

***Original Article***

## **Improvement in the Growth and $\alpha$ -toxin Production of *Clostridium septicum* by Magnesium Sulfate**

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### **ABSTRACT**

*Clostridium septicum*, the anaerobic toxigenic bacterium is the agent that causes dangerous disease in man and animals. There is a lethal toxin of the bacterium namely alpha toxin. The  $\alpha$ -toxin has hemolytic, necrotic and lethal activities. Today, Razi Vaccine and Serum Research Institute of Iran produced the *C. septicum* vaccine in the form of bacterin/toxoid. Because of some problems, the vaccine needs to improve on an industrial scale. The study is going to find an *appropriate* supplement to improve growth and  $\alpha$ -toxin production. Three strains of *C. septicum* (vaccine, NH1 and NH8 strains) were cultured in the basic vaccine media. Magnesium sulfate, Copper, Ferrous, yeast extract, and trace elements plus vitamins' solution were added to the basic vaccine media in different cultures. The effect of the ingredients on the growth was measured by a spectrophotometer and the  $\alpha$ -toxin secretion was assayed by hemolysin test. Growth of the bacterium and  $\alpha$ -toxin secretion were increased by Magnesium (80 mg/l) in NH8 and vaccine strains significantly. The black precipitate was difficult to dissolve in magnesium media that must be solved. Trace elements plus vitamins solution mildly influence on NH1 strain growth and toxin secretion. Other supplements (*Cu*, *Fe*, yeast extract) were not shown any significant changes in the growth and  $\alpha$ -toxin production of *C. septicum*. Overflowing peptone (4%) in the vaccine media, fixes essentials of proteolysis activity, allows the sufficient growth and toxin production without *Cu*, *Fe*, and yeast extract. Due to essentially of Mg for growth, extra magnesium was added for improvement of media culture. The study suggests for Magnesium addition in the *C. septicum* vaccine media during production procedure after precipitation solving problem.

**Keywords:** *Clostridium septicum*, Vaccine media, Alpha toxin, Growth, Magnesium

### **Amélioration de la Croissance et de la Production de Toxine $\alpha$ de *Clostridium septicum* par Magnesium Sulfate**

**Résumé:** *Clostridium septicum* est une bactérie toxigène anaérobie provoquant des maladies dangereuses chez l'homme et les animaux. Il existe une toxine mortelle de la bactérie, à savoir la toxine alpha. La toxine  $\alpha$  a des activités hémolytiques, nécrotiques et mortelles. Aujourd'hui, l'Institut de recherche sur les vaccins et le sérum de Razi en Iran a produit le vaccin contre *C. septicum* sous forme de bactérine / anatoxine. En raison de certains problèmes, le vaccin doit s'améliorer à l'échelle industrielle. L'étude va trouver un complément approprié pour améliorer la croissance et la production de  $\alpha$ -toxines. Trois souches de *C. septicum* (vaccin, souches NH1 et NH8) ont été cultivées dans le milieu vaccinal de base. Du sulfate de magnésium, du cuivre, des ferreux, de l'extrait de levure et des oligo-éléments ainsi qu'une solution de vitamines ont été ajoutés au milieu vaccinal de base dans différentes cultures. L'effet des différents ingrédients sur la croissance a été mesuré par un

spectrophotomètre et la sécrétion de  $\alpha$ -toxine a été testée par test à l'hémolysine. La croissance de la bactérie et la sécrétion de  $\alpha$ -toxine ont été augmentées de manière significative par le magnésium (80 mg/l) dans le NH8 et les souches vaccinales. Le précipité noir était difficile à dissoudre dans les milieux de magnésium qui doivent être résolus. Les oligo-éléments accompagnés d'une solution de vitamines influencent légèrement la croissance de la souche NH1 et la sécrétion de toxines. Les autres suppléments (*Cu*, *Fe*, extrait de levure) n'ont montré aucun effet significatif sur la croissance et la production de l' $\alpha$ -toxine de *C. septicum*. Le surplus de peptone e (4%) dans le milieu vaccinal, fixe l'essentiel de l'activité protéolytic et permet ainsi la production de toxines sans l'ajout de *Cu*, *Fe* et l'extrait de levure. En raison rôle essentiel de *Mg* pour la croissance, du magnésium supplémentaire a été ajouté pour améliorer la culture des milieux. Notre étude suggère l'ajout du magnésium dans les milieux vaccinaux de *C. septicum* pendant la procédure de production après la résolution du problème de précipitation.

