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Rapid Real Time PCR Based Detection of Cell Count in Case of Urinary Tract Infection

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ABSTRACT

Microbial identification and antimicrobial susceptibility testing methods currently used in clinical microbiology laboratories require at least two to three days because they rely on the growth and isolation of micro-organisms. This long, but necessary, delay has enormous consequences on prophylactic usage of antimicrobial drugs. This study was an attempt to reduce this detection time span. Taq Man Real Time PCR has been used as an important tool in the differentiation of Gram nature of bacteria present in UTI patients that allows detection of spiked bacterial 16S rDNA from urine samples within a short span of 5h and also gives us the corresponding cell count of both/either Gram positive and negative organisms present. A standard curve was generated which was used to determine the cell count of control as well as patient samples. Detection could be done in the range of 10^3 to 10^6 cells/mL Patient samples screened clustered either in the allele 1 or allele 2 axes, depending on majority concentration of Gram nature of the micro-organisms. The cell counts for control individuals were scattered within 0 to 10^2, while very few in the range of 10^4. The case was just reverse for patient group, where most of the points were scattered within 10^4 to 10^8. Thus the optimal selection of appropriate antimicrobials (depending on the gram nature) by clinicians, will be gradually improved as an increasing number of rapid molecular diagnostic tools for the detection, identification and characterization of infectious agents become commercially available.

Keywords: Allelic Discrimination Plot, Genotypic Assay, Gram Nature, Realtime PCR, Standard Curve

1. INTRODUCTION

The urinary tract is one of the most common sites of bacterial infection in women (Foxman, 2003; Ishaq *et al*., 2011). These infections also carry the risk of possible progression to bacteremia. The empirical choice of an effective treatment is becoming more difficult as urinary pathogens are increasingly becoming resistant to commonly used antibiotics (Nicolle *et al*., 1996; Barret *et al*., 1999; Mathai *et al*., 2001; Karlowsky *et al*., 2001; Ishaq *et al*., 2011; Dharmadhikari and Peshwe, 2009; Butcu *et al*., 2011; Ultley *et al*., 1988; Leclerq *et al*., 1988; Ishaq *et al*., 2011). Any infection left untreated like UTI, kidney infection, is extremely dangerous and can lead to life threatening conditions such as bacteremia. This is usually a very serious condition that results in death unless prompt appropriate treatment is provided.

One of the major drawbacks of the routine diagnostic methods for pathogen identification in UTI is the long period for detection (48 to 72 h) required in

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culture based methods (Ramlakhan *et al*., 2011). Besides, uncultivable microbes are numerically dominant in biological samples, urine being no exception and therefore have to be detected by culture independent methods (Carroll *et al*., 2000; Belgrader *et al*., 1998; Bittar *et al*., 2008; Bergeron and Ouellette, 1995; Picard and Bergeron, 1999; Tang *et al*., 1997; Ishaq *et al*., 2011). The total count (cultivable as well as non-cultivable bacteria that are alive, but do not give rise to visible growth under non-selective growth conditions) needs to be detected rapidly for prompt medical intervention.

The delay of the microbiology laboratory contrasts with the time required (less than one hour) to get the results from other hospital laboratories or departments, such as biochemistry, hematology and radiology. Indeed, clinical microbiology procedures remain still based on the use of a variety of growth-dependent biochemical tests developed by Pasteur and others during the 19th and 20th centuries. Consequently, physicians rarely consult the microbiology results unless the patient is not responding to the initial antimicrobial therapy, which is based on key information obtained during the first hour after patient admission, thereby excluding any diagnosis based on microbiology results. Clearly, there is a need for rapid and accurate diagnostic tests for use in clinical microbiology laboratories to enable optimal patient management and treatment. Rapid detection and identification of microbial pathogens and their antimicrobial resistance profiles would have a tremendous impact on the practice of medicine by providing physicians with key microbiology results when needed.

The use of rapid molecular diagnostics may provide a solution for treating this disease which has a high morbidity and mortality rate. Molecular biology techniques for correct detection and identification of bacteria is now widely used in clinical microbiology namely 16S rRNA based identification, terminal Restriction Fragment Length Polymorphism (tRFLP), Random Amplification of Polymorphic DNA (RAPD), Real Time PCR (Picard and Bergeron, 2002; Ishaq *et al*., 2011). There are innumerable number of patents (USPTO 20090239248, USPC 4356, US Patent 7205111, US Patent 7662562, US Patent 4693972) stating methodologies for rapid identification of microbes from clinical samples, but none of them mention the sensitivity of detection. Keeping this fact in mind we tried to fine-tune already existing methods to develop a more sophisticated system of detection. Real Time PCR has been used as an important tool in the differentiation of Gram nature of bacteria present in UTI patients using a consensus real-time PCR protocol with a TaqMan probe that allows detection of spiked bacterial 16S rDNA from urine samples within a short span of 5h and also gives us the corresponding cell count of both/either Gram positive and negative organisms present.

