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## تأثير الكريستال فيوليت على الصفات البيولوجية لعنبر الكورائيني أوفيس

عبد المنعم بركات ، طلعت شومان ، رقية عثمان

أختيرت ثمانية عترات من ميكروب الكوريني أوفيس من أربعة  
أجناس من الحيوانات لد راسة تأثير الكريستال فيوليت  
على صفاتها البيولوجية . وجد أنه عند تركيز ١ : ٢٥٠٠٠٠  
لوحظت اختلافات جوهريية في نتائج الاستنبات والخصائص  
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Animal Health Research Institute,  
Ministry of Agriculture, Dokki, Giza,  
Institute Prof. Dr. H. El-Sawah

**EFFECT OF CRYSTAL VIOLET ON THE BIOLOGICAL  
CHARACTER OF C.ovis STRAINS**  
(With 3 Tables)

By  
**A.A. BARAKAT\*, M.T. SHOUMAN\*\* and ROUKAHIA M. OSMAN\***  
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**SUMMARY**

Eight strains of C.ovis from 4 animal species were treated with different dilutions of crystal violet to detect its effect on their biological characters. Treatment with the subinhibitory dilution (1/250,000) caused a noticeable variations in their cultural character, biochemical reactions, and pathogenicity to g-pigs. Vaccinations of 4 groups of g-pig with each of the 4 C.V. attenuated strains gave 91.33% survival. Also challenge by intraocular instillation of virulent SM sheep strain showed that the g-pigs vaccinated with the C.V. buffalo, sheep and goat strains developed observable protection, which prevented completely or partially the development of lesions in the submaxillary lymph nodes and minimize the mortality rate in comparison to the positive controls. Elsewhere, g-pigs vaccinated with C.V. cattle strain induced partial immunity by prolonging the time of survival.

**INTRODUCTION**

GOODMAN and GILMAN (1965) claimed that crystal violet dye was toxic to Gram-positive bacteria and had both bacteriostatic and bacteriocidal effects. In Egypt, AFIFI (1981) treated sheep strains of C.ovis by the addition of 1/750,000 concentration of crystal violet and recorded the attenuation of one strain which induced protection for 63.3% of the vaccinated guinea-pigs after challenge with the homologous strain.

The aim of this study is to investigate the effect of different dilutions of crystal violet on growth and biological characters of C.ovis strains from different animal origin, subsequently their effect as attenuated vaccinal strains in inducing protection.

**MATERIAL and METHODS**

Eight strains of C.ovis were used, from which four were isolated from cases of oedematous skin disease in buffalo and cattle (two strains from buffalo; B<sub>1</sub> and B<sub>2</sub>; and two from cattle; C<sub>1</sub> and C<sub>2</sub>) as well as cases of caseous lymphadinitis in sheep and goats (2 strains from each

\* : Animal Health Research Institute, Dokki, Giza, Egypt.

\*\* : Fac. of Vet. Med., Cairo University, Giza, Egypt.

animal species SM, S<sub>2</sub>, G<sub>17</sub> and G (respectively), by Aerobic Bacteria Section, Animal Health Research Institute, Dokki. All strains were inoculated heavily in nutrient broth and nutrient agar contained one of the following dilutions of crystal violet; 1/1.000.000, 1/750.000, 1/700.000, 1/600.000, 1/500.000, 1/400.000, 1/300.000, 1/250.000 and 1/125.000, then incubated at 37 °C for 24 hours to one week. The growth was checked by the development of colonies on 10% sheep blood agar plates.

The characterization studies of the growing colonies after treatment with the subinhibitory dose was applied on the cultural character and 5 positive biochemical reaction (Table 2). Also the pathogenicity to two groups of g.pigs, group (1) injected with the original strains and group (2) with the treated ones was applied by the subcutaneous injection of 1/4 ml from 24 hrs. old cultures of the tested 8 strains.

