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ORIGINAL RESEARCH ARTICLE

A Three-Tube Scheme for Same-Day Presumptive Identification of *Klebsiella pneumoniae* in Clinical Specimens

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ABSTRACT

A three-tube scheme that comprised a urease tube, motility tube and a gluconate-nitrate composite medium tube was evaluated for its reliability in correctly predicting the identity of *Klebsiella pneumoniae* after six hours of incubation. A total of 33 strains were used to assess the scheme, of which 17 were laboratory stock cultures, 14 were fresh clinical isolates, and two were environmental isolates from soil samples. The three tubes were heavily inoculated to give a density approximating McFarland No. 7 turbidity standard, which is roughly equivalent to a bacterial suspension with a concentration of 21 x 10^8 organisms/ml. The three-tube scheme identified all the five strains of *Klebsiella pneumoniae* tested correctly, but it was only able to accurately identify 23 out of 28 strains that were not *Klebsiella pneumoniae*. The scheme falsely identified five test strains that were not *Klebsiella pneumoniae*. The scheme had a sensitivity of 100 %, a specificity of 82.1 %, a positive predictive value of 50 % and a negative predictive value of 100 %. The scheme should perform better if the distinctive colonial characteristics of isolates on MacConkey agar were considered when predicting making prediction about identity, and a stricter interpretation of the results provided by the scheme was adopted.

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INTRODUCTION

Members of the family *Enterobacteriaceae* constitute the single most important group of bacteria recovered from clinical specimens. As a group, they are responsible for about 80% of all significant Gram-negative rods isolates, more than 70% of all urinary tract infections, about 50% of septicemia and 50% of all significant bacteria in a hospital setting (Edem *et al*., 2001; Kostinek et al., 2005). *Enterobacteriaceae* are known to be responsible for a wide variety of community-acquired infections and nosocomial infections, but members' frequency differs greatly. There are well over 100 species within the large family *Enterobacteriaceae*, but about 80% of all enteric isolates belong to only three species *Escherichia coli*, *Klebsiella pneumoniae*, *and Proteus mirabilis* (Oduro *et al*., 2000).

Targeting laboratory resources to identify these three enteric organisms accurately will ensure efficient service by a laboratory. The genus *Klebsiella* is said to have seven species though experts continue to argue about the taxonomic status of one or two of the listed species. The following species have been accepted as constituting the genus *Klebsiella* by some authorities *Klebsiella pneumoniae*. *K. ozanae*, *K. rhinoscleromatis*, *K. oxytoca*, *K. lerrigina*, *K. planticola* and *K. granulomatis* (Ogiehor *et al*., 2007; Jekayinfa and Olajide 2007).

The most frequently isolated species of *Klebsiella* is *K. pneumoniae* (Asegbeloyin and Onyimonyi, 2007). As the specific epithet implies, K. pneumoniae is an important cause of pneumonia, as several publications of severe *K. pneumoniae* pneumonia testify (Huch *et al*., 2008; Ray and Sivakumar 2009; Akindahunsi *et al*., 1999). However, the pathogenic activities of *K. pneumoniae* are not restricted to the lung. *K. pneumoniae* is second only to *Escherichia coli* as an agent of urinary tract infection (Osho and Dashiell 2002). And there is hardly any anatomical site in which *K. pneumoniae* has not been reported as an agent of severe infection (Asegbeloyin and Onyimonyi 2007).

The most problematic aspect of *K. pneumoniae* is the

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The most problematic aspect of *K. pneumoniae* is the increasing tendency towards multi-drug resistance, making the management of patients with *K. pneumoniae* infection difficult (Harbor and Ogundu 2009; Ray and Sivakumar 2009; Steinkraus 1997). Infections of *Klebsiella pneumoniae* need to be appropriately diagnosed for its management to be beneficial to the patient. Conventional methods of bacterial identification have saved well laboratories in developing countries. However, there is strong advocacy from developed countries to make the commercialisation of bacterial diagnostic services a global thing. Rather than diagnostic laboratories having some control over the production and use of media and reagents for routine bacteriologic diagnosis, the market is flooded with commercial diagnostic kits, which laboratories are encouraged to purchase and use routinely (Ogiehor and Ikenebomeh 2005; Jonathan *et al.*, 2011).