2. MATERIALS AND METHODS

2.1. Genomic DNA Isolation

Bacterial genomic DNA was extracted using Fit Amp Urine DNA Isolation Kit (Epigentek, P-1017-050) from various dilutions (10^8 to 10^1 cells/mL) of urine samples. Cartridge based DNA extraction kit was used for isolation of DNA as per manufacturer's protocol with minor modifications. 900µl of sterile urine (urine samples were filtered by passing them through a 0.22 µ syringe filter) was taken and seeded with culture of gram negative (E.coli) and gram positive (S.aureus) bacteria separately at a concentration of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 10^7 and 10^8 cells/mL. The suspension was centrifuged at 10,000 rpm for 10min (Eppendorf Centrifuge 5418, Rotor eppendorf FA-45-18-11 aerosol tight) at room temperature to pellet down the cells. The supernatant was discarded and the pellet was resuspended in 200 µL of suspension buffer and mixed through pipetting. Then 4 µL of DNA digestion buffer containing enzyme was added and mixed using vortex. The mixture was incubated at 65°C for 1 h. To it 300µl of DNA capture buffer was added and mixed using pipettman. The mixture was transferred to a spin column placed inside a 2 mL collection tube. It was centrifuged at 12,000rpm for 1min (Eppendorf Centrifuge 5418, Rotor eppendorf FA-45-18-11 aerosol tight). The flow through was discarded and the spin column was replaced in the collection tube. The centrifugation step was repeated again and the supernatant was discarded. Then 300 µL of 70% ethanol was added to the spin column and centrifuged at 12,000 rpm for 30 sec (Eppendorf Centrifuge 5418, Rotor eppendorf FA-45-18-11 aerosol tight). The flow through was discarded. Two more washes of 200 µL of 90% ethanol were applied similarly as stated above. This was to remove salts as well as to wash away impurities. The spin column was replaced into a fresh 1.5mLcentrifuge tube and the DNA was eluted using 10 µL of DNA elution buffer.

2.2. Real Time PCR

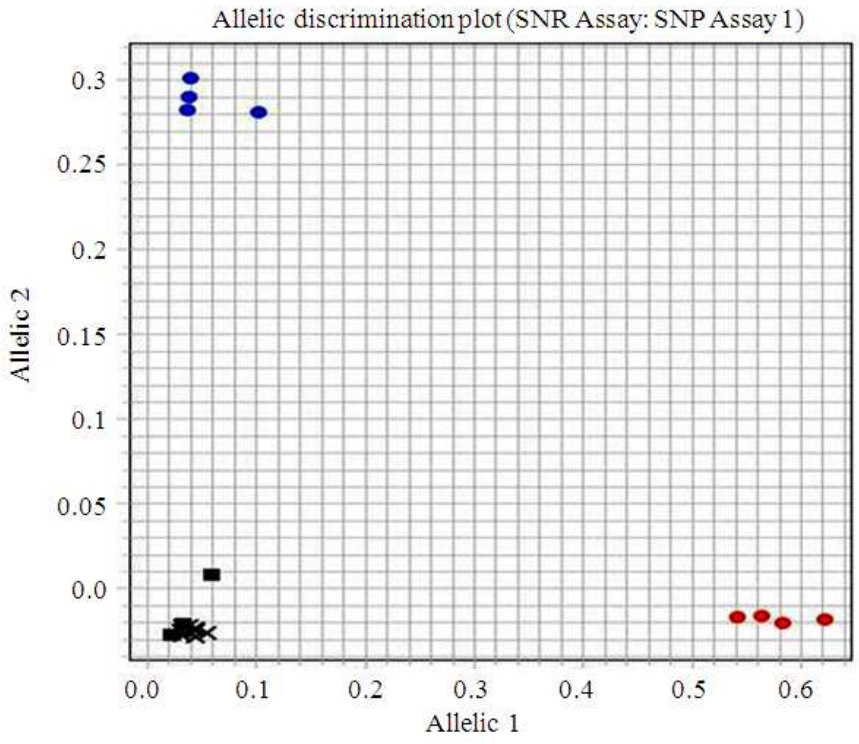
The DNA obtained was directly used for gram nature detection using the TaqMan PCR protocol (Genotypic assay) as reported by Shigemura *et al*. (2005) and Ishaq *et al*. (2011) to check the sensitivity of the assay. The only modification was that for Probe 2, FAM was used in place of TET in order to maintain the compatibility of our Real time PCR Step One system. ROX was used for internal control. The allelic discrimination assay (Genotyping) was set up in a 48 well reaction plate. Each experiment was repeated at least 6 times.

