# Cyanide Media in the Differentiation of Enteric Bacteria

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For more than 20 years the use of media containing potassium cyanide (KCN) has been advocated in the study of enteric bacteria. Braun and Guggenheim (1932) and Braun (1938, 1939) noted that cultures of the *Klebsiella-Aerobacter* group were able to grow in concentrations of KCN which inhibited *Escherichia coli*. Buttiaux (1952) employed the medium of Braun in the study of 280 *Salmonella* cultures and 56 cultures of the Bethesda (Bethesda-Ballerup) group (Edwards, West and Bruner, 1948; Bruner, Edwards and Hopson, 1949). The Bethesda-Ballerup group, as shown by West and Edwards (1954), is composed of cultures of *Escherichia freundii* which ferment lactose slowly or not at all and which, because of the similarity of biochemical reactions, long have been confused with salmonellae. Buttiaux found that the Bethesda cultures grew readily in Braun's medium whereas *Salmonella* were uniformly negative and the two groups could be distinguished with certainty within 24 hours.

While the results obtained by Buttiaux represented a notable contribution to the differentiation of the bacteria, the medium of Braun was time consuming in its preparation and was extremely unstable. It remained for Møller (1954) to devise a medium which could be prepared more simply and which was more stable, being usable for two weeks according to the statement of the author. The results of Møller (1954) and of Kauffmann and Møller (1955) indicated that this medium yielded reliable results in the differentiation of enteric bacteria.

So far as the routine use of KCN medium in the diagnostic laboratory was concerned, Møller's medium failed to fulfil only two requirements. The useful life of the medium was said to be only two weeks when stored at 4 C, and it contained ingredients not readily available in this country. The purposes of the present investigation were threefold: To test the action of Møller's medium on larger numbers of cultures than had hitherto been examined, particularly cultures of the *Salmonella*, Bethesda and Arizona groups; to determine the length of time the medium could be stored and to find a more stable cyanide which could be substituted for KCN; and to substitute a readily available peptone for the Danish peptone employed by Møller.

## Materials and Methods

KCN medium as employed by Møller was used in the investigation of 1981 cultures of the *Salmonella*, Bethesda, and Arizona groups. The cultures composed the type strains of the three groups as well as additional cultures of more frequently occurring serotypes. Among the *Salmonella* cultures tested were all of the presently recognized serotypes as well as at least 10 cultures each of such well known types as *S. paratyphi* A, *S. paratyphi* B, *S. typhimurium*, *S. montevideo*, *S. oranienburg*, *S. cholerae-suis*, *S. newport*, *S. typhi*, and so on. Adequate numbers of the various O groups and serotypes of the Bethesda and Arizona groups also were included in the study.

The medium of Møller was prepared as follows:

| | |
|---|---|
| Peptone (Orthana) | 10 g |
| Sodium chloride | 5 g |
| Potassium phosphate (monobasic) (anhydrous) | 0.225 g |
| Potassium phosphate (dibasic) | 5.64 g |
| Distilled water | 1000 ml |

pH adjusted to 7.6

The medium was autoclaved in flasks. Before use, 1.5 ml of 0.5 per cent KCN solution was added to each 100 ml of the chilled medium. The broth was distributed immediately into sterile 100 x 13 mm tubes in amounts of 1 ml, stoppered tightly with corks which had been soaked in hot paraffin, and stored at 4 C until used. The tubes were inoculated with a 2 mm loopful of a 24-hour nutrient broth culture, incubated at 37 C, and observed for 4 days.

After the above-mentioned cultures were tested in the medium of Møller, varying concentrations of different peptones were substituted for the Orthana peptone used in the original medium and 50 cultures each of the *Salmonella* and Bethesda groups tested in each lot of medium. Various cyanides were tested for differential properties. The keeping qualities of Møller's medium stored at 4 C, 25 C, and 37 C were examined.

