An assessment of inositol fermentation by Klebsiellae and its implication in screening media

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SYNOPSIS Four patterns of inositol fermentation by different isolates of Klebsiellae are described. Inositol fermentation by some isolates may be modified by medium incorporation of acetoin and diacetyl. An inositol—hydrogen sulphide—motility medium, modified by reducing the pH value, increasing the inositol concentration, and the incorporation of acetoin, is described and evaluated as a means of detecting acid production by isolates showing different inositol fermentation patterns.

The implications of Klebsiella spp in nosocomial infections are widely appreciated. However, their differentiation from other genera of the Enterobacteriaceae remains a problem in laboratories dealing with large numbers of bacterial isolates and with limited available resources for extensive biochemical testing. Donovan (1966) proposed a simple screening medium for the differentiation of Klebsiella spp from other Gram-negative bacilli on the basis of motility, hydrogen sulphide production, and fermentation of inositol. Chadwick et al (1974) incorporated inositol fermentation in a proposed comprehensive biochemical identification of hospital Enterobacteria, and Davis and Matsen (1974) used a MacConkey agar with inositol instead of lactose for the detection of Klebsiellae in clinical specimens.

In this laboratory a preliminary evaluation of the Klebsiella screening medium of Donovan (1966) highlighted a number of problems in the interpretation of inositol fermentation and organism motility.

This paper described experiments which demonstrate the variation of inositol fermentation by Klebsiellae on one screening medium (Donovan, 1966). Modifications of this medium were designed to improve the differentiation of Klebsiella spp from other Enterobacteria in 16-24 hour incubations.

Material and methods

ORGANISMS

Clinical isolates
Eighty strains of Klebsiellae, isolated in this laboratory over a three-month period, were ascribed to species of Klebsiella, Enterobacter, and Serratia according to the diagnostic scheme of Cowan (1974). A further 20 clinical isolates of Klebsiella spp were obtained from Ruchill Hospital, Glasgow and included strains of Klebsiella aerogenes (7 in number), K. pneumoniae (4), K. ozaenae (2), K. oxytoca (2), K. atlantae (2), K. edwardsii (1), and K. rhinoscleromatis (2).

Stock cultures
The following 10 cultures were also examined: K. aerogenes NCTC 8172; K. pneumoniae NCTC 9633; K. ozaenae NCTC 5050; K. atlantae NCTC 9496; K. edwardsii NCTC 5054; K. rhinoscleromatis NCTC 5046; Enterobacter aerogenes NCTC 10006; Serratia marcescens NCTC 9940; Providencia B, sub-group B, NCTC 10318; and Providencia sp, NCTC 10286.

MEDIA

All media and filter-sterilized medium constituents were prepared in distilled water. Medium D, described by Donovan (1966), contained (g/l): Tryptone (Oxoid), 10-0; NaCl, 5-0; Agar (Oxoid), 3-0; 2, 3, 5, triphenyltetrazolium chloride, 0-05; FeSO4PO4, 0-3; Na2S2O3·5H2O, 0-2; K2HPO4, 0-3; and inositol, 10-0. Bromothymol blue or bromocresol purple indicators were added as aqueous solutions to give a final concentration of 0-003%. This basal medium was modified by adjustment of pH value with concentrated hydrochloric acid or 10% aqueous sodium hydroxide, adjustment of inositol concentration to 0-5, 1-0 or 2-0%, and by addition of aqueous solutions of sodium acetate, diacetyl or acetoin to give a final concentration of 100 mM (approximately 0-86%), 10 mM or 1 mM.

For the evaluation of strain variation in inositol fermentation medium F was used. This was derived...
from medium D by the omission of 2, 3, 5 triphenyl-
tetrazolium chloride and inositol, and the incorpora-
tion of 1-5% agar and 0-003% bromothymol blue. 
Medium F was modified by additions of sodium 
acetate, diacetyl or acetoin at the concentrations 
given above. Unless otherwise stated, media were 
adjusted to pH 7-2 and sterilized by autoclaving at 
115°C for 10 minutes.

Medium D, and modifications of this medium, 
were distributed in 4 ml amounts in bijou bottles or 
in single compartments of plastic Petri dishes (10 x 
10 cm) which contained a 5 x 5 grid of 18 mm³ com-
partments (Sterilin); medium F was dispensed 
accurately in 10 ml amounts in 100 mm diameter 
plastic Petri dishes. All media were allowed to 
solidify on a level surface to give an agar layer of even 
thickness.

Preparation of inocula and growth 
conditions
Medium D was stab inoculated directly from a colony 
on MacConkey agar.

