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Original Article

Microbiology Section

Rapid Detection of Enterohaemorrhagic *E.coli* Using Phage-Based Bioluminescent Assay

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ABSTRACT

Introduction: There is a need for a reliable, time-saving, and specific detection assay for coliforms, environmental *E. coli*, and Enterobacteriaceae worldwide.

Aim: To innovate a new principle of phage-based rapid diagnostic test for detecting *E. coli* bacteria in short time and low titer.

Materials and Methods: A phage mixture of 200 *E. coli* specific phages, including 22 specific for Enterohaemorrhagic *E.coli* (EHEC), were used in a new detection platform, a phage-based Adenylate Kinase Bioluminescence Assay (AKBA). Ten EHEC *E. coli* and 30 universal *E.coli* isolates were used for AKBA assay.

Results: AKBA showed positive detection of *E.coli* bacteria at 10^3 CFU in just 20 minutes. The phage-based detection was highly specific at strain level of *E.coli*. The sensitivity and specificity of AKBA was 74% and 78%, respectively.

Conclusion: A rapid and strain-specific diagnostic test was prepared for *E.coli* by using coliphages. The significance and impact of the study shows that it might be feasible to formulate a phage-based assay against any Gram negative or positive bacteria using the same approaches of the current AKBA assay with slight modifications.

Keywords: Adenylate kinase, Bacteriophage, Bioluminescence, Rapid diagnostic tests

INTRODUCTION

E. coli detection is considered more specific than the detection of fecal coliforms in water quality testing [1]. Till now, most water and food industry safety laboratories use the time-consuming classical ways of *E. coli* diagnosis which eventually take time between 12 to 24 hour [2,3]. Taken into consideration that water processing plant need to make hundreds of water quality tests per month and due to the impact on public health, a rapid and specific diagnostic test for *E. coli* has become a necessity.

One of the best methods of rapid diagnostic testing relied somehow on the bioluminescent phenomenon of luciferin-luciferase enzyme reaction in the presence of ATP [4,5]. Other scientists used a detectable marker, often the enzyme luciferase, introduced into bacteriophages which can then be used for bacterial detection [6-10]. These assays include a general lysing reagent to break open the bacterial cells and release the intracellular ATP; thus the results only give a measure of microbial load rather than the presence of specific pathogens within the microflora. By using either intact phage or recombinantly produced phage endolysins to replace the general lysing agent, the desired specificity can be added to this test.

The current study was based on a hypothesis that a phage cocktail composed of specific coliphages can be used in adjunction with carefully designed qualitative and quantitative bioluminescent assay to detect *E. coli* bacteria specifically. The performance of the currently used AKBA of luciferin-luciferase was assessed thoroughly in this study.

MATERIALS AND METHODS

This study is a biomedical research on the ability of phage cocktail to detect specifically *E.coli* bacteria; it was conducted as a conjoint study in Institute of Bioscience, Malaysia and Microbiology Department in College of Medicine, Al-Nahrain University, Iraq from the period January 2015 to September 2016.

The research was approved by the ethical committee of University of Putra Malaysia and College of Medicine, Al-Nahrain University, Iraq.

Media

Bacterial dilutions from 18 hour Luria Broth (LB) cultures grown at 37ºC were carried out in Phosphate Buffered Saline (PBS, Oxoid, UK). Luria broth: tryptone 10g/L (HiMedia, Mumbai, India), yeast extract 5g/L (HiMedia, Mumbai, India), and sodium chloride 10g/L (HiMedia, Mumbai, India) at pH 7.2 were used in all the protocols. For plaque assay, the 'soft layer agar' used was LB prepared in Lambda-buffer (HiMedia, Mumbai, India), [11].

Bacterial Strains

Four hundred and thirty clinical isolates of EHEC (85 isolates) and non-EHEC (345 isolates) *E.coli* were provided by Hospital Serdang and Hospital Kajang in Selangor, Malaysia. The bacteria were isolated from diagnosed cases of urinary tract infections, vaginitis, infected wounds, bacteraemia, and from both haemorrhagic colitis and non-haemorrhagic colitis. They were reconfirmed by using Microbact GNB 12A system (Oxoid, UK), a microtitre well-scaled chemical test.

