

In-Vitro Evaluation of Antimicrobial Activities of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Neisseria gonorrhoeae*, and *Candida albicans* Nosodes

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Abstract

Background This study presents the results of the minimum inhibitory concentration (MIC) assay of a series of nosodes: namely *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Neisseria gonorrhoeae*, and *Candida albicans*. Each was tested against its corresponding infection as well as cross infections.

Methods *In-vitro* efficacy of polyvalent nosodes was tested using the MIC assay technique. The nosodes, namely *C. albicans* polyvalent nosode (35c, 100c), *N. gonorrhoeae* (35c), *K. pneumoniae* (35c, 100c), *E. coli* polyvalent nosode (35c, 100c) and *Salmonella typhi* polyvalent nosode (30c, 100c), were tested along with positive and negative controls. Nosodes were studied in different potencies and at 1:1 dilution.

Results *C. albicans* polyvalent nosode 35c, 100c, *N. gonorrhoeae* 35c, and positive control amphotericin B showed inhibition of the growth of *C. albicans* species. *K. pneumoniae* 35c, *E. coli* polyvalent nosode 100c, and meropenem (positive control) showed inhibition of the growth of *K. pneumoniae*; this effect was not seen with ceftriaxone, ofloxacin and amoxicillin antibiotics. *E. coli* polyvalent nosode 30c in 10% alcohol (direct and dilution 1:1) and the positive controls ciprofloxacin, ofloxacin, and amoxicillin showed inhibition of the growth of *E. coli*. The *S. typhi* polyvalent nosode 30c in 10% alcohol showed inhibition of growth of *S. typhi*.

Conclusion This study reveals that the tested nosodes exhibited antibacterial potential against the corresponding micro-organisms and against other selected organisms studied using this assay.

Keywords

- ▶ antibacterial
- ▶ nosodes
- ▶ inhibitory concentration
- ▶ potentized
- ▶ *in vitro*
- ▶ *Escherichia coli*
- ▶ *Klebsiella pneumoniae*
- ▶ *Salmonella typhi*
- ▶ *Neisseria gonorrhoeae*
- ▶ *Candida albicans*

Introduction

The system of homeopathic medicine introduced by Samuel Hahnemann (1755–1843) is based on the Law of Similars, which suggests that any substance having a capacity of producing disease in its crude form also has the capacity to treat a similar disease if administered in a very small dose. The functioning of the homeopathic system of medicine is compa-

able in some respects with hormesis and vaccination (though they differ in their modes of application), and also it involves sourcing the drugs from biological materials, including live and inactivated organisms,¹ their isolates, or diseased materials. Professor Joseph Lux, at the University of Leipzig around the year 1820, introduced a related concept of “isopathy”, suggesting the treatment of infection by the potentized source organisms.²

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A nosode may be used “homeopathically” based on the matching of its symptom similarity with that of the patient, as per a drug proving or pathogenesis. When used against a specific infection, however, it is often termed “isopathy”, falling under the broad umbrella of homeopathy. Various nosodes, such as *Psorinum*, *Medorrhinum*, *Tuberculinum* and *Carcinosin*, are also used for their respective corresponding diseases without formally distinguishing them as “isopathic”.

The category of nosodes is undergoing a revolutionary overhaul of the old drugs (*Mycobacterium tuberculosis* nosode,³ *Cancer* nosode⁴), the introduction of new remedies (HIV nosode,⁵ Hepatitis C nosode,⁶ *Escherichia coli* nosode⁷), as well as their efficacy studies in the laboratory (*Helicobacter pylori* nosode⁸), animal⁹ and *in-vitro* models (*Plasmodium falciparum* nosode¹⁰), and human studies (influenza,¹¹ HIV,¹² and leptospirosis nosode¹³). In an animal trial, piglets in the homeopathy-treated group had significantly less *E. coli* diarrhea than piglets in the placebo group ($p < 0.0001$).¹⁴ In a study, no significant inhibitory effect of non-nosode drugs, such as *Apis mellifica*, *Cantharis*, *Causticum hahnemanni*, *Staphysagria*, *Nux vomica*, *Berberis vulgaris* and *Lycopodium clavatum* in 30c potency, was observed.¹⁵