Medium F was inoculated from 16-hour cultures 
in peptone water. The inoculum broth for each 
strain was adjusted, with sterile peptone water, to an 
optical density of 0-15 at 700 nm, using a Hilger 
spectrophotometer. A sterile cotton wool swab 
(Exogen Ltd, Glasgow) was dipped into the stand-
ardized inoculum, excess broth was expressed against 
the side of the tube, and the swab was streaked three 
ways across the entire surface of the plate. Using a 
sterile cork borer, four 8 mm diameter wells were 
punched halfway along four radii equidistant from 
each other, the agar plugs being removed under 
suction pressure. Four filter sterile solutions con-
taining 10%, 5%, 1%, and 0-1% inositol were pre-
pared, and 100 μl of each solution was dispensed 
into one of each of the four wells in turn. Plates were 
incubated and zones of acid production recorded to 
the nearest millimetre after 6, 9, and 12 hours. Acid 
production was made visible by an indicator colour 
change from blue at pH 7-2 to yellow at pH 6-0.

All incubations were carried out at 37°C.

Results

Strain variation in fermentation of 
inositol
One hundred clinical isolates of Klebsiella and 10 
stock cultures were examined by the methods des-
cribed for their ability to grow and produce acid in 
medium F supplemented with inositol diffusing from 
wells into the surrounding medium. To evaluate the 
reproducibility of the technique six replicate plates of 
each of six strains were examined after 6 and 12 hours.

No significant differences in zone diameters were 
recorded with any one strain.

Klebsiellae varied greatly in the pattern of inositol 
fermentation. Although strain differences occurred 
both in the size of zone produced and in the inositol 
concentrations supporting acid accumulation, the 
major difference was the time of accumulation and 
utilization of the acid products (fig 1).

The results (fig 1) demonstrate that four patterns 
were recorded, which represented strain variation in 
the accumulation and maintenance of acid meta-
bolites. Zones of acid accumulation increased with 
increasing inositol concentration, the majority of 
strains showing acid production from inositol diffus-
ing from wells containing a 10% or 5% substrate 
concentration. Five strains produced acid around 
wells containing 1% inositol while no strain pro-
duced acid around wells containing 0-1% inositol.

The fermentation patterns given by different 
species, groups of species or genera were evaluated 
for the 110 strains under investigation (table I). 
Although no pattern was strictly typical of any one 
species or genus, certain significant correlations were 
noted. K. aerogenes/oxytoca/atlantae/edwardsii pro-
duced acid rapidly and gave fermentation patterns I
Table I  Typical inositol fermentation patterns given by different groups of 110 strains of Klebsiellae.

<table>
<thead>
<tr>
<th>Klebsiellae</th>
<th>Total no. of strains</th>
<th>No. of strains giving specific fermentation patterns1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>K. aerogenes/oxytoca/atlantae</td>
<td>76</td>
<td>8</td>
</tr>
<tr>
<td>K. edwardsii</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>K. ozaenae and related biotypes</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter/Serratia</td>
<td>16</td>
<td>1</td>
</tr>
</tbody>
</table>

1 Typical fermentation patterns I, II, III, and IV outlined in fig 1.
2 Fermentation pattern V was assigned to strains failing to produce acid from inositol under the experimental conditions described.
3 No growth

... and II consistently, the two patterns differing in the time of conversion of acid to neutral fermentation products. K. pneumoniae and K. ozaenae, with one exception, either produced acid later (patterns III and IV) or failed to produce acid during the duration of the experiment (pattern V). Strains of Enterobacter spp and Serratia spp were characterized by fermentation patterns I, II, and III.

These results are consistent with those obtained by fermentation of the same strains in 1% inositol peptone water, when rapid fermentation was characteristic of K. aerogenes/oxytoca/atlantae/edwardsii while acid production by K. pneumoniae, K. ozaenae, and K. rhinoscleromatis was often recorded only after several days' incubation.

A rapid rise in pH value after 12 hours was shown by only 22 strains of the K. aerogenes/oxytoca group. This could be attributed to peptone digestion after the period of maximum acid accumulation.

EFFECT OF SODIUM ACETATE, DIACETYL, AND ACETOIN ON PATTERN OF INOSITOL FERMENTATION

The effect of incorporation in medium F of sodium acetate, diacetyl or acetoin on the fermentation pattern of 25 representative strains of Klebsiella spp was examined.

Sodium acetate, 1 mM, 10 mM, or 100 mM, did not affect the pattern of fermentation or size of zones of acid accumulation. Diacetyl, 10 mM and 100 mM, inhibited the growth of all organisms tested, but diacetyl, 1 mM, substantially increased zone diameters of acid production in 10 of the strains examined without altering the pattern of fermentation. However, diacetyl at this concentration inhibited growth of five of the strains examined.

