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Early Detection of Blood Borne Pathogens and its Antibiotic Susceptibility

Uday Rajesh Titare[1], Dr. Niraj Ghanwate[1], Pradnya Saurkar[1], Ravindra Ashok Sharma[2]

[1]P.G. Department of Microbiology, Sant Gadge Baba Amravati University, Amravati, Maharashtra, India.
[2]Department of Microbiology, R.K. Talreja College, Ulhasnagar- 421003, Maharashtra, India.

Corresponding Author: Uday Rajesh Titare

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ABSTRACT

In this study the evaluation of the new blood culture technique to improve rapid detection for the presence of bacteria in blood by using Tetrazolium dye (TTC) was done. The Blood samples were collected and processed by two different methods i.e. conventional blood culturing method and improved method by using TTC dye. In conventional method the sample was inoculated in blood culture bottle and then plated on blood agar, MacConkey, and chocolate agar and was incubated for 24hrs. After incubation the growth observed was identified and reported. Along with this in Improved TTC dye method the blood was inoculated in tryptone soya broth incubated and then TTC dye was added in it and cherry red color development was considered as positive. In the present study, total 50 blood samples were processed of which 32 samples exhibited growth on media and 18 samples show no growth. From total positive growth samples, 9 were gram positive including *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* and remaining 23 were gram negative rods including *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. Overall, the average time required for growth by conventional method is 51 hours while that of TTC dye method is of 30 hours. In contrast to antibiotic susceptibility test, Microbroth dilution method was easy, reliable, affordable and quick result giving method as compare to standard antibiotic susceptibility test method.

Keywords: Bloodstream infections, Antibiotic susceptibility, Tetrazolium dye

INTRODUCTION

Bloodstream infection is the most severe disease problem in the world; delay in the detection of the bloodstream infection into the patient may lead to dangerous death which is partly due to the inability to rapidly detect and identify bacteria in the early stages of infection [1]. Initial treatment is often empirical because several days may elapse before the presence or absence of Bloodstream infections (BSI) is microbiologically confirmed. Thorough knowledge of risk factors and clinical signs and symptoms that are suggestive of BSI would improve the assessment of the risk of BSI in the individual patient and could lead to a more timely start of an adequate empirical antimicrobial therapy. BSI and associated organ dysfunctions (defined as severe sepsis or septic shock) are a major cause of morbidity and mortality worldwide which can be community or hospital-acquired [2]. It is estimated that 7-18% of patients in the medical department and 7-9% of patients presenting to the emergency department have BSI and that BSI is acquired by up to 19% of patients admitted to the intensive care unit (ICU). With an annual incidence of 100/1, 00,000 patient days and a case fatality rate of 20–50%, BSI

has been declared the third most common cause of death in Germany [3]. Early diagnosis and adequate antibiotic treatment of BSI have been shown to be associated with a substantial reduction in mortality as well as in treatment costs [4]. So the rapid detection of bloodstream infection is most important in today's world. As such there are so many scientists who take so many efforts to minimize the time required to detect the bloodstream infection [5].

The standard time period required to detect the bloodstream infection is 10 days, while that of by using the Castaneda blood culture method does not have much beneficial difference [6]. Antibiotics developed gradually to become more or fully resistant to a number of microbes. Resistance arises through one of three ways: natural resistance in certain types of bacteria; genetic mutation; or by one species of acquiring resistance from another. Resistant microbes are increasingly difficult to treat, requiring alternative medications or higher dosages which may be more costly or more toxic [7]. Minimum inhibitory concentrations (MICs) are considered as the "gold standard" for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing [8].

Therefore, the present study aimed is to develop and compare an improved technique for rapid detection and AST of Ciprofloxacin against blood borne pathogens isolated from infected patients using Tetrazolium dye (TTC).

