

ORIGINAL PAPER

Preparation, standardization and *in vitro* safety testing of *Mycobacterium* nosodes (Emtact- polyvalent nosode)



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Background: Most of the nosodes in the homeopathic pharmacopeia have been sourced from obscure pathological material over a century ago; of which no scientific documentation is available.

Method: A method for preparation and standardization of univalent and polyvalent *Mycobacterium* nosodes (labeled as Emtact), using different strains of *Mycobacterium tuberculosis* was developed. The committee comprising microbiologists, scientist, pharmacist, homeopaths and clinicians had reviewed and approved the method of preparation of nosode. Preparation of the nosode was based on the reference in the Homeopathy Pharmacopoeia of India (HPI), group N-IV. Strains of *M. tuberculosis* viz. Standard strain H37Rv, multi-drug resistant (MDR) *M. tuberculosis*, *Mycobacterium bovis* (BCG vaccine) and *Mycobacterium avium* were identified, procured and documented. Twenty billion viable cells for each strain were taken for Original Stock Nosode (OSN). The original stock was prepared by suspending the microbial cells into water for injection (WFI) (1 ml). As per the Indian Pharmacopoeia (IP) monograph, sterility testing was done for different potencies. Polymerase Chain Reaction (PCR) was performed for 30c potency for detection of any DNA material of the source organisms.

Result: A polyvalent (multi-strain) and univalent *M. tuberculosis* nosodes were prepared for research and clinical use. No growth of *Mycobacterium* was observed in any of the samples above 5c potency. The *in-vitro* testing for nosode (30c) was found to be free from any organism and DNA material.

Conclusion: *Mycobacterium* nosodes sourced from individual strain and polyvalent Emtact nosode *in vitro* testing results found to be satisfactory for its handling and utilization. The nosode seems to be safe and may be tested further *in vivo* to explore its therapeutic application. *Homeopathy* (2016) 105, 225–232.

Keywords: Nosode; *Mycobacterium tuberculosis*; Emtact; Standardization; Polyvalent; Potentization; Safety; PCR

Introduction

Homeopathy is an alternative system of medicine introduced by Samuel Hahnemann, MD, in 1794, in Germany. The homeopathic medicines prepared by using the

process of serial dilution and rigorous shaking, called potentization, have been found to contain nano-particles¹ in a recent study. Nosodes are broad-spectrum, widely used homeopathic preparations sourced from biological materials such as cultures or clinical samples of microorganisms (e.g. bacteria, fungi and viruses) or from parasites, diseased tissues (cancerous tissues), or decomposition products from humans or animals.² This category of drugs has been used in homeopathic profession since 1830 for the treatment of acute as well as chronic diseases.³ Results from Homeopathy Pathogenetic Trials,

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clinical trials and data collection in homeopathic practice show a long and safe track record with the use of nosodes. The manufacturing methods essentially meet pharmacopoeia requirements and must guarantee biological safety. Therefore, patients, practitioners and professional organizations insist on the preservation of nosodes for homeopathic treatment for acute as well as chronic diseases.

As per the European Central Council of Homeopaths (ECCCH) survey, the five most frequently prescribed nosodes⁴ were found to be Tuberculinum (22.6%), Carcininum (20.5%), Medorrhinum (15.0%), Psorinum (14.9%) and Syphilinum (10.9%). The survey was based on rating the importance of nosodes in homeopathy practice by various practitioners. It was found that for 38.2% of patients in the age group of 0–4 years and 5–11 years, nosodes had been indispensable to the improvement of chronic complaints while an average of 41.4% of cases were recorded for the age group of 12 years and above. In the treatment of acute problems, the average percentage of cases where nosodes were considered indispensable was 17%.⁵

Practically all of the nosodes have been sourced from obscure pathological material over a century ago; of which very few literature references^{6,7} are available. The nosodes available presently are largely sourced from the previously potentized (called as back-potencies) preparations which have been passed on from very old pharmacies; having no documented information about the original source materials, thus, presenting many uncertainties. The ill-defined nature of the source, non-reproducibility and limited antigenicity are some of the major drawbacks providing limited immune protection. Also, the organisms have evolved over the time, making it imperative for development of fresher preparations from the recent strains.

