

Methods for Detecting Indole Production by Gram-Negative Nonsporeforming Anaerobes

GUNNAR D. FAY AND ARTHUR L. BARRY

Clinical Microbiology Laboratory, University of California, Davis Sacramento Medical Center, Sacramento, California 95817

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To help select the most appropriate method for detecting indole production with anaerobic gram-negative bacilli, several recommended methods were compared. Indole was measured both quantitatively and qualitatively after varying periods of incubation. Studies evaluated the results obtained in different media, the effect of adding glucose and/or tryptophan, the requirement for strict anaerobiosis, and the effects of reducing the total volume of broth. A 1-ml amount of thioglycolate broth without glucose but with 0.02% tryptophan gave optimal results after 2 to 7 days of incubation in anaerobic (Gas-Pak) jars. The majority of clinical isolates will give strong positive tests after 1 to 2 days but a few require 3 to 7 days of incubation. Prolonged incubation was required more frequently with conventional methods.

In the identification of anaerobic gram-negative nonsporeformers, an important characteristic is the ability to produce indole. Relatively little information is available concerning the production of indole by anaerobic organisms as compared with the amount of information available from studies with aerobes (4, 8, 9, 10).

Anaerobes have been tested for indole after growth in one of several broth media. The lack of standardization is often justified by the generalization that "most basal media contain sufficient tryptophan to be satisfactory for detecting indole production" (6). The laboratory manual of the Virginia Polytechnic Institute (VPI) (11) states that most media, e.g., peptone yeast (PY), indole-nitrite, and chopped meat medium, will support indole production. Dowell and Hawkins (2) recommend an indole-nitrite medium and Loesche et al. (5) used thioglycolate medium supplemented with tryptophan for indole production.

Because recommendations for performing the indole test with anaerobes vary considerably, the amount of indole elaborated in different media was measured quantitatively. Conventional methods using large volumes of media were compared with more convenient tests using small volumes of broth incubated in anaerobic jars. Preliminary investigations were first undertaken to study the effect of adding glucose and tryptophan, and the requirements for strict anaerobiosis.

MATERIALS AND METHODS

To measure indole quantitatively, the method of Wood et al. (12) was used; reference curves were constructed with indole (Calbiochem lot no. 901937) standards prepared in the appropriate medium.

To determine the influence of anaerobiosis on indole production, seven indole-positive *Bacteroides fragilis* strains and one strain of *Escherichia coli* were tested in 10-ml volumes of chopped meat broth (Difco). The medium was prepared as a pre-reduced, anaerobically sterilized (PRAS) medium and as aerobically sterilized broth. After inoculation, the media were incubated at 35 C, and 1-ml volumes were withdrawn and assayed for indole production after 1, 2, and 3 days of incubation. The PRAS media were handled under oxygen-free CO₂ or N₂ gases to prevent oxidation.

To determine the influence of glucose or tryptophan on the production of indole, peptone yeast base was prepared according to the VPI laboratory manual (11) and supplemented with glucose or tryptophan as outlined in Table 2. The broth was prepared in 10-ml volumes; half of the tubes were prepared as a PRAS medium and the other half were sterilized aerobically. Two strains of *B. fragilis* subspecies *thetaiotaomicron* were inoculated into each medium and after 1, 2, and 3 days at 35 C; 1-ml volumes were withdrawn and assayed for indole.

Three media commonly used for indole production by anaerobic organisms were compared in both large and small volumes as described in Table 3. Indole-nitrite medium in 5.0-ml volumes was purchased from Robbins Laboratories. Each test medium was inoculated with 0.05 ml of an overnight broth culture. For the 1-ml volumes of broth, four tubes (13 by 100 mm)

with each kind of medium were inoculated with each test organism and one set of tests was removed from anaerobic jars after 1, 2, 3, and 7 days of incubation at 35 C. At the same time intervals, 1-ml portions were withdrawn from the larger volume tests which were incubated aerobically or under oxygen-free nitrogen gas. On days 1 and 2, each medium was tested by extracting 1-ml volumes of the broth with 1 ml of toluene and then adding 0.5 ml of Ehrlich's reagent. On days 3 and 7 the same extraction was performed; 0.5 ml of the toluene extract was used for quantitation of indole, and the remainder was tested qualitatively with Ehrlich's reagent.

