Preparation and Standardization of *Escherichia coli* Nosodes Sourced from Select *E. coli* Strains

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Homeopathy

Abstract

Background The nosodes are well-known preparations in homeopathy that are sourced from organisms and diseased materials. More than 40 known nosodes have been used in homeopathic practice for over a century. Having identified the need for scientifically developed new nosodes sourced from organisms that are currently prevalent, the preparation of *Escherichia coli* nosodes from different strains of the bacterium is presented in this article.

Materials and Methods *Escherichia coli* strains (*E. coli* ATCC 11775E, ATCC 25922, and ATCC 8739) were identified, cultured, and tested for purity, and 20 billion cells were processed following the nosode preparation method given in the Homoeopathic Pharmacopoeia of India, group N1. Serial dilution and potentization for liquid potency were done up to 30c potency. Nosodes were prepared by two methods: from cell-free extract (endotoxin) and from entire-cell extract.

Result Six nosodes were developed in total. Three univalent nosodes were prepared using individual endotoxins, one from each of the three *E. coli* strains; those three univalent nosodes were also combined as "Trivalent nosode-I". "Trivalent nosode-II" was prepared by mixing entire cells of the three *E. coli* strains. A mix of both Trivalent nosode-I and Trivalent nosode-II was labeled "EC-Polynosode". The safety profile of the potentized nosodes was documented by the non-detectability of traces of source material (absence of contamination, live organisms, or DNA material) through a culture test, sterility test, and molecular testing (polymerase chain reaction).

Conclusion Different variants of *E. coli* nosodes were systematically and scientifically prepared and standardized using the cultures. Homeopathic pathogenetic trials, in-vitro efficacy studies, and clinical evaluation of *E. coli* nosodes (single, trivalent, or polyvalent nosodes) will be required in future.

Keywords
- *Escherichia coli*
- nosode
- potentization
- homeopathy

Introduction

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped, facultative anaerobe that is commonly found in the intestine. Being part of the normal flora of the large intestine, many *E. coli* strains are harmless. Shiga toxin-producing *E. coli* strains,[1] however, can be responsible for food and water-borne diseases, involving diarrhea.[2] There are around 200 different *E. coli* O serotypes producing Shiga toxin, of which over 100 have been associated with human disease.[3] Gastro-intestinal disturbances occur 3 to 4 days after ingestion of the contaminated food or water. Infection can cause mild-to-severe bloody diarrhea (mostly without fever) and sometimes "hemolytic uremic syndrome",[2] characterized by acute kidney failure, bleeding and neurological symptoms, may occur.

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Most of the strains of *E. coli* live naturally in the gut without producing disease. Some of the virulent strains may produce gastroenteritis, hemorrhagic colitis, Crohn's disease, urinary tract infections, or neonatal meningitis.

The nosodes are well-known preparations in homeopathy that are sourced from organisms and diseased materials.[4] More than 40 known nosodes[5] have been used in homeopathic practice for over a century. One of the authors (R.S.) has prepared a series of nosodes, including those for human immunodeficiency (HIV),[6] hepatitis C,[7] *Mycobacterium tuberculosis*,[8] *Plasmodium falciparum*,[9] cancer,[10] and human papillomavirus (unpublished). Nosodes in homeopathy are not primarily used for treating the diseases from which they are sourced but for managing related conditions: for example, *Tuberculinum*[11] from the tuberculosis abscess is used to treat non-tubercular lymphadenitis, migraine, and rheumatoid arthritis.

Literature Review

Some nosodes related to *E. coli* have been found in the homeopathy literature. These nosodes are available for practitioners, and a few of them have been studied for efficacy. *Bacillus coli (Colibacillinum)* 30cH, 200cH, and 1M (from Schwabe India) is a homeopathic nosode prepared from strains of the bacterium *E. coli (Colibacillinum)*.[12]

Julian's *Materia Medica of Nosodes* describes the source, preparation, and remedy picture of *Colibacillinum*. The biotherapeutic *Colibacillinum* is prepared from the lysate obtained from the culture consisting of a mixture of several Colibacilli stocks, with the addition of antiseptic.[13] Three stocks of *E. coli*, Marcy 423, 430, and 431, were used as the starting material. The stocks were controlled for morphological type and biochemical and antigen characteristics, and preserved under lyophilized conditions.