For determination of the standard curve, sterile urine samples (post filtration through 0.22µ syringe filter units by Whatman) were seeded with 1E7 concentration of Gram positive (S aureus) bacteria. Genomic DNA was extracted using Fit Amp Urine DNA Isolation Kit as mentioned above. The DNA was serial diluted (corresponding to 1E1 to 1E7 cells) and real time experiment was set up exactly the similar way as already mentioned above, under Standard Curve option. Patient urine samples were also analyzed based on the standard curve equation to determine the cell count. A total number of 70 non-infected and 89 patient samples were analyzed and their corresponding C_T values for both Gram positive and Gram negative bacteria were noted down.

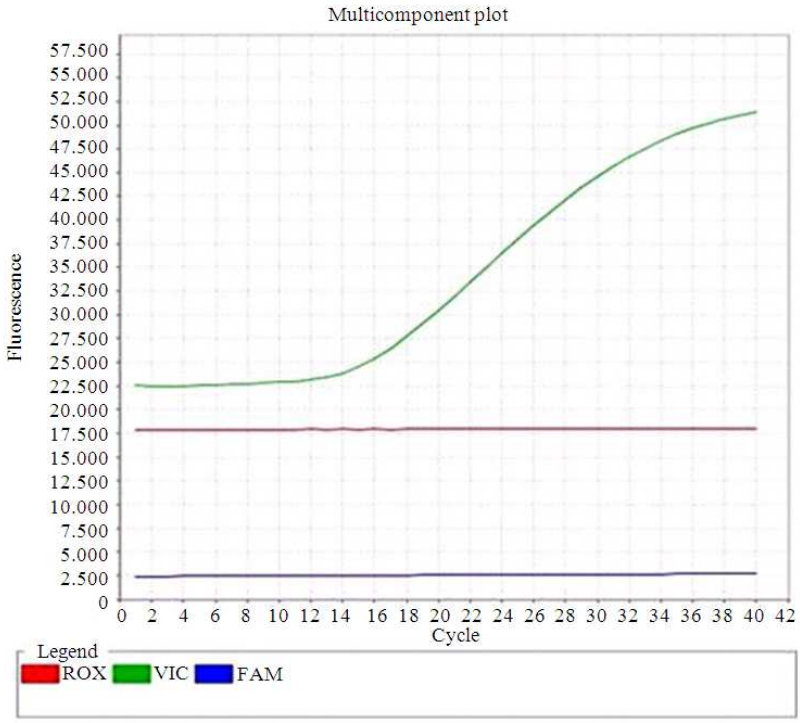
3. RESULTS

Seeded sterile urine was used to check the efficiency of detection/sensitivity of this assay. Detection could be done in the range of 10^3 to 10^6cells/mL as evident from the scatter plot diagrams in **Fig. 1a**. A distinct allelic discrimination plot was obtained that clustered gram positive and gram negative seeded samples in different axes. Patient samples screened clustered either in the allele 1 or allele 2 axes, depending on majority concentration of Gram nature of the micro-organisms present in the infected urine samples.

