Factors affecting the expression of *Clostridium chauvoei* antig used for vaccine preparation and their roles in the imm response

Fayez, M. M., EL-Helw, H. A., Toughan, H. M. & Abdella, Y. A.
Veterinary Serum and Vaccine Research Institute- Abbasia- Cairo

Abstract

The effect of using two different growth media with different incubation period antigen expression of *Clostridium chauvoei* was studied. It was found that in the growth media the incubation period of 26 hours was beneficial for optimum grow *C. chauvoei*. The protein profile of *C. chauvoei* cells revealed that there are 5 pr bands expressed during growth of *C. chauvoei* at molecular weight (200kDa, 170 150kDa, 119kDa and 56kDa), but the intensity of the protein band at 56kDa quite l in culture incubated for 18 hours. The immune response of the already commer used vaccines inoculated in guinea pigs indicated that there is no differen protection percent between the two media, but difference shown at different incub periods in which the protection percentage was 80%, 100%, and 90% at 18hour hours, and 48 hours respectively. Sera collected before challenge from vaccir guinea pigs which resist challenge recognized cellular antigens of *C. chauvoei* a same molecular masses in western blotting.

Introduction

Clostridium chauvoei is an endo-spore forming Gram-positive anaerobic bacte which is the pathogenic agent of blackleg. It is an anaerobic, sporulated, flagell and rod-shaped bacterium. Blackleg is an acute disease characterized by mus inflammation, fetal toxemia, and high mortality rates in ruminants (nearly 100%) apparent primary port of entry is oral and during grazing on pasture contaminate spores. Cases of blackleg can occur year after year on contaminated pastures (B et al., 2009). Several outbreaks have occurred in recent years (Ontiveros Corpus et al., 2008). Moreover, it is considered a toxinfection disease in bovine and ruminants, always lethal and involving considerable economic losses. S bacteriological, biochemical, immunological studies permitted to isolate and identif major soluble antigenic proteins of this bacterium. It's a protein fragment of 70 weight; the 19th fraction is excreted by the bacteria in a suitable culture medium 19th fraction of extracellular medium leads to antibodies production on guinea revealed by the ELISA/antibody test (Mbengue, 2008).

Immunity of *C. chauvoei* is generally considered to be anti-bacterial rather antitoxic, it is therefore important to immunize against *C. chauvoei* with somatic flagellar antigens, although some toxins and other extracellular antigens suc α-toxin, β-toxin, and σ-toxin so blackleg vaccine are used as completely forma cultures (Micalizzi and De Guzman, 1997). Determination of the protective epitop the flagellin of *C. chauvoei* would be important for the development of an effica defined vaccine against blackleg (Kojima et al., 2000). Also, a great variability i protective capacity for different *C. chauvoei* strains has been reported, since bla is considered an enzootic disease, the incorporation of local strains with immunoprotective capacity in vaccine formulations is encouraged (Mattar et al., 20

C. chauvoei produce more than one toxic substance. The number and type of components vary, among other things, with the strain of the organism, the c medium used, and the length of the incubation period (Moussa, 1959). Salts su ferric, ammonium and potassium are factors influencing production of good bac vaccines; with *C. chauvoei* such salts have been found to stimulate fermentatio agressin production (Mhoma, 1985). The relation between culture condition and p expression has been widely reported for a number of factors, such as iron limit phosphate starvation, osmotic and heat stress (Cortinas et al., 1994). It is know for some microorganisms, the expression of different cellular and extra-c proteins, lipoproteins and capsular polysaccharides depends on growth sta cellular density (Mounier et al., 1997; Ramamoorthy and Philipp, 1998). Transitio reversion of *C. chauvoei* flagella may be due to phase variation rather than mutati view of its very high frequency, and also its oscillation between fixed alternatives are characteristic of motility and flagellation (Tamura et al., 1995).