The homeopathic agent *Coli* 30K is a nosode prepared from various strains of *E. coli* bacteria. It is claimed to prevent and cure diseases, such as colibacillosis and mastitis, caused by *E. coli*.[14]

Provings data for *Colibacillinum*[15] and *Bacterium coli*[16] are available. The efficacy of *Coli* 30K was observed to control diarrhea in neonatal piglets: the homeopathically treated group of piglets had significantly less *E. coli* diarrhea than piglets in the placebo group.[17]

It is to be noted that information on microbial characterization of the strains and the methods used in their preparations are not available.

Pathogenic *E. coli* is characterized by major surface antigens, such as somatic (O) antigen (serogroup) and flagellar (H) antigen. More than 400 serotypes of Shiga toxin-producing *E. coli* have been implicated in sporadic human disease.[18] Shiga toxin-producing *E. coli* strains produce Shiga toxins Stx1 and/or Stx2 types of potent cytotoxins. These toxins are believed to have primary virulence factors responsible for human disease. *E. coli* O157:H7 is the prototypical Shiga toxin-producing *E. coli*. We have selected the *E. coli* strains with O:H serogroup.

Resistance

Bacterial species seem to have evolved a preference for some mechanisms of resistance over others. β-lactamase production by several gram-negative and gram-positive organisms is perhaps the most important mechanism of resistance to penicillins and cephalosporins. With the widespread use of oxyimino cephalosporins, such as cefuroxime, cefotaxime, cefmenoxime and ceftriaxone, resistance to the extended-spectrum β-lactam antibiotics has emerged. Because of the increased spectrum of activity, particularly against expanded-spectrum cephalosporins, these enzymes were called extended spectrum β-lactamases (ESBL).[19]

Escherichia coli infections can usually be treated with penicillin or cephalosporin. However, when the bacteria produce ESBLs, they can cause infections that are no longer treatable by these standard antibiotics, necessitating treatment using other options.

Metabolism

Escherichia coli normally remain as benign commensal organisms. However, strains that acquire bacteriophage or plasmid DNA, encoding enterotoxins or invasion factors, can become virulent. There are numerous types of enterotoxins: some of these toxins are cytotoxic, damaging the mucosal cells, whereas others are merely cytotonic, inducing only the secretion of water and electrolytes. Three groups of *E. coli* strains produce enterotoxins, and are associated with diarrheal diseases. The first group of these strains is called enterotoxigenic *E. coli*. The second group of *E. coli* strain has invasion factors and causes tissue destruction and inflammation resembling the effects of *Shigella* (enteroinvasive *E. coli*). The third group of serotypes, called enteropathogenic *E. coli*, is associated with outbreaks of diarrhea in neonatal nurseries, but it produces no recognizable toxins or invasion factors.

Objective

The objective of the study was to scientifically and systematically prepare and document *E. coli* nosodes from different *E. coli* strains, using whole organisms as well as the endotoxins, making univalent, trivalent, and polyvalent variants.

Materials and Methods

Source for the Preparation of Homeopathic Nosode: American Type Culture Collection Strains

1. *Escherichia coli* American type culture collection (ATCC) 11775E isolated from urine; Serovar O1:K1:H7 (coded as "EC-175", univalent nosode).
2. *Escherichia coli* ATCC 25922, clinical isolate; Serotype O6, Biotype 1 (coded as "EC-222", univalent nosode).
3. *Escherichia coli* ATCC 8739 isolated from feces (coded as "EC-339", univalent nosode).
4. Trivalent nosode-I from endotoxin derived from the above three strains (coded as "EC-ET-COMB").