MATERIALS AND METHOD

1. Materials and Methods:

The phlebotomist sanitized his hands immediately before the first contact with the patient, using warm water and soap or disinfectant foams according to the nationally accepted guidelines [9]. This approach ensures that all surfaces touched by phlebotomists before venipuncture are free of pathogens. A requisition form consisting of Name, Gender, Occupation, Date of birth, any medication is taken before, and contact details (address, telephone number) of the patient along with any clinically relevant information about the patient was filled by the patients who volunteered in this study.

The subject's arm was extended and the antecubital fossa or forearm was inspected. After that, the subject was asked to form a fist so the veins are more prominent. A vein of a good size that is visible, straight, and clear was searched. The median cubital vein lies between muscles and is usually the easiest to puncture. Under the basilica vein run an artery and a nerve, so puncturing here runs the risk of damaging the nerve or artery and is usually more painful [10]. The vein should be visible without applying the tourniquet. The tourniquet was then applied about 4-5 finger-widths above the venipuncture site and the vein was reexamined. The site was cleaned with an antiseptic swab for 30 seconds and allowed to dry completely.

Once sufficient blood has been collected, the tourniquet was released before withdrawing the needle. The needle was withdrawn gently and gentle pressure was applied to the site with clean gauze or dry cotton wool ball [11].

Conventional blood culture method:

The bottles were incubated for 24 hours before plating to enhance the growth of bacteria, aerobic bottle (BD BACTEC 442023) was plated on Blood Agar (Himedia MP1301), MacConkey Agar (Himedia M081B), and Chocolate Agar (Himedia M103) (Figure 1.) and incubated in a CO_2 incubator for 24 hours, the anaerobic bottle (BD BACTEC 442193) was plated on blood agar and incubated anaerobically for 48 hours, and the negative bottle was reincubated and tested after 10 days before discarding it as negative culture [12]. If slow-growing organisms like *Brucella spp*. were suspected it was indicated on the requisition form and the culture bottles were further incubated for 2-

4 weeks before being reported out as negative.

Figure 1. Blind Sub-Culturing syringe and drip methods

Initial blood culture results were reported as soon as it exhibited growth. Final results with sensitivity were issued after 24- 48 hours of the initial report. Negative results were issued after 10 days of culture submission (Figure 2.).

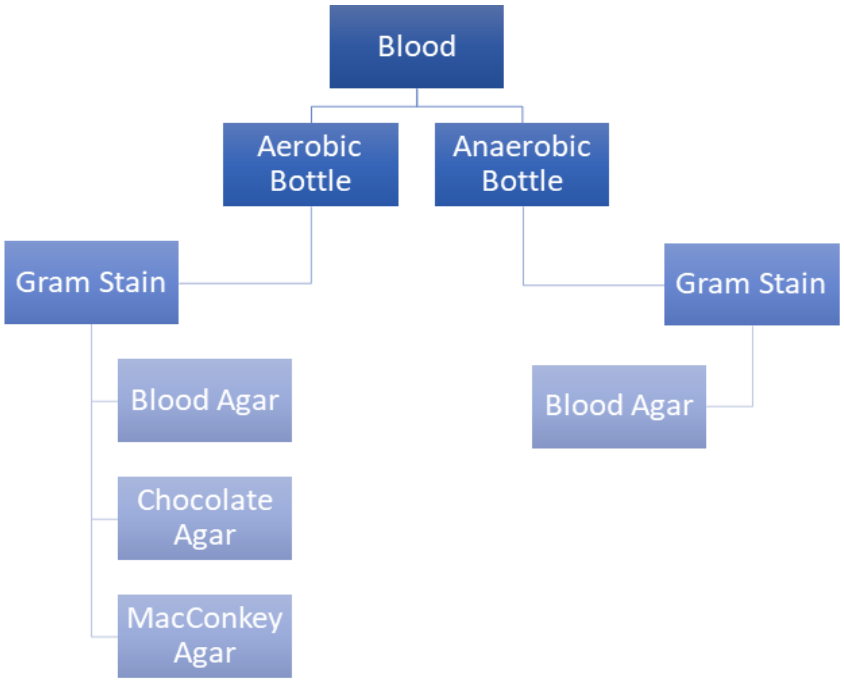

Figure 2. Flow sheet of Blood culture by standard method

Improved method using Tetrazolium dye (TTC):