Immunity to *C. chauvoei* is considered to be mainly anti-cellular, and also antitoxi a role in the immunity against the disease and for this reason there is s information about the immunogenicity of extracellular proteins. In this work variatic protein profiles at different growth phases, immune response by plate agglutinatio and protective capacity of *C. chauvoei* strain against challenge in guinea pigs wester blotting to the sera of vaccinated guinea pig which resist the challenge are reported to establish whether *C. chauvoei* immune-protective antigens are re to the growth phase of different cultures media or not.

Materials and Methods

1-Bacterial Strains:

Local isolated strain of *C. chauvoei* was used for vaccine preparation obtained Anaerobic Research Department, Veterinary Serum and Vaccine Research Institu

2-Animals:

2.1. Guinea pigs

Fifty Guinea pigs weighted about 400-500g, obtained from Veterinary Serum Vaccine Research Institute, where thirty five were used for evaluation of *C. cha* vaccines and fifteen were used for safety test and determination Minimum lethal of spore suspension.

3-Growth media:

Two different growth media were used:

3.1. Medium (1): *C. chauvoei* strains were cultured in a clostridia medium describe **(Cortinas et al., 1994)** it is comprised of proteose-peptone; yeast extract; Mg $7H_2O$; Cysteine; Calcium Chloride; glucose and K_2HPO_4. The last two compor were sterilized separately and added to the basal medium after sterilization. Th was adjusted to 7.3.

3.2. Medium (2): conventional production medium comprised of peptone; so chloride; Di sodium hydrogen phosphate anhydrous; thioglycolate, and glucose.

The two media were inoculated with *C. chauvoei* and incubated at 37°C for 18hr, and 48 hr, at which growth of each was monitored by optical density determinatio 570 nm (OD_{580}) with a Spectronic 20 spectrophotometer (Bausch pLomb, New USA).

4- SDS- Polyacrylamide gel electrophoresis:

SDS-Polyacrylamide gel electrophoresis (PAGE) for growing *C. chauvoei* bact the various conditions was conducted by the discontinuous buffer syst **(Sambrook et al., 1989)** with a 5% Polyacrylamide stacking gel and a polyacrylamide seperating gel. Molecular mass markers (wide range stained m were obtained from Sigma. Gels were run on a Protean vertical slab electroph cell containing Tris-glycine electrophoresis buffer (25mM Tris [pH 8.3], 250mM g 0.1% SDS) (Bio-Rad Laboratories, Richmond, CA, USA) and protein bands visualized by Coomassie Brilliant Blue stain.

5-Vaccine Preparation:

Six types of *C. chauvoei* vaccines were prepared at different growth medi different incubation period according to (Roberts, 2000). Cultures were then inac by using 0.5% formalin incubated at 37°C for a period of 5-10 days for co inactivation and then 1% aluminum potassium sulfate was added as an ad Sterility and safety tests for the prepared vaccines were applied according to (pharmacopoeia, 2009).

6- Potency of prepared vaccines:

6.1. Vaccination schedule:

Thirty five guinea pigs were divided into 7 groups, five guinea pigs for eac groups were vaccinated with 6 types of *C. chauvoei* vaccines and the seventh remained as control group as shown in table (1).

Serum samples were collected from all groups 24 hrs before challenge.

Table (1): Vaccination schedule of different *C. chauvoei* vaccines:

G. pigs group No.	Vaccine No.	Media used for vaccine preparation	Incubation period	First dose	Second dose	Route of vaccination
1	1	Medium(1)	18 hours	5 ml	3 ml	S/C
2	2	Medium(2)	18 hours			
3	3	Medium(1)	26 hours			
4	4	Medium(2)	26 hours			
5	5	Medium(1)	48 hours			
6	6	Medium(2)	48 hours			
7	(control group)	-	-	-	-	-

6.2. The Potency Test:

6.2.1. Plate Agglutination Test:

The antibody titers against *C. chauvoei* in sera of vaccinated guinea pigs challenge was determined by Plate agglutination test according to (Cla Macheak, 1972).

