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Gelatin Liquefaction: a Microtest

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SUMMARY: A slide microtest for gelatin liquefaction is described. Members of the genera *Cloaca*, *Bacillus* and *Clostridium* were tested with varying results. A limitation of the method is its ability to detect only ready-formed enzyme. The main advantage is that small amounts of culture are needed and the organisms may be grown under any selected conditions.

Gelatinase activity in bacteria is detected by methods which often require the progeny of a small inoculum to liquefy relatively huge amounts of substrate at a temperature unfavourable to the growth of the organism. Lewert & Lee (1954), investigating the gelatinase of Helminth larvae, used a thin film of gelatin on a glass slide as substrate, liquefaction (hydrolysis) being shown by clear areas on the fixed and stained slide. The method required very small amounts of material and was quick, so that its possible application as a bacteriological test was considered.

METHODS

Strains from the National Collection of Type Cultures were used as test organisms; the main genera examined were *Cloaca* and *Bacillus*.

Gelatin slides. Clean dry microscope slides were dipped in a melted 5 % (w/v) aqueous solution of gelatin at 37–40°, the excess liquid was drained and the slides stood on end to dry in the air; this left a thin coating of gelatin on the slides. The prepared slides were stored in a Petri dish in the refrigerator. The stock solution of 5 % gelatin could be stored at 4° with 0·1 % (w/v) phenol added as a preservative; the presence of phenol did not affect the gelatinase activity.

Procedure. Drops (*c.* 0·01 ml.) of the sample, usually a broth culture or a heavy suspension made from an agar slope culture grown at the optimal temperature for the organism were placed on the slide together with a suitable control such as uninoculated broth or water. The slide was incubated at 22° for 2–20 hr. in a Petri dish containing a strip of moist filter-paper to minimize evaporation from the drops. As the drops spread when enzyme activity was great, about 8 samples/slide was a convenient number. The extreme ends of the slide were not used because the gelatin drained slightly downwards while the slides were drying. After incubation the gelatin was hardened and the culture killed by immersion in a mixture of 1 vol. formalin (40 % formaldehyde) and 9 vol. saturated aqueous mercuric chloride for 20–30 min.; the slides were then washed in water and stained with any convenient protein stain, usually dilute (1/100) carbol fuchsin. Where hydrolysis had occurred clear areas indicating gaps in the gelatin film were seen (Fig. 1). For rough

quantitative work serial twofold dilutions were made and spotted in order of dilution; for qualitative tests one drop of each culture was used.

Parallel tube liquefaction tests were carried out in stab cultures in nutrient gelatin (containing gelatin, 12 %, w/v; Lemco, 1 %; NaCl, 0·5 %; Evans peptone, 1 %) incubated at 22° and read daily for 30 days.

RESULTS

Effect of incubation time

The sensitivity of the method increased with the time of incubation. Serial twofold dilutions in water of a 24 hr. broth culture of a strain of *Chromobacterium prodigiosum* (NCTC 1377) were incubated on slides for 2, 5 and 21 hr., then fixed and stained. After 2 hr. a clear space corresponded to dilution 1/2; at 5 hr. 1/8 was clear; after 21 hr. clearance occurred at 1/512 and partial clearance at 1/1024; the control (water) was coloured (Fig. 1).

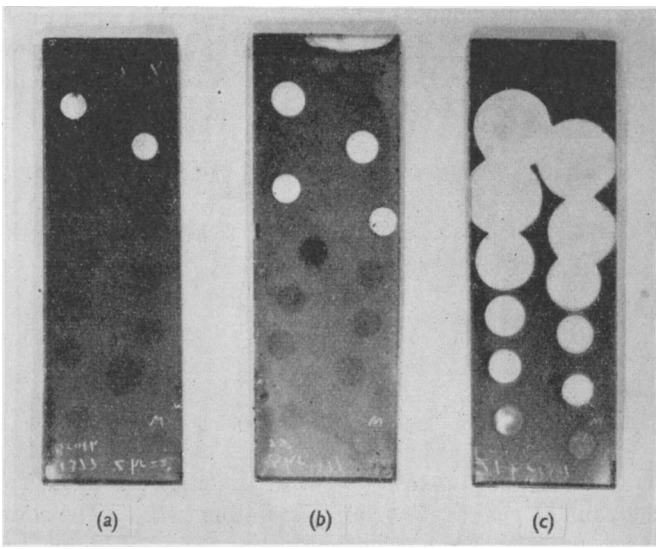

Fig. 1. Effect of incubation time on the sensitivity of the slide test. Lower right of each slide: water. Serial twofold dilutions in water of broth culture of NCTC 1377 placed on a zig-zag line from the upper left to the lower left of each slide. Slides incubated at 22° for (*a*) 2 hr., (*b*) 5 hr., (*c*) 21 hr.

Effect of hydrogen ion concentration and toluene

Since calcium ions are known to enhance gelatinase activity (Haines, 1931) calcium-binding buffer constituents such as citrate and phosphate could not be used; therefore 0·2 N-borate and acetate, and 0·2 M-ethylenediaminedihydrochloride buffers were used in a pH range of 2–9. The test organism was a strain of *Bacillus subtilis* (NCTC 6276) and heavy suspensions in buffer of different pH values were made from an overnight culture on Lemco agar. After 18 hr.

at 22° clearing had occurred in the range pH 5·6–7·7, and partial clearing at pH 5·0, 8·0 and 8·6. Serial dilutions were made in buffers at pH 6·0, 6·8, 7·2 and 7·7 and in distilled water; these were spotted on to slides which were incubated for 18 hr. before being fixed and stained. Clearing occurred at dilution 1/8 in water and in buffers at pH 6·0, 6·8 and 7·2, and at 1/4 in the pH 7·7 buffer. Thus for the gelatinase of NCTC 6276 the optimum pH appeared to be in the range 6·0–7·2, but the unbuffered suspension was as active as the buffered suspensions.

