In vitro behavior of *Mycoplasma gallisepticum* live-type nosode

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ABSTRACT

As a step of a doctoral research project, in this study a live-type nosode was prepared from microorganism *Mycoplasma gallisepticum* strain R (ATCC 93-08/19610) according to Costa model and the rules by Brazilian Homeopathic Pharmacopoeia. Live nosode was tested in vitro to assess safety when used to immunize domestic fowl (*Gallus gallus*) against infection by this microorganism and to investigate its behavior under laboratory conditions. *M. gallisepticum* was not shown to grow in fluid (broth) and solid (plate) modified Frey medium with dilutions 11d, 12d, 20d and 30d. Inhibition halos about 2.0 mm were observed around paper disks impregnated with live-type nosode in microorganism-sown Petri dishes, whereas disks impregnated with conventional antibiotic oxytetracycline exhibited 8.0 mm inhibition halos. Protein assessment by Folin-Lowry method showed protein absence in dilutions 12d and 30d and neither microbial DNA traces were found in PCR assay in dilutions 12d, 20d and 30d.

Key-words: Biotherapy; Chickens; Live nosode; *Mycoplasma gallisepticum*

Introduction

According to Brazilian Homeopathic Pharmacopoeia, biotherapy or nosodes are “medicinal preparations intended for homeopathic use obtained from chemically indefinite biological products: secretions, excretions, tissues and organs, be they pathological or not, microbial products and allergens”. [1] They can be used for prophylaxis purposes, since they correspond to the etiologic agent of some disease used as starting-substance. [2,3] Nosodes are traditionally considered homeopathic medicines because they are prepared according to homeopathic pharmacotechnics using a disease etiologic agent as starting substance; an alternative use to protect and treat animals against various diseases has been tested for several years. [4,5]

The expression live nosode is used to distinguish between nosodes prepared from dead cultures and used in clinical practice according to their pathogenetic effects, and nosodes prepared from non-inactivated (live) cultures; the original model for the latter was live culture of *Neisseria meningitidis*. [6] According to Costa, “dynamized living nosode elicits production of immunizing and blocking antibodies, thus certainly causing exacerbation of natural immunity, and immunological resistance, surveillance and homeostasis”. [3]
Among microorganisms affecting commercial production of chicken, genus *Mycoplasma* is one of the major causes of aviculture harm; this taxon is composed by small prokaryote lacking cell wall organisms. Mycoplasma infections may treated by means of antimicrobial agents in order to improve the overall state of fowl, reduce or eliminate clinical symptoms, reduce or inhibit dissemination of disease and vertical transmission of the etiologic agent, and reduce the mortality rate and the severity of damage caused by pathogenic strains, [8]; however, they may leave residues in treated fowl.

Residue is defined as any compound present in foodstuffs resulting from the metabolism or degradation of veterinary drugs, antimicrobial agents, food additives, heavy metals and pesticides, among others, which pose a risk for dietary safety when their levels surpass the maximum levels recommended by the National Agency of Sanitary Surveillance – ANVISA. [9]

Wilhelm Lux was a veterinarian doctor, professor at Leipzig Veterinary School and contemporary of Samuel Hahnemann, moreover, he was one among the first veterinary doctors to employ biotherapy agents. Lux was called to treat an epidemic of hematic carbuncle; since he lacked homeopathic medicines matching the clinical image of disease, he treated this epidemic with dynamized blood of an ill bovine and obtained positive results. Later on he solved and epidemic of glanders in Hungary by diluting and agitating nasal discharge of an ill horse. In this way, Lux established the basis of biotherapy. [10,11]

**Materials and methods**

**Preparation of live nosode**

Live nosode *Mycoplasmagallisepticum* (strain R ATCC 93-08/19610) – MGR – was prepared in laminar flow using amber-hued vials previously sterilized in autoclave (121°C/30 minutes) according to the technique recommended for live nosodes. [2] Live microorganism culture in suspension containing 3 billion cells per mL was used as starting-substance; serial dilutions up to 30d were performed according to the guidelines of Brazilian Homeopathic Pharmacopoeia. One mL of *M. gallisepticum* culture was diluted in 10.0 mL normal saline, then serial dilution and agitation was performed until 30d. Up to 10d, solvent used was normal saline, 11d was prepared with 5% hydro-alcoholic solution, whereas for higher preparations solvent used was alcohol 60ºGL.

**In vitro inhibition of Mycoplasma growth by live nosode**

To establish the ability of the live nosode to inhibit *M. gallisepticum* growth in vitro, paper disks were impregnated with biotherapy in dilutions 12d and 30d and oxytetracycline aseptically in laminar flow and placed at the center of Petri dishes sown with *M. gallisepticum*, which were incubated at 37°C for 21 days or until characteristic colonies appeared. This test was replicated five times.