Table 1 Escherichia *coli* nosodes

No.	Strain/source material	Variant	Code/label
1.	*Escherichia coli* ATCC 11775E- endotoxin	Univalent	EC-175
2.	*Escherichia coli* ATCC 25922- endotoxin	Univalent	EC-222
3.	*Escherichia coli* ATCC 8739- endotoxin	Univalent	EC-339
4.	*Escherichia coli* ATCC 11775E, 25922, 8739 endotoxins	Trivalent nosode-I	EC-ET-COMB
5.	*Escherichia coli* ATCC 11775E, 25922, 8739 entire cells	Trivalent nosode-II	EC-ECO-COMB
6.	Trivalent nosode-I and Trivalent nosode-II	Polyvalent	EC-Polynosode

Abbreviation: ATCC, American Type Culture Collection.

5. Trivalent nosode-II from entire cells (coded as "EC-ECO-COMB") of the above three strains were prepared and standardized for future research and clinical evaluation.
6. Polyvalent nosode was prepared by combining Trivalent nosode-I and Trivalent nosode-II (coded as "EC-Polynosode").

These six nosodes of the study (showing their strain, variant, and code) are listed in ►**Table 1**.

Morphology

E. coli is a gram-negative, rod-shaped, and approximately 0.5 µm in width by 2 µm in length.

Macroscopy

Colony Characteristics

- Shape: Circular
- Color: Dark purple on eosin methylene blue (EMB) agar and cream on nutrient agar
- Margin: Entire
- Size: Small
- Consistency: Butyrous
- Elevation: Flat
- Opacity: Opaque
- Surface: Shiny

Microscopy

Microscopic appearance showed coccobacilli (small rod)-shaped cells.

Identification of Chemical (Wherever Applicable)

Culture Characteristics

Escherichia coli culture showed cream-colored colonies on nutrient agar after 18 to 24 hours of incubation at 25 to 37°C. *Escherichia coli* showed dark purple colonies when streaked on a selective medium, that is, EMB agar. The colonies have a characteristic green sheen. Culture showed rapid fermentation of lactose and production of strong acids: thus, a rapid reduction in the pH of EMB agar, the critical factor in the formation of the green metallic sheen, was observed.

Biochemical Studies

Biochemical tests were conducted to examine the production of certain enzymes and/chemicals such as catalase, oxidase, lipase, urease, and indole. Enzyme catalase produced by *E. coli* generates bubbles of oxygen when hydrogen peroxide is added to the medium. Indole test detects the ability of bacteria to produce indole from tryptophan. Oxidase test detects the pH change, whereas lipase test detects the ability of bacteria to break down triglycerides. The enzyme urease hydrolyzes urea to ammonia and carbon dioxide, turning the media alkaline and phenol red indicator to pink. The Voges–Proskauer test was performed to check the presence of acetoin in *E. coli*-containing media, using α-naphthol indicator (brownish-red to pink).

Preparation

Method 1: Cell-Free Extract

Three tubes containing 5 mL nutrient broth were inoculated with two to three loopfuls of *E. coli* colonies, and the nutrient broth tubes were incubated at 37°C for 24 hours. The suspension was then centrifuged for 10 minutes at 10,000 rpm. The supernatant was discarded, and the pellet was re-suspended in 1 mL saline. The number of organisms was counted using a hemocytometer, and 6 to 7 billion organisms were obtained in each tube. Twenty billion organisms were considered as the original stock for further work. The preparation was performed as per the method described in Homoeopathic Pharmacopoeia of India (HPI), volume 4, Group N1.[4] Group N1 describes a method for preparation made from cell-free extract of bacteria capable of producing bacterial endotoxins.

A. Under laminar flow, a suspension consisting of 20 billion organisms per mL was obtained.
B. This suspension was then sonicated using a sonicator (Citizen Ultrasonic Cleaner/CUB 2.5) until most of the bacterial cells ruptured. The material was then centrifuged at 10,000 rpm for 30 minutes using a Hettich centrifuge (Remi Lab World; Mumbai, India). The supernatant was filtered through a Seitz filter, and the cell-free extract containing the endotoxin was treated with an equal volume of strong alcohol. This concentration was sealed in separate ampoules and labeled as the primary stock nosode, serving as 1x potency for preparation of homeopathic dilutions. The limulus amebocyte lysate (LAL) test was conducted on cell-free extract, a test for the presence of endotoxin.