**Mots-clés:** *Clostridium septicum*, Milieux vaccinaux, Toxine alpha, Croissance, Magnésium

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## INTRODUCTION

*Clostridium septicum* (*C. septicum*) is an anaerobic gram-positive bacterium. The bacterium is a causative agent of some acute lethal diseases in man and animals. Animal disease syndromes are slightly less known than in man (Vazquez-Iglesias et al., 2017). Broxy and gas gangrene or malignant edema with a high mortality rate toxemia are the important infections in the animals (Gazioglu et al., 2018). *C. septicum* is the cause of acute myonecrosis, meningitis, arthritis, bacteremia, osteomyelitis, myocarditis and other infections in man (Larson et al., 1995; Nanjappa et al., 2015; Mallozzi and Clark, 2016; Ramphal et al., 2018), and are often accompanied with colonic and hematological malignancy in human (Nanjappa et al., 2015; Mallozzi and Clark, 2016; Ramphal et al., 2018). *C. septicum* aortitis is a rare but fatal infection association with occult malignancy (Mizrahi and Halpern, 2016). *Clostridium perfringens* type A, the most commonly isolated of clostridia is in combination with *C. septicum* in 4% of cases (Salari Sedigh et al., 2015). Today, the prevalence of the bacterium is low in man. From 161 asymptomatic community-dwelling stool samples of adults and 192 hospitalized diarrhea patients, all of samples were negative (Chakravorty et al., 2015). There isn't any documented prevalence of *C. septicum*

in Iran. Alpha toxin, the main dangerous toxin, has some activities include necrosis, hemolysis, and lethality (Tweten, 2001; Kennedy et al., 2005; Chakravorty et al., 2015). In fact,  $\alpha$ -toxin is a hemolysin that attaches to a cell receptor called glycosphosphatidylinositol (GPI) and during a cascade process, it causes a cleft in the cytoplasmic membrane, releases the ions from cells, causes cell death (Ballard et al., 1992; Ballard et al., 1993; Gordon et al., 1997; Sellman and Tweten, 1997; Takeuchi et al., 1997; Tweten, 2001; Wichroski et al., 2002). All of the diseases caused by *C. septicum* such as malignant oedema is acute, not more time to treatment except the prevention by the unique solving way, vaccination program. The major immunogenic ingredient of the vaccine, toxoid, is the changed toxin after inactivation. For better vaccine and toxoid production, there is a need for a strong strain of *C. septicum* that produces more toxin. Providing the best conditions for growth and production of the toxin requires several tests. In certain circumstances, *C. septicum* may grow well without much toxin secretion. The growth and toxin production are not correlatives necessarily. Toxin secretion, in addition to the proper growth, is responsible for the gene toxin code activation in the strong strain. Understanding of the best condition for the toxin production leads to the identification and

optimization of the parameters for better vaccine production. In this study, we tried to improve the usual vaccine media by adding iron, copper, magnesium, trace elements-vitamins and yeast extract to affect not only on growth but also on toxin production.

## MATERIAL AND METHODS

**strains.** Two local strains of *C. septicum* called NH1, NH8 plus vaccine strain were used in the study. These three strains (along with seven other strains) were initially confirmed by PCR (Hemmati et al., 2006) and then their nucleic acids of the  $\alpha$ -toxin gene were sequenced (Fathi Najafi and Hemmati, 2007). The results were recorded as EU482197 for strains vaccine strain, EU482188 for NH1 and EU482195 for NH8 strain in the gene bank. The two local strains NH1 and NH8 in the earlier studies showed the highest production of  $\alpha$ -toxin.

