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Real-time PCR Method for the Quantification of Burkholderia Cepacia Complex Attached to Lung Epithelial Cells and Inhibitionn of that Attachment

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Real-time PCR method for the quantitation of *Burkholderia cepacia* complex attached to lung epithelial cells and inhibition of that attachment.

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Running title: QPCR for bacterial attachment

Abstract

Aims: To develop a rapid method to quantify the attachment of the cystic fibrosis (CF) pathogen, *Burkholderia multivorans*, to lung epithelial cells (16HBE14o-) using real-time PCR with a view to monitoring potential inhibition of lung cell attachment.

Methods and Results: Mammalian and bacterial DNA were purified from bacteria attached to lung epithelial cells. The relative amount of bacteria attached was determined by amplification of the *recA* gene relative to the human GAPDH gene, in the presence of SYBR Green®. The method was thoroughly validated and shown to correlate well with traditional plating techniques. Inhibition of bacterial attachment with simple sugars was then evaluated by real-time PCR. Of the sugars examined, pre-incubation of *B. multivorans* with lactose, mannose and xylitol all decreased bacterial adherence to 16HBE14o- cells, while glucose and galactose had no significant effect. Pre-incubation with lactose had the greatest effect, resulting in reduced adhesion to 35% of untreated controls.

Conclusions: This method can be used to quickly and effectively screen novel agents with higher affinities for bacterial adhesins.

Importance and Significance: This method will enable the rapid development of novel agents to inhibit colonisation by this pathogen from the environment.

Keywords: bacterial adhesion; *Burkholderia cepacia* complex; Cystic fibrosis; lung epithelial cells; real-time PCR.

Introduction

Chronic lung infection in cystic fibrosis (CF) patients has a major impact on the mortality and quality of life of these patients. A hierarchy of pathogens are acquired throughout a patient's life and the majority of those acquired in later years are inherently antimicrobial resistant. These include *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex (Bcc). In particular, multiple mechanisms of resistance to multiple antibiotics exist among strains of the Bcc (Mahenthiralingam *et al*., 2005), and consequently this pathogen is rarely eradicated once a patient has been colonised. In spite of strict segregation measures, Bcc continues to be acquired from the environment (Baldwin *et al*., 2007). Rather than antibiotic therapy, a more effective approach would be to prevent colonisation by topical application of biodegradable binding inhibition agents (McClean and Callaghan, 2009).

Previously, it has been shown that Bcc strains bound to many glycolipid receptors, including asialoGM1, asialoGM2 and globosides, on lung epithelia (Krivan *et al*., 1988a; Krivan *et al*., 1988b). However, the complete elucidation and identification of these receptors has yet to be determined. In the absence of clear identification of the actual receptors, screening a range of glycoconjugates for their potential to inhibit or compete with Bcc for binding sites, represents an alternative approach to the discovery of prophylactic treatments to prevent colonization. Efficient identification of lead adhesion-inhibitor candidates requires a quantitative method which is reliable, reproducible and relatively quick to perform. Traditional microbiological techniques for quantitating bacteria can be cumbersome, labour intensive and time consuming due to the extensive dilutions of bacteria that must be prepared, plated and subsequently counted, particularly if a large number of compounds are being analysed. Other potentially more expedient approaches such as using bacterial specific enzyme activity to quantitate bacteria present (e.g. urease) were developed

in our laboratory, but proved unsatisfactory due to an inability to clearly distinguish between the bacterial cells and background due to lung epithelial cells. Enzyme-linked immunosorbent assays (ELISA) were not suitable, due to a lack of commercially available antibody for this pathogen. Furthermore, we have previously found that genetic manipulation of the Bcc strains with GFP or RFP as a means of quantitation of bacteria significantly altered the adhesion phenotype of the mutants (unpublished) and was therefore not suitable either. Therefore, we have designed a rapid, reliable molecular technique for the identification of Bcc adhering to lung epithelial cells *in vitro*.