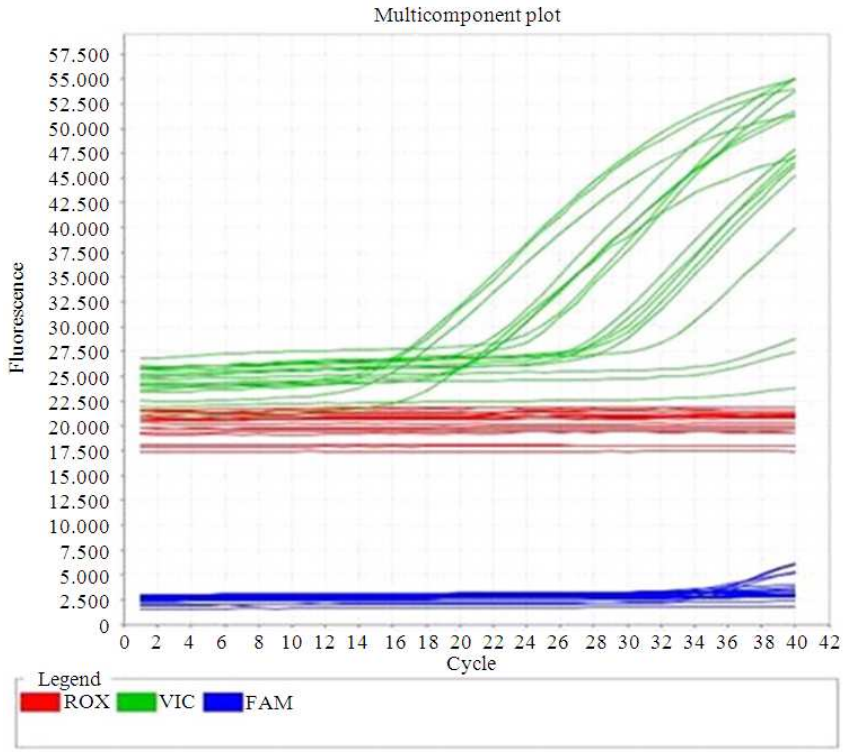
In order to determine the sensitivity of the detection limit, a standard curve was generated using three replicates of each dilution (1E1 to 1E7), by plotting the cell concentration or quantity on the X axis and the C_T value on the Y axis (**Fig. 2a**). The C_T values were also plotted in an Excel worksheet to determine the straight line equation (y = -3.143x+38.44) of the curve. The best fit line (**Fig. 2b**), had a R^2 value of 0.994, which is well within the optimal limit. **Figure 2c** is the screen print of the experimental page setup for standard curve experiment in ABI Step One Real Time PCR instrument, software version 2, showing amplification, multicomponent, raw data plot, besides the standard curve. The plate layout with their respective concentrations and the C_T values for both alleles are also depicted in the diagram. From the standard curve equation, by plotting X (C_T value), the unknown value of Y (cell concentration) was determined. The amplifications obtained for the normal and infected samples are shown in **Fig. 2d and 2e** respectively.



(a)


Multicomponent plot
Fluorescence
57.500
55.000
52.500
50.000
47.500
45.000
42.500
40.000
37.500
35.000
32.500
30.000
27.500
25.000
22.500
20.000
17.500
15.000
12.500
10.000
7.500
5.000
2.500
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Cycle
Legend
ROX VIC FAM

(b)


Multicomponent plot
Fluorescence
57.500
55.000
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50.000
47.500
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27.500
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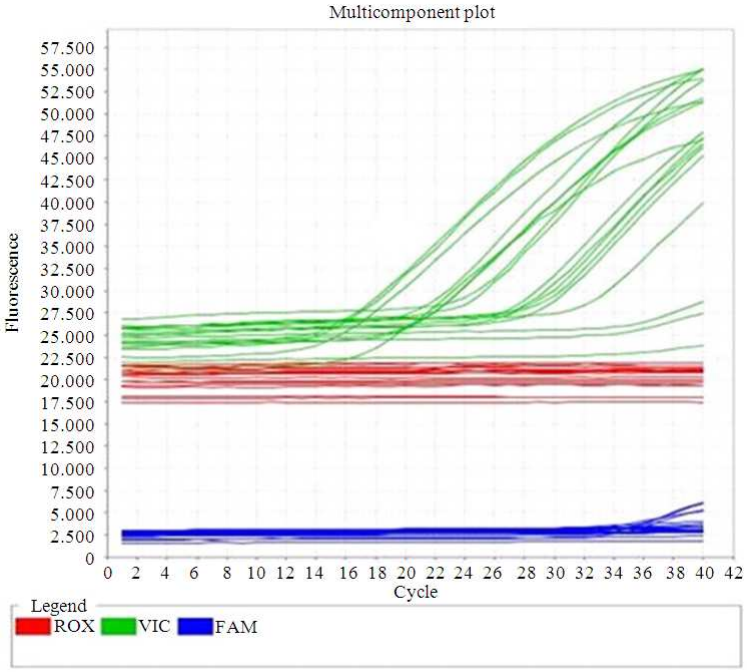
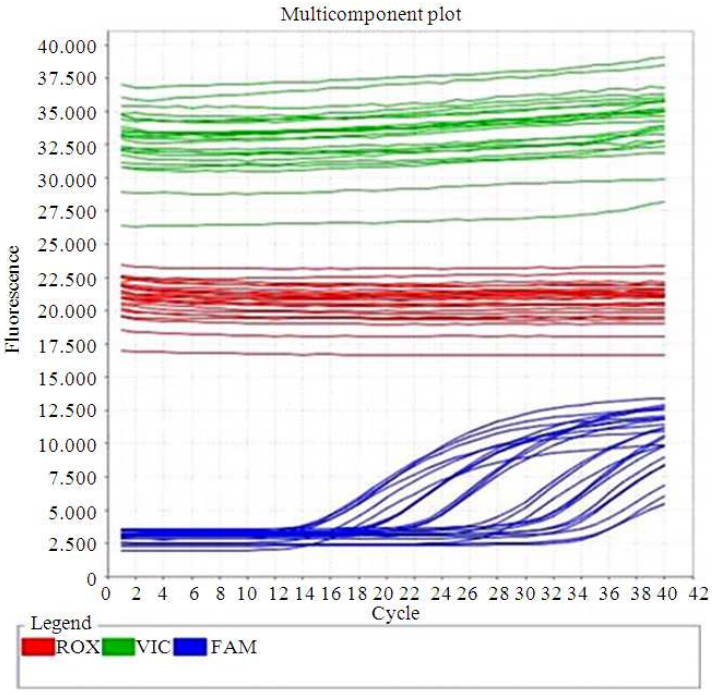
22.500
20.000
17.500
15.000
12.500
10.000
7.500
5.000
2.500
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
Cycle
Legend
ROX VIC FAM

