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Experimental studies of Sereny test and susceptibility test for the pathogenicity of anaerobic propionibacteria[+]

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This is a report of the first experimental study of the application of the Sereny test and drugs susceptibility test to isolated anaerobic propionibacteria from clinical specimens. Of 24 P. acnes strains, two were phase I positive in the Sereny test; of six P. granulosum strains, four were phase I positive and three strains were phase II positive. If the cut-off points of all antibiotics is 6.5 μg/ml, the susceptibility test indicates that propionibacteria are pathogenic. Both the Sereny test and the susceptibility test demonstrate the pathogenicity of propionibacteria.

Key words : Sereny test-propionibacteria

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นราทร ธรรมบุตร, ผดุงศรี วิชิวานิเวศน์, อัญชลี เฉียบฉลาด, อมรรัตน์ ทัศนกิจ. การทดสอบ เซอรินี เทส และความไวต่อยาต้านจุลชีพ เพื่อบ่งชี้พันธุ์โปรปิโอนิบัคเตรีที่ก่อพยาธิสภาพ. จุฬาลงกรณ์เวชสาร 2534 กันยายน; 35(9) : 571-578

ได้ทดลองนำวิธี Sereny test และ Susceptibility test มาใช้ทดสอบเป็นครั้งแรกกับแอนแอโรบิค propionibacteria ที่แยกได้จากสิ่งส่งตรวจทางแพทย์ จาก 24 สายพันธุ์ของ ***P. acnes*** *พบ 2 สายพันธุ์ให้ผลบวก phase I Sereny test ส่วน* ***P. granulosum*** *อีก 6 สายพันธุ์ พบว่ามีหลายสายพันธุ์ที่ให้ผลบวก Sereny test ทั้ง phase I และ phase II. อนึ่งถ้ากำหนดค่า Break point ในการทดสอบยาต้านจุลชีพที่สำคัญ 8 ชนิด ที่ทดสอบกับจุลชีพ propionibacteria เป็น 6.5 μg/ml. จะเห็นได้ว่า susceptibility test ช่วยทดสอบสนับสนุนให้เห็น propioni-bacteria ที่เป็นจุลชีพก่อโรคได้*

In clinical anaerobic pacteriology, the last decade has seen a shift in emphasis from the clostridia to the non-sporing-anaerobes, such as the anaerobic cocci, bacteroides, fusobacteria, campylobacters and propionibacteria.[1]

In contrast with the clostridial diseases, infections due to the non-sporing-anaerobes are less acute in their onset, are prone to chronicity, and do not commonly produce severe toxaemia.[1]

Propionibacteria are anaerobic, short rod, gram-positive, non-spore-forming bacteria, found in normal skin flora and normal flora of the conjunctival sac.[2] Although **Propionibacterium acnes** is an agent associated with acne, ocular infections and endophthalmitis,[2] other propionibacteria usually are considered to be in significant. In our anaerobic laboratory, few carbon-dioxide-dependent facultative anaerobes are found, although propionibacteria have been isolated once in a while from blood cultures, corneal ulcers and even nasal swabs. Up to the present time, a few aspects regarding the pathogenicity of these bacteria to humans remain unclear.

The problem we face is to demonstrate whether isolated-propionibacteria (formerly anaerobic diphtheroids) as well as **P. acnes** are potential pathogens. Fortunately, the Sereny test, which is used occasionally in testing for invasive Shigella strains, was able to demonstrate an inflammatory response to the inoculation of the conjunctival sac of a rabbit with a pathogenic strain, producing purulent keratoconjunctivitis.[3] According to the circumstances mentioned, the experimental Sereny test on the isolated propionibacteria together with the study of the actual minimum inhibitory concentrations (MIC) of the isolates to eight antimicrobial agents may be helpful in evaluating the pathogenicity of the isolated propionibacteria. The objective of our study therefore was to apply the Sereny test with MIC study of these propionibacteria to demonstrated whether the given strains are potential pathogens.

