ACRIFLAVINE VIOLET RED BILE AGAR FOR THE ISOLATION AND ENUMERATION OF KLEBSIELLA PNEUMONIAE FROM ENVIRONMENT AND FOODS

by

Se-Ping Chien

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INTRODUCTION

Klebsiella pneumoniae is an important organism in medically related cases. The occurrence and transmission of this organism in the environment and through the food chain is not well known. One difficulty is the lack of good isolation and enumeration media for detecting this organism as the organism is frequently confused with Enterobacter in primary isolation media.

Dyes have long served as important selective and differential agents in bacteriological media. Studies by Fung and Miller (1973) on the effects of dyes on bacterial growth indicated that Acriflavine may be selective for Klebsiella. Fung and Niemiec (1977) reported that a Acriflavine Violet Red Bile agar was effective for the isolation of K. pneumoniae. That medium contains 0.06% Acriflavine dye in Violet Red Bile agar. However, the usefulness of the medium for enumeration of K. pneumoniae from the environment and food has not been tested.

The purpose of this investigation is to more clearly define the concentration of the dye in the medium for enumeration of K. pneumoniae from the environment and food and also to confirm the usefulness of the medium by identifying suspect cultures with the API-20E system. In addition, a brief survey for antimicrobial effects of some basic, acid and disperse dyes on microorganisms was done in this study. The basic dyes evaluated were many of those known for their growth-inhibiting potential in microbiological media, whereas the acid and disperse dyes represented those most widely used commercially on nylon carpeting.

LITERATURE REVIEW

Effect of Dyes on Microorganisms and Antimicrobial Properties of Dyes

Dye can be defined as an organic compound which contains chromophoric and auxochromic groups attached to benzene rings, the color being attributable to the chromophores and the dyeing property to the salt-forming auxochromes(Conn, 1977). Apart from their application to textiles, dyestuff are used for many purposes, divisible into two classes. The first is the coloration of various materials other than textile, such as paper, paints, foods, drugs, etc. Use of dyes as indicators, or as staining agents in bacteriological and histological work is also based on the light-absorption characteristics of dyes. The second includes noncoloring purposes, depending on the incidental properties of dyes, such as antibacterial and chemotherapeutic activity. Interests in the selective behavior of dyes dates back to Ehrlich's work with methylene blue. The discovery of the selective staining of nerve cells by methylene blue, and later observations that certain dyes stained and paralyzed specific microorganisms, led to the use of dyes as antibacterial and chemotherapeutic agents (Venkataraman,1952).

A striking selective power possessed by gentian violet was brought to light by Churchman (1912). The dye was applied directly to the organisms and its effect was noted after transplanting the stained bacteria to agar. Organisms whose growth is inhibited by gentian violet were called an "violet positive", and those whose growth was not affected by the dye "violet negative". There is some parallelism between violet reaction and gram reaction. He also claimed that the inhibiting action of the gentian violet is more constant when the dye is incorporated with the media than when it is applied directly to the bacterial cells. In the presence of gentian violet, the spores of the violet positive organisms cannot develop; or, if

they become vegetative cells, their growth ceases at that stage, but occasionally, certain "fast" individuals may escape entirely the effect of the dye. The bacteriostatic action of gentian violet on the violet positive organisms is more evident than bactericidal power.

Triphenylmethane dyes constitute a group of substances toxic to bacteria. Crystal violet, malachite green, brilliant green and gentian violet belong to this group. The relationship between the structural chemical factors and the inhibitive effect of aniline and some of its derivatives and of the triphenylmethane dyes on certain bacteria was studied by Kligler (1918). The antimicrobial action of the compounds is a function of the benzene nucleus, the added elements or radicals, their number and in the case of the dyes, probably the quinoid structure of the nucleus. The antiseptic power is enhanced by increasing the number of alky radicals and relative position of the introduced group may be a factor in determining the relative improvement in the effectiveness of the compound. He also found that the simple aniline derivatives, as well as the dyes, are more toxic for the gram-positive than gram-negative bacteria.

Petroff and Gump (1935) used 10 gram-positive and 7 gram-negative organisms to study the bacteriostatic property of some 130 dyes and allied compounds and selected the effective ones for the bactericidal test. Out of 130 compounds studied, 51 were effective for gram-positive and 23 for gram-negative organisms. The most effective for the gram-positive and gram-negative organisms were triphenylmethane dyes and anil quinoline compounds, respectively. Streptococcus as a group are much more resistant to bacteriostasis than other gram-positive organisms and Pneumococcus are the most sensitive ones. Of the gram-negative organisms, the three dysentery organisms studied possessed about similar sensitivities to bacteriostasis. The values of the bactericidal activities, of all

the compounds used in the bacteriostatic test, were of no importance.

Kline (1935) reported a study of the bacteriostatic effect of various concentrations of brilliant green dyes on 5 different organisms (Escherichia coli, Enterobacter aerogenes, Citrobacter sp., Bacillus subtilis and Bacillus cereus). The range of bacteriostasis was that members of the colon group were usually inhibited at between 1:200,000 and 1:400,000, while the spore forming organisms were usually inhibited at from 1:10,000,000 to 1:50,000,000. The variable factors which might influence the results included composition and reaction of the media, concentration of the dye, activity of the organisms, composition of the diluent and size of the inoculum.

The composition, pH and temperature of the growth medium are also factors influencing the antimicrobial activity of dyes in bacteriological media. Miller and Banwart (1965) studied the inhibitory effect of 24 different combinations of brilliant green and bile salt concentrations, using 7 species of microorganisms capable of fermenting mannitol. As the concentration of bile salts was increased, the inhibition of the organisms by brilliant green was decreased. Brilliant green might lose its inhibitory effect on bacteria in the presence of varied amounts of organic material. Studies by Moats et al. (1974) had shown that antimicrobial activity of brilliant green dyes in trypticase soy broth was reduced and ultimately destroyed by prolonged autoclaving at 121°C. Loss of antimicrobial activity is accompanied by decolorization of the dye. Moats and Maddox (1978) further tested four common dyes (crystal violet, ethyl violet, brilliant green and malachite green) as inhibitors of four types of bacteria (Salmonella anatum, Enterobacter aerogenes, Staphylococcus aureus and Bacillus cereus) over the pH range of 5.0-9.0. The sensitivity of the gram-negative organisms (S. anatum and E. aerogenes) to dyes was markedly affected by the pH of the medium. These organisms were roughly 100

times as sensitive to crystal violet and ethyl violet at pH 9 than at pH 5. Above pH 7, brilliant green and malachite green lost their inhibitory properties with these two organisms. Gram-positive organisms (S. aureus and B. cereus) were more sensitive to dyes and results were less affected by pH. A study of the Congo red reaction in bacteria and its differential ability for Rhizobium was undertaken by Hahn (1966). Color differences exhibited by the test organisms in yeast extract mannitol medium were found to be temperature dependent. Also, variation in the composition of the basal medium and storage of the prepared medium before inoculation were factors which influenced the colony coloration. A form to separate Rhizobium from soil organisms can be employed by incorporating Congo red either in a nitrogen-deficient (or in a nitrate medium) with incubation at 25°C to 28°C or in a nitrogen-rich medium with incubation at 37°C. Under these two conditions, Rhizobium will produce white and translucent colonies, respectively. Color differentiation among some test strains of R. meliloti and R. trifolii occurred on the nitrogen-rich agar medium when incubation was carried out at 25°C to 28°C. The Congo red reaction in bacteria is explained as due to the adsorption of Congo red on the surface of the bacteria cell, to the ions which predominate at the surface, and to the production of acid or alkali by the bacterium during growth.

Forty-two dyes (19 acidic, 20 basic and 3 neutral dyes) were used by Fung and Miller (1973) in a rapid screening procedure to test for inhibitory and differential properties against the growth of 30 species of bacteria. Gram-negative organisms exhibited greater resistance to dyes than gram-positive organisms and basic dyes were more inhibitory than acidic and neutral dyes at the same concentration. Shigella spp. in general are more sensitive to basic fuchsin, p-rosaniline and thioflavine TG, whereas Salmonella grew well in these dye media at

appropriate dilutions. Methyl violet B medium inhibited the growth of all Escherichia cultures tested, whereas it allowed all Enterobacter cultures to grow at 1:1,500 dilution. Acriflavine medium at 1:200,000 dilution permitted growth of five Staphylococcus aureus strains and 37 clinical isolates of S. aureus but inhibited all Micrococcus species tested. This paper presented evidence that many dyes not commonly used could be utilized for development of new selective and differential media to separate closely related bacterial taxa.

The incorporation of 69 dyes separately into an agar medium for evaluation of their effects on the quantitative recovery of five serotypes of Yersinia enterocolitica, one strain of Pseudomonas aeruginosa and one strain of Bacillus cereus was described by Schiemann (1979). Certain dyes were evaluated further for their selective properties with five additional serotypes of Y. enterocolitica, three strains of P. aeruginosa and two of Enterobacter spp. Y. enterocolitica was relatively resistant to dyes compared with gram-positive bacteria, but not much different from that of gram-negative organisms. Metanil yellow was the only dye which was tolerated better by Y. enterocolitica than by P. aeruginosa. Also, a great variation in dye sensitivity was found among serotypes of Y. enterocolitica as well as among strains of the same serotype.