A total of 50 clinical isolates were tested by all six methods; 26 of these were indole positive by one or more method. Included were four *Fusobacterium* sp. and 46 *Bacteroides fragilis* (16 subspecies *thetaitaomicron*, 10 *fragilis*, 2 *distasonis*, 1 *vulgatus*, 1 *ovatus*, and 16 unidentified subspecies).

RESULTS

The amount of indole produced in chopped meat incubated under aerobic and anaerobic conditions is presented in Table 1. The control strain of *E. coli* produced indole more rapidly and in greater amounts than any of the *B. fragilis*. The *E. coli* produced less indole under anaerobic conditions than in an aerobic atmosphere, whereas *B. fragilis* produced better growth and greater amounts of indole in an anaerobic atmosphere.

In PY base, the amount of indole produced was greatest when PRAS broth was supplemented with tryptophan (Table 2). The addition of glucose generally interfered with indole production. When indole was produced, it accumulated and continued to increase in quantity during the first 3 days of incubation; in most of the media, indole could not be detected until day 2 or 3 of incubation.

A comparison of six methods for indole production (Table 3) showed that in the smaller volumes of broth, positive results are seen earlier than in larger volumes. However, after 7

TABLE 1. Indole production in chopped meat medium incubated under aerobic and anaerobic conditions

Days of incubation	µg of indole per ml of broth			
	<i>B. fragilis</i> (7 strains) (median [min-max])		<i>E. coli</i> (1 strain) single determination	
	Aerobic	Anaerobic ^a	Aerobic	Anaerobic ^a
1	0 (0-0)	0 (0-1)	8	5
2	0 (0-1)	2 (0-5)	7	5
3	1 (0-5)	3 (1-7)	5	5

^a Tubes gassed with oxygen-free N₂ and sterilized anaerobically.

TABLE 2. Indole production in PRAS PY broth supplemented with glucose or tryptophan, or both^a

<i>B. fragilis</i> strain no.	Tryptophan added (wt/vol) (%)	Glucose (wt/vol) added to PY broth (%)					
		None		0.5		1.0	
		µg of indole per ml after 2 or 3 days incubation ^b					
		2	3	2	3	2	3
2	None	0	5	0	0	0	0
	0.1	8	13	0	0	0	0
22	None	0	2	1	1	1	1
	0.1	8	10	1	1	1	4

^a In parallel studies with aerobically incubated broth, strain no. 22 grew but failed to produce any indole within 3 days, whereas strain no. 2 produced indole only in PY broth without glucose; the concentrations were about 2/3 of that found in PRAS broth.

^b All tests were negative after 1 day of incubation.

days of incubation all the methods gave nearly comparable results, except for tests in aerobically incubated indole-nitrite broth. The amount of indole present after 7 days was usually greater than that found after 3 days. Qualitative tests in chopped meat and thioglycolate supplemented with tryptophan were positive more frequently than in indole-nitrite broth. The greatest quantity of indole was produced in 1 ml of thioglycolate medium without glucose supplemented with tryptophan.

DISCUSSION

Oxygen definitely influences the production of indole by most aerobes. The amount of indole is decreased when facultative organisms are incubated anaerobically. Isenberg and Sundheim (4) found no indole produced by *Enterobacteriaceae* under anaerobic conditions but the *E. coli* included in the present study produced fairly large amounts of indole in an anaerobic environment, although even more indole was produced aerobically. Quantitatively much less indole was produced by *B. fragilis*, especially when precautions were not taken to maintain strict anaerobiosis. *B. fragilis* showed a significant increase in indole production under more anaerobic conditions, presumably because of the improved growth rate. Fry (3) postulated that oxygen may not be involved in indole formation but that the presence of O₂ is necessary for the accumulation of indole. This might partially explain why quantitatively less indole is found with *E. coli* in broth incubated anaerobically. Suassuna and Suassuna (9) tested