(c)

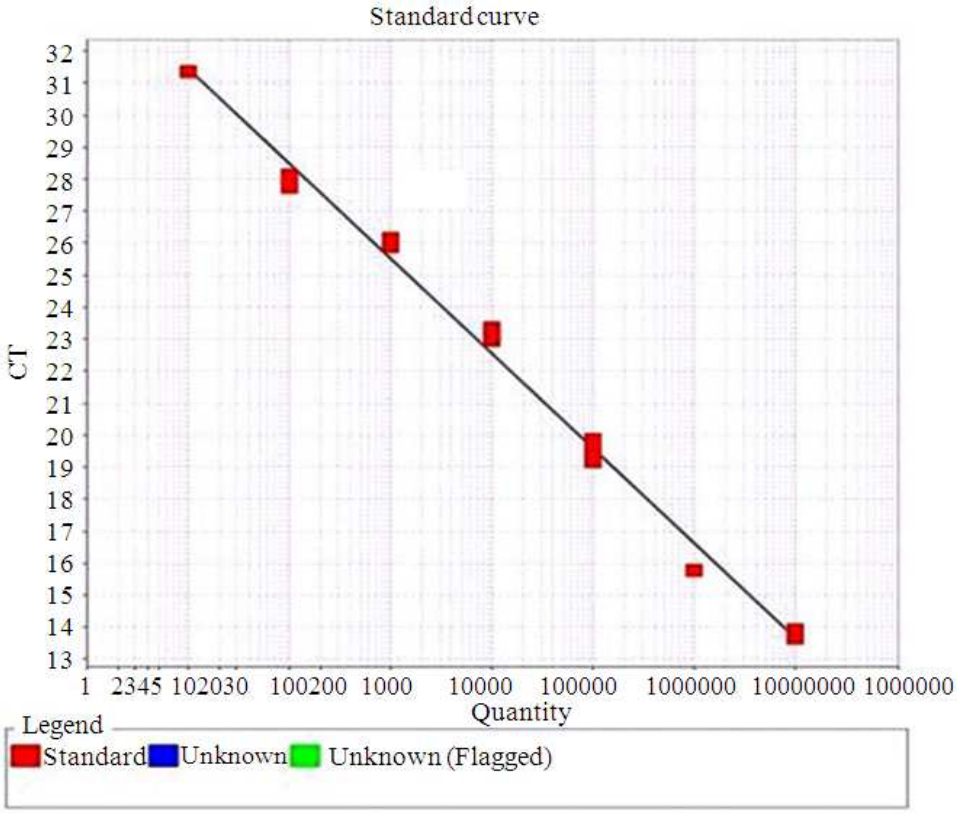


(d)

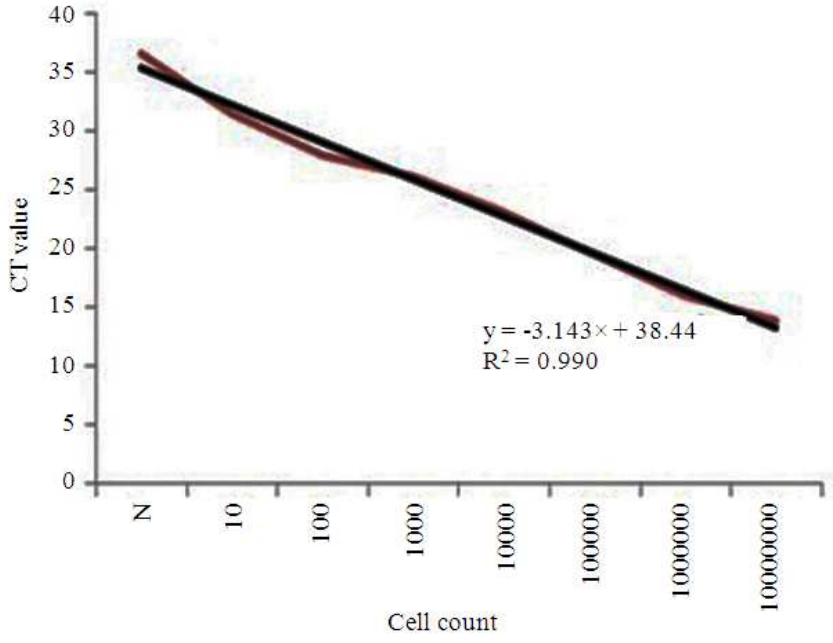


(e)