According to the work done by Holmes (1982), the differentiation of five species of Legionella was facilitated by utilizing buffered charcoal-yeast extract medium containing 0.01% aniline blue in conjunction with a long-wave UV light. Of the five species tested, L. pneumophila, L. micdadei, L. bozemanii and L. dumoffii, when grown on this medium, all produced circular, raised, entire and shiny colonies, but with grey-white, light blue (with grey white periphery), uniform bright blue and dark blue colors, respectively. Only L. gormanii had circular, slightly-convex, bright-blue colonies with a mucoid appearance and viscous texture.

Under long wave (375um) UV light, L. bozemanii, L. dumoffii and L. gormanii produced blue-white fluorescent colonies and yellow-green medium fluorescence. L. pneumophila only had yellow-green medium fluorescence; the colonies did not fluoresce. No fluorescence was observed from the colonies or the medium of L. micdadei. However, for definitive identification by utilizing cellular fatty acid analysis, DNA homology studies or serotyping was suggested by the author.

Lin and Fung (1985) surveyed the effect of 101 organic dyes at various concentrations (1:1,000 to 1:1,000,000 dilutions) on 33 food-related yeast species and tested for inhibitory and differential properties of dyes against yeast groups. Malachite green at 1:250,000 exhibited promising ability to select Candida lipolytica from 12 species of Saccharomyces and 24 species of Candida. Candida lipolytica consistently gave typical white coarsely folded colonies on crystal violet medium at 1:1,000 dilution, while at 1:10,000 dilution, C. lipolytica and C. gropengiesseri showed distinct growth with white and purple colonies, respectively. These dye media can be further developed and applied for isolation of specific yeasts from contaminated food samples. Blue and violet dyes, such as trypan blue, atlantic sky blue FF, Erie brilliant violet B, Eriosin blue B and Jenner's stain, may help counting or differentiation by contributing their characteristic blue or violet to colonies.

Selective and Differential Media for Klebsiella pneumoniae

Klebsiella pneumoniae isolates are frequently confused with strains of Enterobacter aerogenes. Nunez and Colmer (1968) obtained 384 lactose-fermenting isolates from sugar cane and found that 88% of them were IMViC - - + + and would have been classified as Aerobacter aerogenes in the past. By using the classification of Ewing and Edwards (1960), they found that 98% of the so-called

Aerobacter isolates were Klebsiella and 2% were Enterobacter. Ewing (1973) also stated that most cultures submitted to his laboratory labeled as Enterobacter aerogenes were actually K. pneumoniae. This may occur because some workers isolating a lactose-positive coliform with IMViC pattern - - + + automatically identify it as E. aerogenes. At present time, K. pneumoniae is differentiated from E. aerogenes on the basis of motility and ornithine decarboxylase (Krieg & Holt, 1984). Urea, arginine dihydrolase and lysine decarboxylase are also helpful in speciation.

Because of the ubiquitous distribution and pathogenicity of Klebsiella, it is always desirable to have a selective and differential medium specific for the rapid detection and efficient recovery of Klebsiella. Three selective media (methyl violet lactose agar, double violet agar and double violet penicillin agar) for differentiation of Klebsiella pneumoniae from Enterobacter aerogenes on the basis of colonial morphology were evaluated by Campbell and Roth (1975). Strains of Klebsiella pneumoniae isolated from urine, fresh water and fresh produce were tested against other members of Enterobacteriaceae in addition to strains of Aeromonas hydrophila and Pseudomonas aeruginosa. Methyl violet lactose agar was the least inhibitory of the three tested media because of the simplest composition. Double violet agar, containing twice as much methyl violet as methyl violet lactose, as well as crystal violet and bile salts as inhibitors, was more inhibitory than methyl violet lactose agar. Double violet penicillin agar was the most inhibitory medium tested because of the addition of penicillin. These results indicated that double violet agar was the most adequate medium tested for differentiation between pure cultures of Klebsiella and Enterobacter. Klebsiella pneumoniae grew abundantly with 3-5 mm glistening lavender colonies while Enterobacter aerogenes had sparse growth of 1-2 mm, dry purple colonies. Other

species that are frequently isolated from water systems and could be confused with Klebsiella spp. on standard plate count agar, i.e., Escherichia coli or mucoid aquatic Pseudomonas, either did not grow at all or grew sparsely on double violet agar. Campbell et al. (1976b) further reported a field evalution of double violet agar for the isolation and presumptive identification of Klebsiella pneumoniae from water systems. Results had shown that accuracy in correctly picking Klebsiella pneumoniae that were later confirmed by biochemical tests averaged 85.4%.

Stramer (1976) studied the membrane-filtration method which can be used to determine rapidly Klebsiella pneumoniae densities in water with the use of m-FC broth. Findings of this study indicated that Klebsiella pneumoniae occurred repeatedly as light blue, nucleated, mucoid colonies while typical fecal coliforms (i.e, Escherichia coli) appeared dark blue and nonfecal coliforms (i.e, Enterobacter aerogenes) appeared grey-cream in color.

A selective medium containing 0.06% acriflavine in violet red bile agar was developed by Fung and Niemiec (1977) for the isolation and detection of Klebsiella. Klebsiella appeared as 5 to 7 mm mucoid, golden-yellow colonies while Pseudomonas aeruginosa and Enterobacter spp. appeared as small, brown to dark brown colonies. Escherichia coli as well as many other gram-negative and gram-positive organisms did not grow on this medium.

MacConkey-inositol-carbenicillin agar was first devised by Thom (1970) for Klebsiella detection in faeces. Bagley and Seidler (1978) used the similar medium with lower level of carbenicillin than Thom did as a selective medium for primary Klebsiella enumeration. Inositol was used as the substrate which is fermented by all Klebsiella cultures while carbenicillin was added as the primary inhibitor because environmental and clinical Klebsiella gave high resistance to this antibiotic, unlike other Enterobacteriaceae. Klebsiella appeared as pink-to red-

colored colonies on this medium. With pure cultures, nearly 100% recovery was observed and 95% of suspected colonies were verified as Klebsiella with environmental samples. Recovery and percent colony confirmation with MacConkey-inositol-carbenicillin agar were greater than other proposed Klebsiella selective media.

A highly selective medium using potassium nitrate as the specific nitrogen source for the enumeration and isolation of Klebsiella pneumoniae and Klebsiella oxytoca was developed by Wong et al. (1985). Crystal violet, neutral red and Noble agar were also contained in this medium to be the possible sources of assimilable nitrogen. Typical colony was either a 1-to 2-mm-diameter convex rather mucoid pink-to-red colony or a larger colony type which was more watery and pale red with a dark red center. Relatively little colonial growth occurred for any other bacterial genera, and where such colonies did grow, they could be differentiated because of atypical forms. Other Klebsiella spp. were never isolated on this medium. The sensitivity for the recovery of K. pneumoniae and K. oxytoca on Wong modified medium is from 80% to better than 100%. The medium appears to have potential value as a means of assessing the efficiency of treating sewage and monitoring the microbiological quality of vegetables.

Tomas et al. (1986) developed a new medium for the identification and selective recovery of Klebsiella spp. from environmental and clinical sources. MacConkey-inositol-potassium tellurite (MCIK) agar owes its differential capacity to the fact that about 97 to 99% of Klebsiella spp. and only 0 to 1% of Escherichia coli strains were capable of fermenting inositol and hence appear as red colonies. The selectivity of the medium is due to the presence of potassium tellurite since Klebsiella strains showed higher resistance to K_2TeO_3 than did other Enterobacteriaceae able to ferment inositol. With pure cultures, 100% recovery of

Klebsiella spp. and 0% recovery of other gram-negative organisms were observed. One hundred percent of all presumptive colonies were verified as Klebsiella spp.

Isolation and Enumeration of Klebsiella pneumoniae from Environment and Food Products

Klebsiella pneumoniae, also known as Friedlander's bacillus, is a relatively frequent cause of pneumoniae, particularly as a secondary invader in people already suffering from other pulmonary disease. It also causes the same kind of infections as the other gram-negative intestinal opportunists. Urinary tract, peritoneum, meninges and bloodstream are all likely places for their multiplication (Meyer, 1974). Klebsiella pneumoniae is an ubiquitous enteric which has been routinely isolated from food products and environments.

Brown and Seidler (1973) assessed several procedures for isolating and enumerating Klebsiella pneumoniae from vegetables and seeds. They found that nearly 50% of the vegetables and seven out of seven seed samples yielded organisms which biochemically and serologically were identified as Klebsiella pneumoniae and estimated numbers of K. pneumoniae were in the range of 10^3/g of vegetable peel for all trials yielding K. pneumoniae. Seven of the eleven K. pneumoniae serotypes encountered have previously been isolated from human urinary tract and other infections. Wright et al. (1976) also reported that the bacteria found in vegetable salads most frequently and in highest counts belonged to the Klebsiella-Enterobacter-Serratia group of Enterobacteriaceae. The recovery frequency of K. pneumoniae is 46% of samples. Therefore, fresh vegetables and vegetable salads may serve as potential reservoirs for K. pneumoniae for colonization and infection of susceptible patients.