Homeopathic nosodes sourced from any organism can be explored for its prophylactic as well as its therapeutic role in treating the disease caused by the same organism as well as in other conditions. There are over 45 common nosodes sourced from bacteria (*Tuberculinum*, *Diphtherinum*, *Streptococcinum*), viruses (*Morbillinum* [measles], *Variolinum* [smallpox], HIV), parasites (*Psorinum* [scabies]), and diseased tissues (*Carcinosin*). The use of *Psorinum*¹⁶ (sourced from a discharge containing scabies parasites) is not restricted to the treatment of scabies but may also be beneficial against cancer,¹⁷ eczema, anxiety disorders, migraines, and more. HIV nosode and Hepatitis C nosode have shown efficacy not only against HIV or Hepatitis C infections¹⁸ but also against cancer¹⁹ in pre-clinical studies. A literature search reveals that Bacillus Calmette–Guérin (BCG) was first used as immunotherapy to treat superficial bladder cancer.²⁰ Clinical studies showed that the measles, mumps, and rubella (MMR) vaccine is safely and effectively used in the treatment of common warts.^{21,22} This is suggestive that the scope of a vaccine against disease is not restricted to the one it is sourced from, thereby allowing us to explore wider opportunities.

Most of the major nosodes developed during the latter part of the nineteenth century (1870 to 1900) were prepared from the non-characterized clinical samples of infected discharges or tissues (*Tuberculinum*, *Bacillinum*, *Medorrhinum*, *Syphilitinum*, *Diphtherinum*),²³ which did not have microbial categorization. As current knowledge of microbiology allows us to identify different strains of microbes, we have options to make individual nosode from specific strains, and also combine the related strains making bivalent⁵ or polyvalent nosodes such as *Mycobacterium* nosode.³

Treatment of infections as well as antimicrobial resistance continues to be among the major medical challenges. Infectious diseases kill over 17 million people a year.²⁴ Antibiotics, such as ciprofloxacin, erythromycin and ampicillin, may lose their effectiveness as microbes develop resistance. The use of alter-

native methods to treat infections, and to increase the innate immunity of the body to fight against infections, is on the rise. Several homeopathic medicines (*Aconite* 30c, *Arsenicum album* 30c, *Mercurius corrosivus* 30c, *Aspergillus niger*) have been tested for their antibacterial activity in *in-vitro* studies.^{25–28}

This study presents the results of the minimum inhibitory concentration (MIC) assay of a series of potentized (serially diluted and succussed), freshly prepared nosodes: namely *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Neisseria gonorrhoeae* and *Candida albicans* nosodes, tested against their respective infection as well as cross infections. The MIC assay is an *in-vitro* technique to study the lowest drug concentration that prevents visible micro-organism growth. The MIC can be helpful in establishing the level of resistance of a bacterial strain and can substantially affect the decision to use certain antimicrobial agents.

Different methods can be used to assess antibacterial activity. Agar disk diffusion assays are methods that estimate the antibacterial potential of a sample qualitatively with a zone of inhibition. The broth micro-dilution assay is a quantitative antibacterial susceptibility testing method that gives the percentage inhibition of bacteria in a micro-well.²⁹

Methods

Bacterial Strains

Bacterial strains (American Type Culture Collection (ATCC)): *E. coli* (11775E, 25922, and 8739), *Klebsiella pneumoniae* (BAA 2146), *Salmonella enterica Typhimurium* (51812), *Salmonella enterica Typhimurium* (13311), *N. gonorrhoeae* (43069), *C. albicans* (26790), and *C. albicans* (24433 and 60193) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai.

Chemicals

Antimicrobial Agent Stock Solutions (positive controls) ciprofloxacin, ofloxacin, amoxicillin, fluconazole, amphotericin-B, meropenem and ceftriaxone were purchased from the hospital Pharmacy.

