venerology in a Tertiary care hospital, Kolkata, India from April 2013 to October 2014. Slit skin smear examination and microbiological grading of the cases were done followed by in-vitro culture from multi-bacillary cases were done in Dept. of Microbiology. Informed consent and approval of institutional Ethical committee was taken for the study. Purposive random technique was used to select 100 patients with history of clinically suspected Leprosy who were attended OPD in the Department of Dermatology in a Tertiary care hospital at Kolkata, India. Continuous 100 microbiologically proved BL and LL cases were referred from Dermatology OPD to Microbiology Department for follow up study also.
Inclusion criteria has no sex discrimination. Clinically diagnosed cases of leprosy mainly BL and LL as per WHO criteria which include (1) >5 skin lesions, (2) many nerve trunks involvement (3) slit skin smear positive according to Ridley-Jopling classification. Both newly diagnosed and treated multi-bacillary cases were included. Clinically diagnosed cases of tuberculoid leprosy with slit skin smear negative subjects were excluded from the study.
Study design was to determination of incidence pattern, of microbiologically proved leprosy cases including attempt for in-vitro culture of Lepra bacilli experimentally. So Demonstration of Lepra bacilli was done from selected cases by slit skin smear examination and culture attempt was taken from leproma tissue of selected cases using

microscope and Ziehl-Neelsen Staining.

Media prepared for this study

(A) Bi-layered Lowenstein-Jensen Media-lower layer was made up of charcoal phosphate buffer (pH 6.5-6.8), solidified with agar. This layer was used to neutralize toxic metabolites produced by the organism during growth. Upper layer was made up of conventional Egg based Lowenstein-Jensen medium.

Whole eggs were used to provide phospholipid to the desired organism. Malachite green was used to prevent growth of contaminant organisms. McCartney bottles were used to pour the media, as to preserve for long duration. Specimens were taken by slit skin and scrape method from multi-bacillary leprosy cases and inoculated on upper layer of media. Few drops of coconut milk was added over the inoculum. Inoculated media were incubated at 33^0C. Following reagents were used –

a] Lower layer was made up of -

Phosphate buffer: (Cruickshank)

Stock Solutions

A] 0.2 M solution of 31.2g NaH_2PO_4, $2H_2O$ in 1000 ml.

B] 0.2 M solution of 28.39g Na_2HPO_4 in 1000 ml.

At p^H 6.8, 51 ml of A + 49 ml of B, diluted to a total of 200 ml. In this solution, charcoal powder was added at 1% concentration and agar used for solidification at a concentration of about 2 per cent. Whole solution was sterilized by autoclaving. The medium then distributed in 2.5 ml amount in sterile McCartney bottles and screwed the caps tightly on. To form slant bottles were left in horizontally and to form butt bottles were left vertically.

Upper layer was made up of Lowenstein-Jensen Medium:

Mineral salt solution

Potassium Di-hydrogen phosphate, KH_2PO_4 ----------2.4 g

Magnesium sulphate, $MgSO_4$----------0.24 g

Magnesium citrate, $Mg_3(C_6H_5O_7)_2.14H_2O$----------0.6 g

Asparagine----------3.6 g

Glycerol----------12ml

Water----------600ml

The ingredients were dissolved by heating, then autoclaved at 121^0C for 25 min to sterilize.

Malachite green solution: 2 per cent solution of malachite green was prepared in sterile water with sterile technique by dissolving the dye placing in incubator for 1-2 hr.

Preparation of complete Lowenstein-Jensen Medium:

Mineral salt solution----------600 ml

Malachite green solution----------20 ml

Hen's eggs----------20

The eggs used must be fresh (not more than four days old). The eggs were washed thoroughly with a brush and plain alkaline soap in running water for 30 mins. After draining off the water eggs were allowed to dry until the following day. All utensils used to prepare the complete medium were sterilized by autoclaving. The dry eggs were cracked with a sterile knife into a sterile beaker and then beaten them. After mixing the complete medium it was distributed in 2.5 ml amount in sterile McCartney bottles and tightly screw caped on. The bottles were placed horizontally, vertically in the inspissator and heated at $75\text{-}80^0$C for 1 hr. for 3 consecutive days.

Coconut milk preparation: Coconut milk was prepared by dissolving the coconut milk powder which is available in market.

Ingredients: coconut milk solids, maltodextrin, milk protein (sodium caseinate) and stabilizer.

Method of preparation: 3 tbsf (1 tbsf=6g), 6x3=18g of milk powder was added to 150ml of water. After reconstitution coconut milk solution was sterilized by autoclaving.

This media was prepared considering the intracellular growth requirements for Lepra bacilli.