With the advancement in microbiology, histopathology, immunology and medical science; and with the newer strain of organisms available, it necessitates re-visiting and re-developing of nosodes with scientific validation and standardization. Newer preparation will also allow further research using the current *in-vitro* and *in-vivo* studies involving safety, infectivity and possibly mechanism of action.

There are approximately forty five major nosodes⁸ quoted in the literature and about twenty have been found clinically effective in the treatment of infectious and non-infectious diseases. Limited research work on nosodes such as *in-vitro* testing, animal studies, cell line studies, clinical research, veterinary research have been done.⁹

Nosodes sourced from Leptospirosis and dengue have been used in past for prophylactic treatment which have shown noteworthy efficacy.^{10–12}

Hepatitis C nosode¹³ and HIV¹⁴ nosode have been introduced using a standardized method. Many such newer preparations will also encourage further research using the latest methods including but not limited to clinical trials, *in-vitro*, *in-vivo* studies and protein profiling.

Aims and objectives

Preparation and standardization of polyvalent *Mycobacterium* nosode (Emtact) using different *Mycobacterium* strains. *In-vitro* evaluation of safety of Emtact nosode for further research and therapeutic application.

Material and method

Nosode preparation has been based on the guidance in the Homeopathy Pharmacopoeia of India (HPI), Volume IV¹⁵ group N-IV (using microorganism as source material). The standard operating procedure (SOP) was developed for preparation, standardization and evaluation of safety of the nosode. The project review committee comprising microbiologists, scientists, pharmacist, homeopaths and clinicians had reviewed and approved the method of preparation of the nosode. The procedure was executed and implemented under the supervision of investigator and authorized personnel at Haffkine Institute¹⁶ and Life Force.

Steps

Source material: Different *Mycobacterium* strains as mentioned below were employed for the study (Refer Table 1):

- Mycobacterium tuberculosis* Standard strain H37Rv (culture)
- Multi-drug resistant (MDR) *M. tuberculosis* (culture)
- Mycobacterium bovis* (BCG vaccine)
- Mycobacterium avium*

Preparation of stock, dilution and potentization

As per the general method of preparation of nosode stocks, 20 billion viable cells¹⁴ for each strain were selected. In Emtact (*M. tuberculosis* polyvalent nosode), total 20 billion cells (5 billion for each strain) were taken for Original Stock Nosode (OSN). The original stock was prepared by suspending the microbial cells into water for injection (WFI) (1 ml). As per the HPI requirement, 20 billion counts of *Mycobacterium* strains were achieved and used for the preparation of the nosode. Potencies 1c, 2c and further up to 30c were prepared by using individual strains (univalent) as well as combined strains.

Potentization

For preparation of 1c potency, one part (0.03 ml) of OSN was mixed with 99 (2.97 ml) parts of WFI (vehicle), and ten strokes given with potentization machine. The impact parameters of the potentization machine were documented (Torque = 404.3 Nm).² Similarly, potencies up to 6c were prepared using WFI; and stored. For preparation of 6c and above potencies, dispensing alcohol was used in place of WFI. Conventionally, the homeopathic potencies are prepared and stored in dispensing alcohol for long term use.