From 165 dairy products with positive coliform counts examined by Schiemann

(1976), 38 of 607 isolates were accepted as K. pneumoniae. These 38 isolates originated from 25 dairy products (15.2% of total samples examined). Twelve of the products positive for K. pneumoniae were various flavors of gelato, a frozen desert made with pasteurized milk, suggesting the importance of human handling in the introduction of K. pneumoniae. Thirteen different serotypes, including types previously reported in clinical infections, were represented in 25 isolates from 25 products.

Among a variety of raw and processed food materials and waters from river, wells and city supplies surveyed by Rao et al. (1983), K. pneumoniae was most abundant in raw milk (6-8 per cent of coliforms) and vegetables (3-6 per cent of coliforms). Boutin et al. (1985) also reported that K. pneumoniae isolates were recovered in increased numbers from oysters harvested during the warm months of the year from approved and classified waters. But the consistently negative responses with most of the representative oyster isolates examined in animal models and cell systems indicated that environmental strains are not a public health risk.

Klebsiella pneumoniae has been recovered from cases of mastitis (Braman et al. 1973; Jasper et al. 1975). It is defined by Smith et al. (1985) as one of the environmental mastitis bacteria. Bedding material may play a key role in the transfer of environmental pathogens to the cow's udder, because teats are in close contact with bedding and the bacteria it harbors for prolonged periods.

Organisms identified as Klebsiella were isolated from 12 of 30 sawdust cow bedding samples (40% of total) by Newman and Kowalski (1973). Biochemical similarity was noted between sawdust and mastitis milk isolates. Results also indicated that changes in bedding materials from sawdust to sand and straw preceded the decrease in number of Klebsiella isolates.

Rendos et al. (1975) investigated the relationship between microbial

population of common bedding materials and the organisms recoverable from the teat ends. Sawdust, wood shavings and wheat straw as bedding materials were studied. It appeared that sawdust tended to support the highest populations of both total coliforms and Klebsiella and cows bedded on sawdust had the highest number of these organisms on their teat skin. Streptococcus were most numerous on straw-bedded cows while Staphylococcus were more numerous on straw- and sawdust-bedded cows. The higher number of bacteria on teat ends of sawdust-bedded cows may be influenced by the tendency of small sawdust particles to adhere to the teat skin.

Five sterilized bedding materials (fine hard wood chips, recycled dried manure, chopped newspaper, softwood sawdust and straw) and three bacteria species (Escherichia coli, Klebsiella pneumoniae and Streptococcus uberis) were used by Zehner et al. (1986) for a total of 15 bedding and bacteria combinations to study growth of pathogens in bedding materials free from contamination and nutrients from feces and urine. Growth of all bacteria was highest in recycled dried manure, second highest in straw, third in hardwood chips, and lowest in paper and sawdust. Klebsiella pneumoniae showed higher growth peaks than the other two species in all of the bedding materials. Zehner et al. (1986) concluded that clean, damp beddings may support bacterial growth. High bacteria counts under barn conditions are influenced by factors more complex than type of bedding used.

Much has been written in the literature concerning the distribution of Klebsiella pneumoniae in industrial organic wastes and potable or recreational waters. Dufour and Cabelli (1976) described that biodegrading polysaccharide-rich textile finishing plant wastes through the use of a fermentation tank-aeration tower system seems to favor the selective multiplication of bacteria of the genus Klebsiella. The results of the biochemical and mouse pathogenicity studies indicated that there are no substantial differences in these parameters between

effluent and clinical Klebsiella isolates.

Duncan and Razzel (1972) evaluated samples of water, soil and bark from three different forest environments and effluent samples from a pulp and paper mill for total and fecal coliform bacteria. Only a small percentage of the bacteria isolated were found to be Escherichia coli, whereas up to 71% of the isolates were identified as Klebsiella pneumoniae. Knittel et al. (1977) examined total coliform bacteria in pulp and paper mill effluents and found levels in excess of 10^5 organisms per 100 ml. Further evaluation showed 60 to 80% of these isolates to be Klebsiella pneumoniae. Huntley et al. (1976) further confirmed the presence of large numbers of Klebsiella pneumoniae in waters receiving pulp mill effluents. A total of 60% of the isolates were identified as K. pneumoniae, while 33.8% were found to be Escherichia coli.

Natural receiving water is also a habitat of Klebsiella pneumoniae. Two hundred and sixty-six strains of K. pneumoniae were isolated from various natural water sources by Matsen et al. (1974). Reactions with 28 biochemical tests showed percentage responses which were very similar to the summaries of primarily human Klebsiella isolates. In addition, there appeared to be no great differences with respect to the serotypes isolated.

Campbell et al. (1976a) also sampled water from three ponds and a large man-made lake. K. pneumoniae was isolated consistently and in high numbers from eutrophic waters even when environmental stress reduces total numbers. They concluded that mere isolation of the organism as an occasional contaminant probably does not present a hazard to public health.

Although the health significance of environmental Klebsiella strains is being debated, we still need to have good control measures to prevent K. pneumoniae from becoming an emerging pathogen.

The purpose of this investigation was to further refine the acriflavine violet red bile(AVRB) agar medium develped by Fung and Neimiec (1977) for isolation and enumeration of Klebsiella pneumoniae from food and emvironment. Suspect colonies from this AVRB medium were confirmed by API system for Enterobacteriaceae.

MATERIALS AND METHODS

Antimicrobial Properties of Acid, Basic and Disperse Dyes in Agar Media

Dyes

The dyestuffs evaluated were 10 basic dyes and one acid dyes which had been known for their antimicrobial properties in microbiological media. Alos, 22 acid and disperse dyes evaluated had been used industrially to dye nylon carpeting materials. All dyestuffs used are specified in Table 1 with the corresponding Color Index (C.I.) Generic Name and Number. Chemical classification, classical name and source are also listed in Table 1. Stock solutions were made by dissolving 0.1 g of each dye in 10 mL (1:100 dilution) of distilled water.

Microorganisms Tested

Three gram-positive and four gram-negative bacteria, one yeast and two molds were used in the agar medium tests (Table 2). Seven bacteria were grown in tryptic soy broth (Difco) at 37°C for 24 hr, while the yeast culture was grown in YM broth (Difco) at room temperature for 48 hr and molds were maintained on potato dextrose agar (Difco) slant at 21°C for 7 days before they were utilized as inocula in the following screening procedures.

Screening Procedures

Dyes were incorporated into the basal agar medium (tryptic soy agar) at final dilutions of 1:200, 1:1,000 and 1:10,000. Tryptic soy agar without dyes was used as a positive control. Sterilized media were poured into standard (100x15mm) disposable petri dishes (Fisher Scientific Co.), allowed to solidify, and placed in a 32°C incubator overnight before use. The actively growing broth cultures of test bacteria and yeast were introduced into individual wells of a sterile Microtiter

Table 1. Dyes evaluated and their C.I.[1] generic name and number, classification, classical name and source[2]

C.I. Generic Name	C.I. No.	Classification	Classical Name	Source
Basic Dye				
B. blue 9	52015	Thiazine	Methylene blue	DYE
B. green 1	42040	Triarylmethane	Brilliant green	IPC
B. green 4	42000	Triarylmethane	Malachite green	DYE
B. orange 2	11270	Monoazo	Chrysoidine	ACY
B. red 1	45160	Xanthene	Rhodamine	BAS
B. violet 1	42535	Triarylmethane	Methyl violet	ATI
B. violet 3	42555	Triarylmethane	Crystal violet	ACY
B. violet 4	42600	Triarylmethane	Ethyl violet	DSC
B. violet 14	42510	Triarylmethane	Fuchsine	DYE
B. yellow 2	41000	Diphenylmethane	Auroamine	ACY
Acid Dye				
A. blue 9	62055	Anthraquinone		CGY
A. blue 40	62125	Anthraquinone		ATL
A. blue 277		Anthraquinone		CGY
A. blue 324		Disazo		MOY
A. orange 156		Disazo		CKC
A. red 57		Monoazo		CKC
A. red 87	45380	Xanthene	Eosine	BAS
A. red 266		Azo		CKC

Table 1-continued

C.I. Generic Name	C.I. No.	Classification	Classical Name	Source
A. red 299		Disazo		CKC
A. red 337		Monoazo		CKC
A. red 361		Monoazo		CGY
A. yellow 49				MOY
A. yellow 135				ICI
A. yellow 151	13906	Monoazo		BAC
A. yellow 219		Disazo		CKC
Disperse Dye				
D. blue 7	62500	Anthraquinone		CGY
D. blue 26	63305	Anthraquinone		MOY
D. blue 56	63285	Anthraquinone		HST
D. red 17	11210	Monoazo		ATL
D. red 340				ECP
D. red 55		Anthraquinone		CGY
D. yellow 3	11855	Azo		CGY

1: C.I. = 1: Color Index
2: ACY=American Cyanamid Co.; ATI=Atlantic Industries; ATL=Atlantic Chemical Corp.; BAC=Baychem Corp.; BAS=BASF Wyandotte Corp.; CGY=Ciba Geigy Corp.; CKC=Crompton and Knowles Corp.; DSC=Dye Specialties Inc.; DYE=Dyestuff Inc.; ECP=Eastman Chemical Products Inc.; HST=American Hoechst Corp.; ICI=ICI Americans Inc.; IPC=Imperial Chemical Industries; MOY=Mobay Chemical Corp.