**Media.** Magnesium sulfate, copper, ferrous, yeast extract, and trace elements-vitamins' solution were added to the basic vaccine media. Media were used in the study, prepared according to Table 1. The trace element-vitamin solution was used as described for *Clostridium perfringens* vaccine production media. Trace-elements composition contain:  $\text{SO}_4\text{Fe}, 7\text{H}_2\text{O}$ ;  $\text{SO}_4\text{Cu}, 5\text{H}_2\text{O}$ ;  $\text{SO}_4\text{Zn}, 7\text{H}_2\text{O}$ ;  $\text{SO}_4\text{Mg}, 7\text{H}_2\text{O}$ ;  $\text{CL}_2\text{Mn}, 7\text{H}_2\text{O}$  and Vitamins include biotin; thiamin; pyroxidin; B12; *nicotinic* acid in the amount of used for vaccine production (Ardehali and Darakhshan, 1976). All media were sterilized at 121 °C for 15 min using an autoclave. The trace element-vitamin solution was filtered and then added to the sterile medium.

**Culturing.** The strains were converted to an active form after 24 hr incubation in thioglycollate broth at 37

°C. One per cent of the broth was added to the test media, incubated for 24 hours at 37 °C in anaerobic jar contain gas pack A (Merck, German). Then, the growth rate and  $\alpha$ -toxin production were evaluated in different defined media.

**Growth rate.** According to Hasan and Hull's method (Hasan and Hall, 1976), one ml of 24 hr culture solution was centrifuged at 10,000 rpm for 5 minutes and was suspended in 3 ml normal saline and optical density (OD) at 600 nm was achieved by spectrophotometer (Shimadzu, Japan) and calculated in one ml.

**Hemolysin test.** 20 ml of sheep fresh blood was prepared in 3.8% sodium citrate and washed three times in phosphate buffer (pH=7) and centrifuged each time in 1000 rpm for five minutes. Samples were centrifuged at 12,000 rpm for 5 minutes and 100  $\mu\text{l}$  of the supernatant was mixed in one ml of 10%RBC. The positive control was prepared by adding 100  $\mu\text{l}$  of distilled water to one ml of 10%RBC. The negative control was also made by adding 100  $\mu\text{l}$  of PBS to one ml of 10%RBC. All samples incubated at 37 °C in a water bath for 20 minutes. Finally, all of them were centrifuged at 10,000 rpm for three minutes and the OD was determined at 545 nm. The percentage of hemolysis was determined using positive and negative controls as 0 and 100 per cent hemolysis respectively. One hemolysin unit(HU) is equal to reduction of one percent hemolysis of RBC against control positive at one minute in one ml. The test was performed duplicate for different media and strains and the results compared to each other.

## RESULTS

Iron could increase the growth rate and  $\alpha$ -toxin secretion of strain NH1 mildly but had no effect on another two strains. The trace-vitamins solution just for the vaccine strain had a positive effect on the growth

increase the growth and  $\alpha$ -toxin production in vaccine medium. The results show that *C. septicum* is able to supply its iron need from peptone of medium, which can be considered as a rich source of the iron. The