## Results

The results obtained with Møller's KCN medium are given in table 1. Of 900 *Salmonella* cultures tested only 10, or 1.1 per cent, gave evidence of growth within a 4-days observation period. Of these 10 cultures, 4 were stock strains of *S. glostrup*, *S. senftenberg*, *S.* sp. (Type dusseldorf), and *S. kralendyk*. The remaining 6 consisted of 1 culture each of *S. paratyphi* B, *S. derby*, *S. oranienburg*, *S. montevideo*, *S.* sp. (Type muenchen), and *S.* sp. (Type newington) among 458 cultures received for diagnosis which were tested in KCN medium

TABLE 1. *Results obtained with Møller's KCN medium*

| Group | Number Tested | Number Positive | Number Negative |
|---|---|---|---|
| *Salmonella* | 900 | 10 | 890 |
| Bethesda | 580 | 574 | 6 |
| Arizona | 501 | 39 | 462 |

With the exception of the type cultures of *S. glostrup* and *S. kralendyk* which invariably gave positive results with 24 or 48 hours in repeated tests, none of these *Salmonella* cultures yielded visible growth until the third or fourth day of incubation. On the contrary, all of the 574 Bethesda cultures which were positive in KCN medium displayed vigorous growth within 24 hours. The 6 which failed to grow in 4 days were tested in the basic medium without KCN and grew vigorously. The biochemical and serological properties of these 6 cultures were examined and they were found to be typical members of the Bethesda group. It is notable that of 4 strains of O group 22 (West and Edwards, 1954) tested, 3 failed to grow.

The results with Arizona cultures were not so clear-cut as those obtained with salmonellae and Bethesda strains although only 7.7 per cent of the 501 cultures tested gave growth in KCN. The majority of these grew within 24 hours although a few showed no visible growth until the third or fourth day of incubation. Of the 39 positive Arizona cultures, 22 were members of O group 21 (Edwards, West and Bruner, 1947). Only 1 culture of O group 21 which was tested failed to grow. The remaining 17 positive Arizona cultures were scattered throughout a number of O groups. As in the case of the *Salmonella* and Bethesda cultures which gave aberrant results, the purity and identity of positive Arizona cultures were carefully controlled.

Among the cyanides which were examined for possible differential properties were potassium ferrocyanide, potassium ferricyanide, methyl cyanide, benzocyanide, and silver cyanide. Sodium thiocyanate and sodium azide also were tested. Of these substances only silver cyanide was found to be of value in differentiation. When dissolved in 10 per cent sodium thiosulfate solution and added to Møller's base in a final concentration of 1 to 2200, silver cyanide gave results fully as reliable as those obtained with KCN when a large series of *Salmonella* and Bethesda cultures was tested. In order to compare the keeping qualities of the two mediums, tubes of each were stored at 4 C, 25 C, and 37 C and inoculated with salmonellae at intervals. The results of these tests are given in table 2. It is evident that elevated storage temperatures lead to accelerated deterioration of the mediums and that there is little difference in the stability of mediums containing KCN and AgCN respectively. Further, it is apparent that Møller's medium can be depended upon in the differentiation of salmonellae and Bethesda cultures after storage for 30 days.

Since the Orthana peptone used by Møller is not readily available to American workers, the use of several domestic peptones was investigated. These substances were tested at different levels of concentration with a series of 50 cultures each of the *Salmonella* and Bethesda groups. While several of these peptones gave results which were quite good, Bacto proteose peptone No. 3 seemed most satisfactory. Used in a concentration of 0.3 per cent this peptone supported excellent growth of Bethesda strains in KCN medium while salmonellae did not develop. When the peptone concentration was increased to 1 per cent an occasional *Salmonella* culture gave doubtful results, a faint turbidity developed in the tubes after 3 or 4 days' incubation.