Table 2. Test Organisms

Microorganisms	Source	
Gram-Positive Bacteria		
Bacillus cereus	ATCC[a]	14579
Streptococcus faecalis	ATCC	19433
Staphylococcus aureus	ATCC	6538
Gram-Negative Bacteria		
Escherichia coli	ATCC	8739
Klebsiella pneumoniae	ATCC	4352
Pseudomonas aeruginosa	ATCC	13388
Salmonella choleraesuis	ATCC	10708
Yeast		
Candida albicans	ATCC	10231
Molds		
Aspergillus niger	ATCC	6275
Chaetomium globosum	ATCC	6205

a: ATCC = American Type Culture Collection

Plate (Dynatech Lab., Alexandria, Virginia) to form a master plate. From this master plate, organisms were transferred onto the agar surface of the control, basal medium and dye-containing media plates by use of a multipoint inoculation system (Fung and Miller, 1973). All inoculated plates were incubated at 37°C for 24 and 48 hr before observing the results.

Because of distinctive growth characteristics of molds, they are difficult to study in Microtiter Plates. Spore suspensions of molds were made by washing the slants of 7-day-old cultures with 3 mL of sterile distilled water with 2 drops of Tween 80 added (Fung et al., 1977). A sterile swab was used to gently dislodge and transfer the spores onto the dye-containing and control medium plates. Results were read after incubating plates at 21°C for 72 hr. Growth was recorded as positive or negative. All tests were done in 3 replicates.

Acriflavine Violet Red Bile Agar (AVRB) Medium for Selection of Klebsiella pneumoniae

Test Cultures

The named Klebsiella pneumoniae strains and other gram-negative organisms were obtained from the culture collections of the Department of Animal Sciences and Industry, Kansas State University; Mercy Hospital of Pittsburgh, Pennsylvania; Centers for Disease Control, Atlanta, Georgia; and American Type Culture Collection, Rockville, Maryland. Besides K. pneumoniae, other gram-negative cultures included were 2 strains of Citrobacter (C. diversus and C. freundii), 3 strains of Escherichia (2 E. coli and E. sakazaki), 3 strains of Enterobacter (E. aerogenes, E. cloacae and E. geigovia), Hafnia alevi, 2 strains of Proteus (P. mirabilis and P. vulgaricus), Providencia sturartii, 2 strains of Pseudomonas (P.

aeruginosa and P. fluorescens), 2 strains of Salmonella (S. choleraesuis and S. typhimurim), 2 strains of Serratia (S. liquefaciens and S. marcescens) and 2 strans of Shigella (S. flexneri and S. sonnei).

Media Preparation

Violet red bile agar (Difco) was used as a basal medium for the study of selectivity of acriflavine on the test organisms. VRB agar was boiled in 100 mL portions contained in dilution bottles. While the agars were still hot, various amounts (0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 g) of acriflavine hydrochloride (Sigma Chemical Co.) were added to the agar. Pour plates were made from the mixtures. Tryptone glucose extract agar (Difco) was used as a non-selective, control medium. The prepared plates were stable for at least one month in cold storage.

Experimental Procedures

The Klebsiella pneumoniae and other gram-negative cultures were streaked on VRB plates with different concentrations of acriflavine (0.01 to 0.06%) incorporated. Observations of colony development were made after 24, 48 and 72 hr of incubation at 37°C.

Quantitative Recovery Study of Klebsiella pneumoniae on AVRB Media

AVRB Media

AVRB media were made by incorporating acriflavine at 0.01, 0.02 and 0.06% concentrations into basal agar medium (VRB agar). The selective (AVRB) and control (tryptone glucose extract agar) media were poured into petri dishes. Prepared

plates were allowed to dry in 32°C incubator overnight before use.

Tested Klebsiella pneumoniae Strains

Ten Klebsiella pneumoniae strains were used for quantitative recovery study on 0.01, 0.02 and 0.06% AVRB media. Cultures of the test organisms were grown in sterile nutrient broth at 37°C for 24 hr and then diluted with nutrient broth until the solution had an absorbance of 0.28 ± 0.01 at 475 nm in a Spectronic 20 instrument (Bausch & Lomb Optical System, Rochester, New York). K. pneumoniae suspensions were prepared in phosphate buffer and diluted to give colony counts of approximately 10^3-10^4 CFU/mL (Dow Corning Corp., 1979).

Expression of Recovery

Prepared plates were inoculated by surface plating 0.1 mL of Klebsiella pneumoniae suspensions and incubated at 37°C. Counts were usually completed after 24 hr of incubation, but some plates with low counts were incubated for another 24 hr to confirm the results. Percent recovery was calculated as a number of colonies on AVRB medium per number of colonies on tryptone glucose extract (TGE) agar medium.

Recovery of Klebsiella pneumoniae from Artificially Contaminated Meat

Meat samples (ground beef) from grocery stores were inoculated with a selected strain of K. pneumoniae for the recovery study on 0.01% AVRB media. Fresh meat (ground beef) without inoculation was used as a control bacteria count. K. pneumoniae suspension with an absorbance of 0.28 ± 0.01 at 475 nm was prepared as described previously.

Nine gram meat samples were inoculated with 1 mL of K. pneumoniae suspension

and combined with 90 mL of phosphate buffer, then aseptically massaged in a Stomacher (Seward medical, London, England) for 30 sec. Appropriate 100-fold dilutions were made in phosphate buffer. Surface plate counts were done by transferring 0.1 mL of sample homogenate onto 0.01% AVRB and TGE plates. All plates were incubated at 37°C for 24 hr. Percent recovery of K. pneumoniae was calculated as a number of colonies on AVRB(0.01%) medium per number of colonies on TGE agar.

Evaluation of Acriflavine Violet Red Bile Agar in the Isolation of K. pneumoniae from Cow Beddings and Foods

Bedding Sample Preparation

Field evaluation of 0.01% AVRB medium was conducted using bedding materials from Kansas State University (straw and sand) and Pennsylvania State University (sawdust). Ten samples of each 3 different cow bedding materials were obtained from dairy barns. Straw first was chopped to facilitate handling by using alcohol flamed scissors. Five grams of each sample were suspended in 45 mL sterile phosphate buffer, then aseptically mixed in a Stomacher for 30 sec. After appropriate dilutions, duplicate surface platings on 0.01% AVRB and TGE media were done by the procedures described previously. Total count and AVRB count were made after incubation for 24 and 36 hr at 37°C.

Confirmation of Isolates from AVRB Plates by API-20E System

Mucoid, golden yellow or dull yellow colonies suspected to be K. pneumoniae were picked from AVRB plates for biochemical identification tests by API-20E System (Analytab Products Co., Plainview, New York). API-20E System is a

standardized, miniaturized version of conventional procedures for the identification of Enterobacteriaceae and other gram-negative bacteria. It is a ready-to-use, microtube system designed for the performance of 20 standard biochemical tests from isolated colonies of bacteria on plating medium. The API-20E System consists of microtubes containing dehydrated substrates. These substrates are reconstituted by adding a bacterial suspension, incubated so that the organisms react with the contents of the tubes, and read when the various indicator systems are affected by the metabolites or added reagents, generally after 18-24 hr incubation at 35-37°C. Used in conjunction with API Profile Recognition System, it is intended to identify members of the family Enterobacteriaceae and other gram-negative bacteria accurately and easily. Non-suspected K. pneumoniae colonies were also picked randomly for testing.

Survey of Food Samples

Raw milk and mastitis milk samples were obtained from dairy barn and veterinary school, Kansas State University. Hamburger meat samples were obtained from 4 different retail grocery stores. Surfacing plating was done by the procedures described previously. Total count, AVRB count and K. pneumoniae count were made after incubation at 37°C for 36 hr.

RESULTS AND DISCUSSIONS

Antimicrobial Properties of Basic, Acid and Disperse Dyes

All gram-positive and gram-negative bacteria, yeast and molds grew well on Tryptic Soy Agar (Difco), YM Agar (Difco) and Potato Dextrose Agar (Difco) basal medium, respectively. Although the final readings were taken at 24 hr for bacteria, 48 hr for yeast, and 72 hr for molds, little substantial change was observed after 24 more hours of incubation.

The effects of basic dyes on growth of the test organisms were listed in Table 3. Brilliant green, Malachite green, Crystal violet and Ethyl violet, which are all triarylmethane dyes, at 1:200 inhibited the growth of all 10 organisms tested. None of 3 gram-positive bacteria can grow on any basic dyes at 1:200 and 1:1,000. They only grew sparsely on some of these dye-containing media at 1:10,000. Gram-negative were more resistant to dyes than gram-positive bacteria. Of the 4 gram-negative organisms tested, Pseudomonas aeruginosa exhibited strongest capability to grow on dye-containing media. At 1:200, Methylene blue, Chrysoidine, Ethyl violet and Fuchsine successfully selected P. aeruginosa. Rhodamine at 1:200 and Methyl violet at 1:1,000 only allowed sparse growth of Salmonella choleraesuis.