The following media used for the study were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai.

- Nutrient agar for the bacterial strains.
- Sabouraud's agar for the fungal strains.
- *Selective media.*
 - *E. coli*—Eosin Methylene Blue agar.
 - *Klebsiella pneumoniae*—MacConkey agar.
 - *Salmonella enterica Typhimurium*—Xylose-Lysine-Deoxycholate agar.
 - *N. gonorrhoeae*—Thayer Martin agar.
 - *C. albicans*—Bismuth Glycine Glucose Yeast agar.

Method for Inoculum Preparation

- The inoculum was prepared by making a direct broth suspension of isolated colonies selected from a 24 to 48 hour agar plate.
- The suspension turbidity was adjusted to achieve a value equivalent to a 0.5 McFarland turbidity standard

Table 1 McFarland standard

No.	McFarland standard	1% BaCl ₂ (mL)	1% H ₂ SO ₄ (mL)	Approximate bacterial suspension/mL
1.	0.5	0.05	9.95	1.5×10^8
2.	1	0.1	9.9	3.0×10^8
3.	2	0.2	9.8	6.0×10^8
4.	3	0.3	9.7	9.0×10^8
5.	4	0.4	9.6	1.2×10^9
6.	5	0.5	9.5	1.5×10^9
7.	6	0.6	9.4	1.8×10^9
8.	7	0.7	9.3	2.1×10^9
9.	8	0.8	9.2	2.4×10^9
10.	9	0.9	9.1	2.7×10^9
11.	10	1.0	9.0	0

(► **Table 1**—McFarland standard). This resulted in a suspension containing approximately 1×10^8 colony forming units (CFU)/mL for bacterial strains and 1×10^6 CFU/mL for fungal strains.

- The inoculum suspension was diluted in the broth such that, after inoculation, each tube contained approximately 5×10^5 CFU/mL.

Method for McFarland Standard Preparation

For the preparation of 0.5 McFarland standard, 0.5 mL of 1.175% w/v barium chloride (BaCl₂) was added to 85 mL of 1% sulfuric acid (H₂SO₄) and mixed. After mixing, 1% sulfuric acid was further added to make the volume up to 100 mL. Prepared solutions were checked for its optical density at 540 nm range which gives absorbance ranging from 0.12 to 0.19.

Preparation of Positive Controls

Different concentrations of positive controls (antimicrobial agents) ranging from 1 to 256 µg/mL were prepared.

Nosodes: Test Sample Preparations

All organisms were procured from ATCC; each strain was cultured in a cell culture laboratory. Each nosode was sourced from its respective live organism culture. A polyvalent nosode is a combination of more than two strains and preparations were made using different methods. Nosodes were potentized as per the method described in the Homoeopathic Pharmacopoeia of India (HPI). The polyvalent nosodes of *S. typhi* and *E. coli* were prepared by two methods: from the cell-free extract and the entire cells. For the MIC assays, both cell-free extract and the entire cells preparations of *S. typhi* and *E. coli* were combined, respectively.

1. *S. Typhi* and *E. coli*⁶ nosodes were prepared by the following methods:

Method 1: Cell-Free Extract

Twenty billion cells were sonicated using a sonicator (Citizen/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. The supernatant was treated with an equal volume of strong alcohol which serves 1x potency. One part of 1x was diluted up to 100 (1:99 ratio) using alcohol (90% v/v alcohol procured from Merck, Ethanol CAS No. 64-17-5, EC Number 200-578-6) for 2c potency; further potencies were prepared by serial dilution (1:99) and succussion.

Method 2: Entire Cell Extract

The nosode was prepared using 20 billion cells and not from the cell-free extract containing only endotoxin.

2. *Klebsiella pneumoniae* nosode

Twenty billion viable cells were sonicated (Citizen ultrasonic cleaner/CUB 2.5) until most of the cells ruptured. The material was treated with an equal volume of strong alcohol to make 1x potency. The 1x was diluted up to 100 (1:99 ratio) using alcohol (90% v/v alcohol, as above) for 2c potency; further potencies were prepared by serial dilution (1:99) and succussion.