The following *E.coli* reference strains were used, with the same EHEC: non-EHEC ratio, namely 1:5, one EHEC NTCC 129001 and five non-EHEC (two are generic strains; ATCC 12799 and NTCC 9001, three human enteropathogenic strains (EPEC); ATCC 12810, ATCC 25922, and ATCC 35218 as zoonotic). The representative NTCC and ATCC *E.coli* strains together with the clinical isolates of *E.coli* were used in phage isolation, propagation, optimization and breeding as described below. All the strains were maintained on L-agar plates and transferred bimonthly. The cultures were stored at -20°C in 15% glycerol [12].

Bacteriophages

In this study, the wild bacteriophages (phage) were isolated from and specifically passaged from 430 clinical isolates and six reference strains of EHEC and non-EHEC *E. coli* according to IPO-UK Patent Application No. 0822068.3. The term of 'known bacteria' is used in this study to describe the population of bacteria used to isolate,

raise, and design the corresponding coliphages and to differentiate them from other bacteria having termed as 'unknown bacteria'. The phage master mix is prepared from mixing phages together which is composed of 200 highly lytic and specific bred phages and were named EHP-1-200 [13].

SAMPLE PROCESSING

Artificially Inoculated Samples

Ten clinical isolates of EHEC *E. coli* were used artificially in laboratory to contaminate water and lettuce. These clinical isolates of EHEC O157:H7 were obtained from human inpatients of Hospital Serdang and Hospital Kajang in Selangor state, Malaysia. The procedure was done accordingly [14]. Briefly, samples of lettuce were soaked in 500 ml of PBS (Sigma, USA) supplemented with 10^7 CFU/mL of mixed *E. coli* EHEC strains for 2 hour at 20°C, placed in a beaker containing 100 ml of sterile PBS solution and washed carefully for 5 minutes. Centrifugation of the soiled PBS was done for 2500 xg for 5 minutes at room temperature. *E. coli* concentration was measured by the standard plating method on Luria agar for 18 hours. The bacterial titers for both plain water and lettuce washing PBS were adjusted for AKBA assay to be 10^{1-7} CFU/micorplate well.

Adenylate Kinase Bioluminescence Assay (AKBA):

Principle of the Assay:

AKBA was conducted on the same artificially inoculated lab samples of EHEC *E.coli* bacteria in order to apply a reliable extra-rapid assay for bacterial detection. The methods to monitor the hygienic status of food production lines and verify effective cleaning procedures by rapid cleanliness testing using ATP and Adenylate kinase bioluminescence have become widely accepted [15]. The reaction shown below, shows Adenylate kinase as a key intracellular enzyme with a role to equilibrate concentrations of the adenine nucleotides within the cell:

The use of adenylate kinase as a bacterial cell marker in place of ATP is proposed by Squirrell and Murphy [16]. Adenylate kinase is most abundant in the mitochondria of tissues such as liver and muscle in which there is considerable energy turnover. It is present in both eukaryotes and prokaryotes and has a low molecular weight (20±30 kDa).

$$ADP + ADP \underset{Mg^{2+}}{\overset{Adenylate\ kinase}{\rightleftharpoons}} ATP + AMP$$

PROCEDURE

Test Samples

AKBA assay was done using triplicates of each test sample and controls in bioluminescence white 96 microplates (Sigma, USA). The assay was carried out according to Jassim SAA et al., [14]. Fifty µl of 1:1 v/v phage master mix with target bacteria at Multiplicity Of Infection (MOI) 100 were incubated for 30 minutes at 37°C in order to let phages lyse completely target bacteria. Afterwards, 50 µl of 10 mM ADP (Sigma, USA) and 50 µl of buffer (50 mMol/L Tris+ 15 mMol/L $MgCl_2$) at pH 7.4 from Merck, Germany were added to the mixture of phage: bacteria and incubated for 10, 20, 30, and 40 minutes at 37°C. At the end of the AKBA incubation period, 50 µl of a luciferin-luciferase mixture (Calbiochem, USA) in 25 mM Hepes buffer (Merck, Germany) were added in semi-dark environment and within seconds light emission reaction was read using endpoint assay of GloMax 96 microplate luminometer (Promega, USA).