Candida albicans was strongly inhibited by basic dyes. It showed little or negligible growth only on Fuchsine and Auroamine at 1:1,000 and 1:10,000. Brilliant green, Malachite green, Chrysoidine, Methyl violet and Crystal violet completely inhibited the growth of Aspergillus niger and Chaetomium globsum. A. niger could resist Ethyl violet and Methylene blue while C. globsum could resist Fuchsine at 1:200, 1:1,000 and 1:10,000.

None of the acid or disperse dyes had good separation ability of bacteria (Table 4). Gram-negative bacteria grew well on most of the dye-containing media.

Table 3. Effect of basic dyes on organisms in agar medium.

Dye	9			1			4			2			1		
Organism	C. I. Basic Blue (Methylene Blue)			C. I. Basic Green (Brilliant Green)			C. I. Basic Green (Malachite Green)			C. I. Basic Orange (Chrysoidine)			C. I. Basic Red (Rhodamine)		
	A[1]	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Gram-Positive Bacteria															
Bacillus cereus	-[2]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus faecalis	-	-	s	-	-	-	-	-	-	-	-	+	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	s	-	-	-
Gram-Negative Bacteria															
Escherichia coli	-	+	+	-	-	-	-	-	-	-	+	+	-	+	+
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Pseudomonas aeruginosa	+	+	+	-	+	+	-	+	+	+	+	+	-	-	+
Salmonella choleraesuis	-	s	+	-	-	+	-	+	+	-	+	+	s	+	+
Yeast															
Candida albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Molds															
Aspergillus niger	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Chaetomium globsum	-	-	+	-	-	-	-	-	-	-	-	-	-	-	s

1: Dilutions A = 1:200 B = 1:1,000 C = 1:10,000.

2: - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Table 3.- continued

Organism	Dye	1			3			4			14			2		
		C. I. Basic Violet (Methyl Violet)			C. I. Basic Violet (Crystal Violet)			C. I. Basic Violet (Ethyl Violet)			C. I. Basic Violet (Fuchsine)			C. I. Basic Yellow (Auroamine)		
		A[1]	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Gram-Positive Bacteria																
Bacillus cereus		-[2]	-	-	-	-	-	-	-	s	-	-	-	-	-	+
Streptococcus faecalis		-	-	-	-	-	-	-	-	s	-	-	-	-	-	s
Staphylococcus aureus		-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Gram-Negative Bacteria																
Escherichia coli		-	-	-	-	-	s	-	+	+	-	+	+	+	+	+
Klebsiella pneumoniae		-	-	-	-	-	-	-	-	-	-	s	+	-	-	+
Pseudomonas aeruginosa		-	-	s	-	s	+	+	+	+	+	+	+	+	+	+
Salmonella choleraesuis		-	s	s	-	s	+	-	s	+	-	+	+	+	+	+
Yeast																
Candida albicans		-	-	-	-	-	-	-	-	-	-	s	+	-	-	s
Molds																
Aspergillus niger		-	-	-	-	-	-	+	+	+	-	+	+	-	-	+
Chaetonium globsum		-	-	-	-	-	-	-	-	+	s	+	+	-	-	+

1: Dilutions A = 1:200 B = 1:1,000 C = 1:10,000.

2: - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Table 4. Effect of acid and disperse dyes on organisms in agar medium.

Dye	25			40			277			324			156		
Organism	C. I. Acid Blue			C. I. Acid Blue			C. I. Acid Blue			C. I. Acid Blue			C. I. Acid Orange		
	A[1]	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Gram-Positive Bacteria															
Bacillus cereus	-[2]	+	+	-	+	+	+	+	+	+	+	+	-	-	+
Streptococcus faecalis	s	+	+	-	s	s	s	s	s	s	s	s	s	s	+
Staphylococcus aureus	s	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Gram-Negative Bacteria															
Escherichia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella choleraesuis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Yeast															
Candida albicans	+	+	+	+	+	+	+	+	+	+	+	+	s	+	+
Molds															
Aspergillus niger	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Chaetomium globsum	s	+	+	-	+	+	+	+	+	-	+	+	s	+	+

1: Dilutions A = 1:200 B = 1:1,000 C = 1:10,000.

2. - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Table 4. - continued.

Dye	57	87	266	299	337
Organism	C. I. Acid Red	C. I. Acid Red (Eosine)	C. I. Acid Red	C. I. Acid Red	C. I. Acid Red
	A[1] B C	A B C	A B C	A B C	A B C
Gram-Positive Bacteria					
Bacillus cereus	-[2] - +	- - +	- - +	+ + +	- - +
Streptococcus faecalis	- - +	- s s	s s s	+ + +	s s s
Staphylococcus aureus	- + +	+ + +	- s +	+ + +	- - +
Gram-Negative Bacteria					
Escherichia coli	+ + +	+ + +	+ + +	+ + +	+ + +
Klebsiella pneumoniae	+ + +	+ + +	+ + +	+ + +	+ + +
Pseudomonas aeruginosa	+ + +	+ + +	+ + +	+ + +	+ + +
Salmonella choleraesuis	+ + +	+ + +	+ + +	+ + +	+ + +
Yeast					
Candida albicans	+ + +	+ + +	+ + +	+ + +	+ + +
Molds					
Aspergillus niger	+ + +	+ + +	+ + +	+ + +	+ + +
Chaetomium globsum	- + +	+ + +	s + +	+ + +	+ + +

1 : Dilutions A = 1:200 B = 1:1,000 C = 1:10,000.

2 : - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Table 4. - continued.

Organism	Dye	361 C. I. Acid Red			49 C. I. Acid Yellow			135 C. I. Acid Yellow			151 C. I. Acid Yellow			219 C. I. Acid Yellow		
		A[1]	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Gram-Positive Bacteria																
Bacillus cereus		-[2]	-	+	-	+	+	-	-	+	-	-	+	+	+	+
Streptococcus faecalis		-	-	+	s	s	+	-	s	+	-	-	+	+	+	+
Staphylococcus aureus		-	-	+	+	+	+	-	-	+	-	-	+	+	+	+
Gram-Negative Bacteria																
Escherichia coli		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Klebsiella pneumoniae		+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella choleraesuis		+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Yeast																
Candida albicans		s	+	+	+	+	+	-	-	+	-	s	s	+	+	+
Molds																
Aspergillus niger		+	+	+	+	+	+	-	-	+	-	+	+	+	+	+
Chaetomium globsum		s	+	+	s	+	+	-	-	+	-	+	+	+	+	+

1 : Dilutions A = 1:200 B = 1:1,000 C = 10,000.

2 : - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Table 4. - continued.

Dye	7			26			56			17			340		
Organism	C. I. Disperse Blue			C. I. Disperse Blue			C. I. Disperse Blue			C. I. Disperse Red			C. I. Disperse Red		
	A[1]	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Gram-Positive Bacteria															
Bacillus cereus	-[2]	-	-	+	+	+	+	+	+	-	+	+	-	+	+
Streptococcus faecalis	s	s	s	s	s	s	s	s	+	s	+	+	s	+	+
Staphylococcus aureus	-	-	+	+	+	+	+	+	+	-	+	+	s	+	+
Gram-Negative Bacteria															
Escherichia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	s	+	+	+	+	+
Salmonella choleraesuis	+	+	+	+	+	+	+	+	+	s	+	+	+	+	+
Yeast															
Candida albicans	+	s	s	s	s	s	+	s	s	-	-	s	s	s	s
Molds															
Aspergillus niger	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Chaetomium globsum	s	+	+	+	+	+	s	+	+	-	+	+	s	+	+

1 : Dilutions A = 1:200 B = 1:1,000 C = 1:10,000.

2 : - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Table 4. - continued.

Dye	55			3			250		
Organism	C. I. Disperse Red			C. I. Disperse Yellow			C. I. Telon Red		
	A[1]	B	C	A	B	C	A	B	C
Gram-Positive Bacteria									
Bacillus cereus	-[2]	-	+	+	+	+	-	+	+
Streptococcus faecalis	s	s	+	s	+	+	s	+	+
Staphylococcus aureus	+	+	+	+	+	+	+	+	+
Gram-Negative Bacteria									
Escherichia coli	+	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+
Salmonella choleraesuis	+	+	+	+	+	+	+	+	+
Yeast									
Candida albicans	+	+	+	+	+	+	+	+	+
Molds									
Aspergillus niger	+	+	+	+	+	+	+	+	+
Chaetomium globsum	+	+	+	+	+	+	s	+	+

1 : Dilutions A = 1:200 B = 1:1,000 C = 1:10,000.

2 : - = no growth, + = growth, s = sparse growth, data from triplicate samples.

Gram-positive bacteria, the yeast and molds showed better growth than on basic dye-containing media.

In general, basic dyes had stronger inhibitory ability than acid and disperse dyes. Compared with gram-negative bacteria, gram-positive bacteria, yeast and molds were more sensitive to dyes.

Acriflavine Violet Red Bile Medium for Selection of Klebsiella pneumoniae

Acriflavine, a basic dye, is the most powerful antiseptic reagent of the acridine dyes (Fairbrother and Renshaw, 1922).