Blood samples were collected and inoculated into a 100ml blood culture bottle filled with tryptone soya broth (Himedia LQ508) and incubated at 37℃ for 24-48hrs. After incubation, loopful of incubated broth were streaked onto the Blood agar and MacConkey agar plates. Simultaneously, 100 µl of incubated broth was added in the test tube containing 1ml of tryptone soya broth and then incubated for 5-10 minutes at 37℃. After that 100 µl of TTC dye (Himedia MB188) having a concentration of 100 µg/100 µl was added to the test tubes. The sample was considered to be positive if

the cherry red color was observed. But if the cherry red color was not observed then broth was kept for continuous incubation and the same procedure was repeated till it was confirmed that the collected sample was negative [13].

The positive samples exhibited colonies on Blood agar, MacConkey agar plates after 24-48hrs. Then, a smear from colonies on the plate was made and heat-fixed followed by gram staining for identification of its gram nature (i.e. gram positive or gram negative). The colonies were then sub-cultured on nutrient agar slant and further biochemical reactions were performed for the identification of pathogens, the identification was performed according to Bergey`s Manual of Systematic Bacteriology [14].

Antibiotic Susceptibility Testing (AST):
Preparation of Inoculum:

The inoculums were prepared by directly taking colonies of isolates selected from nutrient agar slants. The suspension was then adjusted to achieve turbidity equivalent to 0.5 McFarland turbidity standards. After that the tubes were then incubated for 4 hours then the adjusted inoculums suspension was diluted in MH broth, after the inoculation each tube contained approximately 5 X 10^5 CFU/ml.

Preparation of stock solution of Ciprofloxacin:

The stock solution was prepared by dissolving 0.1g Ciprofloxacin powder (Merck 1134313) into the 10 ml distilled water, i.e. 10 mg/ml stock solution from which 1 ml was added in 99 ml distilled water thus 1 mg/ml first working solution was prepared.

Preparation of different dilutions series of Ciprofloxacin:

Different dilution series/plates of Ciprofloxacin were prepared i.e. 16µg/ml, 32µg/ml, 64µg/ml, 128µg/ml, 256µg/ml, 512µg/ml, and 1024µg/ml from first working solution (Table 1.).

Table 1. Preparation of different dilutions series of Ciprofloxacin

Concentrations of Ciprofloxacin	Concentration taken from First working and stock solution of Ciprofloxacin	Media Concentrations	Inoculums Concentration
16 µg/ml	16µl	984µl	1 µl
32 µg/ml	32µl	968µl	1 µl
64 µg/ml	64µl	936µl	1 µl
128 µg/ml	128µl	872µl	1 µl
256 µg/ml	256µl	744µl	1 µl
512 µg/ml	512µl	488µl	1 µl
1024 µg/ml	102 µg/ml	898 µg/ml	1 µg/ml

AST by using TTC dye:

0.5 MacFarland suspension of each bacterial isolates were prepared by transferring well-isolated colonies from the plates into MH Broth tubes and incubated at 37℃ for 3-4hrs. After incubation 150 µl of this bacterial culture was added into the microtiter plate's wells. In the first row of the microtiter plate, only media was added and it was considered as "Blank". In the second row of the microtiter plate, antibiotic was not added and was considered as "Control". Then, 50 µl of the antibiotic solution was added to the control and all wells in a row as per the concentrations of antibiotic except the wells labeled as Control. After that, the microtiter plate was incubated at 37℃ for 6-8hrs. After incubation 50 µl of TTC dye was added in all wells of each row except the row labeled as Blank once the dye was added the color formation in wells of the microtiter plate was observed (Figure 3.) [15].