Fig. 1. (a) Allelic Discrimination/Scatter plot for seeded urine samples of known concentration. (b and c) shows the amplification of VIC probe responsible for Gram positive bacteria and (d and e) shows the amplification of FAM probe responsible for Gram negative bacteria


Standard curve
CT
32
31
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
1 2345 102030 100200 1000 10000 100000 1000000 10000000 1000000
Quantity
Legend
Standard
Unknown
Unknown (Flagged)

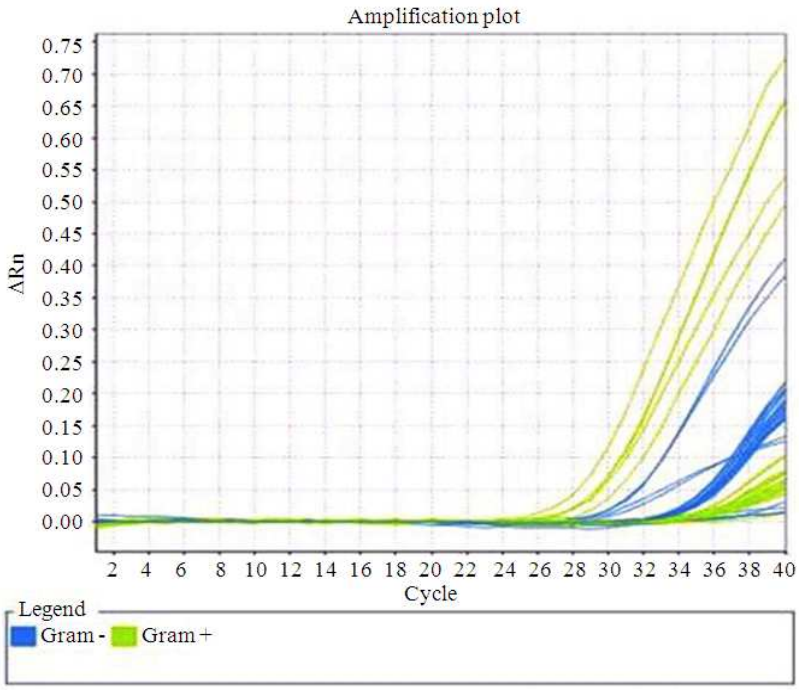
(a)


CT value
40
35
30
25
20
15
10
5
0
y = -3.143× + 38.44
R2 = 0.990
N
10
100
1000
10000
100000
1000000
10000000
Cell count

(b)


Experiment Menu
Setup
Run
Analysis
Experiment: standardB
Type: Standard Curve
Reagents: TaqMan® Reagents
Multiple Plots View
View Plate Layout
View Well Table
Amplification Plot
Standard Curve
Multicomponent Plot
Raw Data Plot

(c)


Amplification plot
ΔRn
Cycle
Legend
Gram -
Gram +

(d)