Materials and methods

a) Test-animals

Young brown New Zealand rabbits weighing about 280 grams each were used as the test animals.

b) Test-strains

Thirty propionibacterium strains were obtained from patients' clinical samples taken in 1990. Propionibacteria were identified by their colonial appearance and anaerobic growth and were speciated according to the method of Marples and McGinley.[4] They were collected on brain heart infusion (BHI) agar (Difco Laboratories, East Molesey, Surrey, U.K.) containing 3% (W/V) additional glucose (BHIg) and 2 μg/ml furazolidone. (BHIgF, Sigma Chemdical Co., Poole, Dorset, U.K.) to inhibit the growth of staphylococci. Incubation was carried out for seven days at 37°C in an anaerobic chamber.[5]

c) Sereny test

Propionibacteria were inoculated into veal infusion broth (Difco) and incubated overnight at 37°C with shaking (250 rpm). Cells were eashed three times and suspended in phosphate-buffered saline (Dulbecco modified formula; Flow Laboratories, Inc., McLean, VA) at a final concentration of approximately 5×10^{12} CFU/ml. Inoculum was standardized based on optical density of 650 nm. The propionibacterium suspensions (0.025 ml) were inoculated into the right eye of the rabbits, and sterile phosphate buffered saline was placed into the left eye as a control. The test was considered positive if there was evidence of conjunctivitis, ulceration, or opacity of the inoculated eyes during five days of observation (phase I). Exudates from the eyes of animals with evidence of keratoconjunctivitis were cultured to confirm the presence of the propionibacteria (phase II). A minimum of two rabbits were inoculated for each strain.[3]

d) Determination of antibiotic susceptibility.

The minimum inhibitory concentration of each antibiotic was determined by agar dilution on BHIg. Inoculated antibiotics were prepared by dilution of a 48 hours broth culture to give a standard inoculum of 10^4 OFU/spot delivered by a multipoint inoculator. Plates were incubated as above but for 72 hours only. The MIC of each antibiotic for each strain was recorded as the lowest concentration yielding no growth or a barely visible haze as determined with the unaided eye.[6,7]

Table 1. Biochemical classification of anaerobic diphtheroids from human skin.[4]

Test	Reaction of strains of		
	group I	group II	
	(C. acnes)	A (C. avidum)	B (C. granulosum)
Phage susceptibility	+	−	−
Haemolysis type	a	B or −	−
DNAase	−	+	+
Gelatinase	+	+	+
Casein hydrolysis	+	+	−
Indole	+	−	−
Nitrate reduction	+	−	−
Sucrose	−	+	d^+
Sorbitol	d^+	−	−
Trehalose	−	+	d
Tween 80	−	d	d^-

+ = Reaction positive; − = reaction negative; d = reaction variable;
d^+ = reaction variable, usually positive; d^- = reaction variable, usually negative.

The following eight pure antibiotics were used in this study :

1. Chloramphenicol (CH) from Achdron Drug Company.
2. Cifoxitin (CX) from Merck, Sharp and Dohme Company.
3. Clindamycin hydrochloride (CD) from Upjohn Company.
4. Erythromycin (EM) from Abbott Phamaceutical Company.
5. Penicillin G (PC) from Glaxo Phamaceutical Company.
6. Imipenem (IM) from Merck, Sharp and Dohme Company.
7. Tetracycline hydrochloride (TH) from Pfizer International Company.
8. Metronidazole (MD) from Siam Pharmaceutical Company.

Result

Out of 30 propionibacteria strains, 24 **C. acnes** and six **C. granulosum** strains isolated from eye swabs, pus swabs, nasal swabs and hemocultures. Only six isolated **C. acnes** strains were phase I positive according to the Sereny test. Only three **C. granulosum** strains were phase II positive according to the Sereny test (Table 2).