The attenuated treated strains, used as vaccinal ones, were prepared from strains B<sub>1</sub>, G<sub>5</sub>, SM and G<sub>25</sub> by growing in media of CAMERON et al. (1972). The resultant growth was harvested, centrifuged and washed for 3 times by sterile phosphate buffer, then standardized by Wellcome opacity tube No. 3 (= 750 million bacteria /ml for C.acne) in sterile buffer of pH 7.2, then checked with purity. The injected number of the vaccinal strains must be not less than 1.5 x 10<sup>8</sup> bacteria/ml (CAMERON et al. 1972).

In the vaccination experiment, 4 groups (each contained 6 g.pigs) were injected subcutaneously in the thigh region with 2 ml from each of the 4 prepared standardized strains treated with 1/250.000 crystal violets (Groups 3, 4, 5 and 6 respectively). The positive control group 7 (2 g.pig/strain) were injected with the same number i.e 750 million bacteria /ml and with the same dose (2 ml) of the original strains in phosphate buffer without crystal violet. The negative control group 8 was injected with the phosphate buffer contained the used dilution of crystal violet. The animals were put under strict observation for one month with recording any changes to determine the safety of the attenuated strains.

The survived vaccinated g.pigs and negative controls were challenged by intraocular instillation of one drop of 24 hours broth culture of C.ovis SM sheep strain (BARAKAT et al. 1974). The challenged g.pigs were observed for another month, where the dead or slaughtered animals were sacrificed for the presence of P.M. lesions.

## RESULTS

The 8 strains of C.ovis could not be able to develop colonies on nutrient agar with any concentration of crystal violet, even after heavy inoculation and incubation for one week at 37 °C on the other hand, the organism grew in crystal violet nutrient broth from 1/100.000 to 1/250.000 dilutions (Table 1). Subculturing of the strains grown in 1/250.000 crystal violet broth showed on sheep blood agar minute, pinheaded opaque whitish colonies, some of them were nonhaemolytic and others with narrow zone of haemolysis under them. Biochemically, the catalase test remained after treatment always positive, while the other 4 reactions were altered to negative (Table 2).

The pathogenicity of the original strains showed that the bovine strains skilled the guinea-pigs within 2 days with large oedematous swelling at the site of injection and severe congestion in internal organs. The ovine strains caused death of 6 guinea-pigs within 3-13 days and the survival of the other 2 animals till the end of the experiment. All animals showed abscesses at the site of injection and spleen. Comparatively, the crystal violet treated strains (C.V. strains) did not induce deaths in the injected guinea-pigs during the period of the experiment. Post-mortem examination of the slaughtered injected guinea-pigs with C.V. strains revealed no lesions

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in 14 animals while 2 animals showed lesions. One guinea-pig injected with C.V.S<sub>2</sub> sheep strain, another one with C.V.B<sub>8</sub> buffalo strain, both showed slight lesions at the site of injection (Table 2). Generally, it was found that the C.V. strains had a limited morbidity to guinea-pigs (2/16) in comparison to that of original strains (16/16). It must be mentioned that 2 cases died during the first days of the experiment without showing any P.M. lesions i.e. non-specific causes.

The vaccination experiment by injection of the 4 treated strains (C.V.B<sub>A</sub>, C.V.C<sub>5</sub>, C.V.SM and C.V.G<sub>25</sub>) in g-pig groups 3,4,5 and 6 respectively revealed the following results. All members of positive control group 7 died within 24 - 48 to 5 days with showing the typical lesions. Comparatively, the death and morbidity rates in the vaccinated groups were considerably low, as only 2 vaccinated g-pigs (one with C.V.B and the other with C.V.SM) died within the first week with showing mild lesions at the site of injection, i.e. morbidity and death rates were 8.33% each. The other 22 survived g-pigs indicated that the safety rate for the crystal violet vaccines reached 91.66% and were furtherly used for challenge experiment.