TABLE 3. Quantitative and qualitative estimates of indole produced by 26 indole-positive gram-negative nonsporeforming anaerobes in six different test systems^a

Test medium, volume, and incubation conditions	µg of indole per ml		Cumulative % ^a positive Ehrlich's reagent			
	Median ^a (min-max)		1 day	2 days	3 days	7 days
	3 days	7 days				
Thioglycolate without glucose with 0.02% tryptophan						
10 ml, aerobic	5 (0->20)	9 (1->20)	12	81	96	100
1.0 ml, Gas-Pak jar	11 (0->20)	11.5 (0->20)	69	92	96	100
Indole-nitrite broth						
8.0 ml, aerobic	2.5 (0->20)	3 (0->20)	46	77	85	85
5.0 ml, flushed with O ₂ -free N ₂ gas	6.5 (0->20)	8 (0->20)	85	96	96	96
1.0 ml, Gas-Pak jar	5.5 (0->20)	6.5 (0->20)	77	89	96	96
Chopped meat broth						
10 ml, aerobic	1 (0-13)	3 (1-14)	39	81	92	100

^a An additional 24 strains were negative by all six methods after 7 days of incubation; percentage and median values were calculated after excluding results with the negative strains.

several *Enterobacteriaceae* for indole production under aerobic and anaerobic conditions and concluded that indole is produced in buffered media anaerobically as well as aerobically. They suggested that the positive influence of aeration on indole production was mostly due to increases in cell mass rather than an inferior enzyme activity under anaerobic conditions.

The negative effect of glucose on indole production appears with *B. fragilis* as well as with *E. coli*, an effect which has been studied by many investigators. Suassuna and Suassuna (9) concluded that glucose did interfere with indole production but qualitative test results were altered primarily with weakly positive groups of organisms. The two *B. fragilis* tested in this study (Table 2) demonstrate the importance of this observation. Strain no. 2 was qualitatively negative for indole in broth containing glucose, whereas strain no. 22 produced traces of indole in all anaerobically incubated glucose-containing media, but not in the same media grown aerobically.

The addition of tryptophan and omission of glucose stimulated indole production. One *B. fragilis* isolate showed a fivefold increase in indole after 3 days, and the other isolate showed a 2.6-fold increase in indole when 0.1% tryptophan was added to the PY base. Furthermore, addition of glucose to this medium neutralized the effect of tryptophan.

Comparison of some of the commonly used methods for indole production by anaerobic gram-negative bacilli (Table 3) demonstrated the need for standardization of methods. Indole-nitrite broth incubated aerobically gave the

largest number of false-negative results even after 7 days of incubation. If held long enough, the other methods and media showed nearly comparable results. However, preliminary tests performed after 1 to 2 days were positive more frequently in the small volumes of media incubated in anaerobic jars than in the larger volumes incubated aerobically. Thus, prolonged incubation is required less frequently in small volumes of broth than with the more conventional test methods.

Quantitatively, chopped meat medium gave the lowest average yield of indole and Loesche's medium (thioglycolate without glucose supplemented with tryptophan) produced the most indole. Of the 26 indole-positive organisms assayed after 3 days, six were negative in chopped meat and seven were negative in the aerobically incubated indole-nitrite broth. The increased growth under anaerobic conditions is presumably responsible for the increased production of indole.

The deleterious effect of nitrite on indole production has been described by Beam (1) and Smith et al. (7). If the nitrate is reduced to nitrite and if the nitrite accumulates in sufficiently large amounts, it will decrease the production of indole. Only one of the indole-positive anaerobes used in this study was capable of reducing nitrate, and its capacity to produce indole was not inhibited in the nitrate-containing medium. Perhaps the amount of nitrite produced by this organism was insufficient to inhibit indole production.

In summary, anaerobic gram-negative nonsporeforming bacilli produced the greatest

amount of indole when grown anaerobically in a glucose-free medium supplemented with tryptophan. Small volumes of broth incubated in anaerobic jars gave positive results earlier than larger volumes incubated aerobically.

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