C. Liquid potency: One part of the 1x preparation was diluted up to 100 (1:99 ratio) using alcohol in a clean glass bottle and potentized using an electromechanical potentizer, as described previously,[20] with a well-defined force parameter at every step of the potency. For example, 12,099 Nm torque was applied to prepare 30c potency. Subsequent potencies were prepared, labeled, and safely stored for future use.

Method 2: Entire Cell Extract

The nosode was prepared using entire *E. coli* cell extract and not from the cell-free extract containing only endotoxin. Step B differs from method 1 as described below:

B. This suspension was sonicated using a sonicator (Citizen Ultrasonic Cleaner/CUB 2.5) until most of the bacterial cells ruptured. The material was then centrifuged at 10,000 rpm for 30 minutes using a Hettich centrifuge and then treated with an equal volume of strong alcohol (91% v/v). This concentration was sealed in separate ampoules and labeled as primary stock nosode. This serves as 1x for preparation of homeopathic dilutions.

Storage

Preparations below 6c potency were stored at 5°C and not allowed to freeze.

Caution

All nosodes were handled with care and followed aseptic conditions for preparation of nosode up to 4c potency.

Standardization

With the objective of standardizing the potencies with respect to the growth of *E. coli* strain on specialized media (viability testing), the following testing was conducted:

Detection of *Escherichia coli* Strains

Reagents used: EMB agar and nutrient agar.

Samples: EC-Polynosodes of 1x, 2c, 3c, 4c, 5c, 6c and 7c potencies.

Positive control: Three *E. coli* ATCC strains (11775E, 25922, 8739).

Test Procedure

Ten microliters of the given nosode preparations (prepared in water for injection) was streaked on EMB agar plates. The plates were incubated at 37°C for 24 hours for the culture growth. Ten microliters of *E. coli* culture grown in the nutrient broth was also streaked as a positive control.

Molecular Test

The polymerase chain reaction (PCR) test for *E. coli* nosodes 6c and 12c was performed using the CFX-96 model (Bio-Rad) with the objective of tracing whether any nucleic material was present in the two dilutions. The detection of the target is based on the principle of real-time PCR: in this, the progress of DNA amplification during a PCR can be monitored in real-time PCR by measuring the release of fluorescent "dyes" during the amplification.

Table 2 Biochemical tests

No.	Biochemical test	Expected results	Obtained results
1	Catalase	+	+
2	Oxidase	−	−
3	Acetoin	−	−
4	Lipase	−	−
5	Urease	−	−
6	Indole	+	+

Sterility Testing

Escherichia coli nosode was tested for sterility as per the requirements of the Indian Pharmacopoeia (IP). Standard test cultures for the tests, such as aerobic bacterial culture, anaerobic bacterial culture and fungal culture, were used as positive controls. Standards, nosodes, and inhibition controls were incubated for 14 days. The analytical results were reported by the testing laboratory as compliant for sterility testing as per the IP.[21]

Results

Escherichia coli strain was identified by microscopy, macroscopy, and biochemical tests. Results of biochemical tests showed the presence of catalase and indole and the absence of oxidase lipase, urease and acetoin (►**Table 2**). *Escherichia coli* was isolated from urine (EC-175), feces (EC-339) and the clinical isolate (EC-222).

Cell-free extract: Three univalent nosodes from each strain (EC-175, EC-222, EC-339), and the trivalent nosode-I using endotoxin of three strains (EC-ET-COMB), were prepared.

Entire cell extract: The trivalent nosode-II using entire cells (EC-ECO-COMB) of three strains, and the polyvalent nosode combination of trivalent nosode-I and II, were prepared and standardized for future research and clinical evaluation. The LAL test showed gel clot formation, indicating the presence of protein coagulation due to endotoxin.