A] p^H of 6.5-6.8 was chosen as mycobacteria inhibit phagosome maturation and prevent acidification of phagosome. Mycobacterium vacuoles acquire the lysosomal membrane protein (LAMP-1) but not vesicular proton adenosine triphosphate (ATPase) which is responsible for phagosomal acidification. These processes help in maintaining a static intracellular growing condition within a p^H range of 6.5-6.8.Mycobacterium also inhibits phago-lysosome fusion. A previous study done with M. Bovis BCG showing that, a host protein CORO 1A also known as TACO (Tryptophan aspartate containing coat protein) accumulates on the phagosomal membrane thereby inhibits phago-lysosome fusion.

B] Phospholipid and lipid were supplemented within the media as - In lepromatous leprosy lesion, the foamy changes are due to accumulation of host derived lipids such as oxidized phospholipids and cholesterol esters. These lipids are stored in non-membrane bound cytoplasmic organelles – known as lipid bodies or lipid droplets. Lipid droplets are lipid storage organelles and constitute lipid sources for many biological processes such as synthesis and secretion of inflammatory mediators.

In BCG and M. leprae lipid droplets constitute site for eicosanoid synthesis, leads to increased production of PGE_2 by infected macrophages. Which decrease Th 1 response and bactericidal activity to wards intracellular organisms. This ultimately helps in intracellular survival of Mycobacterium.

Incubation temperature was at 33^0C. As Lepra bacilli prefer to settle at cooler parts of body. Lepra bacilli was successfully cultured in-vivo within nine-banded armadillo within a temperature range of 28 – 33^0C.

[B] Modified Davis and Mingioli minimal medium: following reagents were used –

Glucose, sterile 10% solution--------- ----------20 ml.

Dipotassium hydrogen phosphate, K_2HPO_4---------- 7 gm.

Potassium dihydrogen phosphate, KH_2PO_4 ----------3 gm.

Sodium citrate, $Na_3C_6H_5O_7.2H_2O$----------0.5 gm.

Magnesium sulphate, $MgSO_4.7H_2O$----------0.1 gm.

Ammonium sulphate, $(NH_4)_2SO_4$----------1 gm.

Agar ----------2%

Activated charcoal ----------1%

Distilled water----------1L.

Egg yolk of hen's egg----------20 pc.

Malachite green solution----------20 ml.

Coconut milk.

Trace element solution

Ferrous sulphate, $FeSO_4.7H_2O$----------0.5 gm.

Zinc sulphate, $ZnSO_4.7H_2O$----------0.5 gm.

Manganese sulphate, $MnSO_4.3H_2O$----------0.5 gm.

Sulphuric acid, H_2SO_4, 0.1 N----------10

ml.
Distilled water----------1L.

Trace element solution 5 ml. and Sodium chloride 0.9% solution were added per liter of medium. Glucose and malachite green solution was added as a sterile solution after the reminder of the medium was autoclaved. thus a p^H range of 6.5-6.8 was maintained. Medium poured in McCartney bottles in the form slant. Test samples were taken by slit skin and scrape method then inoculated on slant of the medium. Few drops of coconut milk was overlaid on inoculums.
Medium incubated at 33^0C and examined at periodic interval.
[C] Lowenstein-Jensen Medium with coconut milk overlaid on inoculums.
Inoculation and incubation was done in the same process as described previously.

Materials used in this for overlaying

a. Coconut milk
b. Restoraderm

Skin restoring, body moisturizer used for very dry itchy skin.
Composition–aqua, glycerin, caprylic/capric triglyceride, Helianthus annuus seed oil, pentylene glycol, Butyrospermum parkii butter, cyclopentasiloxane, cetearyl alcohol, sorbitol, biphenyl alcohol, glyceryl stearate, allantoin, arginine, caprylyl glycol, ceteareth-20, cetyl alcohol, citric acid, dimethiconol, disodium EDTA, disodium ethylene dicocamide, PGE-15 disulfate, glycerol stearate citrate, hydroxypalmitoyl sphingosine, niacin amide, panthenol, sodium hyaluronate, sodium PCA, sodium polyacrylate, tocopheryl acetate.

c. Heat killed atypical Mycobacteria: Suspension of atypical mycobacterium was killed by autoclaving, then the filtrate used as overlay.

Results

Total numbers of bacteriologically confirmed leprosy cases in this study were eighty (80).

Table 1: Distribution of Bacteriologic classification of Leprosy cases.

Cases of Leprosy	Numbers	Percentage %
Multi-bacillary	34	42%
Pauci-bacillary	46	58%
Total	80	100%

Table 2: Distribution of leprosy cases among newly diagnosed and treated groups.

Leprosy cases	Newly diagnosed	Treated cases	Total
Multi-bacillary	23	11	34
Pauci-bacillary	28	18	46
Total	51	29	80

Distribution of bacteriologic classification of Leprosy cases have been shown in table 1. More numbers of Pauci-bacillary (58%) cases were found than Multi-bacillary (42%) cases. Pauci-bacillary cases are more in both newly diagnosed cases as well as treated cases.