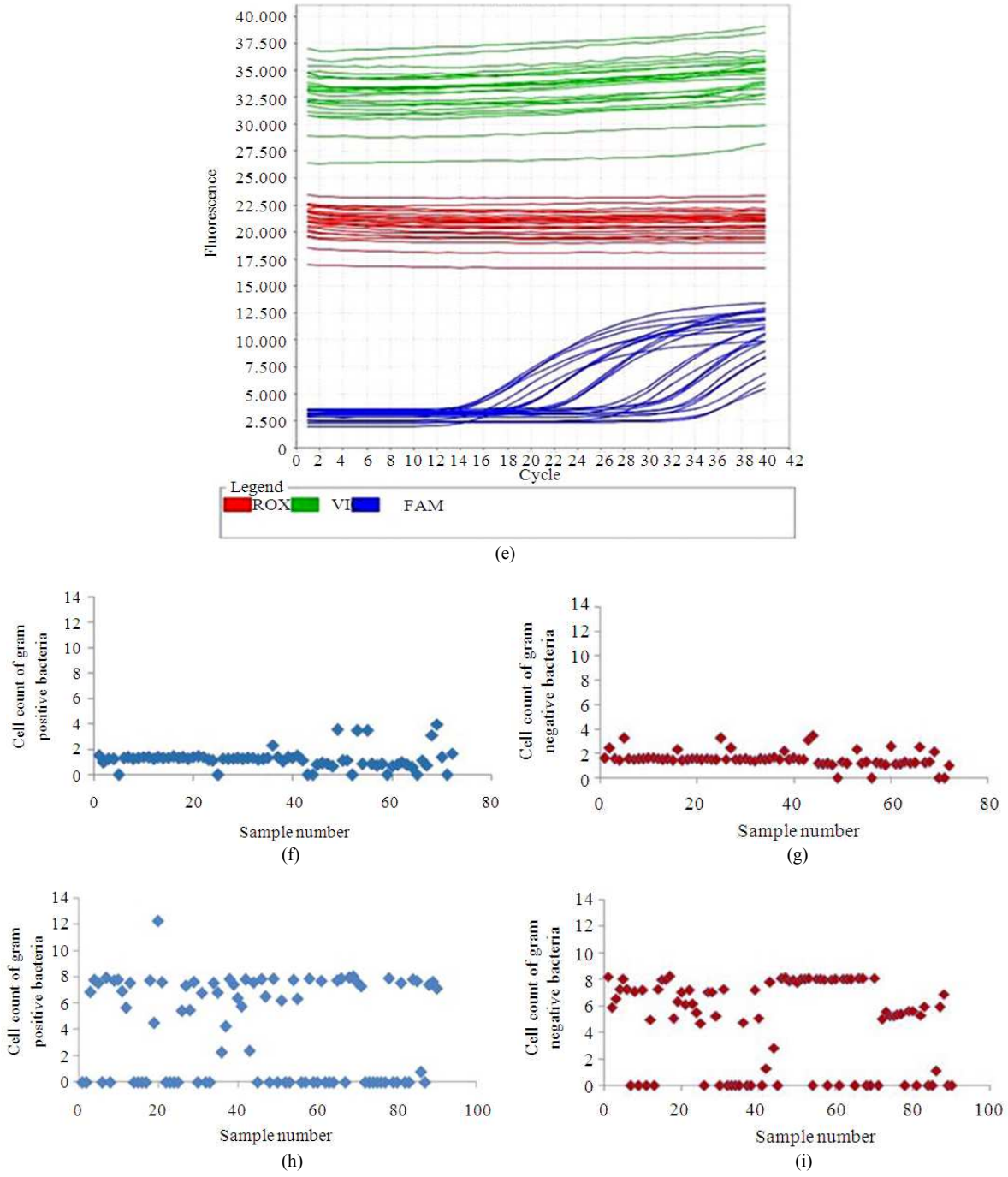


Fig. 2. (a) Represents the standard curve as obtained in step one software version 2 ABI PCR instrument by performing standard curve experiment and (b) depicts the same standard curve after plotting them in an excel worksheet (c) Show the screen print of the entire experimental setup (d) Show late amplification of normal urine samples and (e) is the graphical representation of variable amplification of the two alleles for random patient urine samples (f and g) have been plotted with sample number on the X axis and their cell count calculated from their corresponding CT values by putting them in the standard curve equation on the Y axis, with normal samples, while (h and i) are their infected sample counterpart