The effect of 0.06% acriflavine on growth of selected K. pneumoniae strains, Enterobacteriaceae and other organisms has been studied by Fung and Niemiec (1977). In this study, we retested and refined the AVRB medium for the identification and selective recovery K. pneumoniae from environmental and food sources.

Eleven K. pneumoniae strains from diverse sources grew equally well on VRB agar with various amounts of acriflavine (0.01 to 0.06%) incorporated and showed no differences in colonial morphology except strain ATCC 4352 (Table 5). K. pneumoniae grew as a large, mucoid and glistening colony, generally about 3 to 5 mm in diameter, and appeared with a puffy center on AVRB media. Colonies were either typical golden-yellow as Fung and Niemiec (1977) observed or dull-yellow in color. Color variation might be due to strain characteristics. As shown in Table 5, although most Klebsiella grew on AVRB agar with 0.04, 0.05 and 0.06% acriflavine, growth was poorer and the colonies were smaller than at lower concentrations (0.01, 0.02, and 0.03%). Higher concentrations of acriflavine probably have a certain inhibitory effect on growth of K. pneumoniae. For this reason, the lowest acriflavine concentration (0.01%) was used throughout the remaining experiments.

Table 5. Effect of different concentrations of acriflavine in VRB[1] agar on growth of selected *Klebsiella pneumoniae* strains

Organisms	Source	Violet Red Bile Agar + Acriflavine at					
		0.01%	0.02%	0.03%	0.04%	0.05%	0.06%
Klebsiella pneumoniae	CDC[2]	+[3]	+	+	S	S	S
Klebsiella pneumoniae	CDC Type 2	+	+	+	S	S	S
Klebsiella pneumoniae	KSU	+	+	+	S	S	S
Klebsiella pneumoniae	KSU	+	+	+	S	S	S
Klebsiella pneumoniae	KSU	+	+	+	S	S	S
Klebsiella pneumoniae	ATCC 4352	-	-	-	-	-	-
Klebsiella pneumoniae	MHP Urine	+	+	+	S	S	S
Klebsiella pneumoniae	MHP Urine	+	+	+	S	S	S
Klebsiella pneumoniae	MHP Urine	+	+	+	S	S	S
Klebsiella pneumoniae	MHP Wound	+	+	+	S	S	S
Klebsiella pneumoniae	MHP Sputum	+	+	+	S	S	S

1: VRB = Violet Red Bile Agar

2: CDC = Center for Disease Control, KSU = Kansas State University, ATCC = American Type Culture Collection, MHP = Mercy Hospital of Pittsburgh

3: + = good growth, convex, mucoid golden yellow or dull yellow colonies with 3-5 mm diameter, S = sparse growth, - = no growth

K. pneumoniae ATCC 4352 did not grow at all on AVRB media. This strain was cited as a test organism under the name Escherichia coli with Simmons citrate and malonate negative reactions (American Type Culture Collection, 1982). Similar growth characteristics to E. coli might be the reason of this culture not growing on AVRB media. In addition, long cultivation time in laboratory (as in a culture collection center) could cause some strains to lose certain activities.

Table 6 showed the color reactions and growth responses of selected Enterobacteriaceae and other gram-negative organisms on 0.01% AVRB medium. All test organisms grew well on non-selective Tryptone Glucose Extract(TGE) agar. C. diverse, P. vulgaricus, P. sturartii, S. choleraesuis and S. typhimurim gave similar growth characteristics on this media, i.e., pale transparent and small colonies 1 mm or less in diameter. C. freundii, H. alevi, P. fluorescens, S. liquefaciens and S. flexneri grew sparsely with similar small and pale transparent colonies appearing after 48 hr. E. aerogenes grew well on this medium, but colonies were usually small, dark brown in color. E. cloacae produced brown, flat and larger (5 mm) colonies with concave center. E. geigovia grew only sparsely, forming a few small-sized, brown colonies. Two E. coli strains had slightly larger (3 mm) colonies that were brown in color. E. sakazaki had heavy growth with big (6-8 mm) and flat colonies. Colonies of S. marcescens were similar to those of E. coli. P. mirabilis, P. aeruginosa and S. sonnei exhibited no growth on the plate even after incubation for 72 hr.

Violet red bile agar, designed for enumerating coliform bacteria, does not allow the growth of gram-positive bacteria. As expected, several gram-positive bacteria streaked on 0.01% AVRB medium showed no growth (data not shown).

Fung and Niemiec (1977) concluded that 0.06% AVRB was appropriate for isolation and detection of Klebsiella. We demonstrated that at lower concentrations

Table 6. Effect of acriflavine on growth of selected *Enterobacteriaceae* and other gram-negative organisms

Organisms	Source	TGE[2]	VRB+ Acriflavine at 0.01%	Remarks
Citrobacter diversus	CS[1]	+[3]	+	small, pale transparent (< 1mm)
Citrobacter freundii	CDC	+	s	small, pale transparent
Enterobacter aerogenes	ATCC 13042	+	+	small, dark brown (1mm)
Enterobacter colacae	ATCC 13047	+	+	flat, brown, concave center (5mm)
Enterobacter geigovia	KSU	+	s	small, brown
Escherichia coli	ATCC 8739	+	s	brown (3mm)
Escherichia coli	ATCC 11775	+	+	brown (3mm)
Escherichia sakazaki	KSU	+	+	big, flat, brown (6-8mm)
Hafnia alevi	CDC	+	s	small, pale transparent
Proteus mirabilis	CDC	+	-	
Proteus vulgaricus	CDC	+	+	small, pale transparent
Providencia sturartii	CS	+	+	small, pale transparent
Pseudomonas aeruginosa	ATCC 133388	+	-	
Pseudomonas fluorescens	ATCC 13525	+	s	small, pale transparent
Salmonella choleraesuis	ATCC 10708	+	+	small, pale transparent
Salmonella typhimurim	ATCC 23566	+	+	small, pale transparent
Serratia liquefaciens	CDC	+	s	small, pale transparent
Serratia marcescens	ATCC 13380	+	+	brown
Shigella flexneri	CDC	+	s	small, pale transparent
Shigella sonnei	CDC	+	-	

1: CS = Cathra System, CDC = Center for Disease Control, ATCC = American Type Culture Collection, KSU = Kansas State University.

2: TGE = Tryptone Glucose Extract Agar.

3: + = good growth, s = sparse growth, - = no growth.

(to 0.01%) better growth of K. pneumoniae was obtained (see Table 5). At 0.01% acriflavine, colonial size and morphology of K. pneumoniae were distinctive when compared with the other gram-negative organisms which can grow at this low concentraion. In our judgment, 0.01% acriflavine is the most adequate concentration tested for differentiating between pure cultures of K. pneumoniae and other gram-negative organisms and for enumeration of K. pneumoniae from food and environmental samples.

The most important advantage of this selective medium over other proposed selective Klebsiella media is simplicity. No complicated components are needed as compared with Wong's modified medium (Wong et al., 1985) which had more than 10 added ingredients. No autoclave sterilization is required before use in this AVRB media. Relatively untrained laboratory personnel could prepare it with little practice. Another advantage is that acriflavine had a longer and more constant potency. Poured and uninoculated plates can be stored in 4°C for at least 2 months without any contamination, as compared with MCIC medium (72 hr at 4°C according to Bagley and Seidler, 1978.)

Quantitative Recovery Study of Klebsiella pneumoniae on AVRB Medium

The percent culture recovery for all types of K. pneumoniae on 0.01, 0.02, and 0.06% AVRB agar were presented in Table 7. The recovery rate of K. pneumoniae averaged 73, 55 and 34% at 0.01, 0.02, and 0.06%, respectively. Although recovery rate on 0.01% AVRB varied from 45.2 to 98.9% between strains, clinical strains had stronger recovery capability than laboratory strains.

According to Rao et al. (1983), Klebsiella species were found in several types of food materials with K. pneumoniae being the most abundant. K. pneumoniae

Table 7. Comparative recovery of *Klebsiella pneumoniae* by surface plating on selective acriflavine violet red bile agar media

Organisms	Source	% Culture recovery[1] with VRB + Acriflavine at		
		0.01%	0.02%	0.06%
Klebsiella pneumoniae	CDC[2]	52.0	23.0	1.9
Klebsiella pneumoniae	CDC Type 2	63.9	65.7	53.7
Klebsiella pneumoniae	KSU	65.0	62.3	17.7
Klebsiella pneumoniae	KSU	54.7	35.2	10.2
Klebsiella pneumoniae	KSU	45.2	21.9	3.6
Klebsiella pneumoniae	MHP Urine	83.4	59.4	44.5
Klebsiella pneumoniae	MHP Urine	82.1	61.8	43.3
Klebsiella pneumoniae	MHP Urine	94.7	75.0	60.6
Klebsiella pneumoniae	MHP Wound	98.9	96.1	86.3
Klebsiella pneumoniae	MHP Sputum	88.4	45.7	22.8

1: Percent recovery calculated as number of colonies on AVRB per number of colonies on tryptone glucose extract agar.

2: CDC = Center for Disease Control, KSU = Kansas State University, MHP = Mercy Hospital of Pittsburgh.