Controls and Standards

Before adding luciferin-luciferase mixture, the positive control was diluted 1:50 to avoid Benzalkonium Chloride (BKC) (Merck, Germany) inhibitory effect on luciferase enzyme. Triplicates of both negative controls (incompatible phage: bacteria mixtures) and positive controls, bacteria with BKC at 0.5 mg/mL for 15 minutes were prepared. The dilution factor was (1:50) for bioluminescence readings. To get the Relative Light Units (RLU) of the bioluminescence reaction, linear regression equation was used for the standard curve of the standard ATP solution [14].

Interpretation of the Assay

The differences in RLU values were evaluated in terms of significance between; RLU *test1* and RLU*test2*, RLU*negCon1* and RLU*negCon2*, RLU*posCon1* and RLU*posCon2* (for each ADP incubation period and for each bacterial concentration). The difference of samples magnitude (ΔRLU) of *test* (ΔRLU*test*), negative control (ΔRLU*negCon*) and positive control (ΔRLU*posCon*) were compared with each other. The target bacteria positive detection was considered when RLU*test2* was significantly higher than RLU*test1* and the ΔRLU *test* was close to ΔRLU*PosCon* and significantly higher than ΔRLU*NegCon* ($p<0.01$). In addition, both the sensitivity and specificity of AKBA assay were checked [14].

STATISTICAL ANALYSIS

The data analysis of the current study was done using SPSS version 12.0 and Microsoft Excel 2000. The linear regression equation of the standard curve was used to measure the RLU of bioluminescence which was repeated at every run depending on the level of ATP in AKBA assay. The used equation for linear regression was Y = a + bX, where X is the explanatory variable and Y is the dependent variable; (b) is the slope of the line; and (a) is the intercept. The The mean ΔRLU- *test*, *-PosCon* and *-NegCon* values at different incubation times and for different bacterial titers in AKBA assay was compared using Student's t-test. The AKBA assay sensitivity and specificity were calculated from the test and the negative control samples as AKBA results were compared with that of the standard plating method (golden standard). The significance for Pearson (r) and t- tests was considered as p-value less than 0.01.

RESULTS

AKBA was conducted on the same ten EHEC-artificially inoculated samples. The positive detection of target bacteria was achieved when the difference between RLU*test1* and RLU*tets2* was significant and ΔRLU *test* was insignificantly lower than ΔRLU*posCo*n and significantly higher than ΔRLU*negCon*.

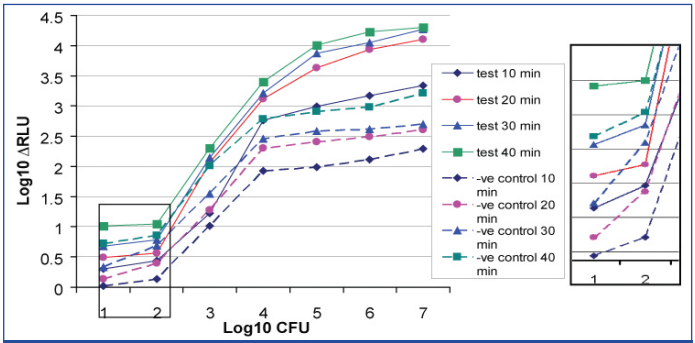
AKBA was conducted on the same isolates of *E. coli* bacteria using the same phage master mix for 10, 20, 30, and 40 min incubation times at 37°C [Table/Fig-1]. The used EHEC concentrations were of wide range 10^2 to 10^7 CFU/well [Table/Fig-2]. The minimal threshold of *E. coli* concentration detected by AKBA was 1000 CFU/well at incubation time 20 minutes at sensitivity and specificity 74% and 78%, respectively. For the bacterial concentration 10^4 CFU/well, AKBA was capable to detect target bacteria within just 10 minutes at relatively lower sensitivity/specificity, 72%/78%. Nevertheless, 20 minutes incubation period of ADP, at bacterial concentration 10^4 CFU/well, resulted in higher sensitivity/specificity, 85%/83%. The sensitivity of positive detection in AKBA, at 10^{3-4} CFU/well, was increasing with assay incubation period. However, the rate of increase slowed down after incubation period of 30 minutes. On the other hand, the specificity of positive detection in AKBA, at 10^3 CFU/well, was slightly increasing with assay incubation time while, at 10^4 CFU, it was decreasing with assay incubation time. This indicates that specificity of AKBA assay does not decrease with increase of assay incubation time at lower bacterial concentrations, namely less than 10^4 CFU. On the contrary, it decreases clearly with the increase in assay incubation time at higher bacterial concentrations providing evidence that diluting samples to 10^{3-4} CFU/well at incubation periods 20-30 minutes are optimal for AKBA assay in terms of sensitivity and specificity. Moreover, there was no difference in terms of ΔRLU and sensitivity/specificity between water and lettuce samples.