Potencies up to 6c were stored at Haffkine Institute, at temperature between 4°C and 6°C and not allowing freezing. The bio-safety measures were followed during

Table 1 Characteristics of different strains

Characteristic	Strain I	Strain II	Strain III	Strain IV
Category Strain	Wild type bacilli <i>Mycobacterium tuberculosis</i> standard strain H37Rv	Attenuated bacilli <i>Mycobacterium bovis</i>	Wild type bacilli <i>Mycobacterium avium</i> IMTECH 1723 (MAV)	Wild type bacilli <i>Mycobacterium tuberculosis</i> drug resistant strain confirmed by drug susceptible test (DST) Haffkine Institute, Mumbai
Procured from	Foundation of Medical Research, Worli, Mumbai	TUBERVAC BCG vaccine which is manufactured by Serum Institute of India Ltd.	Institute of Microbial Technology, Chandigarh	
Morphology Culture characteristics	Rod shaped organisms Colonies are characteristically rough and appear to be formed serpentine growth that piles up in the center. They are non-chromogenic (cream to buff color), grow at 35–37°C, discrete or confluent, slightly raised, grayish yellow growth, with finely granule surface.	Rod shaped organisms Colonies are characteristically smooth and tiny. They grow at 35–37°C.	Rod shaped organisms Rod shaped organisms, usually have predominantly smooth spreading, serrated edge, transparent, thin and domed colony forms on primary isolation. Occasionally rough strains are seen. More than one colony form may be found within the same culture. Colonies sometimes shows varying shades of pale pigment as they become older.	Rod shaped organisms Colonies are characteristically rough and appear to be formed serpentine growth that piles up in the center. They are non-chromogenic (cream to buff color), growth a 35–37°C, discrete or confluent, slightly raised and grayish yellow growth, with finely granule surface.
Shape	Serpentine	Irregular	Irregular	Serpentine
Color	Buff	Buff	White	Buff
Margin	Serrated	Serrated	Serrated	Serrated
Consistency	Dry	Dry	Dry	Dry
Elevation	Slightly raised	Raised	Slightly raised	Slightly raised
Surface	Rough	Smooth	Smooth	Rough
Biochemical	No acid produced in sugar media, gives positive reactions in the niacin neutral red and nitrate reduction test. But a negative reaction in the Tween.	This species, the least reactive in the tests, gives a positive result only in the room temperature and the semi-quantitative catalase tests. <i>M. bovis</i> is usually niacin negative but an occasional strain can be weekly niacin positive. The pyrazin-amidase test and sensitivity to thiphen-2-carboxylic acid hydrazide (TCH) and aid in separating it from <i>M. tuberculosis</i> . Confirmation by rabbit inoculation is rarely required.	They are niacin negative; nitrate negative, Tween hydrolysis negative at 10 days, urease negative and pyrazina-amidase positive.	No acid produced in sugar media, gives positive reactions in the niacin neutral red and nitrate reduction test, but a negative reaction in the Tween 80 hydrolysis and aryl sulphatase tests. Forms abundant catalase and peroxidase. Produces amidase for urea, nicotinamide and pyrazinamide.
Sugar acid	–	–	–	–
Niacin	+	–	–	+
Neutral red	+	–	–	+
Nitrate reduction	+	–	–	+
Tween hydrolysis	–	–	–	–



the process. The transfer of the material for preparation of subsequent potency was done in controlled, aseptic environment (in chamber with burners and UV/laminar flow) by single operator. The potencies up to 6c were prepared in Haffkine Institute and transfer of material from one potency to another was done in bio-safety cabinet. Post prep-

aration, all bio-waste materials were disposed by the bio-waste management agency.

Etmact 1c, 2c, 3c, 4c, 6c, 8c, 10c, 12c, 15c, 20c and 30c potencies were tested for microbial growth on Lowenstein–Jensen medium. Sterility testing as per IP monograph was done. Etmact 6c and 30c were evaluated for



Photo 1 The preparations were tested for growth of individual strain for 21 days along with positive and negative controls.

safety and sterility testing.¹⁷ Detection of DNA material by Polymerase Chain Reaction (PCR) was performed for 6c and 30c. Medicines thus prepared were labeled and stored appropriately.