(MHP Wound strain, see Table 5) which had the highest recovery capability on AVRB media from our preliminary study was inoculated into hamburger meat for recovery study.

Raw meat without K. pneumoniae inoculation had average bacterial counts of 4.5×10^4 CFU/g on non-selective agar (TGE agar) and of 4.0×10^2 CFU/g on AVRB agar. However, no typical K. pneumoniae colonies (golden- or dull-yellow) were founded on AVRB plates from the natural raw meat . The distinct color reaction of K. pneumoniae helps to distinguish them from other gram-negative bacteria on AVRB medium. K. pneumoniae suspension with viable cell count of 1.3×10^8 CFU/mL was inoculated into meat samples (Table 8). Recovery counts from the meat indicated an average of 6.4×10^7 CFU/mL of organisms on TGE and 4.1×10^7 CFU/mL on AVRB agar. Recovery rate averaged 81% (AVRB count/TGE count x 100%). Since these numbers are far greater than the non-inoculation meat counts (4.5×10^4/g for total counts and 4.0×10^2/g for AVRB count), we ignored these latter numbers in the calculations. Since heavy K. pneumoniae inoculum was seeded into meat, TGE and AVRB counts were considered as K. pneumoniae counts.

From the results shown in Table 7 and Table 8, AVRB medium is capable of giving a resonable percentage of recovery of K. pneumoniae from pure cultures and artifically contaminated foods.

Auxillary data indicated that meat particles trap bacteria and only allow 32% true recovery from the meats inoculated. For example, the concentrate inoculated into meat was calculated to be 1.3×10^8 CFU/mL, but the number of organisms obtained from meat was 4.7×10^7 CFU/mL.

Table 8. Viable cell counts and recovery rate of Klebsiella pneumoniae from artifically contaminated meat on AVRB medium

Treatment	Total[a] Count	K. pneumoniae (AVRB)Count	Recovery[b](%)
Standard[c]		1.3×10^8	
1[d]	2.2×10^7	2.1×10^7	95
2	5.5×10^7	3.9×10^7	71
3	5.3×10^7	2.9×10^7	55
4	3.0×10^7	3.6×10^7	120
5	1.4×10^7	1.3×10^7	93
6	2.1×10^8	1.1×10^8	52

a: Viable cell counts are expressed as CFU/mL, data represents average of 2 replicates.
b: Percentage of recovery calculated as K. pneumoniae count divided by total count from the same meat sample.
c: Standard = K. pneumoniae inoculum with an absorbance 0.28 $\pm$ 0.01 at 475nm.
d: 6 replicates of meat samples inoculated with K. pneumoniae .

Evaluation of Acriflavine Violet Red Bile Agar In the Isolation of K. pneumoniae from Cow Beddings and Foods

Pure culture techniques are necessary for the development of selective media, but field testing is essential in determining efficiency in practical use. Previous studies from Fung and Niemiec (1977) showed that AVRB medium can be used to isolate K. pneumoniae from cow bedding. Therefore, straw, sawdust, and sand as bedding materials were obtained for our field evaluation of AVRB medium. Logarithm of geometric means of bacterial populations in 3 different beddings are listed in Table 9. Total count was higher in straw and sawdust than in sand, but differences between straw and sawdust were not significant. AVRB counts in bedding materials were largely E. coli , a much smaller proportion of Klebsiella pneumoniae and variable numbers of other gram-negative organisms. K. pneumoniae count was obtained by counting golden- or dull-yellow colonies. Difference was significant for AVRB count with sawdust supporting the larger population than straw and sand. The K. pneumoniae count was significantly higher in sawdust than in sand and straw. In general, sawdust had the highest count from AVRB and K. pneumoniae counts while straw had highest total count, intermediate AVRB count and lowest K. pneumoniae count. Sand seemed to be the best bedding material with lowest bacteria counts.

This is in general agreement with the work of Rendos et al. (1975), who found that sawdust appeared to support the highest concentration of both total coliforms and Klebsiella while straw had largest populations of Streptococcus and Staphylococcus. These data indicated the value of the AVRB medium for one-step enumeration of K. pneumoniae from environmental samples.

API-20E Biochemical Tests

Sixty golden- or dull-yellow colonies suspected to be K. pneumoniae were

Table 9. Logarithms of geometric means[1] of bacterial populations in 3 different beddings

Bedding Material	Total[2] Count	AVRB Count	Klebsiella pneumoniae
Straw	9.33^{a}	$4.38^{a,b}$	1.30^{b}
Sawdust	8.87^{a}	5.27^{a}	2.99^{a}
Sand	7.29^{b}	3.52^{b}	1.32^{b}

1: means based on 10 observations .
2: viable cell counts are expressed as $LOG_{10}CFU/g$.
a,b:means within bedding columns with different superscripts are significant different ($P<.05$).

picked at random from AVRB plates for testing in API-20E ststem. API-20E System is a highly reliable commercial system for the identification of Enterobacteriaceae with 90% accuracy in speciation of cultures. (Cox et al., 1977). The results of the biochemical tests obtained with 60 isolates (15 groups) were given in Table 10. Of the 60 mucoid, golden- or dull-yellow colonies studied, 44 were determined to be K. pneumoniae by API-20E Profile Recognition System, 7 were characterized as biotypes of K. pneumoniae which were indole positive. Some variable biochemical reactions for arginine dihydrolase, ornithine decarboxylase, citrate, urease, indole and inositol tests were obtained from these 51 colonies. Accuracy in correctly picking suspected K.pneumoniae that were later confirmed by API biochemical tests was calculated as 85% (51/60 x 100%).

Nine isolates suspected to be K. pneumoniae from AVRB colony morphology could not key out from API profile system, but they were not identified as other members of Enterobacteriaceae. Most of those atypical isolates exhibited gelatin, hydrogen sulfide and ornithine decarboxylase positive reactions. The reason might be mixed cultures obtained from AVRB plates, since we did not purify the isolates before performing the API tests. Also, since API Profile Recognition System was based on the biochemical reactions obtained from clinical specimens, variance probably existed in environmental isolates.

Thirty colonies believed not to be K. pneumoniae were also randomly isolated and analyzed. All of them were shown to be non-K. pneumoniae (Table 11). Escherichia coli, which appeared as dry, brown colonies with red ring, was the major organism isolated from AVRB plates. Abundant numbers of Enterobacter species were also found in bedding materials. They produced variable colony types which were not very similar to those of their pure cultures (described in Table 6), but they were distinguished from K. pneumoniae. Pseudomonas species exhibited very

Table 10. API-20E biochemical identification of suspected *Klebsiella pneumoniae* isolates obtained from AVRB plates

Suspected *K. pneumoniae*	No. of isolates	Biochemical Characteristics[1]																					Remarks
		OPNG	ADH	LDH	ODH	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OXI	
Group 1	20	+	-	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-	*K. pneumoniae*
Group 2	8	+	-	+	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-	*K. pneumoniae*
Group 3	8	+	-	+	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-	*K. pneumoniae*
Group 4	3	+	-	+	-	-	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	-	*K. pneumoniae*
Group 5	2	+	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	+	+	+	+	-	*K. pneumoniae*
Group 6	2	+	-	+	+	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-	*K. pneumoniae*
Group 7	1	+	+	+	-	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-	*K. pneumoniae*
Group 8	4	+	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+ indole
Group 9	3	+	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+ indole
Group 10	3	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	nonidentifiable
Group 11	2	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	nonidentifiable
Group 12	1	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	nonidentifiable
Group 13	1	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	nonidentifiable
Group 14	1	+	-	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	nonidentifiable
Group 15	1	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	nonidentifiable
Total	60																						

1: OPNG = o-nitrophenyl-ß-d-galactoside; ADH = arginine dihydrolase; LDC = lysine decarboxylase; ODC= ornithine decarboxylase; CIT = citrate; H_2S = hydrogen sulfide; URE = urease; TDA = tryptophane deaminase; IND = indole; VP = Vogues-Proskauer; GEL = gelatin; GLU = glucose; MAN = mannitol; INO = inositol; SOR = sorbitol; RHA = rhamnose; SAC = saccharose; MEL = Melibiose; AMY = amygdalin; ARA = arabinose; OXI = oxidase

2: + = positive reaction, - = negative reaction

Table 11. API-20E biochemical identification of non-suspected *Klebsiella pneumoniae* isolates obtained from AVRB plates[1]

Organism	No. of isolates	ONPG	ADH	LDH	ODH	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OXI
Escherichia coli																						
Group 1	8	+	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	-	+	-
Group 2	2	+	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	+	-
Enterobacter aerogenes	1	+	-	-	+	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-
Enterobacter agglomerans																						
Group 1	2	+	-	-	-	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	-
Group 2	1	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	+	+	+	-
Group 3	1	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	-
Group 4	1	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	+	-
Enterobacter cloacae																						
Group 1	2	+	+	-	+	+	-	-	-	-	+	-	+	+	-	+	+	-	+	+	+	-
Group 2	1	+	+	-	+	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-
Enterobacter spp.	1	+	-	-	+	-	-	-	-	-	+	-	+	+	-	-	+	+	+	+	+	-
Klebsiella oxytoca	2	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-
Kluyvera spp.	1	+	-	-	+	+	-	-	-	+	-	-	+	+	-	-	+	-	+	+	+	-
Serratia odorifera	2	+	-	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	-
Pseudomonas spp.	3	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
Fluorescent pseudomonas spp.	2	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Total	30																					

1: Symbols and data presentation are the same as in legend Table 10.

small and light brown colonies similar to background growth.