Table 1. Cell count raw data of the patient urine samples

Subjects	Gram positive cell count to the power of 10	Gram negative cell count to the power of 10	Subjects	Gram positive cell count to the power of 10	Gram negative cell count to the power of 10
1	0.000000000	8.184713376	53	7.767515924	0.000000000
2	0.000000000	5.859872611	54	6.340764331	8.025477707
3	6.853503185	6.506369427	55	0.000000000	8.000000000
4	7.773885350	7.222929936	56	0.000000000	7.974522293
5	7.541401274	7.996815287	57	7.866242038	0.000000000
6	7.939490446	0.000000000	58	0.000000000	7.961783439
7	0.000000000	7.076433121	59	0.000000000	7.987261146
8	7.726114650	0.000000000	60	7.691082803	0.000000000
9	7.792993631	7.187898089	61	0.000000000	8.035031847
10	6.914012739	0.000000000	62	0.000000000	8.000000000
11	5.668789809	4.964968153	63	0.000000000	7.987261146
12	7.554140127	0.000000000	64	7.729299363	0.000000000
13	0.000000000	7.264331210	65	7.875796178	8.047770701
14	0.000000000	7.961783439	66	0.000000000	8.070063694
15	0.000000000	7.980891720	67	7.952229299	0.000000000
16	0.000000000	8.219745223	68	8.041401274	0.000000000
17	7.732484076	5.057324841	69	7.585987261	8.092356688
18	4.509554140	6.315286624	70	7.283439490	0.000000000
19	12.242038220	7.022292994	71	0.000000000	4.980891720
20	7.601910828	6.089171975	72	0.000000000	5.531847134
21	0.000000000	7.175159236	73	0.000000000	5.235668790
22	0.000000000	6.124203822	74	0.000000000	5.232484076
23	0.000000000	5.471337580	75	0.000000000	5.312101911
24	0.000000000	4.697452229	76	0.000000000	5.375796178
25	5.433121019	0.000000000	77	7.891719745	0.000000000
26	7.328025478	7.044585987	78	0.000000000	5.621019108
27	5.480891720	7.031847134	79	0.000000000	5.611464968
28	7.624203822	5.197452229	80	7.547770701	0.000000000
29	0.000000000	0.000000000	81	0.000000000	5.286624204
30	6.780254777	7.261146497	82	0.000000000	5.939490446
31	0.000000000	0.000000000	83	7.802547771	0.000000000
32	0.000000000	0.000000000	84	7.681528662	0.000000000
33	7.541401274	0.000000000	85	0.786624204	1.108280255
34	6.808917197	0.000000000	86	0.000000000	5.910828025
35	2.280254777	4.735668790	87	7.401273885	6.882165605
36	4.248407643	0.000000000	88	7.624203822	0.000000000
37	7.837579618	0.000000000	89	7.127388535	0.000000000
38	7.417197452	7.197452229			
39	6.385350318	5.063694268			
40	5.764331210	0.000000000			
41	7.815286624	1.302547771			
42	2.388535032	7.770700637			
43	7.573248408	2.834394904			
44	0.000000000	0.000000000			
45	7.834394904	8.047770701			
46	6.515923567	8.101910828			
47	0.000000000	7.859872611			
48	7.856687898	7.961783439			
49	0.000000000	7.748407643			
50	6.207006369	8.009554140			
51	0.000000000	7.993630573			
52	0.000000000	8.057324841			

The Gram positive and Gram negative C_T values were separately plotted for both normal and patient groups (**Fig. 2f to 2i**). For control individuals, most of them are scattered within 0 to 10^2, while very few in the range of 10^4, but not above that. The case is just reverse for patient group, where most of the points are scattered within 10^4 to 10^8.

4. DISCUSSION

The passive reference dye signal ROX was absolutely constant during the entire experiment. Gram positive samples clustered on the Allele 1 axis of the scatter plot, while Gram negative ones clustered on the allele 2 axis. The probe VIC (allele 1) was responsible for amplification of Gram positive specimens, while FAM (allele 2) for Gram negative ones. Sterile urine sample seeded with a known concentration of Gram positive bacteria shows amplification of only the VIC probe (**Fig. 1b**) in case of single or multiple samples seeded with the same Gram nature at different concentrations (**Fig. 1c**). The case is exactly the same for Gram negative seeded samples which shows only amplification of FAM probe (**Fig. 1d and 1e**), while VIC and ROX are relatively constant. Thus we can say that there was no experimental error.

Without even looking at the CT values in case of control and patient samples (**Fig. 2f-2i**), one can easily infer from the graph that, there is considerable less amplification in case of normal samples as compared to the infected ones. In case of infected samples amplification starts after 12cycles only while in control individuals there is no amplification till almost 26th cycle.

Another important notable feature was that, the few points which lie towards 0 to 10^1, in Gram negative plot in the patient group have a higher cell concentration value in the Gram positive plot and vice versa; i.e., those samples are not infected by high concentration of both Gram nature organisms, but only one of either types. For example, in patient 1 and 2, the gram positive cell count was 0 in both cases, while their respective gram negative counts were 10^8 and 10^5. Similarly in case of the last two sample numbers 88 and 89, the gram negative cell count was 0, but the corresponding gram positive count was 10^7 for both. The raw data with cell count value has been provided in **Table 1** (highlighted), where sample numbers exhibiting this phenomenon have been highlighted (showing 0 in one gram nature plot and a higher cell count value in the other gram nature plot).

Thus, infections caused by non-cultivable bacteria can also be detected by using this culture independent assay. The detection time is drastically brought down from 72h to less than 5h, thus allowing quick administration of antibiotics. The exact cell number of micro-organisms causing the infection can also be determined from the standard curve equation, without the hassle of cultivating them.

5. CONCLUSION

A major goal in diagnosis and treatment of patients especially female patients suffering from UTI is the ability to rapidly detect the characteristics of infecting microbes. We have used Real Time PCR in the differentiation of Gram nature of bacteria present in UTI patients using a consensus real-time PCR protocol that allows detection of spiked bacterial 16S rDNA from urine samples within 5h along with the corresponding cell count of both/either Gram positive and negative organisms present. The similar technique could be used for pathogen detection in case of Septicemia.

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