3. *N. gonorrhoeae* and *C. albicans* nosodes were prepared by the trituration method

An isolated cell palate of 20 billion cells of respective organisms (*N. gonorrhoeae*/*C. albicans*) was triturated with *Saccharum lactis* powder to make 1x preparation. 1x to 6x potencies were prepared by maintaining a 1:9 ratio. Two parts of 6x were diluted up to 100 (1:99 ratio) by using 50% water for injection and 50% alcohol, and potentized to achieve 4c potency. As per description in the HPI¹ and the Homeopathy Pharmacy textbook³⁰ the preparation of liquid potencies is made by converting 6x to 4c. Two parts of 6x were taken to compensate the possible material loss during the process. One part of 4c potency was diluted using 99 parts of alcohol to arrive at 5c potency. Likewise, serial dilutions were done to prepare further potencies using dispensing alcohol.

All the potencies by succussion were made using an electromechanical potentizer imparting a standard and quantifiable force parameter³¹ (for example, 30c potency = 12,099 Nm, 35c = 14,115 Nm, 100c = 40,330 Nm).

E. coli polyvalent nosode (35c, 100c), *K. pneumoniae* (35c, 100c), *S. typhi* polyvalent nosode (30c, 100c), *N. gonorrhoeae* (35c), and *C. albicans* polyvalent nosode (35c, 100c) were used for the MIC experiments.

Method for the MIC Assay

- The stock solutions of the antimicrobial agent/s were prepared in the required concentrations.
- Two-fold dilutions of the antimicrobial agent/s were prepared volumetrically in the broth.
- The inoculum was prepared by making a direct broth suspension of isolated colonies selected from a 24 to 48 hour agar plate, and the adjusted inoculum suspension was diluted in the broth such that, after inoculation, each tube contained approximately 5×10^5 CFU/mL.
- One milliliter of the adjusted inoculum was added to each tube containing 1 mL of antimicrobial agent and to the positive-control tube containing only broth and then mixed.

- The inoculated tubes were then incubated at 37 °C for 24 to 48 hours in an incubator.
- Growth in the tubes was checked at 24 and 48 hours.
- The amount of growth in the tubes containing the antimicrobial agent/s was compared with that observed in the positive control tubes.
- The lowest concentration at which no growth was observed in the tube was recorded as the MIC.
- For confirmation, a loop of the broth was taken from the 48 hour-old tubes and streaked on agar plates containing Nutrient agar and selective media for each organism respectively under study. The presence or absence of the growth on the agar plate was documented after 24 and 48 hours. The absence of growth indicated the inhibitory activity of medicine studied.

In-vitro efficacy of polyvalent nosode was tested using the MIC assay technique at the Department of Pharmacology, Nair Hospital, Mumbai. The polyvalent nosodes were tested along with positive and negative controls. Nosodes were studied in different potencies at 1:1 dilution. Nosodes were prepared using the vehicle 90%, 30% and 10% alcohol.

The nosodes prepared from each organism were tested for their antimicrobial activity against the same organism, as well as against a few other organisms (cross-activity). For example, *E. coli* polyvalent nosode 100c was tested against the organisms *E. coli* and *K. pneumoniae*.

Results

The MIC assay results showed that the test samples at 24 and 48 hours of incubation, *E. coli* polyvalent nosode 30C in 10% alcohol (direct and dilution 1:1), and the positive controls ciprofloxacin, ofloxacin and amoxicillin, could inhibit the growth of organisms of the *E. coli* species (→ **Table 2A**).

K. pneumoniae 35c and *E. coli* polyvalent nosode 100c could inhibit the growth of organisms of *K. pneumoniae* species when used directly. *K. pneumoniae* (35c, 100c), *E. coli* polyvalent nosode (35c, 100c), and meropenem (positive control) have shown inhibitory activity against *K. pneumoniae* species growth; however, the antibiotics (ceftriaxone, ofloxacin, and amoxicillin used as positive control) did not exhibit similar inhibitory activity (→ **Table 2B**).