The color and morphology differences are more marked as investigators become familiar with the medium and cultural reactions on it. We have noticed increased accuracy in choosing suspect colonies as experience with the medium is gained.

FOOD SAMPLE SURVEY

Among 3 types of food samples examined (Table 12), none of them were positive for K. pneumoniae. Raw milk had very low bacterial count as compared with the data shown by Rao et al. (1983) that fresh raw milk had 10^4-10^7/mL of total count and 100% of samples containing Klebsiella. Probably, our raw milk sample size was not big enough to give any conclusion. K. pneumoniae is defined as one of the environmental mastitis milk bacteria (Smith et al., 1985). However, the mastitis milk samples in our survey did not show any growth of K. pneumoniae. Among 7 samples, 3 of them had no bacteria growth at all, the reason might be antibiotics treatments on mastitis cows. Raw hamburger meat from different grocery stores also contained no K. pneumoniae.

Although studies have implicated Klebsiella in clinical conditions, its role as an indicator of pollution has not been seriously considered. A brief survey here has shown that raw milk and meat are free from K. pneumoniae contaminated at source, and since they are exposed to high temperature during pasteurization and cooking, respectively, the products are usually safe from viable bacteria. The risk of ingesting K. pneumoniae through these materials is minimal.

Table 12. Occurrence of K.pneumoniae among total bacteria in various food samples

Type of samples	Samples tested(No.)	Total[a] count	AVRB count	K.pneumoniae count
Raw milk	1	10^3	0-10	0-10
Mastitis milk	7[b]	10^4-10^7	10^1-10^5	0-10
Raw hamburger meat	20[c]	10^2-10^5	10^2-10^4	0-10

a: Viable cell counts were expressed as CFU/g or mL.
b: Among 7 samples examined, 3 of them were treated with antibiotics, the cell counts were based on the other 4 samples.
c: Twenty samples were from 4 batches of raw meat in 4 different grocery stores.

CONCLUSIONS

Antimicrobial Properties of Basic, Acid and Disperse Dyes:

1. Brilliant green, Malachite green, Crystal violet and Ethyl violet, which are all triarylmethane dyes, at 1:200 inhibit the growth of all 10 organisms tested.
2. Methylene blue, Chrysoidine, Ethyl violet and Fuchsine successfully select Pseudomonas aeruginosa at 1:200.
3. None of the acid and disperse dyes tested show good separation ability of bacteria.
4. Basic dyes have stronger inhibitory ability than acid and disperse dyes.
5. Compared with gram-negative bacteria, gram-positive bacteria, the yeast and molds are more sensitive to dyes.

Acriflavine Violet Red Bile agar for selection of Klebsiella pneumoniae

1. 0.01% Acriflavine in VRB agar is most suitable for enumeration of K. pneumoniae from cultures as well as from cow bedding.
2. On this 0.01% AVRB medium, K. pneumoniae grows as large mucous and golden - or dull-yellow colonies. Gram-positive organisms are inhibited and some gram-negative organisms can grow but can easily be distinguished from the typical K. pneumoniae due to small colony sizes and different colors.
3. With pure culutures, 45.2 to 98.9% recovery of K. pneumoniae was observed. From artifically contaminated meat, recovery rate averaged 81%.
4. Accuracy in correctly picking suspected K. pneumoniae colonies on AVRB plates that were later confirmed by API-20E biochemical tests was calculated as 85%. Those not confirmable colonies might have been mixed cultures or unusual strains. Atypical colonies on our medium were identified as other enterics and not K. pneumoniae.
5. K. pneumoniae count was significantly higher in sawdust than in sand and

straw cow bedding while straw had highest total count and lowest K. pneumoniae count. Sand seemed to be the best bedding material with lowest bacteria counts.

6. The most important advantage of this selective medium over other proposed selective Klebsiella media is simplicity. No complicated components are added and no autoclaving is required. In addition, the 0.01% AVRB medium has a longer and constant potency.
7. Among 3 types of food samples (raw milk, mastitis milk, and raw hamburger meat) examined, none of them are positive for K. pneumoniae. The risk thus of ingesting K. pneumoniae through raw milk and meat is minimal.

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APPENDIX

Table A-1 Bacterial counts[1] from straw cow beddings

Sample	Total Count	AVRB Count	K.pneumoniae
1	9.48	4.76	3.95
2	9.40	4.81	1.00*
3	9.63	4.85	1.00
4	9.00	3.90	1.00
5	9.04	1.00	1.00
6	9.34	4.68	1.00
7	9.04	4.99	1.00
8	9.32	4.11	1.00
9	9.49	5.66	1.00
10	9.57	5.04	1.00
mean	9.33	4.38	1.30
S.D.[2]	0.23	1.28	0.93

1:Cell counts are expressed as LOG_{10}CFU/g bedding
2:S.D.= standard deviation
*:Every "1.00" showing in this table means viable cell counts smaller than 10 CFU/g bedding

Table A-2 Bacterial counts from sawdust cow bedding[1]

Sample	Total Count	AVRB Count	*K.pneumoniae*
1	8.81	3.94	2.30
2	9.23	5.32	1.00
3	8.97	6.18	4.70
4	9.26	5.54	1.00
5	8.20	3.99	2.54
6	8.08	4.36	1.70
7	9.08	5.32	3.81
8	9.11	4.69	2.70
9	9.00	6.28	4.00
10	8.95	7.08	6.11
mean	8.87	5.27	2.99
S.D.	0.41	1.04	1.66

1: symbols and data presentation are the same as in legend Table A-1.

Table A-3 Bacterial counts from sand cow bedding[1]

Sample	Total Count	AVRB Count	K.pneumoniae
1	7.86	4.36	2.18
2	5.40	1.00	1.00
3	6.94	3.28	1.00
4	8.11	3.90	1.00
5	6.78	4.82	3.00
6	7.62	4.11	1.00
7	7.91	5.15	1.00
8	6.32	1.00	1.00
9	7.87	4.11	1.00
10	8.08	3.43	1.00
mean	7.29	3.52	1.32
S.D.	0.90	1.44	0.70

1: symbols and data presentation are the same as in legend Table A-1.

ACRIFLAVINE VIOLET RED BILE AGAR FOR THE ISOLATION AND ENUMERATION OF KLEBSIELLA PNEUMONIAE FROM ENVIRONMENT AND FOODS

by

Se-Ping Chien

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ABSTRACT

Eleven basic and acid dyes which are known for their antimicrobial properties in microbiological media and 22 acid and disperse dyes which are used industrially to dye nylon carpeting were incorporated into basal medium individually at various concentrations (1;200, 1:1,000 and 1:10,000) to test their effects on growth of 3 gram-positive bacteria and 4 gram-negative bacteria, 1 yeast and 2 molds. Of 33 dyes tested, some triarylmethane dyes (e.g. Brilliant green, Malachite green, Crystal violet and Ethyl violet) inhibited the growth of all 10 organisms at 1:200. Basic dyes had stronger inhibitory ability than acid and disperse dyes and none of the acid or disperse dyes showed good separation ability of bacteria. Compared with gram-negative bacteria, gram-positive bacteria, the yeast and molds were more sensitive to dyes.

Acriflavine, a basic dye, is the most powerful antiseptic reagent of the acridine dyes. The effect of 0.06% Acriflavine on growth of selected K. pneumoniae strains, Enterobacteriaceae and other organisms have been studied by Fung and Niemiec (1977). In this study, we retested and refined the AVRB medium for the identification and selective recovery of K. pneumoniae from environmental and food sources. The results indicated that 0.01% acriflavine in VRB agar is most suitable for enumeration of K. pneumoniae from cultures as well as from food and cow beddings. On this medium, K. pneumoniae grows as large mucous and golden- or dull-yellow colonies. Gram-positive organisms are inhibited on this medium and some gram-negative organisms can grow but can easily be distinguished from the typical K. pneumoniae colonies due to small colony sizes and different colors.

With this medium, we found that 85% of the typical K. pneumoniae colonies on our medium obtained from cow beddings were confirmed to be correct by the API-20E system. Those not confirmable colonies might have been mixed cultures or unusual

strains. Atypical colonies on our medium were identified as other enterics and not K. pneumoniae. Percentage recovery of K. pneumoniae on 0.01% AVRB showed that this medium is capable of giving a reasonable percentage of recovery of K. pneumoniae from pure cultures (45.2 to 98.9%) and artifically contaminated foods (81%). Among 3 types of food samples (raw milk, mastitis milk and raw hamburger meat) surveyed, none of them were positive for K. pneumoniae. The risk thus of ingesting K. pneumoniae through these materials is minimal.

The most important advantage of this selective medium over other proposed selective Klebsiella media is simplicity. No complicated components were added and no autoclave sterilization was required. In addition, this medium had a longer and constant potency.