Set of bacteria	Bact. Conc. CFU (Log_{10})	ADP incubation period: 10 (min) ΔRLU** *test* sn/sp*	10 (min) ΔRLU *posCon* sn/sp	10 (min) ΔRLU *negCon* sn/sp	20 (min) ΔRLU *test* sn/sp	20 (min) ΔRLU *posCon* sn/sp	20 (min) ΔRLU *negCon* sn/sp	30 (min) ΔRLU *test* sn/sp	30 (min) ΔRLU *posCon* sn/sp	30 (min) ΔRLU *negCon* sn/sp	40 (min) ΔRLU *test* sn/sp	40 (min) ΔRLU *posCon* sn/sp	40 (min) ΔRLU *negCon* sn/sp
Mean readings of 10 water-inoculated EHEC *E.coli* in triplicates (30 readings)	100 (2)	2.74	10.45	1.36	3.63	12.58	2.51	6.16	18.53	4.88	11.23	22.59	7.4
	1000 (3)	16.7	48.51	10.45	**114.85 74/78**	124.93	18.73	**138.36 84/80**	133.94	35.7	**198.54 88/85**	245.6	101.54
	10000 (4)	**579.94 72/78**	684.99	84.41	**1327.63 85/83**	1418.6	201.62	**1634.83 93/80**	1694.72	288.49	**2511.5 92/73**	2391.37	604.6
Mean readings of 10 lettuce-inoculated EHEC *E.coli* in triplicates (30 readings)	100 (2)	3.27	8.27	1.8	4.29	11.85	3.11	8.1	17.37	5.83	24.3	31.41	19.11
	1000 (3)	21.4	39.57	17.52	**97.39 80/78**	112.5	26.57	**147.19 90/81**	139.42	42.5	**172.48 90/84**	179.82	111.52
	10000 (4)	**738.48 73/79**	802.5	190.21	**1478.99 88/85**	1461.35	263.82	**1722.9 95/81**	1805.93	323.63	**1971.5 95/78**	2021.52	143.63

[Table/Fig-1]: Phage-based AKBA assay ΔRLU*test*, ΔRLU*PosCon*, and ΔRLU*NegCon* values for 2 sets of 10 known EHEC bacteria at titers adjusted to 2, 3, and 4 ^{log10} CFU/well. The positive detections typed in bold with sensitivity, specificity of the positively detected *E.coli*

*sn/sp: sensitivity/specificity for the positive detections

**: differences between RLU*test1* and RLU*tets2*, RLU*negCon1* and RLU*negCon2*, and RLU*posCon1* and RLU*posCon2* were mentioned as ΔRLU*test*, ΔRLU*negCon*, and ΔRLU*posCon*, respectively.



[Table/Fig-2]: A diagram shows the plotting of log_{10} ΔRLU*test* (continuous plot) and ΔRLU*NegCon* (dashed plot) in AKBA assay versus log_{10} CFU/well for 10 known EHEC samples at ADP incubation times 10, 20, 30, and 40 min. Dark bordered rectangle area is magnified showing, no minimal detection threshold at bacterial titer 100 CFU. The greater difference between log_{10} ΔRLU*test* and log_{10} ΔRLU*NegCon* the higher positive detection achieved.