Tables 3 and 4 demonstrate the susceptibility patterns (MIC) of the isolated 24 **C. acnes** and six **C. granulosum,** respectively, with eight antimicrobial agents. The most active drugs at MIC_{90} (of **P. acnes**) were Clindamycin and erythromycin while at MIC_{90} (of **P. granulosum**) the most active was imipenem.

When the cut-off points of the antibiotics are calculated at a minimum og 6.25 μg/ml, the resistance to multiple drugs of anaerobic propionibacteria can be tabulated are shown in Table 5.

There were 10 propionibacteria resistant to seven drugs (6.25 μg/ml); 7 strains resistant to six drugs (12.5 μg.ml); three strains resistant to three drugs (50 μg/ml) and two strains resistant to two drugs (25 μg/ml).

Table 6. shows that only 26.67% of the total were propionibacteria only whereas 73.33% were a combination of aerobes and propionibacteria. In the propionibacteria only isolaties alone, only six **P. acnes** and two **P. granulosum** were isolated; in the polymicrobial group, 18 **P. acnes** and four **P. granulosum** were recovered.

Table 2. The isolated anaerobic propionibacteria from the patients clinical specimens and the result of the Sereny test.

Clinical diagnosis	Clinical specimens and No.of cases (n)	Isolated propioni-bacteria			Result of the Sereny test		
					Positive		Negative
		Pc*.	Pg**.	Pa***.	Phase I	Phase II	
Conjunctivitis	eye swab (4)	4	–	–	–	–	4
Endophthalmitis	eye swab (2)	2	–	–	–	–	2
Orbital cellulitis	eye swab (4)	4	–	–	–	–	4
Canaliculitis	eye swab (2)	2	–	–	1	–	1
Corneal ulcer	eye swab (2)	–	2	–	2	2	–
Axilla abscess	eye swab (2)	–	2	–	1	1	1
Alae nasi infection	nasal swab (4)	4	–	–	1	–	3
Open cornedo	pus swab (2)	–	2	–	1	–	1
Acute lymphoid leukomia	hemocul-ture (4)	4	–	–	–	–	–
Mitral stenosis (After TEE+ performance+)	hemocul-ture (4)	4	–	–	–	–	–
TOTAL STRAINS		**24**	**6**	–	–	–	**16**

*Pc = Propionibacterium acnes., **Pg = Propionibacterium granulosum.
***Pa = Propionibacterium avidum.
+TEE = Trans esophageal echocardiogram.

Table 3. Susceptibility distribution of 24 P. acnes strains.

Drugs	MIC (μg/ml)												
	0.025	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	MIC_{90}
1 CH	8	5	1	3	2	2	1	–	1	1	–	–	0.81
2 CX	10	3	1	3	3	1	2	–	1	–	–	–	0.03
3 CD	7	3	3	2	2	2	1	1	1	1	1	–	0.06
4 EM	9	1	3	1	2	1	1	2	1	1	1	1	0.06
5 PG	4	8	2	1	1	2	1	2	2	1	–	–	3.86
6 IM	14	7	1	–	1	–	1	–	–	–	–	–	0.025
7 TH	7	1	3	1	2	2	1	1	3	2	–	1	0.096
8 MN	12	1	2	1	2	1	1	2	1	1	–	–	0.025

Table 4. Susceptibility distribution of six P. granulosum strains.

Drugs	MIC (μg/ml)												
	0.025	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50	MIC_{90}
1 CH	2	2	–	–	–	–	1	1	–	–	–	–	2.05
2 CX	4	–	–	1	–	1	–	–	–	–	–	–	0.53
3 CD	3	–	1	1	–	–	1	–	–	–	–	–	1.03
4 EM	4	–	–	1	–	–	–	1	–	–	–	–	1.6
5 PG	3	–	1	–	–	1	–	–	1	–	–	–	3.23
6 IM	4	–	1	–	1	–	–	–	–	–	–	–	0.27
7 TH	4	–	–	–	–	1	–	1	–	–	–	–	1.6
8 MN	3	1	1	–	–	–	–	1	–	–	–	–	1.6

Table 5. Patterns of resistance to multiple drugs in propionibacteria.