Polymerase chain reaction test for *E. coli* nosodes 6c and 12c was performed. The test results were reported by an external laboratory as "not detected".

No growth of *E. coli* was observed when 1x to 7c potencies were grown on EMB agar and nutrient agar. Growth was observed only in the positive control used (*E. coli*: see ►**Table 3**, ►**Fig. 1** (*Eco 11775E*) and ►**Supplementary Fig. 1** (*Eco 25922* and *Eco 8739*; online only)).

Discussion

The *E. coli* nosodes were prepared by two methods: as per the HPI using the cell-free extract of endotoxin; and using the entire cell along with endotoxin. Considering that *E. coli* is an extraintestinal pathogenic (ExPEC) bacterium, all *E. coli* cells were sonicated and potentized with due care. Pathogenicity of the ExPEC strain is related to bacterial colonization and virulence, including adhesins, toxins, iron acquisition factors,

Table 3 Results of growth in culture media

No.	Test sample	Observation		
		24 h	48 h	72 h
1.	1x potency	–	–	–
2.	2c potency	–	–	–
3.	3c potency	–	–	–
4.	4c potency	–	–	–
5.	5c potency	–	–	–
6.	6c potency	–	–	–
7.	7c potency	–	–	–
8.	Combination 6c potency	–	–	–
9.	Combination 7c potency	–	–	–
10.	Positive control (*Escherichia coli*)	+	+	+

[+] Growth observed; [–] No growth observed.

lipopolysaccharides, polysaccharide capsules and invasins, which are usually encoded on pathogenicity islands (PAIs), plasmids, and other mobile genetic elements.[22] The entire *E. coli* cell, with the PAIs and genetic elements, is potentized to preserve the virulence factor.

The biotherapeutic *Colibacillinum* is prepared from the lysate obtained from the culture consisting of a mixture of three Colibacilli, with the addition of antiseptic stocks (Marcy 423, 430, 431).[12] As per the European method, the nosodes are prepared by exposing the medium containing the organisms to heat. *Colibacillinum* nosode mentioned in Julian's *Materia Medica of Nosodes with Repertory* is prepared using such a method. In this method of heating, the suspension of organisms is heated at 56°C for 45 minutes; endotoxins collected are again heated at 75°C for 1 hour.[13] In our experience, the exposure to heat may lead to denaturation of proteins,[23] thereby jeopardizing the very objective of preparing the nosode.

Our previous experiments have shown specific anti-disease activities of some nosodes, such as *P. falciparum* nosode,[8] cancer nosode,[24] HIV nosode,[6] and hepatitis C nosode,[7] in cell-line and human models. More nosodes need to be investigated in this direction, strengthening the fundamental homeopathic principle of the Law of Similars. *Escherichia coli* nosodes from the selected strains need to be explored for their potential anti-*E. coli* effects as well as against the range of conditions that they may have the capacity to induce. Similarly, microbial studies to evaluate the disease-producing effects, homeopathic pathogenetic trials for assessing their effects in potentized form, in-vitro efficacy studies, and clinical evaluation of *E. coli* nosodes (single, trivalent, or polyvalent) will be required to be performed in future.

Microbiology was at an early stage of development when the term "nosode" was introduced originally. None of the major nosodes (*Psorinum*, *Medorrhinum*, *Syphilinum*, *Tuberculinum*, *Bacillinum*, *Morbillinum*, *Diptherinum*, *Variolinum*, *Pyrogen*, and *Lyssin*) was sourced from a pure culture of organisms. Also, the nosodes were sourced from diseased discharges (presumably containing organisms) such as *Tuberculinum* and *Syphilinum*, diseased tissues (*Carcinosin*), healthy secretions such as *Lac caninum* (sarcodes), and from healthy tissues such as *Thyroidinum*. The time has come to redefine the glossary and classify the nosode category.