Table 3: Sex distribution of Leprosy cases.

Leprosy cases	Male	Female	Total
Multi-bacillary	29	05	34
Pauci-bacillary	32	14	56
Total	61	19	80

Table 4: Geographic distribution of Leprosy cases

Leprosy cases	Rural areas	Urban areas	Total
Multi-bacillary	28	6	43

Male subjects are affected more than female and pauci-bacillary cases are seen more in both sex as shown in Table No.3. Maximum cases are from rural areas (68). Though 12 cases are seen from urban areas (as in Table 4).
In-vitro culture in artificially prepared media shows the growth of a Corynebacterium species. Further Gene sequencing was done. The strain is of Corynebacterium ureicelerivorans.

Phylogenetic Tree

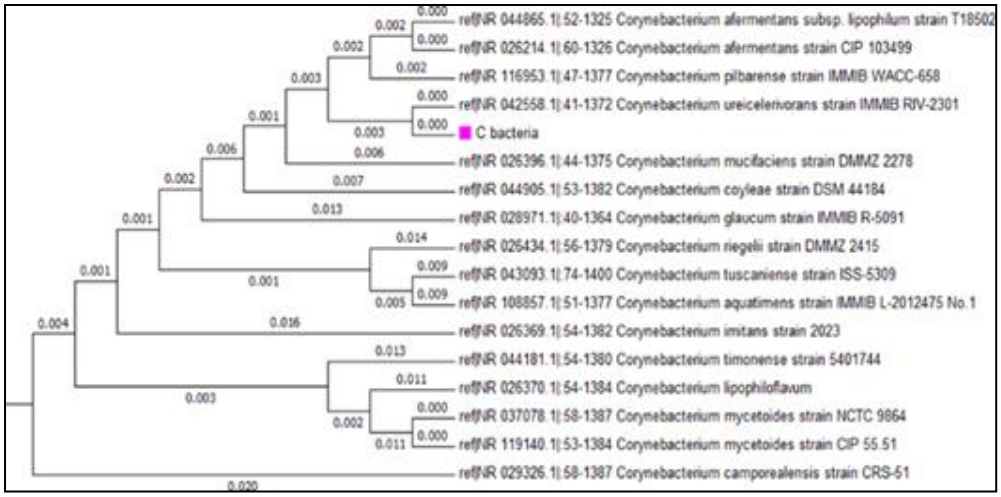

Fig 1: Evolutionary relationships of 16 taxa

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.16701561 is shown. (Next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and

missing data were eliminated. There were a total of 1236 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Conclusion: The strain is of Corynebacterium ureicelerivorans.

Conclusions

The National Leprosy Eradication Programme was based on early detection of cases, short term multi-drug therapy, and health-education and rehabilitation activities recommended by WHO. NLEP provided free domiciliary in endemic districts through specially trained staff and in moderate to low endemic districts through mobile leprosy treatment units and primary health care personnel. As a Result the number of cured cases increased progressively over years.

India has achieved the "leprosy elimination target by 2005". But some states are still having higher prevalence rate. These states are Bihar, Jharkhand, Orissa, Chhattisgarh, Uttar Pradesh and West Bengal. In India, leprosy scene is passing through an important phase of transmission-from a high burden to a low burden country. Though, new leprosy cases are still being reported from different tertiary care hospital.

The main portal of exit and entry for transmission of Lepra bacilli is the nose, hence spread being via nasal secretions and droplets. Leprosy is associated with lower socioeconomic status, close contact with an index case especially of lepromatous leprosy, lack of BCG vaccination and male preponderance. So, socioeconomic improvements in nutrition, housing and sanitation are key factors in altering immune susceptibility of leprosy transmission. Developing appropriate health promotion health promotion strategies, to increase self-reporting at the earliest stage for diagnosis and treatment of leprosy.

The elimination strategy cannot depend on the health service alone; require involvement of other sectors like participation of local community leaders in improving public awareness, availability of treatment and reducing stigma of the disease.

Maintaining research activities in leprosy are needed to identify optimal methods for vaccine development, early diagnosis and treatment of leprosy. As one drawback has remained from the time of discovery of the organism to the times of the present-day leprologists i.e. the organism has not yet been grown in artificial culture media.

However, many workers attempted in the past and there have been several reports of cultivation in-vitro, but none has been confirmed. Though, Lepra bacilli have been grown successfully in wild armadillo, mouse foot pad. After studying the previous worker's reports regarding in-vitro cultivation and considering growth characteristics in natural habitat, at the end of my work I have isolated a Corynebacterium species in my developed cultural media. This organism has very slow growing character and close similarities with mycobacterium. Like many other previous workers, my isolated organism's growth character also mimics an illusive in-vitro growth of Mycobacterium leprae. But on Genomic sequencing, it reveals strain is of Corynebacterium ureicelerivorans.

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