Quantitative real-time PCR was selected as a method for development due to its speed and specificity. The method was developed based on that of Candela *et al.,* (2005), however substantial alterations to the method were required in order to adapt it to the quantitation of Bcc on lung epithelial cells. *B. multivorans* was the focus of these studies as it has recently become the most frequently isolated *Burkholderia* species on both sides of the Atlantic and is predominantly acquired from the environment (Baldwin *et al*., 2008). The r*ecA* gene was used to quantify the number of bacterial cells and the human *GAPDH* gene was used as a housekeeping gene to normalise for differences in DNA isolation. The real-time method was successfully optimised, validated and used to assess the ability of simple sugars to reduce the adhesion of *B. multivorans* to lung epithelial cells.

Materials and Methods

Bacterial strain and Epithelial Cells

B. multivorans strain LMG13010, which is a member of the International *Burkholderia cepacia* Working Group panel, was used throughout. It was obtained from the BCCM/LMG, University of Ghent, Belgium and was routinely grown on *Burkholderia cepacia* specific agar (BCSA), (Henry *et al*., 1997) or in Luria–Bertani (LB) broth at 37 ^{o}C. Overnight cultures were grown to an $O.D._{600nm}$ of 0.6 and then diluted to a given concentration. The lung epithelial cell line 16HBE14o^{-} was kindly donated by Dr. Dieter Gruenert (University of California, San Francisco, UCSF) and maintained in fibronectin-vitrogen coated flasks containing minimal essential medium (MEM) plus 10 % (v/v) FBS as described previously (Cozens *et al*., 1994). For adhesion assays, 16HBE14o^{-} cells were seeded on coated 24 well plates at 2.5×10^{5} cells ml^{-1} in supplemented MEM in the absence of antibiotics and incubated overnight at 37^{o}C in 5 % CO_2. The bacteria (~5x 10^{6} CFU) were then applied to the cells in MEM only and the plate was centrifuged at 700 *g* for 5 min, to facilitate bacterial attachment. The plate was incubated at 37^{o}C in the presence of 5% CO_2 for 30 min and then washed vigorously three times with PBS to remove unbound bacteria.

Relative quantitation of *B. multivorans*

This method was based on a method relating to detection of bifidobacteria on Caco-2 gastrointestinal epithelial cells (Candela *et al*., 2005). The original method amplified bacterial DNA directly from a cell lysate using 5 % (w/v) SDS in 0.5 mol l^{-1} NaOH. However, due to issues with reproducibility when trying to quantitate bacterial DNA with this method, most likely as a result of lack of reproducibility in extraction of the bacterial template, an alternative strategy was developed. These modifications involved: 1) DNA extraction using the Wizard® Genomic DNA purification kit (Promega) as outlined in the

manufacturer's instruction, to purify the template prior to amplification; 2) inclusion of poly acryl DNA carrier (Molecular Research Centre, Inc., 5ul, as per manufacturer's instructions); and 3) a reference gene, GAPDH, was also used to normalise the level of bacterial DNA present with that of human DNA. Forward and reverse primers to the *B. multivorans recA* gene and to the human *GAPDH* gene were designed (Table 1). Amplification was carried out in a final volume of 20µl containing 10 µl SYBR Green PCR Mix (Roche), 379 nmol l^{-1} *GAPDH* primers or 190 nmol l^{-1} *RecA* primers, and 4 µl of DNA. The plate was centrifuged briefly for 1 min at 700 *g*. Real-time PCR was carried out on 7300 Real-Time PCR System (Applied Biosystems). The samples were amplified in triplicate as follows: 10 min at 95^{o}C for denaturation, followed by 40 cycles at 95^{o}C for 15 sec and 66^{o}C for 1 min. The data was collected during the 66^{o}C annealing/elongation phase. A dissociation step was carried out for melting curve analysis.

Efficiencies of GAPDH and recA amplification

The Pfaffl equation (Pfaffl, 2001) was used to determine results incorporating the relative efficiencies of amplification of the target and reference genes. Real-time PCR efficiencies (E) were acquired using a five-point standard curve with both *GAPDH* and *recA* gene primers to determine the slope. The corresponding efficiencies were calculated according to the following equation: Efficiency (E) = ($10^{\wedge(-1/slope)}$). Using these calculated efficiencies, the relative amount of bacterial DNA was determined compared to human *GAPDH* based on the cycle threshold values of bacterial versus human genes (Pfaffl, 2001).

