ORIGINAL ARTICLE

In vitro adhesion assay of lactic acid bacteria, *Escherichia coli* and *Salmonella* sp. by microbiological and PCR methods

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Abstract

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***In vitro* adhesion assay using *Lactobacillus reuteri* KUB-AC5 as a test strain has been studied by applying simple PCR reaction together with image analysis and plate count techniques. Critical factor affecting the PCR method was quality and quantity of DNA. The cell lysis technique was modified to optimize this method. Thus, lysozyme and proteinase K were added to lyse the cells, followed by SDS solution to obtain a complete cell lysis. Only PCR products from total cells (TC) were obtained, with low consistency, but none from cells bound to mucus (BC) at either 0.1 or 0.5 mg/mL concentration. It was hypothesized that the attached cells might not be extracted into the cell suspension. Therefore, 1% SDS solution and 0.1M NaOH were used directly in the extraction. As expected, PCR products were observed when both TC and BC were used as a DNA template. Adhesion appeared at a wide range of 0-45%, with low consistency. Therefore, a simple microbiological method (plate count) was used. The extraction of bound cells into cell suspension**

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was critical in this method. Extraction times of 20, 60, 120 and 150 min were tried. Results showed that maximum cell number was obtained with 120 min extraction. *L. reuteri* KUB-AC5, *L. reuteri* KUB-AC16, *L. reuteri* KUB-AC20, *L. salivarius* KUB-AC21, *L. acidophilus* KV-1, *Escherichia coli* E010, *Salmonella* sp. S003, *E. coli* ATCC8739, and *S. typhimurium* ATCC 13311 exhibited adhesion activity of 21.6%, 0.8%, 5.7%, 1.1%, 23.1%, 10.7%, 10.3%, 4.4% and 3.2%, respectively. Among the 9 types of microorganisms tested *L. acidophilus* KV-1 and *L. reuteri* KUB-AC5 showed higher adhesion activity than the others.

Key words : adhesion assay, microbiology assay, *Lactobacillus*, PCR, *Escherichia coli*, *Salmonella* sp.

Lactic acid bacteria (LAB) have long been used for preservation of food and feed. More recently, a great deal of interest has been focused on some members of LAB strains for use as probiotics (Fuller, 1989; Salminen *et al*., 1996a). Probiotic is defined as microbial cell preparations or components of the cells that have a beneficial effect on the health and well-being of the host (Salminen *et al*., 1999). The main criteria for selecting probiotic strains are their acid and bile tolerance, survival through the gastrointestinal tract, ability to adhere to and colonize intestinal surface, antagonism against pathogens and good technological properties (Salminen *et al*., 1996b). Our laboratory has isolated LAB from chicken intestine and primarily screened for their antagonism against *Escherichia coli* and *Salmonella* sp. which are resistant to several antibiotics used as chicken growth promotion (Nitisinprasert *et al*., 2000). Two interesting strains, *Lactobacillus reutri* strain KUB-AC5 and KUB-AC16, were recently characterized for their high tolerance to acid, bile and temperature while retaining their adhesion ability.

The *in vitro* adhesion assay has been studied using different techniques such as ELISA assay (Roos *et al*., 2000), radioactive assay with [^{3}H} label (Ouwehand *et al*., 2001), microscopy method using diagnostic glass slide and cell staining with methylene blue (Edelman *et al*., 2003), and fluorescence assay by fluorescein isothiocyanate labeling (Edelman *et al*., 2003). Recently, Polymerization Chain Reaction (PCR) technique has become widely used due to its convenience and high sensitivity. Huijsdens *et al*. (2002) has quantified bacteria adhering to gastrointestinal mucosa by real time PCR. The assay showed a very high sensitivity in which 1 cfu of *E. coli* and 9 cfu of *Bacteroides vulgatus* could be detected. However, the reagents and machine to perform the real time PCR reaction are very expensive. Another simple PCR reaction called Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique has been proposed by Pintado *et al*. (2003) using primers 16S rDNA to determine mixed cultures of LAB and analyze their quantities by image analysis. They showed that 1-15 ng of target DNA involved in PCR reaction and the intensity of PCR products had good correlation. Taking advantage of PCR technique mentioned above, we have modified the adhesion method using simple PCR reaction with image analysis and compared it with the microbiological (plate count) method. The results are presented in this paper.

Materials and Methods

Microorganisms, culture media and conditions

L. reuteri KUB-AC5, *L. reuteri* KUB-AC16, *L. reuteri* KUB-AC20, *L. salivarius* KUB-AC21 and *L. acidophilus* KV-1 were obtained from the culture collection of Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University. *E. coli* E010 and *Salmonella* sp. S003 were obtained from Betagro Agro-Group Plc. Co. Ltd. A de Man Rogasa and Sharpe (MRS, Difco) medium was used to grow all LAB strains while a nutrient broth (NB) was used to grow *E. coli* and *Salmonella* sp. One colony each was inoculated into 5 mL of broth and incubated at 37ºC for 16 h. The cultures obtained were further studied in adhesion experiments.