6.2.2. Challenge test:

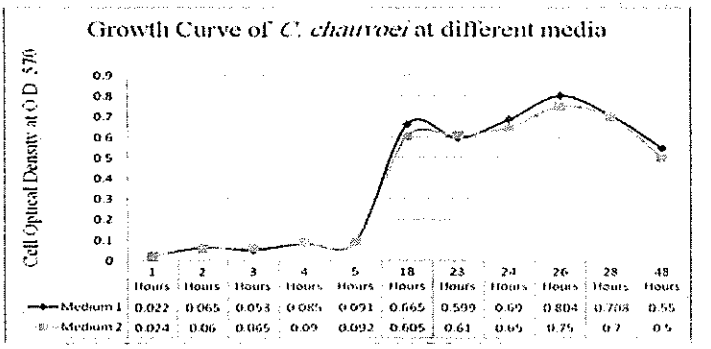
Fourteen days after the second dose, both vaccinated and non-vaccinated gr guinea pigs were challenged with 0.1ml intramuscularly of spore suspensio *chauvoei* containing guinea pigs 10 LD_{50} according to (Micalizzi and De G 1997).

7- Immunoelectroblotting (Western Blot):
Enzyme assisted immunoelectroblotting was done according to (Rybicki and Pu 1996), to sera of vaccinated guinea pigs with freshly electrophoresed S polyacrylamide gel of *C. chauvoei* bacterin antigens.

- Antigens were blotted on nitrocellulose membrane
- Serum samples were applied (first antibody)
- Anti-guinea pig peroxidase conjugated were applied (second antibody)
- Blots were visualized by Diaminobenzidine (insoluble substrate) (sigma D4293).

Results and Discussion

An effective amounts of *C. chauvoei* bacterins is the amount required to genera sufficient circulating antibody to prevent or reduce disease symptoms. It is us expressed in terms of opacity or absorbency units (O.U. or A.U., respectively). T units are based on the optical density (O.D.) of the culture, as measured at a sui wavelength. The O.D. value is then multiplied by the volume of the culture in one of vaccine as shown in *C. chauvoei* (Roberts, 2000).
Figure (1): Growth curve of *C. chauvoei* during various incubation periods using types of growth media (1) and (2):

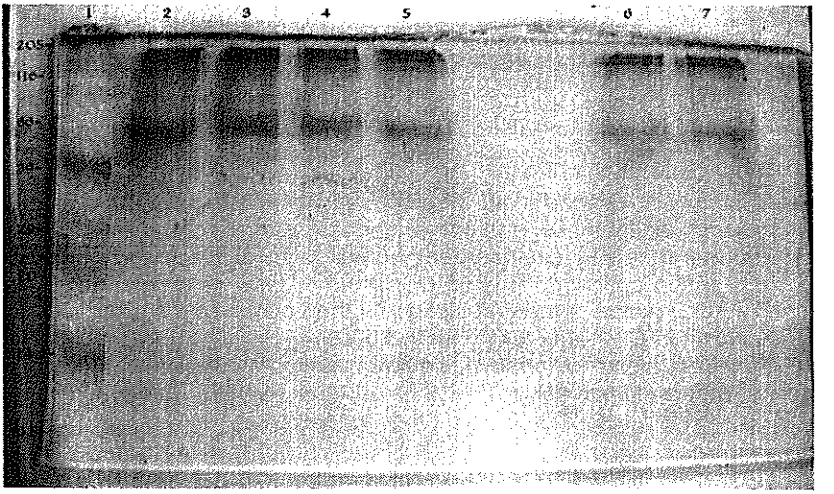


The effect of incubation periods and different clostridial media on the growth o *chauvoei* revealed that the incubation period of 26 hours and medium supplied glucose, yeast extract, magnesium sulphate, and calcium chloride (medium 1) beneficial for optimum growth of *C. chauvoei* as shown in figure (1).