No. drugs	Cut-off points* (μg/ml)	Drugs	No. of strains	From table
7	6.25	CH,CX,CD EM,PG,TH NM	10	3
6	12.5	CH,CD,EM PG,TH,NM	7	3
3	50	EM,PG,TH	3	3
2	25	CD,EM	2	3
1	6.25	PG	1	4

* Cut-off points are designations above which an organism is considered resistant and below which an organism is classified as susceptible.

Table 6. Bacterial findings from the clinical samples.

Isolated	No. of isolated strains/total of isolates	Distribution of isolated propionibacteria		
		Pc*	Pg*	Pa*
Propioni-bacteria only	8/30 (26.67%)	6	2	–
Mixed aerobes and anaerobes	22/30 (73.33%)	18	4	–
Total strains	30	24	6	–

*Pc = **Propionibacterium acnes**
Pg = **Propionibacterium granulosum**
Pa = **Propionibacterium avidum**

Discussion

As mentioned by AT Willis, anaerobic diphtheroids or propionibacteria were considered insignificant (contaminants) if they were isolated from swabs and other clinical specimens.[1] Moreover, these carbon-dioxide-dependent facultative anaerobes are normal inhabitants of the skin. However, this original article is the first application of the Sereny test to demonstrate the pathogenicity of anaerobic isolates. Young brown New Zealand rabbits were used because of their substantial white conjunctival area. Because of the positive reaction of hyperemia, keratoconjunctivitis is easily detectable (Fig.1). From Table 2, many strains of **P. acnes** showed positive phase I only in the Sereny test. By contrast, few **P. granulosum** demonstrated positive phase II in the Sereny test. The isolated **P. acnes** may be more sensitive to oxygen in the environment than the isolated **P. granulosum;** therefore, it was not possible to isolate **P. acnes** from the rabbits exudated. However, the positive phase I reaction demonstrates sufficiently well that the anaerobe is a potential pathogen and may induce an unknown potent toxin.

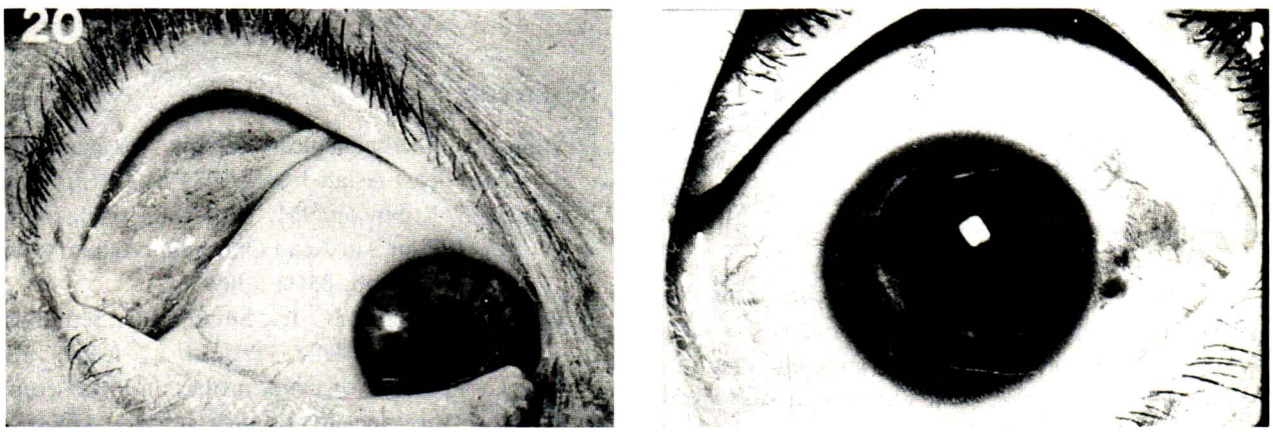

Figure 1. Sereny test showing :
a) Negative test
b) Positive phase I with keratoconjunctivitis

In our laboratory, unfortunately, **P. avidum** was not identified in any clinical sample. Consequently, **C. avidum** is either rare or it is not a normal inhabitant in the Thai people (Table 2).