S. typhi polyvalent nosode 30c in 10% alcohol (direct and dilution 1:1) and the positive controls ciprofloxacin and ofloxacin could inhibit the growth of organisms of *S. typhi* species at 24 hours, but the effect was not continued further until 48 hours (→ **Table 2C**).

C. albicans polyvalent nosode (35c, 100c), *N. gonorrhoeae* (35c), and positive control amphotericin B could inhibit the growth of organisms of the *C. albicans* species (→ **Table 2D**).

N. gonorrhoeae polyvalent nosode in 10% and 30% alcohol could not inhibit the growth of *N. gonorrhoeae*. Positive controls, ceftriaxone and ofloxacin B could inhibit the growth of *N. gonorrhoeae* organisms (→ **Table 2E**).

Photographs of the results showing the growth of *E. coli* and *S. typhi* are provided as → **Supplementary Figs. S1** and **S2**, available online only.

Discussion

Evaluation of the direct microbicidal efficacy of potentized drug substances, particularly the nosodes in the cell line model, has been a relatively under-explored method in medical research. Earlier, we presumed that the homeopathic medicines would induce a therapeutic immune response only if administered to the host. Research by other scientists and by the authors has shown antimicrobial effects against malaria^{8,9} and *H. Pylori*.⁷ Both possibilities call for further exploration.

Alcohol (10%, 30% and 90%) as vehicle control, water, and positive and negative control were used for the comparison. Nosodes prepared in 90% alcohol were tested for their inhibitory action in a pilot study before this experiment to investigate whether 90% alcohol (vehicle) itself has microbial inhibitory activity. It was noted that for *E. coli* and *S. typhi*, respective organism growth was inhibited by 90% vehicle alcohol: hence nosodes prepared in 10 and 30% alcohol were used as test samples.

It is interesting to note that *K. pneumoniae* (35c, 100c) and *E. coli* polyvalent nosode (35c, 100c) have shown inhibitory activity against *K. pneumoniae* species growth, even when the antibiotics (ceftriaxone, ofloxacin, amoxicillin) did not induce the inhibitory effect. Qualitatively, nosodes had shown inhibitory activity similar to the antibiotics ciprofloxacin, ofloxacin, amoxicillin, meropenem and ceftriaxone in some of the experiments, a finding that might lead to a great deal more investigative research in this area.

At the prescriber's discretion, nosodes can be used isopathically (against the specific infection) or homeopathically as per the symptom totality. The terms Nosode (Νοσῶδες, an adjective, meaning *that which causes disease*) and Sarcode (Σαρκῶδες, meaning *flesh-like*) were introduced about 150 years ago before the advancement of microbiology, endocrinology and histopathology. These medicines might be considered as sarcodes (if used isopathically), but in this study the preparations have been sourced from microbes (pathogenic agents) and so they are termed here as nosodes. After having developed new drugs from the specific, characterized, strains of viruses (HIV, Hepatitis C), bacteria (*M. tuberculosis*, *E. coli*), parasites (*P. falciparum*), fungi (*C. albicans*), and well-defined diseased tissues (*Cancer nosode*⁴), the principal investigator of this project envisages a need for a developed terminology for this category of biological products, as the terms "nosode" and "sarcodes" do not justify the qualitative standards of the entire new range. The scientific, professional, regulatory and pharmaceutical bodies may consider new terminology in the future.

Cross-activity was tested for those organisms that have some known organ affinity. For example, *E. coli* and *N. gonorrhoeae*, having urinary tract infection affinity, were examined against each other. A limitation of this study is that we have not tested the cross-activity of different nosodes against all the organisms. More studies are planned in order to validate the results.