The traditional practice of a laboratory organising and controlling the quality of diagnostic media is now being considered as archaic or obsolete, hard on the heel of the advocacy to get rid of in-house production of diagnostic items is the trend toward Gene-based diagnostic techniques, which is being vigorously promoted in the scientific literature (Alexopoulus *et al.*, 1996). A phylogenetic approach to classification and identification is being promoted as being far better than the phenetic-based methodologies that have served so well for decades (Ogiehor and Ikenebomeh 2005; Aguoru *et al.*, 2014; Ogiehor and Ikenebomeh 2005; Obadina *et al.*, 2009; Georgia and Olufunmilayo 2016).

It is proper that laboratory personnel resist the trend towards modernisation which is too costly, unaffordable, and unsustainable in third-world laboratory settings. Laboratory personnel should continue to focus on research projects that seek to improve conventional diagnostic modalities that are affordable and reliable. Routine diagnostic laboratories often require 72 hours between the time a specimen is collected and the time a report of definitive identification is submitted to the clinician. It is advantageous if this turnaround time can be shortened so that a reasonably accurate presumptive identification can be given on the same day the organism is recovered on primary isolation media.

Klebsiella pneumoniae is one of the commonly encountered pathogens with peculiar physiologic characteristics that should permit a smaller set of media for fairly accurate presumptive identification. It is also an organism that exhibits some rapid reactions that do not necessarily require 24 hours of incubation to read if heavy inoculum is used to inoculate the media.

With heavy inoculation of the three-test medium which are motility medium, Urease as well as Gluconate-nitrate medium, the rapid reaction allows for reading results within six hours of incubation. This will thereby help to cut down the time frame from the conventional series of fifteen biochemical test for identification of *Klebsiella pneumoniae* by a day or thereabout. By cutting off a day from the conventional 3-4days of proper identification time, appropriate antimicrobial therapy can be started earlier. Such early therapy has been shown to favourably affect the outcome of infections, especially in hospitalised patients who are seriously ill.

MATERIALS AND METHODS

Collection of Samples

A total of thirty-three bacterial test strains were used in evaluating the three-tube scheme containing Urease, motility and gluconate-nitrate medium. Of these, seventeen were laboratory stock cultures of different known bacteria; fourteen were fresh clinical isolates gotten from urine and swab analysis which were sourced from Ahmadu Bello University Teaching Hospital Zaria, Kaduna State; two were environmental stock known isolates collected from soil samples. All the samples were transported to the Ahmadu Bello University Microbiology laboratory for analysis. All the 33 isolates were inoculated on MacConkey & CLED media and were incubated aerobically at 37°C for 24 hr. After 24 hr incubation, the cultures plates were examined Morphologically, microscopically and later they were subjected to series of biochemical test which include indole test, methyl red test, motility, voges - proskauer test, citrate utilization test, urease test, sugar fermentation tests to know there tentative identity so as to test the scheme performance.

Evaluation Procedure

The isolates were then coded with laboratory numbers so that the investigator was blinded to the identity of the strains to ensure the objectivity of the results. The three tubes Urease, Motility and Gluconate-nitrate were heavily inoculated to give a density approximating McFarland No. 7 turbidity standard, which is roughly equivalent to a bacterial suspension with a concentration of 21×10^8 organisms/ml.

Urease test

Urea agar slants were prepared and heavily inoculated with freshly culture of bacteria isolate, then incubated at 37 ºC for 6 hrs. Changing medium color to purple – pink indicates a positive result as described by (Atlas *et al.*, 1995).

Motility test

Semi solid motility medium were prepared and inoculated by single stabbing with a needle inoculated with the isolates, and then incubated at 37 ºC for 6 hrs. Movement away from the stab line or a hazy appearance through the medium indicated a motile organism as described by (Cruickshank *et al.*, 1975).