Acetoin, 1 mM, did not influence acid accumulation or the pattern of inositol fermentation. At a concentration of 10 mM and 100 mM acetoin affected 15 of 25 strains by markedly increasing zone diameters of acid production and/or by supporting acid accumulation around wells containing inositol of lower concentration. This stimulation of acid production occurred with strains of K. aerogenes, K. oxytoca, K. edwardsii, K. atlantae, and K. rhinoscleromatis. Only trace amounts of acid production were detected by all strains of K. pneumoniae and some strains of K. ozaenae on medium F supplemented with acetoin. Acetoin, 10 mM or 100 mM, did not inhibit growth of any of the strains examined.

The characteristic effect of acetoin (100 mM) on the pattern (I) of inositol fermentation by K. aerogenes is shown in figure 2.

Fig 2  The influence of acetoin (100 mM) on acid accumulation and pattern of fermentation of K. aerogenes on medium F, with available inositol diffusing from a well containing (a) 10% inositol and (b) 5% inositol. The accumulation of acid was determined by measurements of zone diameters of indicator colour change: ○—○, medium F; ●—●, medium F + acetoin (100 mM).
Modification of Inositol—Hydrogen Sulphide—Motility Medium

Technique

Medium D, dispensed in bijou bottles and compartmented Petri dishes, was stab inoculated with each of 110 strains of Klebsiellae.

Variation in light transmission through the glass of bijou bottles made weakly motile organisms and highly diffuse outgrowths difficult to detect. Similarly, acid production could be overlooked if the acid products did not diffuse some distance from the line of stab inoculation.

Both problems were overcome by the use of compartmented Petri dishes. After incubation plates could be viewed by placing them between the eye and a light source. This allowed the detection of minimal zones of indicator colour change due to acid production and aided the detection of motility. All strains which gave positive fermentation reactions and/or motility reactions in bijou bottles gave the same result in compartmented Petri dishes.

Modification of basic medium

The variation in inositol fermentation pattern and the influence of acetoin on these patterns have been described. These results demonstrated that medium modification by alteration of inositol concentration and incorporation of acetoin could influence acid accumulation. Variation in the medium pH value, which could be influenced by inositol fermentation and peptone digestion, was also examined.

The effects of medium pH value and inositol concentration on detection of inositol fermentation by 110 strains of Klebsiellae are summarized in Table II. The results of preliminary investigations demonstrated that no improved detection of inositol fermentation was achieved with inositol concentrations in excess of 2%, which is shown here to give the best detection of inositol-fermenting strains. In 18-24-hour incubations, the incorporation of a 2% inositol concentration in a medium adjusted to pH 6.6 enhanced the accumulation of acid metabolites from inositol.

The indicator used, bromothymol blue, changed from green at pH 6.6 to yellow at pH 6.0 or below. This colour change, although less pronounced than a change from blue to yellow (Donovan, 1966), did not result in error in detection of inositol fermentation.

In parallel experiments using this modified medium (inositol, 2%; pH 6.6), bromocresol purple, 0.003%, was incorporated as the indicator of acid accumulation. Under these conditions only 60% of strains, whose acid production was detected by a colour change of bromothymol blue, could be confirmed as inositol-fermenting strains.

Influence of acetoin

Acetoin (100 mM) was incorporated into medium D (inositol 2%, pH 6.6). In parallel stab inoculations of 110 strains in media with and without acetoin, a significant difference in acid accumulation during 16-24-hour incubations was noted (table III). Acetoin stimulated acid production by strains of K. aerogenes, K. oxytoca, K. ozaenae, and K. pneumoniae, and allowed detection of acid production by eight strains which were negative in control inoculations.

Discussion

It has been shown that in medium F supplemented with inositol, four patterns of inositol fermentation were recorded. The patterns may reflect quantitative differences in acid accumulation and utilization by strains which have the same or different capacities for growth on this medium. However, since this investigation involved an evaluation of acid production on medium D and medium F only, the undoubted strain variation in growth need not be considered here. It would not be expected that the patterns of acid accumulation and utilization

<table>
<thead>
<tr>
<th>Klebsiellae</th>
<th>Total no. of strains</th>
<th>Number of inositol-fermenting strains detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inositol 2% (pH)</td>
<td>Inositol 1% (pH)</td>
</tr>
<tr>
<td></td>
<td>6.6</td>
<td>6.8</td>
</tr>
<tr>
<td>K. aerogenes/oxytoca/</td>
<td>76</td>
<td>70</td>
</tr>
<tr>
<td>atlantae/edwardii</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>K. ozaenae and related</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>biotypes</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>K. rhinoscleromatis</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Enterobacter/Serratia</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>97</td>
</tr>
</tbody>
</table>