## DISCUSSION

It is axiomatic that any new test applied to a family of bacteria should be investigated in relation to all the groups or genera within the family. It usually is found that a given test is of greater practical value in the differentiation of certain of these groups than of others. Such is the case with the KCN test. Although Møller (1954) has demonstrated that the KCN test is of value in the differentiation of the several groups of enteric bacteria, it is obvious that the greatest value of the test lies in its application to the *Salmonella*, Arizona, and Bethesda groups. Hitherto it was not possible rapidly to distinguish these organisms by biochemical methods; whereas in other groups in which the KCN

TABLE 2. *Growth of salmonellae in stored cyanide mediums*

| Supplement | Storage Temperature | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 C | | | | | 25 C | | | | | 37 C | | | | |
| | Days of storage | | | | | | | | | | | | | | |
| | 7 | 20 | 28 | 40 | 50 | 4 | 8 | 12 | 20 | 28 | 4 | 8 | 15 | 20 | 28 |
| KCN* | 0/20‡ | 0/20 | 0/20 | 0/20 | 7/95 | 0/20 | 2/20 | 1/20 | 1/20 | 1/20 | 0/20 | 2/20 | 8/95 | 22/95 | 52/95 |
| AgCN† | 0/20 | 0/20 | 0/20 | 0/20 | 3/95 | 0/20 | 1/20 | 1/20 | 1/20 | 1/20 | 0/20 | 1/20 | 11/95 | 16/95 | 49/95 |

* Møller's medium supplemented with 1.5 ml. of 0.5 per cent KCN solution per 100 ml.

† AgCN dissolved in 10 per cent sodium thiosulfate solution and added to Møller's medium at a concentration of 1 to 2200.

‡ Numerator, number of tubes showing growth; denominator, number of tubes inoculated.

test is helpful in differentiation, reliable differential tests previously were available.

Further, it is obvious that the identity of a given organism should be judged by the pattern of its reactions in only a selected few. However, in the routine work of a diagnostic laboratory it often is necessary to use a minimal number of biochemical tests to eliminate an unknown organism as a member of a pathogenic group or to establish its identity as a probable pathogen. This need becomes acute in the study of enteric bacteria, in the identification of which large numbers of biochemical tests and prolonged observation may be required. If a test can be devised which is practical and easily applied and which will differentiate accurately and rapidly two groups of bacteria which occur frequently and which otherwise are difficult to distinguish, it is of great value in the diagnostic laboratory. It is believed that the KCN test as modified by Møller fulfils such a need in the differentiation of salmonellae and those strains of *E. freundii* which ferment lactose, sucrose, and salicin slowly or not at all (Bethesda-Ballerup group). It has been found in the present work that readily available materials may be used in the medium and that it can be stored for at least 30 days before it deteriorates. While Møller found that the medium was most effective in differentiation when one loopful of a fresh broth culture was used as inoculum, this is not a deterrent to its use in routine work. In most laboratories a suitable broth is inoculated for indol production. If, after overnight incubation, preliminary examination of biochemical reactions indicates that a culture in question may belong either to the salmonellae or to the Bethesda group, a tube of KCN medium may be inoculated from the broth culture which is to be used for the indol test. In this way the medium may be inserted into the usual series of biochemical tests when most needed without adding notably to the load of work performed. Obviously, it will be most useful in the study of those cultures which resemble salmonellae, but which fail to agglutinate in *Salmonella* serums. As judged by the cultures received in this laboratory as reference specimens, the organisms of the Bethesda group occur with great frequency both in normal and diseased persons and very often are confused with salmonellae. In view of this situation the use of KCN medium for differentiation in the diagnostic laboratory is strongly recommended.

## SUMMARY

The KCN medium of Møller was used in the study of 1981 cultures of the *Salmonella*, Arizona and Bethesda groups. Of 900 *Salmonella* cultures, only 10 were able to grow in the medium and of these only 2 developed within 48 hours. Of 580 Bethesda cultures, 574 or 99 per cent grew within 24 hours. Among 501 Arizona strains tested, 39 grew in the medium. Of the 39 positive Arizona cultures 22 were members of one O group. It was found that KCN medium could be stored safely for 30 days at 4 C and that domestic peptones could be used in its preparation. Its use in the diagnostic laboratory is recommended.

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