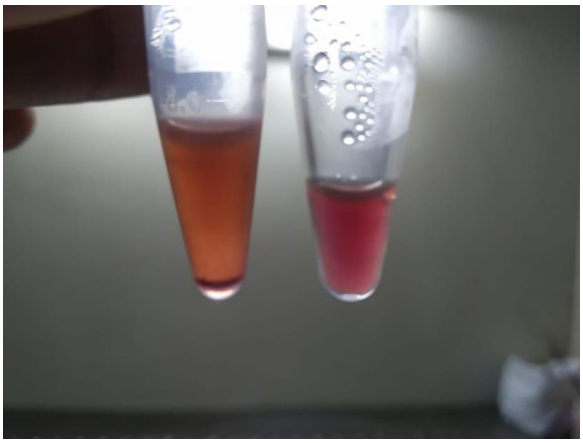

Figure 3. The positive culture indicated by the reduction of "2,3,5-Triphenyl-2*H*-tetrazolium chloride (TTC) salt" to the red coloured reduced product called Formazon

AST by Standard method:

150 µl of 0.5 McFarland suspension of bacterial isolates were added in Eppendorf tubes containing 50 µl antibiotic solution of different concentrations and then it was incubated at 37℃ for 24hrs. After 24hrs of incubation, one loopful of suspension was streaked on Nutrient agar plates. These plates were then incubated at 37℃ for 24hrs.

RESULTS

Table2. Data of collected blood samples

Sr. No.	Sample No.	Gender	Age	Organism identified
1	B1	M	17	-
2	B2	F	23	*K.pneumoniae*
3	B3	F	22	-
4	B4	F	27	*K.pneumoniae*
5	B5	M	33	*S.haemolyticus*
6	B6	F	22	*K.pneumoniae*
7	B7	F	25	-
8	B8	M	28	*K.pneumoniae*
9	B9	M	47	*K.pneumoniae*
10	B10	F	27	*E.coli*
11	B11	M	34	*P.aeruginosa*
12	B12	M	21	-
13	B13	M	24	*K.pneumoniae*
14	B14	F	31	*P.aeruginosa*
15	B15	F	39	*S.aureus*
16	B16	M	28	-
17	B17	F	24	*P.aeruginosa*
18	B18	F	44	-
19	B19	M	39	*P.aeruginosa*
20	B20	F	28	-
21	B21	M	32	*E.coli*
22	B22	F	37	-
23	B23	M	44	*K.pneumoniae*
24	B24	M	24	-
25	B25	M	21	*E.coli*
26	B26	F	18	*P.aeruginosa*
27	B27	M	25	*S.epidermidis*
28	B28	M	19	-
29	B29	M	30	*S.aureus*
30	B30	M	42	*P.aeruginosa*
31	B31	M	32	*S.aureus*
32	B32	M	22	-
33	B33	F	38	-
34	B34	M	42	*S.aureus*
35	B35	M	40	*K.pneumoniae*
36	B36	M	39	*S.aureus*
37	B37	F	20	-
38	B38	F	23	*K.pneumoniae*
39	B39	M	24	-
40	B40	M	25	*E.coli*
41	B41	M	30	*S.aureus*
42	B42	F	41	-
43	B43	F	28	*P.aeruginosa*
44	B44	M	24	*S.aureus*
45	B45	F	29	*P.aeruginosa*
46	B46	F	30	-
47	B47	M	41	-
48	B48	F	22	*S.haemolyticus*
49	B49	F	43	-
50	B50	F	30	*K.pneumoniae*

In the present study, a total of 50 clinical specimens of Blood were collected aseptically from various hospitals and pathology labs in Amravati processed by the standard procedure for isolation and identification of various microorganisms. During the collection of patients, history was recorded. Different species were isolated from blood samples (Table 2.) A total of 50 specimens were collected from patients with clinical evidence of blood infection. The subject includes 27 males and 23 females. From the above data, it was noted that the patients at the age of 21-30 are most infected from a blood infection. When the data was compared it was found that Males were more infected when compared to females. Blood samples were collected from various hospitals and pathology labs in Amravati. The specimens were processed for standard morphological and cultural characteristics from the bacterial colonies on blood agar, MacConkey agar plates inoculated by the blood specimen and confirmed by gram staining and biochemical test.