Validation of real-time PCR quantification compared with traditional microbiological methods

B. multivorans strain, LMG13010 was grown to an $O.D._{600nm}$ of 0.6 and serially diluted to a final concentration range of 200 to $2x10^7$ CFU ml^{-1}. Each dilution was applied to 2.5 x 10^5 16HBE14o$^-$ cells and lysed in either DNA lysis buffer (Wizard® Genomic DNA purification kit (Promega)) or cell lysis buffer (PBS, 10 mmol l^{-1} EDTA, 0.25 % Triton X-100). The DNA was isolated and quantified using real-time PCR described above. For the microbiological method, the resulting lysate was serially diluted in Ringer's solution and quantified by viable counts on LB agar after 48 h. Correlation curves were plotted of bacterial dilution against the relative CFU obtained after logarithmic transformation and relative quantity based on real-time PCR.

Sugar Competition Assays

Sterile solutions (1 to 20 mmol l^{-1}) of glucose, galactose, mannose, xylitol or lactose were prepared in MEM. An overnight culture of LMG13010 was grown to an $O.D._{600nm}$ of 0.6 and diluted to a final concentration of 5 x 10^6 CFU ml^{-1}. Bacteria were pre-incubated with the sugar solutions for 30 min at 37^{o}C with gentle shaking and then applied to the 16HBE14o- cells. The plate was centrifuged at 700 *g* for 5 min and incubated at 37^{o}C in the presence of 5% CO_2 for 30 min. Cells were washed vigorously three times with PBS to remove unbound bacteria. Real-time PCR was used for relative quantification or plate counts were obtained from serial diluted cell lysates as described previously.

Results

Detection using amplification direct from cell lysate.

Initial studies aimed to determine the levels of adherent bacteria by absolute quantitation, based on an adaptation of a previous method (Candela *et al*., 2005) and involved using a standard curve to enumerate unknown amounts of this strain attached to lung cells. The original method involved direct amplification of bacterial DNA from a lysate prepared from mammalian cells plus attached bacteria. However, results indicated that the lysis buffer did not appear to lyse the bacteria adequately or that the sample was too complex for adequate polymerisation, as indicated by high Cycle threshold (Ct) values ($\geq$35 cycles for 2 x 10^5 bacteria). In addition, the standard curves obtained from triplicate samples over a range of CFU ml^{-1} were not linear (typical r^2 = 0.247, data not shown). Furthermore, major issues with reproducibility were encountered with some replicates of many samples not being amplified, indicating that even where adequate lysis took place, the lysis buffer was not suitable for reliable amplification. A comparable standard curve prepared with stepwise dilutions of purified bacterial DNA resulted in a straight-line curve with a reported r^2 value of greater than 0.97, indicating that the issues were associated with the matrix and not due to the target DNA or primers.

Modification of real-time PCR method

The method was adapted in two ways to improve the accuracy and reproducibility of bacterial quantification. Firstly, a DNA extraction step including the use of a DNA carrier was employed to improve the quantity and quality of the template. It was also decided to use a relative quantification method with the human *GAPDH* gene as a reference gene in order to improve the reproducibility, particularly at lower CFU levels. The *GAPDH* gene controlled for differences in total DNA extraction, as its quantitation should be constant for all wells

plated with the same lung cell number. Strict conditions were employed in this assay to prevent the *recA* bacterial primers binding non-specifically to the human template. This included a low concentration of primer (190nM) and a very high annealing and elongation temperature of 66^{o}C despite primer melting temperatures of approximately 59^{o}C. The specificity of the amplification was demonstrated by melting curve analysis (Fig 1a). Only one fluorescence peak was detected even at minimal levels of *recA* template (from 200 CFU). The product size of the amplicon was also verified on a 2% agarose gel (Fig 1b).