Gluconate-nitrate composition broth

The nitrate reduction test is a qualitative procedure for determining the ability of bacteria to reduce nitrate into nitrite. The isolates were heavily inoculated into the gluconate-nitrate incorporated broth and incubated at 37 ºC for 6 hrs and then tested for the presence of nitrite and gluconate oxidation (Buxton, 2011).

Based on the results obtained, a prediction was made as to whether the test organisms would be *Klebsiella pneumoniae* or not *Klebsiella pneumoniae*. Each test organism was then identified using a wide range of biochemicals tests that included; Acidification of Kliger iron agar, Alkalinisation of the Arginine medium, malonate utilisation, aesculine hydrolysis, gelatine hydrolysis, indole production, Lysine decarboxylase, Ornithine decarboxylase tests and Voges Proskauer reaction. The identity of each isolate was then compared with the prediction based on the three schemes. The specificity, sensitivity, positive predictive value, and negative predictive values were then calculated and tabulated.

RESULTS AND DISCUSSION

The three tube scheme tends to analyses whether it can be able to correctly identify *Klebsiella pneumoniae* from other members of the *enterobacteriaceae* because of it peculiar characteristics of urease positive, motility negative and gluconate-nitrate positive thereby reducing the conventional series of 15 or more biochemical test for its identification. In tropical and developing countries like Nigeria, bacterial identification is traditionally achieved by carrying out labour intensive and time consuming homemade biochemical assays which may not be useful in a situation where results are urgently needed for medical diagnosis (Cheesebrough, 2000; Abdessalam *et al*., 2010; Iroha *et al*., 2011). In addition, not all microorganisms are reliably identified by biochemical methods. These methods often give wrong results in an unacceptable rate (Ayeni *et al*., 2015). A major advantage of the scheme is the rapid turnaround time used in accurate identification of the organism compared with conventional techniques. It requires only a few test and has a simple sample preparation process (Panda *et al*., 2014).

All the five strains of *Klebsiella pneumoniae* tested were detected as *Klebsiella pneumoniae* based on the results of the three-tube scheme, only twenty-three out of the twenty-eight test strains that were *not Klebsiella pneumoniae* (strains were correctly detected as not being *Klebsiella pneumoniae*). Five non-*Klebsiella pneumoniae* strains were falsely identified as *Klebsiella pneumoniae* strains. Therefore, the three-tube scheme's sensitivity is 100 %, but its specificity is 82.1 %. The positive predictive value of the three-tube scheme is 50 %, and the negative predictive value is 100 %

The results obtained showed that the three-tube scheme can recognise *Klebsiella pneumoniae* when it is present. Still, it also demonstrated that it could mistakenly consider some organisms as *Klebsiella pneumoniae* when they are not. A 50 % positive predictive value is low. It means that when the scheme suggests that an isolate would be *Klebsiella pneumoniae*, it could only be considered correct five times out of ten. At the same time, it is reasonably good in indicating organisms that appear to be *Klebsiella pneumoniae* but are not based on the results of three tube scheme alone. So, 50% of the time, it may wrongly ascribe the identity of *Klebsiella pneumoniae* to organisms that are not.

Table 1: Predictive value of a three-tube scheme for same-day identification of *Klebsiella pneumoniae* in laboratory specimens

Number of bacterial tests	True positive	False positive	True negative	False negative	Sensitivity%	Specificity%	Positive predictive value of three-tube scheme	
							Positive%	Negative%
33	5	5	25	0	100	82.1	50	100

Table 2: Performance of bacterial test strains in a three-tube scheme for same-day presumptive identification of *Klebsiella pneumoniae*