In the present study, a total of 50 blood samples were processed. Out of 50 samples, 32 samples show growth on media and 18 samples show no growth. From total positive growth samples, 9 were gram positive including *Staphylococcus aureus, Staphylococcus haemolyticus, and Staphylococcus epidermidis* and the remaining 23 were gram negative rods including *E. coli, Pseudomonas aeruginosa, and Klebsiella pneumonia.* The sample numbers B1, B3, B7, B12, B16, B18 , B28, B32, B33, B37, B39, B42, B46, B47, B49 do not show growth on media even up to 6 days and are considered as negative while, others all samples are positive (Figure 4.).

The minimum time required to detect the presence of bacteria in sample by the conventional method is 24 hours (Figure 5.). Overall, the average time required for growth by conventional method is 51 hours which is much greater for treatment of the patient and may lead to severe problems and even death due to lack of proper treatment.

Figure 4. MIC of isolates by standard/ reference method

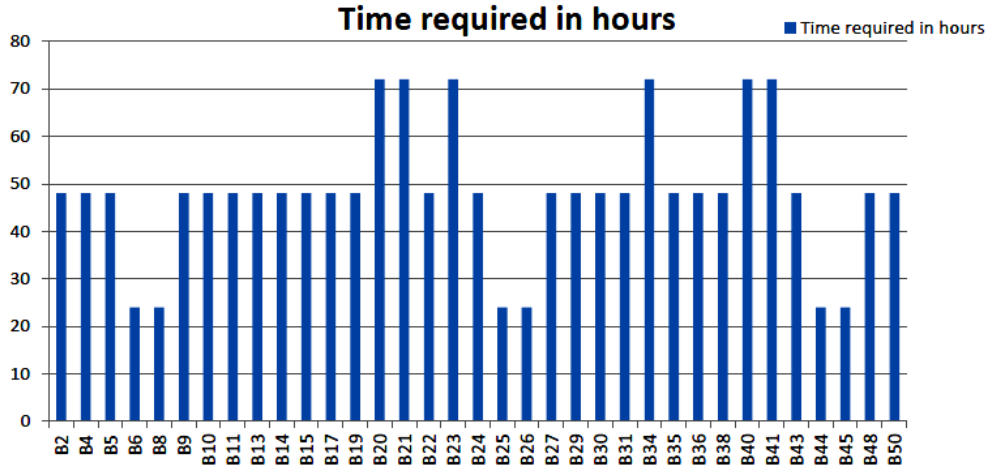

Figure 5. Graphical representation of time required by standard method

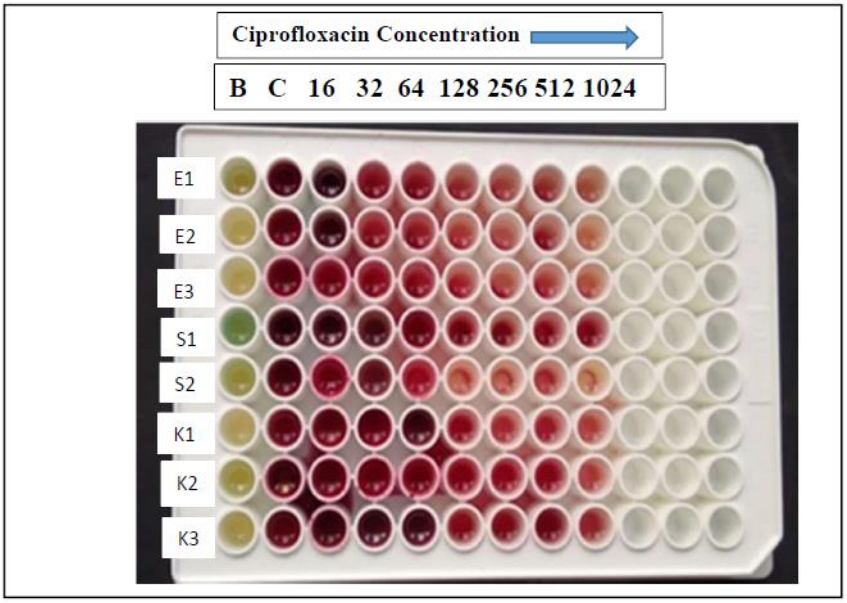

Figure 6. Microtitre plate showing result of MIC of isolates by Broth microdilution method