**Table 1.** list of media with ingredients, pH and sterilization condition

no	Media name	Ingredients	pH	Sterilization
1	vaccine production basic medium	peptone 4%, cysteine hydrochloride 0.05%, sodium chloride 0.5%, glucose 0.5%	7.5	121°C for 15 min
2	vaccine production basic medium+ Fe	Basic medium plus 1 mg/l FeSO4	7.5	121°C for 15 min
3	vaccine production basic medium+Cu	Basic medium plus 0.5 mg/l CuSO4	7.5	121°C for 15 min
4	vaccine production basic medium+Mg	Basic medium plus 80 mg/l MgSO4	7.5	121°C for 15 min
5	vaccine production basic medium+yeast extract	Basic medium plus 0.25% Yeast Extract	7.5	121°C for 15 min
6	vaccine production basic medium + trace elements-vitamins	Basic medium plus 0.7% of trace-elements + vitamins: (SO4Fe,7H2O; SO4Cu,5H2O; SO4Zn,7H2O; SO4Mg,7H2O; CL2Mn,7H2O Biotin; Thiamin; Pyroxidin; B12; Nicotinic acid)	7.5	121°C for 15 min(trace-elements + vitamins after filtration added to sterile medium)

and secretion of the toxin. While the growth and  $\alpha$ -toxin secretion of NH8 and NH1 strains would not have affected by the trace-vitamins solution. Adding yeast and copper extracts did not stimulate the growth and secretion of the toxin compare to the control (Figures 1 and 2). Magnesium could significantly increase the growth rate and the  $\alpha$ -toxin in the NH8 strain. The Mg showed a similar effect on the vaccine strain, but it is not as high as the NH8 strain. The growth and the toxin of NH1 strain did not affect by Mg compare to basic control medium (Figures 1 and 2).

## DISCUSSION

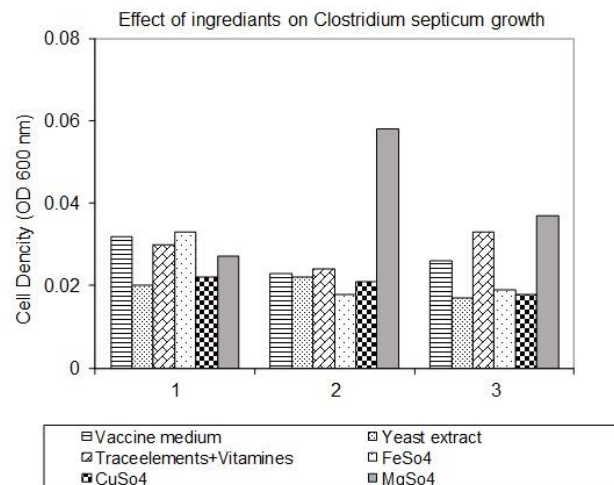
The study is going to increase growth and toxin production of *C. septicum* by adding iron, copper, magnesium, trace elements and yeast extract to the usual vaccine media. Some of the ingredients have a side effect on the bacterium growth in upper concentration like copper. Although the growth and toxin production are not correlatives necessarily, the pattern of the growth rate and  $\alpha$ -toxin production as the results, like together in the most tests. The iron is necessary in one mg/l concentration for the growth and  $\alpha$ -toxin secretion of *C. septicum* (Smith, 1975). According to results of the study, the iron could not

vaccine strain of *Cl. perfringens* can only produce enough toxin by adding the trace element-vitamin solution in vaccine production procedure. Each ingredient of the solution is a necessary cofactor to activate of the toxins secretion in *Cl. perfringens*. Base on the result of the study the trace element-vitamins solution could not increase the growth and  $\alpha$ -toxin secretion of *C. septicum* significantly in vaccine media except a little on vaccine strain. Generating a large amount of toxin in vaccine production process means producing a proper vaccine after the inactivation of toxin, changed to toxoid. However, as the trace-vitamin solution cost and the long process of its preparation and sterilization, and the little increase in the growth and production of toxin, there is no economic logic to use expensive solution for little benefit. But *if there was no toxin production in C. septicum except with the trace-vitamin solution as like as Cl. perfringens vaccine, it could be advisable.* Copper (Cu) is an essential cofactor in enzymes that catalyze redox reactions in all aerobic organisms. Cu not only cannot stimulate growth and toxin secretion of *C. septicum* in comparison to the basic vaccine medium but also decreased them. Copper has an antibacterial activity but the copper concentration used in the study is necessary for some