Test strain designation	Sources of strains	Test results in a three-tube scheme				Prediction of strain as *K. pneumoniae*	Identity of the test strain
		Gluconate oxidation	Nitrate reduction	Motility	Urease		
VM1	SC	+	-	-	-	Negative	*Alcaligenes faecalis*
CCNA	EI	-	+	+	-	Negative	*Pseudomonas stuzeri*
CNR	EI	-	+	+	-	Negative	*P. aeuginosa*
39A	SC	-	+	+	-	Negative	*A. Hydrophila*
BP3	SC	+	+	+	-	Negative	*P. aeruginosa*
MHO3	SC	+	+	+	-	Negative	*P. aeruginosa*
SM3	SC	+	+	-	+	Positive	*Serratia marcenscens*
K3806	CI	+	+	-	+	Positive	*P. aeruginosa*
K3813	CI	-	+	+	-	Negative	*E. coli*
DAN	CI	-	-	-	-	Negative	*Staphylococcus* sp.
K3812	CI	-	+	-	-	Negative	*E. coli*
K3826	CI	-	+	+	-	Negative	*E. coli*

Table 2: Continued

Test strain designation	Sources of strains	Test results in a three-tube scheme				Prediction of strain as *K. pneumoniae*	Identity of the test strain
		Gluconate oxidation	Nitrate reduction	Motility	Urease		
K3810	CI	-	-	-	-	Negative	*C. violaceum*
CI2058	CI	+	+	-	+	Positive	*K.pneumonia*
CI2069	CI	+	+	-	+	Positive	*K. pneumonia*
845	SC	-	+	-	-	Negative	*S. samara*
VM3	SC	+	+	-	+	Positive	*Serratia marcenscens*
305	SC	-	+	-	-	Negative	*E. coli*
36A	SC	+	+	-	+	Positive	*K. pneumonia*
SH2	SC	-	+	-	+	Positive	*K. pneumonia*
SMM1	SC	+	+	-	+	Positive	*Serratia marcenscens*
SMM2	SC	+	+	-	-	Negative	*Serratia marcenscens*
DS1A	SC	+	+	-	-	Negative	*E. coli*
DS1B	SC	+	+	-	+	Positive	*K. pneumonia*
SAI	SC	-	+	-	+	Positive	*Staphylococcus sp.*
AO1A	SC	-	+	-	-	Negative	*Salmonella sp.*
AO1B	SC	-	+	+	-	Negative	*Salmonella sp.*
VM2	SC	+	+	-	-	Negative	*Alcaligenes faecalis*
RECTa	SC	-	+	+	-	Negative	*E. coli*
RECTb	SC	+	+	-	-	Negative	*P. aeruginosa*
KP5	CI	+	+	+	-	Negative	*P. aeruginosa*
KWS	CI	+	+	-	+	Positive	*P. aeruginosa*

Key: SC= *Stock cultures*; CI= *Clinical isolates*; EI= *Environmental isolates*; + = *positive*; - = *negative*

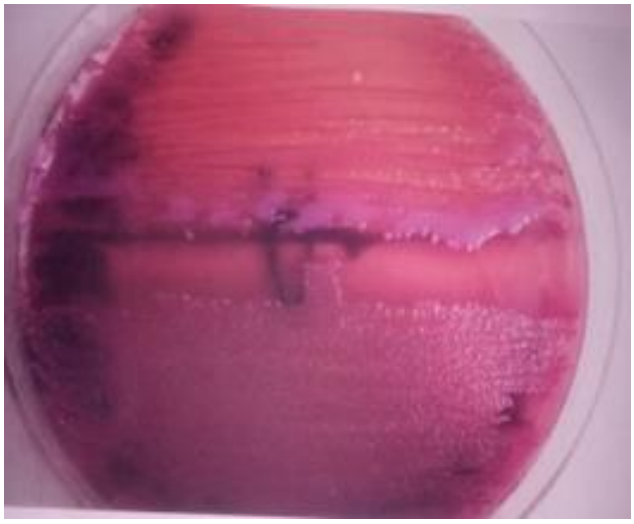

Plate I: Two urine specimens plated on half-plate each of MacConkey. Heavy confluent growth of mucoid, lactose fermenters suggestive of *Klebsiella pneumoniae*. Colonial characteristics on a primary isolation medium are highly suggestive of final identification