The sample numbers B1, B3, B7, B12, B16, B18, B28, B32, B33, B37, B39, B42, B46, B47, B49 were detected as negative while remaining samples were detected as positive with the Standard/conventional blood culture method, the same results were found with that of the improved blood culture method using Tetrazolium dye (Figure 6.).

The invention or output of this improved blood culture technique is that the growth appears much earlier than that of the Standard/conventional blood culture method. The minimum time required for the detection of the presence of bacteria into the sample is only 24 hours while the maximum samples detected as positive within 48 hours; the average time period required for the detection of the presence of bacteria is 30 hours (Figure 7.).

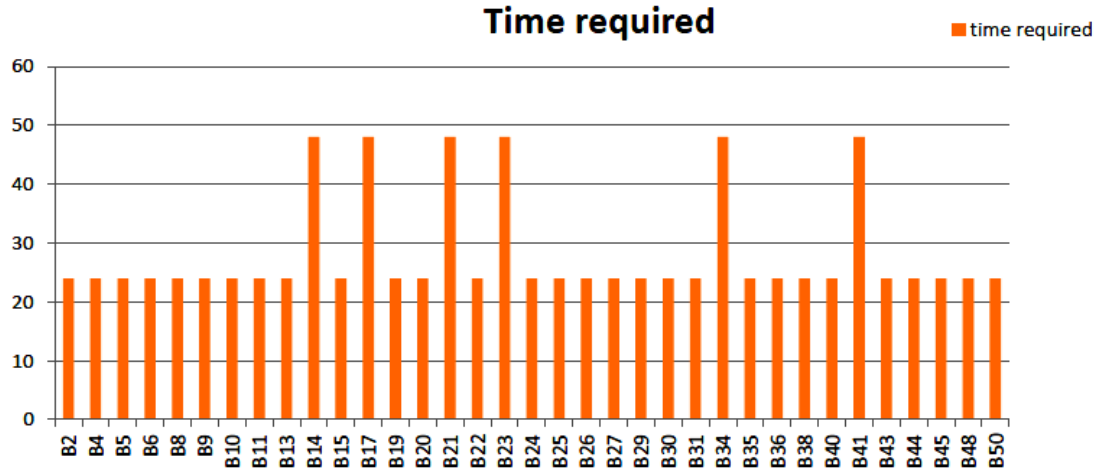

Figure 7. Graphical representation of time required by improved method

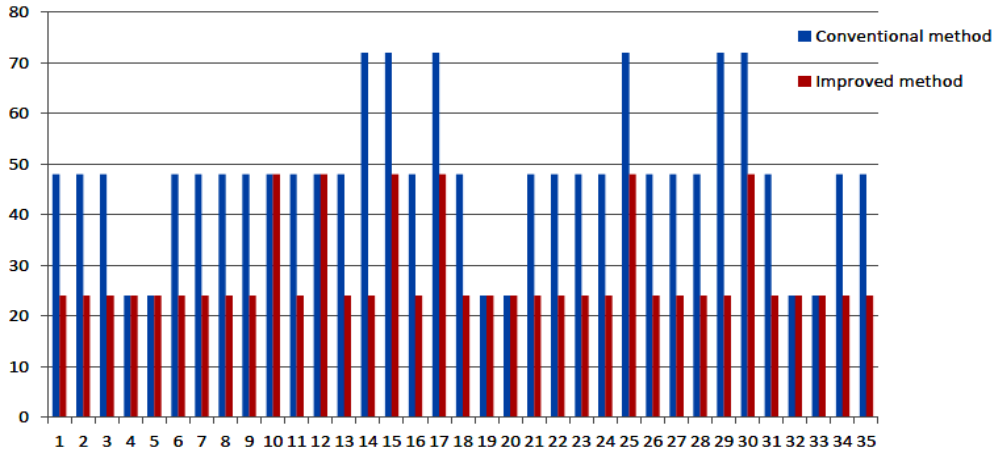

Figure 8. Graphical representation of time difference between standard and improved TTC dye method