The protein patterns of *C. chauvoei* cultivated in various incubation periods using types of growth media were analyzed in order to establish whether proteins differentially expressed during the growth cycle in vitro, the protein profiles show high percentage of similarity among different types of media, as shown in figure (2) table (2) , there are 5 protein bands expressed during growth of *chauvoei* at molecular weight (200kDa, 170kDa, 150kDa, 119kDa and 56k although there is no difference in expression of protein on different types of media

the intensity of the protein band at 56kDa quite lower in culture incubated for than incubated for 26 hours and 48 hours.

Figure (2): SDS-PAGE Protein profiles of *C. chauvoei* cultivated in various ir periods using two types of growth media (1) and (2)



Lane (1): Molecular weight marker – lane (2): cells from medium (1) at 26 hrs - (3): cells from medium (2) at 26 hrs - lane (4): cells from medium (1) at 48 hrs (5): cells from medium (2) at 48 hrs - lane (6): cells from medium (1) at 18 hrs (7): cells from medium (2) at 18 hrs

Table (2): Protein analysis of *C. chauvoei* cells at different incubation periods on SDS-PAGE

		Protein concentration					
Rows	M. W.	Medium 1 26 hrs	Medium 2 26 hrs	Medium 1 48 hrs	Medium 2 48 hrs	Medium 1 18 hrs	Me 18
R1	200 kDa	14.093	10.96	10.963	8.842	8.1197	8.6
R2	170 kDa	9.32	9.399	8.2069	9.1796	7.639	6.3
R3	150 kDa	6.393	6.0687	6.0437	5.243	4.576	3.3
R4	119 kDa	3.9017	3.412	2.579	2.439	2.3915	1.7
R5	56 kDa	14.267	12.663	12.619	11.8	9.7122	9.5

M. W.: Molecular weight in kDa (kilo Dalton)

The vegetative cells of *C. chauvoei* possess two known agglutinogens, a he somatic O antigen common to all strains, and a heat-labile flagellar H antige antigen has been shown to be unimportant as a protective antigen, whereas, strains, the heat stable O antigen appears to be the main protective antigen (and Hamilton, 1975).

The prepared *C. chauvoei* vaccines when inoculated in guinea pigs revealed t agglutination antibody titer against *C. chauvoei* in sera of vaccinated guinea p before challenge appeared to be similar in the two media at the same incubation per as shown in table (3) and the most higher titer for both media was at 26ho incubation.

Table (3): Agglutinating antibody titer of *C. chauvoei* in sera of vaccinated guinea pigs

Types of vaccines	Agglutination titers of vaccinated guinea pigs*
Vaccine (1)	0.5 µl
Vaccine (2)	0.005 µl
Vaccine (3)	0.04 µl
Vaccine (4)	0.5 µl
Vaccine (5)	0.005 µl
Vaccine (6)	0.04 µl
Control group	-

* Agglutination titer is defined as the number of micro liters (µl) of serum required provide definite agglutination (75%) with 30µl of standard antigen.

The prepared *C. chauvoei* vaccine at different incubation periods and different gro media when inoculated in guinea pigs revealed that there is no difference in protec between the two media, but the difference was shown at different incubation period which the protection % was 80%, 100%, and 90% at 18hours, 26 hours, and 48 ho respectively and all of control group were die as shown in table (4).

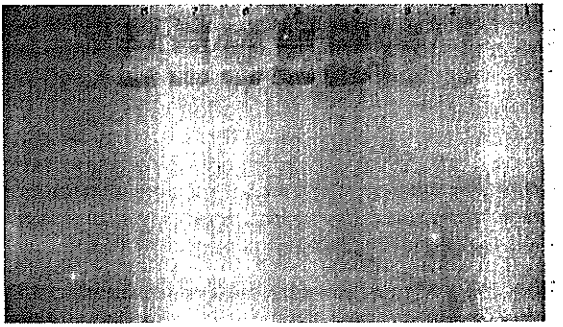
Table (4): Challenge test of vaccinated guinea pigs with 10 LD_{50} spore suspensior *C. chauvoei*