Treatment of the bacterial suspension with toluene for 1–2 hr. before testing did not enhance the gelatinase activity of 3 strains of *Cloaca cloacae* and 1 strain of *Bacillus coagulans*.

Tests on Cloaca cloacae

Sixty-two strains of *Cloaca cloacae* were tested as heavy suspensions from overnight Lemco agar cultures, the slides being incubated for 18–21 hr. The results, together with those of the routine gelatin stab tests, are summarized in Table 1. It can be seen that strains which liquefied gelatin stabs most slowly were the least likely to give positive reactions on slides. Two serum-digesting strains which were included in the series both liquefied gelatin stabs in 3 days and were positive in the slide test.

Table 1. *Gelatinase tests on strains of* Cloaca cloacae

Days to liquefaction in gelatin stab	No. of strains and slide reaction	
	+	−
1–5	14	0
6–10	4	2
11–13	1	4
14–20	2	14
21–30	3	5
Negative at 30 days	0	13

Slide tests and stab culture tests on 152 strains of *Klebsiella* spp. and 31 strains of the Alkalescens-dispar group were all negative. The slide tests for this series were done on suspensions prepared from 18–20 hr. Lemco agar slopes.

Tests on Bacillus *species*

Strains from 12 species of *Bacillus* (*B. alvei*, 5; *B. brevis*, 1; *B. cereus*, 5; *B. circulans*, 4; *B. coagulans*, 2; *B. licheniformis*, 4; *B. lentus*, 1; *B. macerans*, 1; *B. megaterium*, 2; *B. pumilus*, 6; *B. sphaericus*, 1; and *B. subtilis*, 14) were tested as unwashed suspensions from overnight Lemco agar slopes. The results (Table 2) do not show the same relation between slide reaction and time to liquefaction in stabs as was found with *Cloaca cloacae*. An attempt to correlate gelatinase activity with serum digestion showed that a higher proportion of slide-test positives occurred among the serum-digesting strains than among the non-digesting (serum-negatives), and the majority of serum-negative, slide-positive strains were able to liquefy gelatin stabs within 10 days.

Table 2. *Gelatinase tests on members of the* Bacillus *group*

Days to liquefaction in gelatin stab	Slide reaction	No. of strains and	Serum digestion +	Serum digestion −
1–5	+	10	6	4
	−	0	0	0
6–10	+	10	6	4
	−	3	3	0
11–13	+	4	3	1
	−	1	0	1
14–20	+	5	4	1
	−	1	1	0
21–30	+	4	2	2
	−	4	0	4
Negative at 30 days	+	0	0	0
	−	4	1	3

Tests on Clostridium *species*

Tests on a few strains of *Clostridium* spp. were carried out with drops from cultures grown for 1–4 days in Robertson's meat broth; two 8-day cultures were also tested (*C. tetanomorphum* and *C. sphenoides*). The results are shown in Table 3. *C. welchii* was particularly active, 5 of the 6 strains tested giving positive slide reactions within 135 min. The stab tests on Clostridium strains were incubated under anaerobic conditions at 37° and were read after refrigeration.

Table 3. *Gelatinase tests on species of* Clostridium

Species	No. of strains and reaction in: Tube at 37° +	Tube at 37° −	Slide test +	Slide test −
C. welchii type A	6	0	6	0
C. oedematiens type A	2	0	(2)	0
C. oedematiens type B	2	0	(1)	1
C. sporogenes	2	0	1, (1)	0
C. tetanomorphum	0	2	0	2
C. botulinum	1	0	1	0
C. sphenoides	0	1	0	1
C. putrefaciens	0	1	0	1

( ) indicates a weak reaction.

DISCUSSION

Among the limited number of strains tested, gelatinase activity as shown by the slide and stab culture tests was roughly parallel, and the proportion of strains showing a positive result in the slide test was greatest among those strains which most quickly liquefied gelatin in stab cultures; this relationship was more marked in *Cloaca cloacae* than in the *Bacillus* group. Many of the

Bacillus strains were able to digest serum, and the majority of these were gelatin-slide positive irrespective of the stab-liquefaction time; with the serum-negative strains the activity on gelatin slides decreased in parallel with that of the stab cultures in the same way as *C. cloacae* (serum-negative). This suggests that the serum-digesting enzymes of *Bacillus* species may reinforce the gelatinase activity in some strains but are not entirely responsible for it.

The results in Tables 1 and 2 show that the serum-negative strains which liquefied gelatin slowly did not give positive slide tests. Conditions on the slides do not favour the growth of organisms, therefore only enzymes which are present in appreciable amounts at the time of testing are detected, and slight or 'adaptive' activity does not give positive results. Attempts to make the test more sensitive by using preformalized slides at a higher temperature on the lines of the gelatinase test of Kohn (1953) were not successful. Within the limitations outlined, however, the gelatin slide technique may be of use as a bacteriological test, particularly for anaerobes, since these react under the aerobic conditions of the test.

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