As shown in Figure 8. the comparative data of the two methods performed in the laboratory in which there is a very greater time difference between the standard / conventional method and improved TTC dye method. By using the newly improved method, the samples B2, B4, B5, B9, B10, B11, B13, B15, B19, B21, B22, B23, B24, B26, B27, B28, B29, B31, B33, B36, B37, and B38 gives positive result before 24 hours than that of standard/ conventional method. While, samples B20, B30 give positive results before 48hours than that of the standard/ conventional method. So the new blood culture technique is evaluated and by this newly evaluated technique, the time period is reduced up to 24 hrs (An average) than that of the traditional methods of blood culture.

DISCUSSION

In the present study, we discussed the early detection of blood-borne pathogens and their antibiotic susceptibility using Ciprofloxacin as an antimicrobial agent/drug against different isolates obtained from blood samples. As enlisted in Table 3., a total of 50 blood samples were collected and streaked on blood agar, MacConkey agar for isolation of organisms. Isolates were confirmed by gram staining and biochemical tests.

Table 3. Characterization of bacterial isolates from blood samples by standard method

Organisms Identified	Sample No.	Colony Characteristics		Gram Staining	Confirmatory Tests
		Blood Agar	MacConkey Agar		
K. pneumonia	B2, B4, B6, B8, B9, B13, B23, B35, B38, B50	Small, Circular, Colorless, Transparent, Irregular	Small, Circular, Pinkish, Translucent, Regular	Gram negative rods	I = -ve MR = -ve VP = +ve C = +ve
S. haemolyticus	B5	Large, Whitish, Spreaded, Opaque, Irregular	No Growth	Gram positive cocci	I = -ve MR = -ve VP = +ve C = -ve
E. coli	B10, B21, B25, B40, B48	Small, Circular, Whitish, Opaque, Regular	Small, Circular, Yellow, Tranparent, Regular	Gram negative rods	I = -ve MR = +ve VP = -ve C = +ve
P.aeruginosa	B11, B14, B17, B19, B26, B30, B43 , B45	Small, Circular, Whitish, Opaque, Regular	Large, Circular Colourless, Opaque, Regular	Gram negative rods	I = -ve MR = -ve VP = -ve C = +ve
S. aureus	B15, B29, B31, B34, B36, B41, B44	Small, Circular, Whitish, Opaque, Regular	No Growth	Gram positive cocci	I = -ve MR = +ve VP = +ve C = +ve
S.epidermidis	B27	Small, Non-Pigmented, Non-Hemolytic	Pinpoint, Circular, Red. Opaque, Regular	Gram positive cocci	I = -ve MR = -ve VP = +ve C = -ve

In Figure 8., the time required for growth and time difference between standard/conventional blood cultures techniques were compared with that of the improved blood culture method using tetrazolium dye. It was found that the newly evaluated technique reduces the time period up to 24hrs (approx) than that of the traditional methods of blood culture. In Table 4., the MIC of ciprofloxacin for isolates were enlisted and in Table 5. A comparison of ciprofloxacin MICs by standard method and Micro broth dilution method (TTC Dye) in terms of major and minor error and % agreement is summarized.

Table 4. Minimum inhibitory concentration by standard method and Microbroth dilution method using TTC dye.