Pfaffl Method of Quantification

Primer efficiencies for both the target and reference genes were calculated by amplifying triplicate standards on five point standard curves in three independent experiments. Correlation coefficients were greater than 0.99 in each case (Fig 1c). The average slopes and the subsequent efficiency value for the human *GAPDH* and bacterial *recA* genes are shown in Table 1. According to criteria described by Pfaffl (2001), the inter- and intra-assay coefficients of variation for each primer measured over the range of concentrations examined in the standard curve (Table 1) are considered low for this assay.

Validation of Real-Time Quantitative Method and Comparison to Traditional Microbiological Techniques

To validate the real-time method of quantitation, a range of 10-fold dilutions of bacteria were mixed with human cells and the combined DNA was isolated in order to confirm that there was no interference from the human cells, or any PCR inhibitors in the extracted DNA. The real-time PCR technique was able to reliably detect bacterial DNA down to ~200 CFU and a correlation coefficient value of R^2 = 0.9997 was obtained (Fig 2A) over this 5 log range. Concomitantly, duplicate samples of a range of CFU of bacteria (200 to 2x10^7 CFU) were

added to epithelial cells and were lysed using an EDTA/ Triton X-100-based lysis buffer. The resulting lysate was serially diluted and plated on LB agar for 48 h. The number of CFU obtained confirmed that the ten-fold dilutions were comparable to that of the real-time PCR method (Fig 2B).

Adhesion of *B. multivorans* to Lung Epithelial Cells: Sugar Competition Studies

Sugar competition studies were used to determine if the method could be applied to quantitation of the inhibition of adhesion of *B. multivorans* to lung epithelial cells in the presence of a series of mono- and di-saccharides. Bcc strains have been shown to bind to the sugar groups of gangliosides (Krivan *et al*., 1988b). Pre-incubation of the bacteria with sugar molecules could therefore block receptors on the bacterial cell, inhibiting subsequent binding. Competition with lactose was most effective of all 5 sugars examined, showing a concentration-dependent inhibition of adhesion at concentrations from 10 mmol l^{-1} to 20 mmol l^{-1} (Fig 3). At the highest concentration, the level of bacterial adhesion was 35% of the control ($p<0.001$). Pre-incubation with mannose and xylitol both had an effect at a single concentration (5 mmol l^{-1} and 10 mmol l^{-1}, respectively), again reducing adhesion to approximately 60% of the control ($p<0.05$) in both cases. Statistically significant effects were not observed at the other concentrations examined. Pre-incubation of *B. multivorans* with glucose had no effect over a range of concentrations and although galactose appeared to inhibit adhesion at a concentration of 20mmol l^{-1} to levels approximately 60% of that of the control, this was not statistically significant. The coefficients of variation for both intra-assay variability and inter-assay variability were low for the control untreated samples (<2.33 % for intra-assay variability and <3.61 % for inter-assay variability for both genes). ANOVA analysis demonstrated statistically significant inhibition of bacterial attachment with simple sugar molecules as shown in Fig 3.

For the purposes of comparison, three of the sugars, mannitol, xylitol and lactose were also tested using traditional plating methods. These data were comparable to the real-time method and confirmed that lactose inhibited the % bacterial cell binding at 10 mmol l^{-1} ($P<0.01$) and 20mmol l^{-1} ($P<0.001$), however neither mannitol nor xylitol showed any significant effect on *B. multivorans* binding.

Discussion

We have designed a rapid, reliable molecular technique that can be utilised *in vitro* to detect *B. multivorans* adhering to epithelial cells. This technique, which was validated down to a level of 200 CFU of bacteria, has been shown to yield comparable results to those of the traditional plate count method. It was also less cumbersome and more reliable than the traditional method, due to the poor growth of Bcc strains.