Table 2 MIC assay results

No.	Name of drug tested	Conc. of drug tested	Time	
			24 hours	48 hours
A. <i>Escherichia coli</i> species			24 hours	48 hours
1.	Ciprofloxacin	1 µg/mL	-	-
2.	Ofloxacin	2 µg/mL	-	-
3.	Amoxicillin	8 µg/mL	-	-
4.	<i>E. coli</i> polynosode 30c in 10% alcohol	Direct	-	-
5.	<i>E. coli</i> polynosode 30c in 10% alcohol	1:1 dilution	-	-
6.	Alcohol 10%	Direct	+	+
7.	Alcohol 10%	1:1 dilution	+	+
B. <i>Klebsiella pneumoniae</i> species			24 hours	48 hours
8.	Ceftriaxone	4–256 µg/mL (at different concentrations)	+	+
9.	Ofloxacin	16–128 µg/mL (at different concentrations)	+	+
10.	Amoxicillin	8–128 µg/mL (at different concentrations)	+	+
11.	Meropenem	16 µg/mL	-	-
12.	<i>K. pneumoniae</i> 35c	Direct	-	-
13.	<i>K. pneumoniae</i> 100c	Direct	-	-
14.	<i>E. coli</i> polynosode 35c	Direct	-	-
15.	<i>E. coli</i> polynosode 100c	Direct	-	-
16.	Alcohol 90%	Direct	+	+
C. <i>Salmonella typhi</i> species			24 hours	48 hours
17.	Ciprofloxacin	1 µg/mL	-	-
18.	Ofloxacin	2 µg/mL	-	-
19.	<i>Salmonella typhi</i> polynosode 30c in 10% alcohol	Direct	-	+
20.	<i>S. typhi</i> polynosode 30c in 10% alcohol	1:1 dilution	-	+
21.	Alcohol 10%	Direct	+	+
22.	Alcohol 10%	1:1 dilution	-	+
D. <i>Candida albicans</i> species			24 hours	48 hours
23.	Amphotericin B	8 µg/mL	-	-
24.	<i>C. albicans</i> polynosode 35c	Direct	-	-
25.	<i>C. albicans</i> polynosode: 100c	Direct	-	-
26.	<i>Neisseria gonorrhoeae</i> 35c	Direct	-	-
27.	Alcohol 90%	Direct	+	+
E. <i>Neisseria gonorrhoeae</i> species			24 hours	48 hours
28.	Ceftriaxone	16 µg/mL	-	-
29.	Ofloxacin B	16 µg/mL	-	-
30.	<i>N. gonorrhoeae</i> 35c	Direct	+	+
31.	<i>N. gonorrhoeae</i> 100c	Direct	+	+
32.	Medorrhinum 35c	Direct	+	+
33.	<i>E. coli</i> polynosode 35c	Direct	+	+
34.	<i>E. coli</i> polynosode 100c	Direct	+	+
35.	Sample 1 NG 35c	Direct	+	+
36.	Alcohol 90%	Direct	+	+
37.	Negative control		+	+

Abbreviations: -, no growth; +, growth.

Conclusion

This study reveals that the nosodes exhibited antibacterial potential against the corresponding micro-organism and against other selected organisms studied using this assay.

Highlights

- The study presents the results of the minimum inhibitory concentration (MIC) assay of a series of nosodes: namely *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Neisseria gonorrhoeae* and *Candida albicans*.
- *E. coli* polynosode 100c, *K. pneumoniae* 35c, *S. typhi* polynosode 30c, *N. gonorrhoeae* 35c, and *C. albicans* polynosode 35c and 100c have shown microbial growth inhibition.
- This study reveals that the nosodes exhibited antibacterial potential against the micro-organisms tested.

Supplementary material

Supplementary File 1 Images of results showing growth of *Escherichia coli*.

Supplementary File 2 Images of results showing growth of *Salmonella typhi*.

Authors' Contributions

Renuka Munshi provided the laboratory facility and contributed to the laboratory work and manuscript writing. Gitanjali Talele contributed to the laboratory work and manuscript writing. Rajesh Shah (the principal investigator) is the inventor of the drugs; he also developed the concept, supervised the laboratory work, and did the principal manuscript writing.

Conflict of Interest

One of the authors (Rajesh Shah, the principal investigator) has a patent pending for these nosodes.

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