

## Short Paper

# Measuring of free endotoxin in alum-precipitated vaccine of haemorrhagic septicaemia by limulus amebocyte lysate test

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## Summary

Haemorrhagic septicaemia (HS) vaccine which is prepared in Razi Institute is used in endemic areas of Iran. Aluminum-hydroxide gel was used as adjuvant for preparing this vaccine. Post-vaccinal shock reactions were the main complaint after use of this vaccine. In a previous study, we could improve the vaccine by alum-precipitation *Pasteurella multocida* cells and removing the liquid phase. In this study, the amount of free endotoxin in aluminum-hydroxide and alum-HS vaccines was determined. It was found that endotoxin level was considerably decreased from 0.22 EU/ml to 0.03 EU/ml after alum-precipitation.

**Key words:** Haemorrhagic septicaemia vaccine, Endotoxin, Shock reaction, LAL test

## Introduction

Pasteurellosis in cattle and buffalo is a specific form of acute and fatal disease commonly referred to as “haemorrhagic septicaemia” (HS) which is caused by *Pasteurella multocida* serotypes B2 and E2 (De Alwis, 1993). HS is considered economically as the most important disease in the South East Asia, including Indonesia, Philippines, Thailand, Malaysia, the Middle East, North East, Central and South Africa (Rimler and Rhoades, 1989; Verma and Jaiswal, 1998). In Iran, the disease has an enzootic nature and has been responsible for some bovine mortality. HS is endemic in Khouzestan, Guilan, Mazandaran, Ardebil, and West and East Azerbaijan (Kaweh *et al.*, 1960; Baharsefat and Firouzi, 1977).

Vaccination is the accepted method for control of HS throughout the world. Aluminum-hydroxide gel adjuvant vaccine is used for prevention of HS in Iran. The vaccine is prepared from a local isolate of *P. multocida* B2 in fermentor (Razi Institute,

Karadj, Iran). An anaphylactic shock reaction has been reported following administration of aluminum-hydroxide gel HS vaccine in cattle and buffalo (Vesal and Maleki, 2000; Moazeni Jula, 2001). We have improved the vaccine by precipitation of bacterial cells with alum and substitution of the liquid phase by sterile normal saline solution. The experiments showed that the improved vaccine is enough safe and potent to use as a tool for HS prevention in the field (Jabbari and Moazeni Jula, 2002, 2004). As it is well-known that the main anaphylactic agent of such killed bacterial vaccines is the free endotoxin, the objective of the present study was to determine the changes in the endotoxin level during the process of HS vaccine preparation.

## Materials and Methods

### Vaccine preparation

The modified alum-precipitated vaccine was prepared as explained previously (Jabbari and Moazeni Jula, 2002). Briefly, a

dense culture ( $4.5 \times 10^9$  CFU/ml) of *P. multocida* serotype B2 (local isolate) was prepared in triptose phosphate broth. The bacterial cell was precipitated by adding a 10% alum solution. The supernatant was discarded and replaced by sterile normal saline. The prepared vaccine was kept at 4°C until use. The safety trials of the vaccine were conducted in mice, rabbits, guinea pigs, cattle and buffalo. The methods and results of safety tests have been explained elsewhere (Jabbari and Moazeni Jula, 2002).

### Limulus amebocyte lysate test

The limulus amebocyte lysate (LAL) test for measuring the level of free endotoxin was conducted by a commercial standard kit, manufactured by Charles River Endosafe Company (USA). The assay was performed using chromogenic microtiter method according to the manufacturer's directions.

## Results

The results of LAL test for measurement of endotoxin level are shown in Table 1. It was found that the amount of lipopolysaccharide (LPS) in HS vaccine was considerably decreased from 0.25 EU/ml to 0.03 EU/ml, after alum-precipitation.

**Table 1: The results of chromogenic endpoint LAL test method, showing the endotoxin concentration in HS vaccine**

Sample	Absorbance at 405 nm	Endotoxin concentration EU/ml
<i>P. multocida</i> (8 hrs) culture	0.481	0.16
Formalized culture	0.697	0.25
Aluminum-hydroxide HS vaccine	0.570	0.22
Improved (alum) HS Vaccine	0.198	0.03

## Discussion

Shock reactions following administration of HS aluminum-hydroxide adjuvant vaccine have been previously reported (Bain, 1963; Rhoades and Rimler, 1987). It was demonstrated that LPS (endotoxin) of Gram-negative bacteria was able to produce anaphylactic reactions in tested animals. Injection of 50–100 µg/kg of *E. coli*

endotoxin induced sever anaphylactic reactions in calves. The severity of the shock reactions in response to a given dose of endotoxin was related to the degree of previous sensitization (Wray and Thomlinson, 1972a, b).

In Iran, aluminum-hydroxide gel vaccine induced shock reactions in 3 to 12% of vaccinated animals (Vesal and Maleki, 2000; Moazeni Jula, 2001). In a previous study, we have improved the vaccine by precipitation of bacterial cells with potassium aluminum sulfate (alum) and substitution of the liquid phase by sterile normal saline solution. The vaccine passed the safety tests in laboratory animals (mouse, guinea pig and rabbit) according to OIE standard protocols (OIE standard commission, 2000). Also the improved vaccine was safe in farm animals. No noticeable reactions were happened after vaccination of 1,229 cattle and 775 buffaloes in Khouzestan province (Jabbari and Moazeni Jula, 2002). Our findings in the present study showed that the LPS concentration was considerably reduced in the improved HS vaccine (Table 1). However, the accepted amount of endotoxin level in HS vaccine were not still reported.

We used the chromogenic endpoint LAL test for measuring the endotoxin level in HS vaccine. This test is the most sensitive and specific means to measure the bacterial endotoxin (Ogikubo *et al.*, 2004). The LAL is a reagent prepared from the washed blood cells (amebocytes) of *Limulus polyphemus*, the horseshoe crab. The LAL contains an enzyme system which is activated in the presence of endotoxin. The activated enzymes split off para-nitro aniline (pNA) from the chromogenic substrate to produce a yellow colour. The pNA release is measured photometrically at 504 nm which gives the opportunity to quantify the amount of existing endotoxin in the system (Dawson, 1999). There was a coordination between endotoxin level of HS vaccine measured by LAL test and clinical observations in farm animals, as reported previously (Jabbari and Moazeni Jula, 2002).

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