**In vitro test of live nosode innocuousness**

Dilutions 1d, 10d, 11d, 12d, 20d, 30d of biotherapy were tested for *M. gallisepticum* growth by means of culture in fluid and solid Frey medium. After inoculation, samples were incubated at 37°C for 21 days; they were rated negative after this time elapsed.

**Protein dosage**

Protein was dosed by means of Folin-Lowry method [12] in mother solution and dilutions 1d, 12d and 30d.
Polymerase chain reaction (PCR)

Dilutions 1d, 5d, 10d, 11d, 12d, 20d, 30d and mother solution (MS) were tested for presence of M. gallisepticum DNA fragments; DNA was extracted from samples by means of phenol/chloroform method following Sambrook et al. [13] After DNA extraction, each sample was concentrated in ethyl alcohol according to Zeugin & Hartley, [14] then centrifuged, the sediment was suspended in Tris-EDTA buffer pH 8.0 and stored at 20º C. DNA amplification reaction was performed in thermocycler for 40 cycles using primers B1 5’ CGT GGA TAT CTT TAG TTC CAG CTG C 3’ and B2 5’ GTA GCA AGT TAT AAT TTC CAG GCA T 3’ (481 pb), to detect M. gallisepticum. Amplified products were added tracking buffer and subjected to electrophoresis in 1.5% agarose gel submerged in TBE O.5X buffer. [13] After electrophoresis, gel was stained with ethidium bromide, results were visualized in a transilluminator under ultraviolet light and photographed.

Results and discussion

In vitro test of live nosode innocuousness

No growth of M. gallisepticum was observed in cultures with dilutions 11d, 12d, 20d and 30d, whereas dilutions 1d and 10d exhibited microorganism growth. This agrees with Costa, [2] who stated that live nosode is innocuous from dilution 12d onwards. Inactivation of MGR is due to the change of solvent from normal saline to alcohol 60º GL (Figure 1, Table 1).

Table 1: Growth of MGR 19610 colonies in several dilutions

<table>
<thead>
<tr>
<th>Dilution/Day</th>
<th>1st day</th>
<th>3rd day</th>
<th>5th day</th>
<th>12th day</th>
<th>17th day</th>
<th>21st day</th>
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</thead>
<tbody>
<tr>
<td>1d</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10d</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>20d</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30d</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Figure 1: Growth of MGR 19610 living nosode in Frey medium
These results agree with Costa, [2] who stated that dilution 12d is absolutely innocuous as to the possibility of causing contamination, therefore, the proposed live nosode in dilution 30d is safe for use.

Results also agree with Siqueira, [15] who assessed cell alterations induced by a new live-type nosode on lines MDCK e j774.g8 and concluded that hemagglutination was able to detect virus titers < 2 HAU/25 μl in supernatant of fertilized eggs inoculated with Influenzinum RC (Roberto Costa), thus confirming absence of infectious virus in dilution 30d. The viral titer found showed that no viral particles able to cause infection were present in dilution 30d.

**In vitro inhibition of Mycoplasma growth by live nosode**

Inhibition halos about 2.0 mm were observed around disks impregnated with live nosode, whereas disks impregnated with tetracycline exhibited 8.0 mm inhibition halos (Figure 2).

![Image of Petri dishes](image1.png)

**Figure. 2.** Inhibition halo in Petri dishes inoculated with *Mycoplasma gallisepticum* and disk impregnated with MGR 19610 live nosode 30d (on the left); inhibition halo in Petri dish with Frey medium inoculated with *Mycoplasma gallisepticum* and disk impregnated with oxytetracycline (on the right).

Costa et al [5] concluded that biotherapy was not able to interfere in the growth rate of yeasts when they developed and assessed the in vitro effects of a new Roberto Costa-type biotherapy against oral candidiasis. In this study, however, 2.0 mm halos were observed, which were considered non-conclusive.

**Protein dosage and polymerase chain reaction (PCR)**

Protein dosage by means of Folin-Lowry method showed absence of protein in dilutions 12d and 30d (Fig. 3).

![Image of protein dosage graph](image2.png)

**Figure 3:** Protein dosage in mother solution and dilutions 1d, 12d and 30d of MGR 19610 live nosode
No DNA fragments compatible with *M. gallisepticum* were found in dilutions 12d, 20d and 30d, whereas microorganism DNA was found in dilutions 1d, 5d, 10d, 11d and mother solution (MS) (Table 2).

Table 2: Presence of microbial DNA residues in different dilutions of MGR 19610 live nosode.