Challenge by intraocular instillation of the 22 survived g-pigs and the 5 negative control group 8 with the virulent SM sheep strain showed variations in mortality, morbidity and protection rates (Table 3). At first, 4 g-pigs from the 5 animals of group (8) died within 5-10 days with severe congestion with large abscesses at both sides of the submaxillary lymph nodes. On other instances, from the 22 challenged vaccinated g-pigs 8 died within 10 - 17 days without showing lesions, i.e. non-specific reaction, 6 died within 15 - 24 days with local abscesses in one submaxillary lymph node, while the survived 8 g-pigs till the end of the experiment developed only localized small abscess in one side of the same lymph node. Generally, it was found that the morbidity rate was 63.63%, while the death rate was limited (27.27%).

Concerning the challenged vaccinated groups in relation to the protective power of the 4 C.V. strains and the morbidity of the virulent SM sheep strains, it was found from Table (3) that the buffalo C.V.B<sub>A</sub> strain (group 3) and goat C.V.G<sub>25</sub> strain (group 6) could induce complete immunity to 40% and 50% of the challenged guinea-pigs respectively. On the other hand, the cattle C.V.C<sub>5</sub> induced only slight immunity in 16.67% as it could not prevent the morbidity of the challenged SM sheep strain in 5 guinea-pigs. Moreover, in group 5 the animals were challenged and vaccinated with the homologous strain (C.VSM sheep strain), 2 guinea-pigs died due to non-specific causes and the 3 survived animals showed only localized small abscess in one side lymph node.

## DISCUSSION

In this work, no colonies of the 8 strains of C. ovis could be grown on nutrient agar with crystal violet, while there were variable degrees of growth within 24 - 48 hours in nutrient broth contained different dilutions from 1/1000.000 to 1/250.000. GOODMAN and GILMAN (1965) and WILSON and MILES (1975) stated that crystal violet stain had strong bacteriostatic and bacteriocidal action on Gram-positive bacteria, but some members of streptococci and staphylococci were more resistant. Accordingly, it can be suggested that C. ovis strains are more sensitive to crystal violet than staphylococci and streptococci.

The character of the treated C. ovis strains with the subinhibitory dilution of crystal violet (1/250.000) showed a considerable variations in the size and character of colonies, biochemical activities and loss of pathogenicity to g-pigs. As from the 5 positive biochemical reactions of C. ovis 4 deviated to negative except catalase test (Table 2). Also the 4 C.V. strains of cattle and goat were unable to produce any lesions, while that of buffalo and sheep strains showed relatively noticeable decrease in pathogenicity to g-pigs.

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HARD (1969) stated that the application of relatively attenuated strain of live C. ovis in immunizing procedure was most successful in terms of the resultant degrees of resistant to challenge. Also, BARAKAT *et al.* (1974) concluded that dead vaccine gave no protection, while a solid immunity against C. ovis infection could not be obtained unless a live-attenuated vaccine was used. Accordingly, in this work vaccination of g-pigs with a dose contained  $1.5 \times 10^9$  bacteria /ml (CAMERON *et al.* 1972) of the C.V. strains showed the survival of 91.33% of the vaccinated g-pigs without showing any lesions at the site of injection. Comparatively, the positive controls injected with the original strains died within 48 hrs. to 5 days with severe typical lesions.

The results of challenge experiment showed in general that the C.V. strains limited to great extent the development of lesions in all g-pigs vaccinated except group 4 that vaccinated with C.V. cattle strain (C<sub>5</sub>). At the same the injection with the C.V. strains prolonged the survival time in all vaccinated g-pigs in comparison to the positive control group. Moreover, the mortality as a measurement of immunity is of no value due to the occurrence of deaths without any P.M. lesions. These findings supported the suggestion of HARD (1969) who claimed that the evaluation of immunity was measured by the suppression of lesion formation.