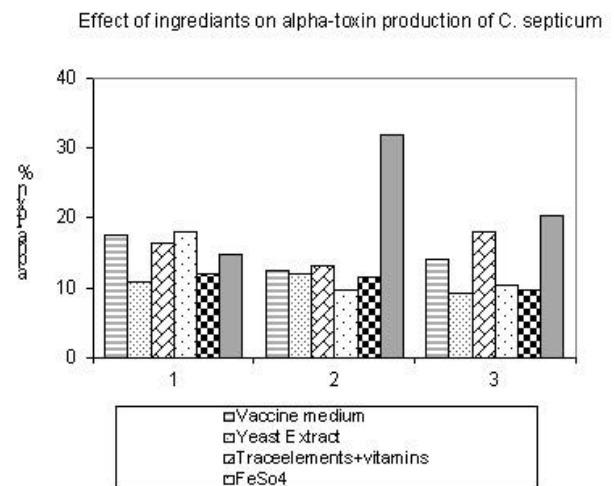
enzymes activity in the bacteria (Ladomersky and Petris, 2015). Base on the result, there is no reason to add yeast extract in the *C. septicum* vaccine basic media for any change in growth and toxin production. Magnesium (80 mg / L) could significantly increase the growth rate and the  $\alpha$ -toxin production of NH8 strain. Mg also had the same effect on the vaccine strain. Although Mg increases the growth and toxin production, the use of Mg has a problem. Some black sediment was seen in magnesium-containing media after autoclaving in the bottom of the flax. The black sediment, in addition, to make an inappropriate appearance of the production, can create a blockage into the needle during the vaccine injection. Magnesium could be recommended for *C. septicum* vaccine production and its problem must be solved. If magnesium would have been a perfect effect on the vaccine potency test as the same as the effect on the  $\alpha$ -toxin production, it seems it is valuable to solve the problem with the black sediment. It is believed that the vaccine media is the proper medium for toxin production of *C. septicum*. Hasan and Hall also noted that casein hydrolyzed in a concentration of 13.7 g/l fixed all the need of the bacterium to amino acids. Also, the peptone (0.05%) and yeast extract (0.05%) fixed all the need of *C. septicum* to vitamins (Hasan and Hall, 1976). *C. septicum* has enormous enzymatic abilities, which could absorb the various growth factors (except magnesium) from the basic vaccine media. This means that the bacterium can fix all the requirements for the growth and toxin secretion, using the high peptone of the medium (4%). However, the glucose (0.5%) seems to be important to start faster growth and the cysteine hydrochloride (0.05%) to create suitable regenerative conditions for the anaerobic growth.

Since the use of magnesium could increase the toxin production of *C. septicum* as the same as the NH8 strain, it can remove the disadvantages of using it, include the cost of raw materials and resolve of the sediment. Although a new substance utilization in vaccine production procedure requires extensive

research, as a suitable candidate, the use of magnesium in the medium of *C. septicum* recommended.



**Figure 1.** Effect of ingredient changes in basic vaccine medium on growth of *Clostridium septicum* NH1(No.1), NH8(No.2) and vaccine(No.3) strains



**Figure 2.** Effect of ingredient changes in basic vaccine medium on  $\alpha$ -toxin secretion of *C. septicum* NH1(No.1), NH8(No.2) and vaccine(No.3) strains

### Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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## Authors' Contribution

Study concept and design: Hemmaty, M.; Fathi Najafi, M.

Acquisition of data: Hemmaty, M.; Zibae, S

Analysis and interpretation of data: Fathi Najafi, M.; Navidmehr, J.; Hemmaty, M.

Drafting of the manuscript: Hemmaty, M.

Critical revision of the manuscript for important intellectual content: Fathi Najafi, M.; Hemmaty, M.; Farhoodi, M.

Statistical analysis:

Administrative, technical, and material support: Fathi Najafi, M.; Navidmehr, J.; Zibae, S.; Hemmaty, M.

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