Results

Polyvalent *M. tuberculosis* nosode (Emtact) sourced from *M. tuberculosis*, MDR strain, *Mycobacterium bovis* and *M. avium*; as well as univalent nosodes from individual strains have been prepared and standardized as per applicable regulatory, ethical and bio-safety guidance. Pure cultures of each strain were obtained from authentic sources (Haffkine Institute, Mumbai; FMR, Mumbai; IMTECH, Chandigarh), each strain was subjected to sub-culturing on to Lowenstein–Jensen medium (LJ medium) at Haffkine Institute to obtain required count of cells (Refer Photo 1).

Results of testing of nosodes on LJ medium

The samples (nosode preparations of MTB, MBV, MAV, MMDR and Emtact) were cultured on to the LJ medium to

check for the growth of Mycobacterial cells. The slants were observed on days 3, 7, 10, 14, 21 and 28. The study was terminated on the 28th day after a final observation (Table 2). The standard strains (Original Stock Solutions) were used as control.

Mycobacterial colonies were observed from 10th day onwards in 1c of MTB and Emtact and from 14th day onwards in 1c of MBV, MAV, MMDR strains. The 2c potency of MTB exhibited colonies on 10th day. All the samples (MTB, MBV, MAV, MMDR and Emtact) of 2c potency, 3c potency and 4c potency registered growth on 14th day, 21st day and 28th day, respectively (Refer Photos 2 and 3). No growth was observed in any of the samples above 5c potency until the final observation taken on the 6th week of incubation (Refer Photo 4). No growth was observed in Emtact 6c and 30c potency prepared in WFI, tested on LJ medium (Refer Photo 5).

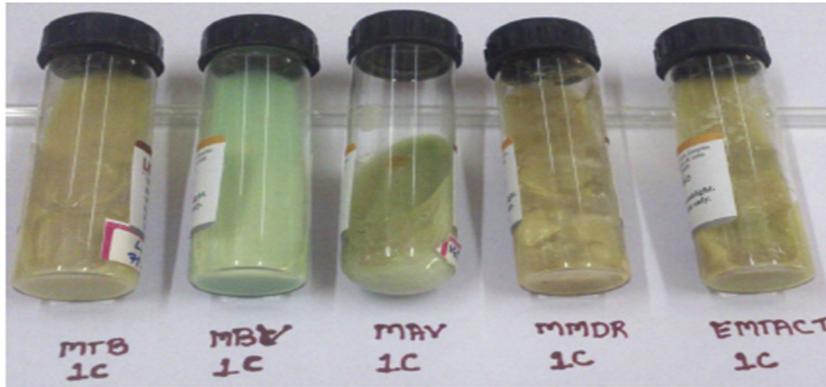
Molecular detection for *Mycobacterium* DNA by PCR

The 30c potency made by using WFI and alcohol medium as vehicle control of all the samples (nosode

Table 2 Observation of colonies on LJ medium

Nosode	Potency														
	1c	2c	3c	4c	5c	6c	7c	8c	9c	10c	15c	20c	25c	30c	
MTB	G (10 days)	G (10 days)	G (21 days)	G (28 days)	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	
MBV	G (14 days)	G (14 days)	G (21 days)	G (28 days)	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	
MAV	G (14 days)	G (14 days)	G (21 days)	G (28 days)	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	
MMDR	G (14 days)	G (14 days)	G (21 days)	G (28 days)	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	
Emtact	G (10 days)	G (14 days)	G (21 days)	G (28 days)	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	

Key: Nosode: MTB = *Mycobacterium tuberculosis*, MBV = *Mycobacterium bovis*, MAV = *Mycobacterium avium*, MMDR = multi-drug resistant *Mycobacterium tuberculosis*, Emtact = MTB + MBV + MAV + MMDR. **G:** growth. **NG:** no growth.