Types of vacines	No. of inoculated Guinea pigs	No. of survived Guinea pigs	Protection %
Vaccine (1)	5	4	80%
Vaccine (2)	5	5	100%
Vaccine (3)	5	5	100%
Vaccine (4)	5	3	60%
Vaccine (5)	5	5	100%
Vaccine (6)	5	5	100%
Control group	5	0	0%

The stationary phase is the most suitable for obtaining protective immunogens ir *chauvoei* strains of different protective capacity, since the highest protection and titers were obtained in this phase (Mattar et al., 2002). Partially purified cell-f supernatants and those concentrated 10 times by ultrafiltration (C-CFS), obtaine the early stationary phase of growth, and induced a strong immunoprotec response, even at low doses, that was more marked for *C. chauvoei* strain AT 10092. With C-CFS (Concentrated- Cell Free Supernatants) formulations, a c relationship was observed between IgG titres, protective capacity and concentratio the antigen doses, indicating a specific immune response (Mattar et al., 2007).

Differences in sera immunoreactivity and protective capacity obtained in d growth phases in vaccinated guinea pigs that resist challenge were tested by W blot. It was possible to identify five immunodominant bands, which reacted v tested sera, indicating that the vaccination of guinea pigs with 6 type of vaccines to the production of antibodies that recognize 200kDa, 170kDa, 150kDa, 119kD 56kDa *C. chauvoei* proteins in SDS as shown in figure (3). So it could be assum they are conserved antigens of *C. chauvoei*, deserving to put in considera potential candidates for *C. chauvoei* vaccine preparation. This result co accordance with (Tanaka et al., 1987) where they done immunoblotting anal purified flagella with five monoclonal antibodies, they found all the five mon antibodies reacted with several proteins, showing one clear band of 56 kDa, corresponded to the flagellin monomer, and at least three weak bands rangir 120-200 kDa are polymeric forms of the flagellin monomer, as described previou

Figure (3): Western blotting of sera of vaccinated guinea pigs against *C. chauvoei* antigens



So from the previous study, it could be concluded that constituents of medium c yeast extract; $MgSo_4.7H_2O$; Calcium Chloride and Potassium Chloride and inc at $37^{o}C$ for 26 hours is beneficial for expression of *C. chauvoei* antigens, v useful in protection against blackleg, so it should be recommended for prepar blackleg vaccine.

References

Bagge, E., Lewerin, S.S. & Johansson, K.E. (2009). Detection and identification by PCR of C *chauvoei* in clinical isolates, bovine feces and substrates from biogas plant. Acta Vet Scar 8.

British Pharmacopoeia (2009). "Veterinary Supplement." Strasburg: Council of Europe, 2 9780113227990.

Chandler, H.M. & Hamilton, R.C. (1975). The preparation of immunogenic cell walls from highly strain of *Clostridium chauvoei*. J. Gen. Microbiol, 88, 27-35.

Claus, K. D. & Macheak, M. E. (1972). Preparation of a *C. chauvoei* antigen and determination of immunity by plate agglutination test. Am. J. Vet. Res., 5, 1045-1052.

Cortinas, T.I., Micalizzi, B. & de Guzman, A.M.S. (1994). Influence of culture conditions on gr protective antigenicity of *Clostridium chauvoei*. J. Appl. Bacteriol., 77, 382-387.

Kojima, A., Uchida, I., Sekizaki, T., Sasaki, Y., Ogikubo, Y., Kijima, M. and Tamura, Y. (2000). Cloning expression of a gene encoding the flagellin of *Clostridium chauvoei*. Veterinary Microbiology. 359-372.

Mattar, M. A., Cortinas, T. I., & de Guzman, A.M.S. (2002). Immunogenic protein variations of *Clostri chauvoei* cellular antigens associated with the culture growth phase. FEMS Immunology and Me Microbiology , 33, 9-14.