The concentrations of simple sugars that were required to inhibit bacterial attachment are considerably high. However, it has been recently demonstrated that inhalation of simple sugars (fucose and galactose), administered to patients at concentrations of 0.1 mol l^{-1} each, was a safe and effective measure to reduce *P. aeruginosa* counts in CF patients (Hauber *et al*., 2008). Inhibition of adherence of several respiratory pathogens by common oligosaccharides has also previously been demonstrated (Thomas and Brooks, 2004). Maximal inhibition of the order of 1 log of CFU applied was achieved with concentrations of oligosaccharides in the millimolar range. However, their method relied on culture of serial dilutions of bacterial-epithelial cell lysates. Many pathogens are difficult to culture, giving unreliable quantification of low bacterial numbers (Huijsdens *et al*., 2002). The method described in this study will be more reliable for these types of species. It is hoped that novel glycoconjugates can be designed with better affinities for bacterial adhesins which may show even greater efficacies, reducing attachment by several logs. The linearity of this method over 5 log CFU range clearly demonstrates the suitability of this method to screen such novel glycoconjugates as potential inhibitors of bacterial attachment. In particular, the efficacy of inhibitors that reduce adhesion by several log of CFU will be quantifiable using this method. Furthermore, it will not be necessary to apply extremely high (and not necessarily clinically relevant) levels of bacteria in order to enable accurate detection of inhibition in order to test these agents.

We propose that the technique described herein could also be adapted for a number of other *in vitro* assays carried out to determine the virulence of bacteria e.g. adhesion, host cell invasion, translocation across the epithelium or intracellular growth. All of these assays routinely rely on microbial culture as their endpoint and are inherently variable (McClean and Callaghan, 2009). Primers can be designed against any bacterial species and their levels interacting with host cells quantified. Displacement of colonising bacteria by competing bacterial pathogens could also be quantified by a method such as this. In the future, we will use this method for screening of novel carefully designed glycoconjugates with potentially higher affinities for bacterial adhesins.

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Table 1: Sequences of primers used in the real-time PCR assay and respective primer analysis data.

Target species	*Gene*	*Primer*	*Primer sequence (5' to 3')*	*Slope*	*Efficiency*	*Intra-assay CV(%)*	*Inter-assay CV(%)*
B. multivorans	*recA*	Forward	TGGACACGACCTGGATGT	3.872	1.81	<2.76	<4.53
		Reverse	ATGACCGCCGAGAAGAG				
Human	*GAPDH*	Forward	CCCCTTCATACCCTCACGTA	3.206	2.05	<1.92	<3.83
		reverse	GACAAGCTTCCCGTTCTCAG				

* CV: Coefficient of variation. Each value represents the mean of three independent experiments.