1c Potencies of Nosode showing growth on L.J medium at 14th day of incubation

Photo 2



3c Potencies of Nosode showing growth on L.J medium at 21st day of incubation

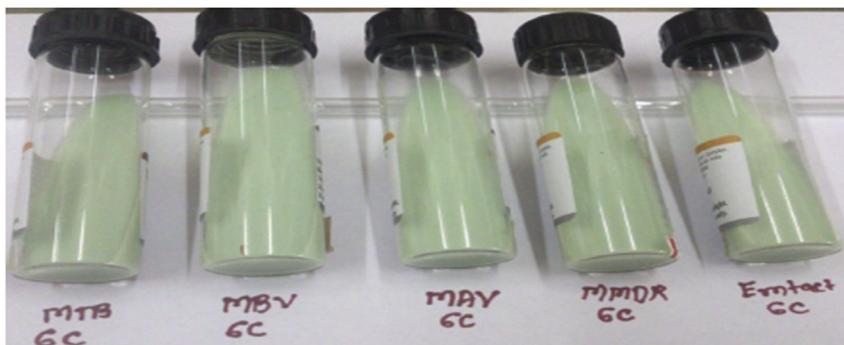
Photo 3

preparations of MTB, MBV, MAV, MMDR and Emtact- which is mix of MTB, MBV, MAV, MMDR) was used for molecular assessment of presence of Mycobacterial DNA. DNA from the samples of the nosode was isolated using commercially available kit. This was followed by conventional Nested PCR of IS6110 gene. The band for outer primer amplicon was expected as 553 bp and that for the inner amplicon was expected at 285 bp. As shown in [Figures 1 and 2](#), no bands were observed in any of the

samples (nosode preparations of MTB, MBV, MAV, MMDR and Emtact). DNA of H37Rv standard strain of tuberculosis was used as positive control (PC). The PC exhibited their respective bands as expected.

Sterility testing

The sterility of Emtact nosode was performed by direct inoculation method as per Indian Pharmacopoeia (IP 2010).



6c Potencies of Nosode showing No Growth L.J medium at 30th day of incubation

Photo 4



6c and 30c potency in WFI showing no growth on L.J slants

Photo 5



Figure 1 Detection of Tuberculosis IS6110 sequence (556 bp Outer Product) from the DNA extracted by using nosode preparations of 30c potency prepared in water for injection (WFI) and sac lac (alcohol medium) by PCR. Key: Lanes L: Ladder, NC: Negative control, PC: Positive control (H37Rv standard culture DNA), Lane MTB: *Mycobacterium tuberculosis*, Lane MBV: *Mycobacterium bovis*, Lane MAV: *Mycobacterium avium*, Lane MMDR: multi-drug Resistant *Mycobacterium tuberculosis*, Lane Emtact; (MTB + MBV + MAV + MMDR).



Figure 2 Detection of Tuberculosis IS6110 sequence (285 bp Inner Product) by PCR, from the DNA extracted by using nosode in 30c potency prepared in water for injection (WFI) and alcohol as medium. Key: Lanes L: Ladder, NC: Negative control, PC: Positive control (H37Rv standard culture DNA), Lane MTB: *Mycobacterium tuberculosis*, Lane MBV: *Mycobacterium bovis*, Lane MAV: *Mycobacterium avium*, Lane MMDR: multi-drug Resistant *Mycobacterium tuberculosis*, Lane Emtact; (MTB + MBV + MAV + MMDR).

Table 3 Observations of sterility testing

Sr. No.	Test	Set I		Set II	
		Medium 1	Medium 2	Medium 1	Medium 2
1.	Positive aerobic bacterial control: <i>Staphylococcus aureus</i> ATCC 25923.	+	+	+	+
2.	Positive anaerobic bacterial control: <i>Bacteroides vulgatus</i> ATCC 8482.	+	+	+	+
3.	Positive fungal control: <i>Candida albicans</i> ATCC 10231.	+	+	+	+
4.	Test 1: 6c Emtact nosode.	–	–	–	–
5.	Test 2: 30c Emtact nosode.	–	–	–	–
6.	Inhibition control 1: <i>Staphylococcus aureus</i> + 6c Emtact nosode.	+	+	+	+
7.	Inhibition control 2: <i>Staphylococcus aureus</i> + 30c Emtact nosode.	–	–	–	–
8.	Inhibition control 3: <i>Bacteroides vulgatus</i> + 6c Emtact nosode.	+	+	+	+
9.	Inhibition control 4: <i>Bacteroides vulgatus</i> + 30c Emtact nosode.	–	–	–	–
10.	Inhibition control 5: <i>Candida albicans</i> + 6c Emtact nosode.	+	+	+	+
11.	Inhibition control 6: <i>Candida albicans</i> + 30c Emtact nosode.	–	–	–	–
12.	Medium control	–	–	–	–