Plate II: The three-tube scheme for the presumptive identification of *Klebsiella pneumoniae*. The result after 6 hours of inoculation. L-R: Urease +, gluconate +, motility -. Profile predictive of *Klebsiella pneumoniae*

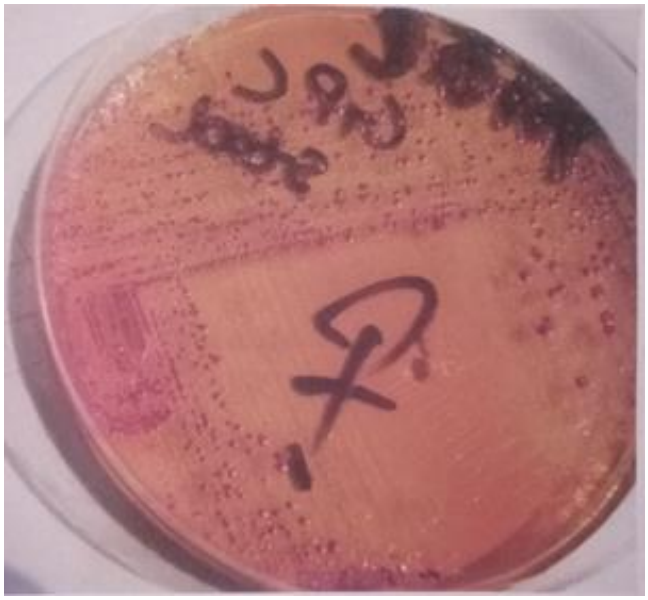

Plate III: **Primary MacConkey plate of a wound swab** showing heavy growth of the mixed culture of lactose and non-lactose fermenters. The lactose fermenters are not mucoid, suggesting they are not likely to be *Klebsiella pneumoniae*. Colonial characteristics on a primary isolation medium are highly suggestive of final identification.

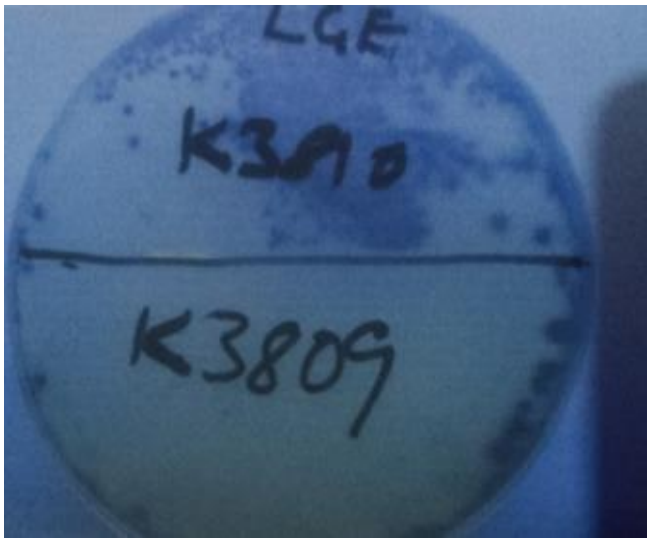

Plate IV: Two urine specimens plated on liver extract based gelatin-egg yolk medium. Specimen K3810 shows moderate growth of *Chromobacterium violaceum* while specimen K3809 showed no growth. Colonial characteristics on a primary isolation medium are highly suggestive of final identification

Conclusion

The results obtained on the surface are rather unsatisfactory concerning the scheme's low positive predictive value; this is apparent rather than real. In the actual situation on the bench, the positive predictive value is likely to be higher when the colonial characteristics of isolates are factored into the final prediction of the true identity of an isolate.

The scheme needs to be further evaluated using a larger number of test strains and incorporating the suggested modification of criteria for positive prediction highlighted above.

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CONFLICT OF INTERESTS

The authors declare no conflicting interests.

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