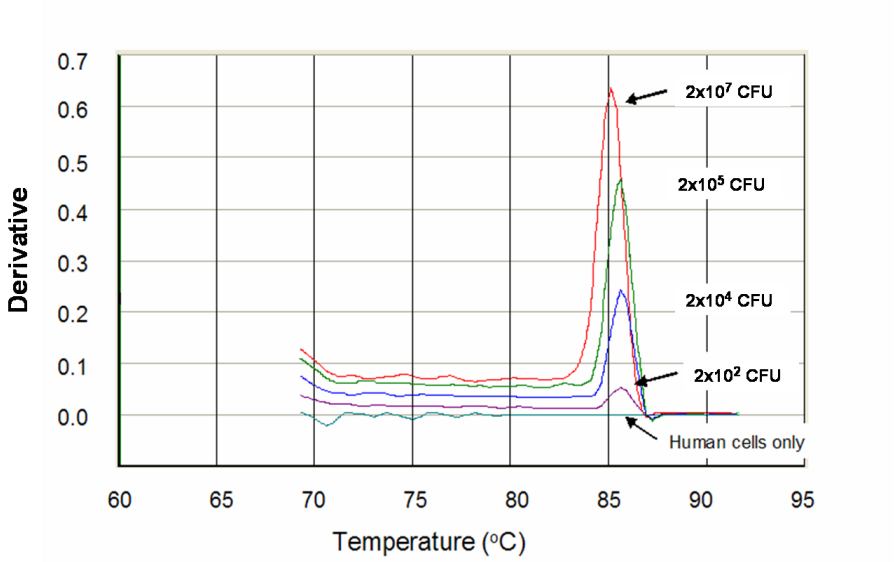
Legends

Figure 1. A) Melting curve analysis on samples containing a range of bacterial CFU (0 to 2 x 10^7) in the presence of human lung cells. A single fluorescence peak demonstrates *recA* primer specificity; no fluorescence is detected from human cells in the absence of bacterial template. B) Agarose gel (2%) of *GAPDH* (2 to 4) and *recA* gene (5 to 7) PCR products. Lane 1: 100bp marker, Lanes 2 & 5: no template control; lanes 3 & 6: product from 2 x 10^7 CFU in the presence of lung epithelial cells; Lanes 4 and 7: epithelial cells only. C). Serial dilutions of 2 x 10^7 CFU were applied to 2.5 x 10^5 human cells to demonstrate the linear range of the assay for both primers, recA (♦) and GAPDH (■).

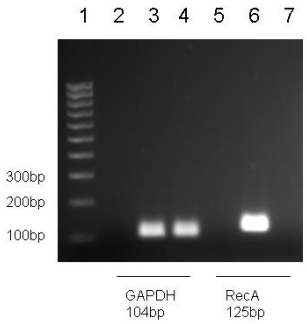
Figure 2: Correlation between the bacterial counts obtained in each assay by either relative quantification as determined by Real-time PCR (●) or the absolute quantification as determined by traditional plating methods using colony counts (o) and the actual bacterial CFU applied from three independent experiments. The dotted line represents the theoretical values that should be obtained based on the numbers of bacteria applied.

Figure 3. Competition studies with simple sugars as determined by real-time PCR analysis (A) or plate counts (B). Pre-incubation with simple sugars inhibited subsequent adhesion of *B. multivorans* to lung epithelial cells. Bars represent the mean % binding relative to inhibitor free control (100%) as follows: 1mM (dark); 5mM (white); 10mM (striped) and 20mM (bar) from three separate experiments for each monosaccharide. Error bars represent the standard error of the mean (SEM). * $p<0.05$, ** $p<0.01$, ***$p<0.001$ using a one-way ANOVA and Tukey's post-test (excluding values at 1mM concentration), compared to inhibitor free control.

Figure 1.
A)


Derivative
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
2x10^7 CFU
2x10^5 CFU
2x10^4 CFU
2x10^2 CFU
Human cells only
60
65
70
75
80
85
90
95
Temperature (°C)

B)


1 2 3 4 5 6 7
300bp
200bp
100bp
GAPDH
104bp
RecA
125bp

C)