+: Growth observed; –: No growth observed; Medium 1: Soya-bean casein digest medium; Medium 2: Fluid thioglycollate medium.

Soya-bean casein digest medium and fluid thioglycollate medium were used for the same.

Observations and results: Refer Table 3.

Interpretation

No microbial growth was observed in any of the test samples. Emtact nosode of potency 6c and 30c was found to be sterile. Emtact nosode of potency 6c and 30c was found to be sterile and devoid of any bacterial or fungal contamination.

Discussion

Since Tuberculinum, a well-known old homeopathic nosode in practice since 1879,⁷ sourced then from suspected tuberculosis infected material (mucus of a patient suspected to have tuberculosis by Swan in 1879), or from the lung tissues of a patient suspected to have suffered from tuberculosis, or from Tubercular glands from slaughtered cattle² are no more re-producible as their method of preparation was not standardized. The investigators recognized the necessity to standardize the method and prepare a polyvalent nosode using the recent strains of *Mycobacterium* organisms, which are different than those un-characterized organisms possibly used in the old Tuberculinum and Bacillinum nosodes.

Handling of the source material containing viable *M. tuberculosis* organisms and assuring the safety of the final nosode, were some of the challenges from regulatory and safety point of view; which were resolved by suitable bio-safety preparatory measures and by confirming the absence of any infective material beyond 6c potency of Emtact nosode.

This nosode standardization method is likely to encourage re-making of all other old nosodes as well as the introduction of new nosodes.

The concept of polyvalent nosode sourced from multiple strains is relatively new in homeopathy, simply because of the fact that organism-based nosodes have hardly been introduced to the profession in the last fifty years. The investigator has introduced Hepatitis C nosode¹³ and HIV nosode¹⁴ sourced from multiple strains.²

Further studies such as homeopathic pathogenetic trial (drug proving), animal studies, human trial and *in-vitro* studies could be carried out using this nosode.

Safety of organism-based nosodes, which are, administered orally need safety check in spite of its ultra-diluted nature. Homeopathic nosodes available in the market for oral use do not undergo any safety check, due to its ultra-diluted nature; safety testing is not prescribed in the regulation. However, the investigators suggest it to be a part of the protocol to have safety testing in place for the nosodes. Sterility and infectivity tests were carried out for various potencies.

Conclusion

M. tuberculosis polyvalent nosode (Emtact) sourced and prepared from individual strains underwent *in-vitro* testing, of which the results were found to be satisfactory for handling and utilization. The nosode seems to be safe and may be tested further *in-vivo* to explore its therapeutic application.

Conflict of interest

Homeopathy India Pvt Ltd sponsored this project. The sponsor has played no role in interpretation of results.

Patent related

Patents are pending with Rajesh Shah and he solely holds all the rights.

Author contributions

- 1. Study conception and design:** Rajesh Shah.
- 2. Experimentation and acquisition of data:** Suvarna Joshi, Sandeepan Mukerjee, Shashikant Vaidya and Gitanjali Talele.
- 3. Analysis and interpretation of data:** Suvarna Joshi, Sandeepan Mukerjee, Shashikant Vaidya, Gitanjali Talele and Abhay Chowdhary.

4. **Drafting of manuscript:** Rajesh Shah, Suvarna Joshi, Sandeepan Mukerjee, Shashikant Vaidya, Gitanjali Talele and Abhay Chowdhary.

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