Isolates	Concentrations of antibiotic (Ciprofloxacin)													
	16µg/ml		32µg/ml		64µg/ml		128µg/ml		256µg/ml		512µg/ml		1024µg/ml	
	Broth	Dye	Broth	Dye	Broth	Dye	Broth	Dye	Broth	Dye	Broth	Dye	Broth	Dye
E.coli (1)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
E.coli(2)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
E.coli(3)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve
S .aureus (1)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
S haemolyticus (1)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
K. pneumoniae(1)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
K. pneumonia (2)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
K. pneumonia (3)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
K. pneumonia (4)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
K. pneumonia (5)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
P. aeruginosa (1)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
P. aeruginosa (2)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve
P. aeruginosa (3)	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
P. aeruginosa (4)	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve

Table 5. Comparison of Ciprofloxacin MIC by Standard method and Broth Microdilution method along with errors and % agreement with reference method.

Sr. No	Isolates	Standard method	Improved method	Major errors	Minor errors	% agreement
1	*E.coli (E1)*	128µg/ml	128µg/ml	0	0	100%
2	*E.coli (E2)*	128µg/ml	128µg/ml	0	0	100%
3	*E.coli (E3)*	256µg/ml	256µg/ml	0	0	100%
4	*S. aureus*	1024µg/ml	1024µg/ml	0	0	100%
5	*S.haemolyticus*	128µg/ml	128µg/ml	0	0	100%
6	*K. pneumoniae (K1)*	128µg/ml	128µg/ml	0	0	100%
7	*K. pneumoniae (K2)*	1024µg/ml	1024µg/ml	0	0	100%
8	*K. pneumoniae (K3)*	1024µg/ml	1024µg/ml	0	0	100%
9	*K. pneumoniae (K4)*	256µg/ml	128µg/ml	128	0	72%
10	*K. pneumoniae (K5)*	1024µg/ml	1024µg/ml	0	0	100%
11	*P.aeruginosa(P1)*	128µg/ml	128µg/ml	0	0	100%
12	*P.aeruginosa(P2)*	512µg/ml	256µg/ml	256	0	66%
13	*P.aeruginosa(P3)*	128µg/ml	128µg/ml	0	0	100%
14	*P.aeruginosa(P4)*	512µg/ml	256µg/ml	256	0	66%

In the broth microdilution (BMD) method using TTC Dye all isolates MICs were correctly identified with the naked eye, suggesting that visual reading could be sufficient to interpret the results. Technically, the broth microdilution (BMD) method was easy to perform with very rapid results. In this study, we developed an affordable and reliable method for the detection of ciprofloxacin susceptibility tests for blood-borne pathogens. The minimum time required to complete all the investigations with the BMD method for isolates was 5-6 hrs. Ideally, antimicrobial treatment should be based on the knowledge of two features, first, the relevant organism's susceptibility to the drugs available and, second, the amount of the drug attainable at the site of infection. The agar dilution method is not commonly used in clinical laboratories because it is technically cumbersome. Furthermore, mistakes in the addition of drugs to media are frequently made and difficult to recognize [16].

CONCLUSION

After the whole study of the bloodstream infection, it is found that the bloodstream infection is a very severe disease and delay in the diagnosis may lead to the patient's death too. So the early detection is very important to treat the patient with proper treatment which can help to save the patient we have worked on this problem to overcome the danger of bloodstream infection.

The time required for the detection of the blood-borne pathogen with that of the Standard method is much lengthy, Even the Castaneda blood culture technique is not much useful and gives results at the nearly same time as that of the Standard method. When we perform our newly improved method, we found that the detection of the blood-borne pathogen by the improved technique can be achieved much time earlier than that of the Standard method and Castaneda blood culture bottle method. In contrast to the antibiotic susceptibility test, the Micro broth dilution method was an easy, reliable, affordable, and quick result giving method as compared to the standard antibiotic susceptibility test method. It is concluded that the goal of this study was to give improve the blood culture method for early detection of bloodstream infection and develop reliable antibiotic susceptibility methods for treatment of infections in this era of ever-evolving antimicrobial resistance. So our final findings show that the new blood culture technique is evaluated and by this newly evaluated technique the time period is reduced up to 24 hrs (average) than that of the traditional method.

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Ethical Approval: Approved

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