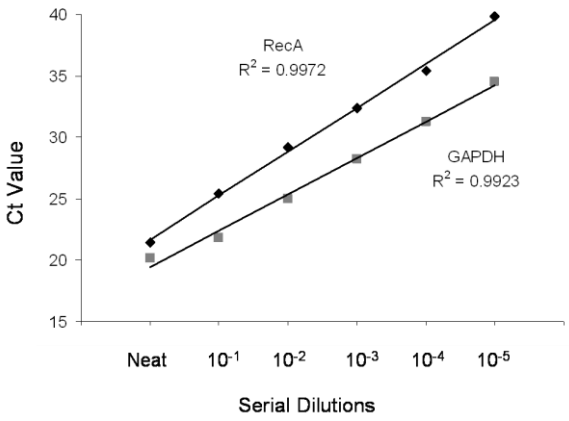
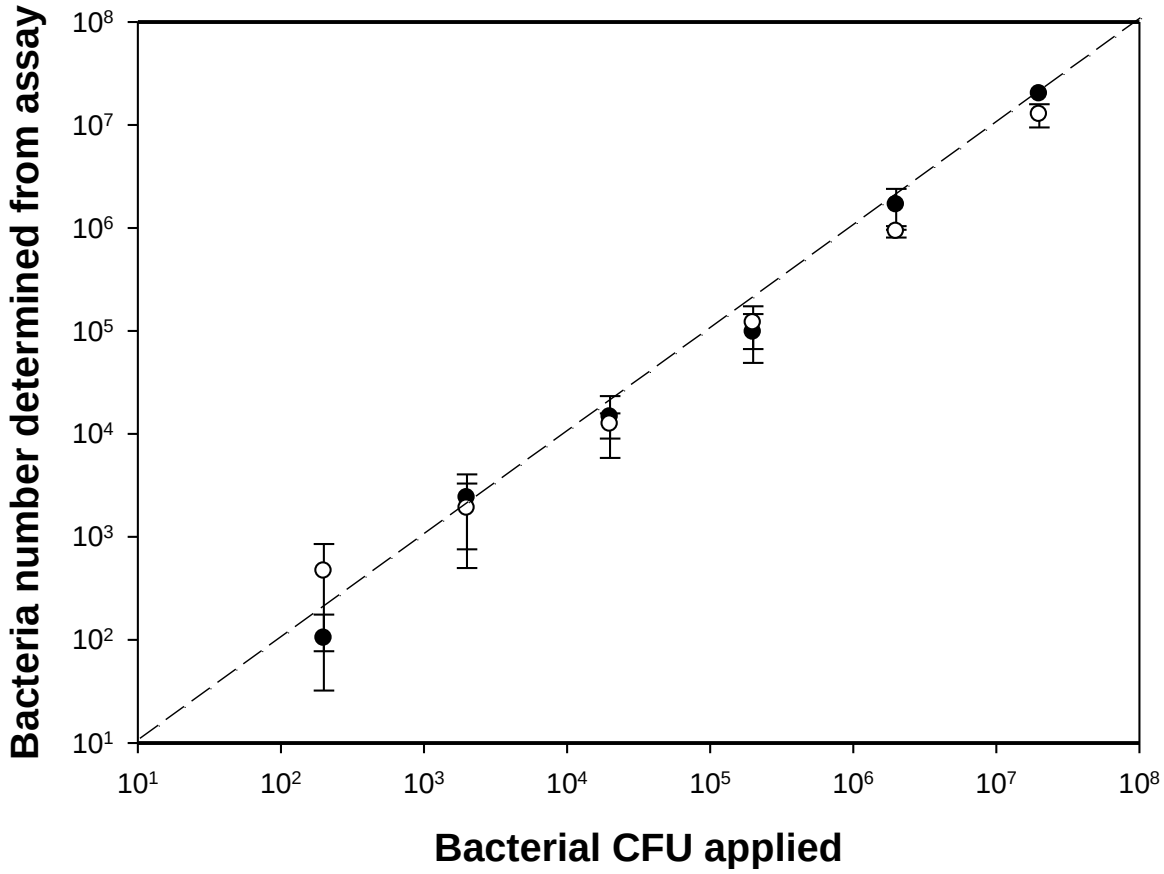
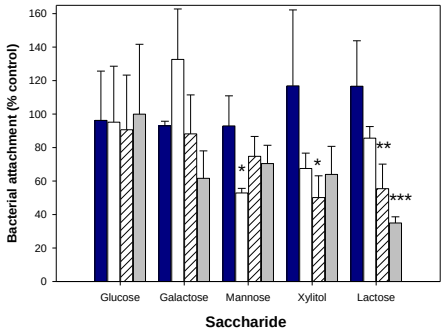

Ct Value
40
35
30
25
20
15
RecA
R2 = 0.9972
GAPDH
R2 = 0.9923
Neat
10-1
10-2
10-3
10-4
10-5
Serial Dilutions

Figure 2


Bacteria number determined from assay
Bacterial CFU applied
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8

A


Bacterial attachment (% control)
160
140
120
100
80
60
40
20
0
*
*
**
***
Glucose
Galactose
Mannose
Xylitol
Lactose
Saccharide

B

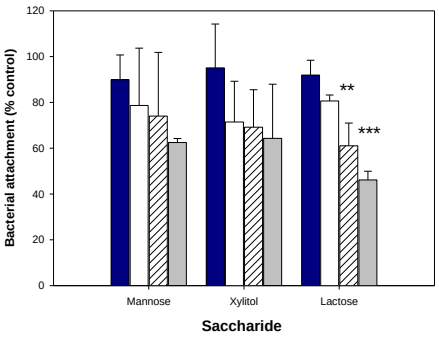

Bacterial attachment (% control)
120
100
80
60
40
20
0
**
***
Mannose
Xylitol
Lactose
Saccharide

Figure 3