Mattar, M. A., Cortinas, T. I., & Stefanini, A. M. (2007). **Extracellular proteins of** *Clostridium chauvoe* **protective in a mouse model.** Acta Veterinaria Hungarica 55 (2), 159-170.

Mbengue, M.B. (2008). Isolation and identification in Senegal of the most immunogenic protein so antigen of a *Clostridium chauvoei* strain. Bull Soc Pathol Exot., 101(1), 3-4.

Mhoma, J.R.L., (1985). Observations on the influence of media composition on the efficacy of vacc derived from some *Clostridium chauvoei* strains. Bull. Aim. Hlth. Prod. Afr., 33, 117-121.

Micalizzi, B. & De Guzman, A. S. (1997). Immunogenicity of an extract of *Clostridium chauvoei* in gu pigs. Clin. Inf. Dis., 25(suppl 2), s171-s172.

Moussa, R.S. (1959). Antigenic formulae for *Clostridium septecum* and *Clostridium chauvoei*. J. Pa Bacteriol., 77,341-350.

Mounier, J., Barant, F.K. & Sansonetti, P.J. (1997). Secretic Shigella flexneri Ipa invasins on contact epithelial cells and subsequent entry of the bacterium into cells are growth stage dependent. Ir Immun., 65, 774-782.

Ontiveros Corpus Mde, L., Hernandez, A. L., Lopez, M. J. & Tenorio, G. V. (2008). Prevention of Blackle an immunogen of *Clostridium chauvoei*. Ann N Y Acad Sci., 1149, 303-305.

Ramamoorthy, R. & Philipp, M.T. (1998). Differential expression of Borrelia burgdorferi proteins d growth in vitro. Infect. Immun., 66, 5119-5124.

Roberts, D. S. (2000)."Multicomponent clostridial vaccines using saponin adjuvants." U. S. Patent 608 Ed. U. S. Patent 6083512.

Rybicki, E. P. & Purves, M. (1996). "Enzyme Assisted immunoelectroblotting ." Molecular Bio Techniques Manual (Third Edition), Ed. Coyne, V. E., James, M.D., Reid, S. J., Rybicki, E. P.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual (Se Edition)."

Tamura, Y., Tanaka, M., Aoki, A., Ogikubo, Y. & Takahashi, T. (1995). Reversible expression of motility flagella in *Clostridium chauvoei* and their relationship to virulence. Microbiology, 141, 605-610.

Tanaka, M., Hirayama, N. & Tamura, Y. (1987). Production, characterization, and protective effe monoclonal antibodies to *Clostridium chauvoei*. . Infect. Immun., 55, 1779-1783.

خص العربي

امل المؤثرة علي ظهور أنتيجينات ميكروب الكلوستريديم شوفياي المستخدم في ح اللقاح ودورها في الاستجابة المناعية

ود فايز-حامد عادل الحلو – حاتم توغان – ياسر أحمد عبد الله

بحوث الأمصال واللقاحات البيطرية

ذه الدراسة تم استخدام نوعين من المستنبتات البكتيرية لنمو ميكروب الكلوستريديم شوفياي علي فترات تحضين ة وهي ١٨، ٢٦ ، ٤٨ ساعة وقد وجد أن كلا المستنبتين أعطي نسبة نمو عالية عند فترة التحضين لمدة ٢٦ ساعة. يل الكهربي للبروتينات وجد أن الميكروب أعطي خمسة من البروتينات عند الوزن الجزيئي ٢٠٠، ١٧٠، ١٥٠ ، ٥٦. وعند تحصين الأرانب الهندية باللقاحات المحضرة خلال فترة تحضين (١٨، ٢٦، ٤٨ ساعة) وجد أنها ، نسبة حماية ٨٠%، ١٠٠%، ٩٠% علي الترتيب. وهذا ما أثبت بالتحليل اللطعي لمصل الأرانب الهندية المحصنة أنها تفاعلت مع نفس الأوزان الجزيئية للبروتينات المحللة كهربائيا.