Actually, the cut-off points of an antibiotic are those recommended by the Food and Drug Administration of the United States. Such cut-off points are available for all antibiotics. However, at present, one cannot determine exactly the value of cut-off points of propionibacteria against many antimicrobial agents.[8]

From Tables 3,4 and 5, it is estimated that the cut-off points of **P. acnes** or **P. granulosum** to the current antimicrobial agents are about 6.25 ug/ml; many propionibacteria are resistant to the given antimicrobial agents. Our study demonstrates the tendency of resistant propionibacteria to be potential pathogens. Moreover, EA Eady et al. inducibly or constitutive supports our finding (Tables 3,4 and 5) that there are many propionibacteria resistant to macrolide (e.g. erythromycin) and lincosamide (e.g. clindamycin) antibiotics which, in the treatment of acne patients, are associated with therapeutic failure.[9]

From Table 6, the number of propionibacteria only that were isolated (26.67%) was less than that of the propionibacteria and mixed aerobes (73.33%). The findings revealed that propionibacteria contain no invasive substances. In other words, Table 6 demonstrates clearly that anaerobic propionibacteria alone are not completely pathogenic. In order to be complete pathogens, anaerobic propionibacteria require other components from other aerobes; therefore, more than half of the isolated strains were polymicrobial agents. (Diagram 1)

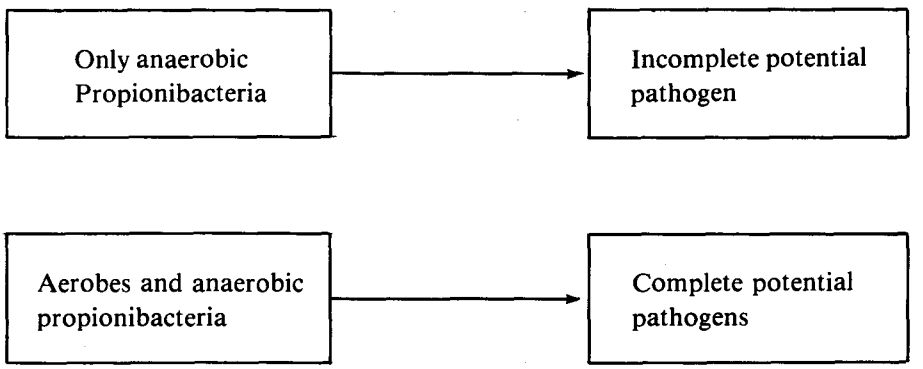

Diagram 1. Showing polymicrobial effects.

Although the propionibacteria resistant to many antibiotics were not correlated with the Sereny test-positive strains, our application of the test at least demonstrated the potential of the propionibacteria to become pathogens (Tables 3,4 and 5).

A continuous scale of invasiveness could be drawn up for microorganisms. One end of this scale would be occupied by toxin producers such as tetanus or diphtheria; the other, by highly invasive organisms such as anthrax or plague, with staphylococci and streptococci in between. However, a part of the invasiveness of microorganisms may be attributed to certain components that protect the bacteria from phagocytosis and destruction. Such surface substances may be polysaccharide capsule (e.g., **Bacteroides fraqilis** or **Haemophilus influenzae).** How can one prove that a give microorganism really causes a disease? Traditionally, the causative relationship between a microorganism and a disease is established by "Kock postulates".[7] Although these postulates were adequate in proving the cause of some bacterial diseases, they had to be modified for other infections.[7]

In our situation, the Sereny test is meaningful in considering whether or not a propionibacterium is a potential pathogen. Our experimental Sereny test is the first application of this test to anaerobes.

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