DISCUSSION

The current world is in urgent need for a reliable, time-saving, and specific detection assay for coliforms, environmental *E. coli*, EHEC, and Enterobacteriaceae. We exploited the ultimate specificity conferred by using highly specific and lytic phages against *E. coli* bacteria in preparing high sensitivity/specificity assay, namely AKBA assay. Therefore, this assay was designed to be a specific rapid diagnostic testing for *E. coli* bacteria or any other Gram negative or positive bacteria. Although, the methodology of AKBA is not new, the use of a mixture of highly specific and lytic phages, 172 designed phages including 22 EHEC-specific phages, against *E. coli* bacteria is considered innovative.

The minimal threshold of EHEC concentration detected by AKBA was 10^3 CFU/well at incubation time 20 minutes at sensitivity/specificity 74% and 78%, respectively. This is a remarkable achievement taken into consideration, the detection of coliforms or pathogenic bacteria in water can be done by using filters to trap bacteria. In case the contamination level was 1 bacterium per 100 ml, 1000 bacteria can be collected from filtrating 100 liters. Collectively, 20 minutes for AKBA assay along with 40 minutes for the filtration and washing steps render the total period of time needed to yield positive result only one hour. In addition, the AKBA sensitivity/specificity was shown to be good. Other advantages of AKBA, it is cheap and portable test that can be used in the field. However, the use of luciferin-luciferase enzyme complex is not so easy requiring careful conditions of storage. Nevertheless, there is an advantage of using AKBA, it can detect both Gram negative and positive bacteria; thus, AKBA can be used for almost all bacteria.

It has been found that rapid and sensitive detection of *E. coli* is essential for surveillance, sanitary supervision, and minimizing the outbreak of infection [17]. The detection of *E. coli* in water and environment is usually carried out by three methods; routine bacterial cultures, PCR analysis, and immunoassay [18]. The laborious and expensive culture methods require a minimum of 2-3 days to perform [19]. Although PCR assays may be useful for the examination of human or animal fecal samples, for example, Meng J et al., described a PCR technique that could detect as few as 25 CFU of *E.coli* within 3 hour [20], their usefulness for diagnosis is limited due to their inability to differentiate between viable and non-viable bacteria [21]. For immunoassays, although sensitive, these assays are laborious, expensive, and cannot definitely differentiate between viable and dead cells [22]. On the other hand, the current AKBA assay is relatively simple and rapid; it targets only the viable cells at unrivalled specificity due to the use of *E.coli*-specific phages. Using microplate bioluminescence device for AKBA assay guarantees the ability to conduct at least 30 tests per hour including the negative and positive controls. This technique is recommended for the largest public water systems where at least 480 samples of water per month must be examined to ensure water cleanliness [23]. Nevertheless, the practical applications of the designed AKBA is not limited to water and vegetables, as experimented in this study. Similar to the sample processing of vegetables done in this study, AKBA can be used for fruits, meat, fish, etc. Moreover, with little modifications of sample processing, it can easily be used for detecting bacteria in hospital or wound swabs as well as detecting bacteria in samples of body fluids such as urine, stool, blood, or pleural fluid.

LIMITATION

The current study is tailored for one species namely Enterohaemorrhagic *E.coli*. Very low bacterial counts might be below the sensitivity threshold of the AKBA assay. Concentrating or trapping methods for bacteria-contaminated samples might be needed.

CONCLUSION

Altogether, this study revealed a novel and rapid phage-based diagnostic testing of Gram negative bacteria and *E.coli* in particular, namely AKBA assay which revealed a detection limit of 10^3 CFU at 20 minutes. This detection limit is suitable for water testing by detecting trapped bacteria of tens of liters of filtered water. In comparison with other rapid diagnostic methods, AKBA, was shown to be of

high value. Moreover, AKBA assay is simple, relatively cheap, and portable.

It is recommended to conduct further studies evaluating phage-based bioluminescent assay for the quantitative detection of other bacteria rather than *E.coli* and in different samples.

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