Concerning the cross protection between the challenge virulent SM sheep strain and C.V. homologous and the 3 heterologous strains, it was found that the C.V. cattle (C<sub>5</sub>) strain could not protect completely any challenged g-pigs. The C.V. buffalo and sheep strains induced 40% complete protection rate, while the C.V. goat strains protected 50% of the vaccinated animals (Table 3). From such results, it can be concluded that there is great cross immunologic reaction between sheep, goat and buffalo strains of C. ovis, while there is limited relationship between the sheep and cattle strains. Such conclusion was attained after the results of agar-gel precipitation test on the antigenicity of C. ovis strains from various hosts (BARAKAT *et al.* 1983). They found that the precipitating lines of sheep and goat strains were completely identical. Moreover, the buffalo strains shared a faint line with that of sheep strains, but the cattle strains exhibited variable reactions with that of sheep and buffalo strains.

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Table (1): The effect of crystal violet on various *C. ovis* strains

Strain	Different concentrations of crystal violet																			
	1/1000,000		1/750,000		1/700,000		1/600,000		1/500,000		1/400,000		1/300,000		1/250,000		1/200,000		1/125,000	
	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
B 8 B A	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
G 1 G 5	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
S M S 2	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
G 17 G 25	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-

S: Solid media. L: Liquid media +: Growth of the organism -: No growth of the organism

Table (2): The biochemical variations and changes in pathogenicity in *C. ovis* grown in 1/250,000 crystal violet.

Strain	Biochemical reactions					Pathogenicity for guinea pigs	
	Glucose	Sucrose	Urea hydrolysis	Nitrate test	Catalase test	Time of death or slaughtering	P.M. findings
B 8	-	-	-	-	+	D 1/2 S 1/2 2 days 2 weeks	No P.M. lesions Slight swelling, congestion at site.
B A	-	-	-	-	+	S 1/2 S 1/2 D.R. 25%	No P.M. lesions M.R. 25%
C 1	-	-	-	-	+	S 1/2 S 1/2 1 week 2 weeks	No P.M. lesions
C 5	-	-	-	-	+	S 1/2 S 1/2 D.R. 0%	M.R. 0%
S M	-	-	-	-	+	S 1/2 S 1/2 1 week 2 weeks	No P.M. lesions
S 2	-	-	-	-	+	S 1/2 S 1/2 D.R. 0%	Abcess at site of injection. M.R. 50%
G 17	-	-	-	-	+	D 1/2 S 1/2 3 days 2 weeks	No P.M. lesions
G 25	-	-	-	-	+	S 1/2 S 1/2 D.R. 25%	M.R. 0%
Control 1/250,000 crystal violet broth	-	-	-	-	-	S 1/2 S 1/2 D.R. 0%	No P.M. lesions M.R. 0%

D: Dead      S: Slaughtered      +: Positive reaction      -: Negative reaction      D.R.: Death rate      M.R.: Morbidity rate



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Table (3): Challenged guinea pigs vaccinated with 1/250,000 crystal violet treated strains

Strain	No. & time of death	Changes after challenge		P.m. findings	Mortality rate		Protection rate	
		Death No.	Rate %		No.	%	No.	%
B A Group 3	D 1/5	7	60%	-ve	3/5	60%	2/5	40%
	D 1/5	11		-ve				
	S 2/5	18		+ve				
G 5 Group 4	S 2/5	30		+ve				
	D 1/6	15	83.33%	+ve	5/6	83.33%	1/6	16.67%
	D 1/6	17		-ve				
	D 1/6	21		+ve				
	D 1/6	23		+ve				
S M Group 5	D 1/6	24		+ve				
	S 1/6	30		+ve				
	D 1/5	4	40%	-ve	3/5	60%	2/5	40%
G 25 Group 6	S 3/5	30		-ve				
	D 1/6	8	66.67%	-ve	3/6	50%	3/6	50%
	D 1/6	9		-ve				
Negative control Group 8	D 1/6	11		-ve				
	D 1/6	19		-ve				
	S 2/6	30		+ve				
	D 1/5	5	80%	+ve	5/5	100%	-	-
Negative control Group 8	D 3/5	10		+ve				
	S 1/5	30		+ve				

+ Access in submaxillary lymph nodes. - Negative lesions. M Mortality rate D Dead. S Slaughtered.