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>Result</th>
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<tbody>
<tr>
<td>MS</td>
<td>+</td>
</tr>
<tr>
<td>1d</td>
<td>+</td>
</tr>
<tr>
<td>5d</td>
<td>+</td>
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<td>10d</td>
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<td>11d</td>
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<td>12d</td>
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<td>20d</td>
<td>-</td>
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<tr>
<td>30d</td>
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Reduction of protein amount with dilution increase was expected, since there is serial decimal dilution from mother solution onwards, reaching landmark 0.00 μg/mg (absence of measurable protein) from the 12th dilution (12d) onwards, where there is neither presence of microbial DNA nor microorganism growth.

These results agree with the findings of several authors, such Arenales, [16] who studied the viability of homeopathy in veterinary medicine applied to fowl production and concluded that homeopathic medication is exclusively energetic, since there is no matter in medicines, and Siqueira, [15] who studied cell alterations induced by a new live-type nosode on lines MDCK e J774.G8, and concluded that readings above 4d were all similar up to 30d and also similar to water 1d, which confirms that there are not anymore viral macromolecules such as nucleic acids or proteins in this dilution. Already Hahnemann had stated the medicines become more powerful when they are diluted and agitated, because this process increases the medicine efficacy, although from the theoretical point of view molecules are no longer present. [17]

Conclusions

On the grounds of this study results, we conclude that MGR 19610 live nosode may be administered to fowls in dilution 30d with no risk of causing infection, since there was no in vitro microbial growth from dilution 12d onwards and the following dynamizations eliminate the presence of matter detectable in protein dosage and PCR assays.

References


Comportamento *in vitro* do bioterápico tipo nosódio vivo *mycoplasmagallisepticum*

**RESUMO**

Como parte do experimento de uma tese de doutorado, um bioterápico do tipo nosódio vivo utilizando o microrganismo *Mycoplasmagallisepticum* estirpe R (ATCC 93-08/19610) foi preparado de acordo com o paradigma desenvolvido por Costa, observando as normas da Farmacopeia Homeopática Brasileira. O nosódio vivo foi testado *in vitro* com a finalidade de avaliar a segurança de sua utilização para imunizar galinhas domésticas (*Gallus gallus*) contra a infecção pelo microrganismo *M.gallisepticum*, e também conhecer seu comportamento sob condições de laboratório. Não houve crescimento de *M. gallisepticum* no cultivo em meio de Frey modificado líquido (caldo) e sólido (placa) das diluições 11d, 12d, 20d e 30d, enquanto que as diluições 1d e 10d mostraram crescimento do microrganismo. Foram observados halos de inibição de cerca de 2,0 mm em torno de discos de papel impregnados com o nosódio vivo em placas de Petri cultivadas com o microrganismo, enquanto os discos impregnados com o antimicrobiano convencional oxitetraciclina apresentaram halos de inibição de 8,0 mm. A dosagem de proteína pelo método de Folin-Lowry identificou a ausência de proteínas nas diluições 12d e 30d, assim como também não foram encontrados traços de DNA microbiano na prova de PCR para as diluições 12d, 20d e 30d.

**Palavras-chave:** Bioterápico; Galinhas; Nosódio vivo; *Mycoplasmagallisepticum*.

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Conducta *in vitro* de nosodio vivo de *mycoplasmagallisepticum*

**RESUMEN**

Una etapa de un proyecto de doctorado, en este estudio fue preparado un nosode vivo del microorganismo *Mycoplasmagallisepticum* cepa R (ATCC 93-08/19610) según el modelo de Costa y las normas de la Farmacopea Homeopática Brasileña. El nosode vivo fue testead *in vitro* para determinar su seguridad cuando utilizado para inmunizar aves domésticas (*Gallus gallus*) contra la infección por este microorganismo e investigar su conducta en condiciones de laboratorio. Fue observado que *M. gallisepticum* no creció en medio modificado de Frey líquido (caldo) y sólido (placa) con las diluciones 11d, 12d, 20d y 30d. Fueron observados halos de inhibición de aproximadamente 2,0 mm alrededor de discos de papel impregnados con el nosode vivo en placas de Petri sembradas con el microorganismo, mientras fueron observados halos de inhibición de 8,0 mm en los discos impregnados con el antibiótico convencional oxitetraciclina. La medición de proteínas mediante el método de Folin-Lowry evidenció ausencia de proteínas con las diluciones 12d y 30d y ausencia de vestigios de DNA microbiano por PCR con las diluciones 12d, 20d y 30d.

**Palabras-clave:** Bioterápicos; Pollos; Nosode vivo; *Mycoplasmagallisepticum*.