Clinically, the nosodes are usually used in higher potencies (1M, 200c or 30c), and it is not recommended in any potency below 6c. In our earlier studies with *M. tuberculosis*

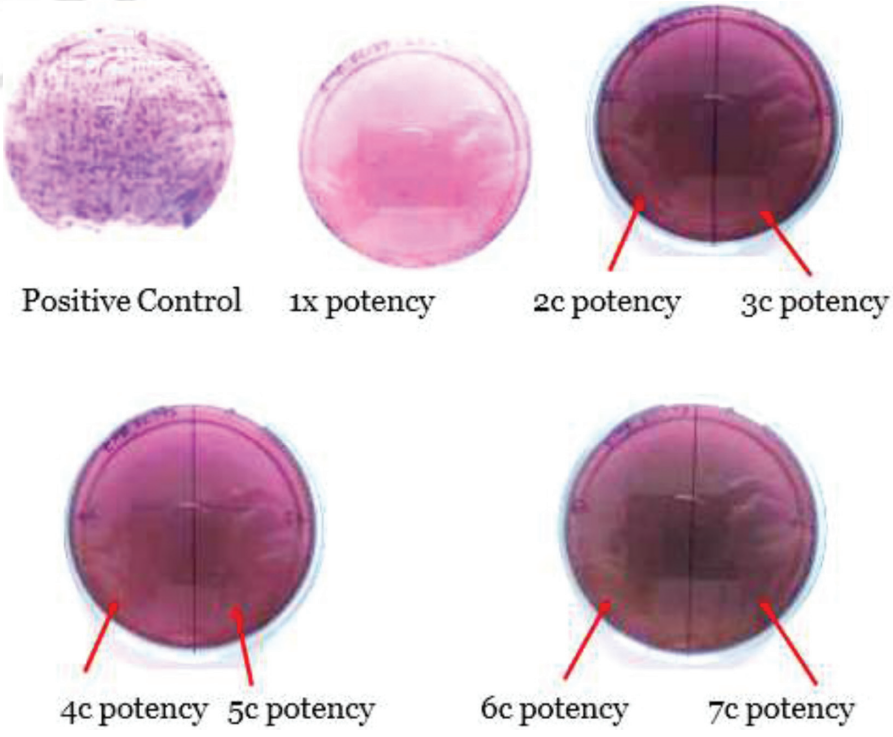

Fig. 1 Results of growth in culture media (*Escherichia coli Eco11775E*).

nosode, a culture test on the Löwenstein–Jensen medium was done with the objective to check the presence of source organism. The test results showed traces of the source material up to 5c potency.[7]

In the current study, we have used only three of the many possible strains of *E. coli*. More strains of the organisms, as well as other endotoxins, could be explored in future.

The challenge of antibiotics' resistance to antibacterial agents against *E. coli* compels us to examine newer approaches in the treatment against this bacterium. The prospective therapeutic and prophylactic role of such nosodes may be examined using suitable experimental models.

Conclusion

Different variants of *E. coli* nosodes were systematically and scientifically prepared, and standardized using the cultures. Homeopathic pathogenetic trials, in-vitro efficacy studies, and clinical evaluation of *E. coli* nosodes (single, trivalent, or polyvalent) will be required in future.

Highlights

- There is a recognized need for the scientific development of nosodes from currently prevalent organisms.
- Strains of *E. coli* (ATCC 11775E, ATCC 25922 and ATCC 8739) and their endotoxins were selected and standardized for preparing *E. coli* nosodes, using single strains and their combinations.
- The safety of *E. coli* nosodes using culture, sterility, and molecular tests was established.
- These *E. coli* nosodes can be explored in pre-clinical and clinical efficacy evaluation.

Supplementary File
Supplementary Fig. 1 Results of growth in culture media. (**A**) *Eco 25922*. (**B**) *Eco 8739*.

Authors' Contributions

Renuka Munshi developed the laboratory facility, conducted the research, and reviewed the manuscript. Gitanjali Talele contributed to laboratory work and co-wrote the manuscript. Rajesh Shah is the principal investigator, who developed the drug, conceptualized the study, supervised the laboratory work, and co-wrote the manuscript.

Conflict of Interest

Dr. Rajesh Shah has a patent-pending for a nosode prepared from the organisms.

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