Table II Influence of medium pH and inositol concentration on detection of inositol fermentation by Klebsiellae after 18-24 hour incubations
An assessment of inositol fermentation by Klebsiellae and its implication in screening media

<table>
<thead>
<tr>
<th>Klebsiellae</th>
<th>Total no. of strains</th>
<th>Acid production(^1) by Klebsiellae</th>
<th>- Acetoin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+ Acetoin</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ + +</td>
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<tr>
<td></td>
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<td>+ +</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

| K. aerogenes/oxytoca/atlantae | 76 | 63 | 9 | 3 | 1 | 46 | 12 | 12 | 6 |
| K. pneumoniae | 5 | 0 | 0 | 3 | 2 | 0 | 0 | 1 | 4 |
| K. ozaenae and related biotypes | 10 | 0 | 6 | 3 | 1 | 0 | 0 | 8 | 2 |
| K. rhinoscleromatis | 3 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| Enterobacter/Serratia | 16 | 2 | 12 | 2 | 0 | 2 | 13 | 1 | 0 |
| Total | 66 | 28 | 11 | 5 | 49 | 26 | 22 | 13 |

Table III Influence of acetoin (100 mM) on acid production by Klebsiellae in modified medium \(^1\) after 18-24-hour incubations

\(^1\)Medium, containing 2% inositol, was adjusted to pH 6.6

\(^4\)Acid production was recorded as -- (no indicator change), + indicator change along line of stab inoculation, + + clear zone of indicator change, and + + + complete indicator change within individual inoculation compartment

... observed would necessarily be reproduced on other nutrient media.

The four patterns observed allowed the strains of Klebsiellae examined to be gathered loosely into three groups on the basis of the time of accumulation and utilization of acids derived from fermentation. K. aerogenes, K. oxytoca, K. atlantae, and K. edwardsii accumulated acid rapidly and could be divided into two groups on the basis of the time of utilization of the acid metabolites (patterns I and II) while K. pneumoniae and K. ozaenae (patterns III and IV) accumulated acid slowly.

These patterns, although not influenced by sodium acetate, which had been shown to influence acetoin accumulation by Klebsiellae (Bryn et al., 1973), were influenced by medium incorporation of diacetyl and acetoin. Acetoin was selected as a medium additive because alone it stimulated acid accumulation of a substantial number of strains without limiting the growth of any. It is probable that both acetoin and diacetyl, at the concentrations used here, may influence acid accumulation and subsequent utilization either by an end product inhibition of action, or repression of synthesis, of enzymes associated with the conversion of acid metabolites of inositol metabolism to neutral products.

In the light of the patterns of fermentation encountered and the influence of acetoin on acid accumulation, the inositol - hydrogen sulphide-motility medium described by Donovan (1966) was modified with the aim of enhancing the detection of inositol-fermenting strains in 18-24-hour incubations.

The results obtained confirmed the widely accepted value of increasing the carbohydrate concentration (2%) (Stuart et al., 1949; Lowe and Evans, 1957) and further demonstrated the value of reducing the pH change (0.6 unit) required to give a clear indicator colour change.

The merit in adopting the minimal pH change which could give a clear result was confirmed by comparing the value of the indicators bromothymol blue and bromocresol purple in a medium at pH 6.6. Only 60% of strains which gave a detectable acid reaction with bromothymol blue (yellow, pH 6.0) also gave a detectable acid reaction when bromocresol purple was used (yellow, pH 5.2). This demonstrated that the increased detection of acid-producing strains at pH 6.6 was due to the reduced pH change required to cause an indicator colour change, and not to enhanced accumulation of acid at this pH value.

The incorporation of acetoin in the screening medium enhanced acid accumulation by representative strains of all Klebsiella spp and enabled the acid-producing potential of eight of 13 otherwise negative strains to be expressed.

The results indicate that a modified screening medium containing 2% inositol, 0.86% (100 mM) acetoin, and adjusted pH 6.6 increased the detection of acid production from inositol by Klebsiellae. However, only a relatively small number of slow inositol fermenters, K. pneumoniae and K. ozaenae, were included in this study, and the demonstration of acid production by these species using the method described cannot be considered to be satisfactorily solved by the medium modifications adopted. This indicates the limitations of using inositol fermentation as a criterion for the identification of these species.

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