In vitro adherence Assay

Mucus was prepared from 45 d broiler intestine by the modified method of Roos *et al.* (2000). By scraping the inside of the intestine with a spatula, the material was removed and collected in 200 mL ice-cold phosphate buffer solution (PBS) (g/L: NaCl, 8.0; KCl, 0.2; $Na_2HPO_4.2H_2O$, 1.44; KH_2PO_4, 0.2; pH 7.3). The suspension was first centrifuged at 11,000g for 10 min and at 26,000g for 15 min in order to remove cells and particulate matters. The crude mucus was subsequently lyophilized and kept at -20ºC until use. The mucus solution for adhesion experiments was prepared at the concentration of 0.1 and 0.5 mg/L in 50 mM Na_2CO_3 buffer pH 9.7 as required. A 150 µL sample of the mucus solution was transferred into polystylene titer plate or Eppendorf tube (E-tube) and incubated overnight at 4ºC. The bacteria were grown in either MRS or NB broth medium for 16 h at 37ºC, washed once in PBST (PBS supplemented with 0.05% Tween 20) and diluted to OD_{600} = 0.5 in the same buffer. A 150 µL sample of bacterial suspension was added to each well or E-tube and incubated for 1 h at 37ºC. The well or E-tube was then washed 3 times with 150 µL of PBST to remove unattached bacteria. The bacteria adhered to the mucus was directly analyzed by PCR reaction and microbiological methods.

DNA extraction

Two methods, assigned as method A and B were modified from the methods of Pintado *et al.* (2003) and Ouwehand *et al.* (2001), respectively. For method A, the mucus with adhering cells or whole cells was suspended in 180 µL of TE, 20 µL of 10 mg/mL lysozyme and 10 µL of 20 mg/mL proteinase K. The well-mixed reaction was incubated at 37ºC for 30 min. The cells were lysed by the addition of 20 µL of 20% sodium dodecyl sulfate (SDS) and incubated at 55ºC for 1 h. One volume of MATAB/NaCl (2% alkyl trimethyl ammonium bromide and 3M NaCl) was later added and the mixture incubated at 65ºC for 30 min. All proteins and cell debris were then removed by phenol-chloroform. DNA pellet was precipitated by isopropanol precipitation and dissolved in 25 µL of Tris-EDTA buffer (TE).

For method B, the bacterial cells which adhered to the mucus and the whole cells were first released and lysed with the mixture of 1% SDS and 0.1 M NaOH (150 µL) by incubating at 60ºC for 1 h. Subsequently, 10 µL of proteinase K (20 mg/mL) was added and incubated for 60 min at 55ºC. To facilitate better separation, one volume of MATAB/NaCl was later added and incubated at 65ºC for 30 min. The removal of impurities and DNA precipitates were carried out as in method A. The DNA pellet was subsequently dissolved in 25 µL of TE.

PCR reaction

One µL of purified DNA was amplified with primers gc338f and 518r spanning the V3 region of the 16S rDNA (Muyzer *et al*., 1993). The 25 µL mixture contained 1 µL of template DNA, 0.25 µM of each primer, 200 µM of deoxynucleotide triphosphate (dNTP), 2.5 µL of 10xPCR buffer with Mg (Fermentas, Co.) and 1.5 U of Taq polymerase (Fermentas, Co.). The basic condition for PCR reaction was carried out as follows: 1 cycle of 5 min DNA denaturation at 94ºC; 30 cycles of 1 min denaturation at 94ºC, 1 min primer annealing at 65ºC, and 1 min primer extension at 72ºC. The tubes were then incubated for 10 min at 72ºC to complete the reaction. A 10 µL sample of the amplification products were analyzed by electrophoresis in 1.2% agarose gel and qualified the size using 1 kb DNA ladder (Invitragen Tech-Unesm, USA) as a marker.

Image analysis and adhesion efficiency determination

The gels were scanned and band intensities were quantified with Scanner software. The adhesion efficiency (A_e) was determined as intensity from adhesion condition (I_a) in 100% of intensity from total cell (I_t) used as shown in the following equation:

$$A_e = I_a/I_t \times 100$$

Microbiological assay

In vitro adherence assay was performed as mentioned elsewhere. One mL of saline solution was used to suspend the bound cells from E-tube. Bacterial populations were assayed by the standard plate count method using MRS agar with 0.5% $CaCO_3$ and nutrient agar to determine LAB and Gram negative bacteria (*E. coli*, *Salmonella* sp.), respectively. Then, decimal dilutions of the sample were distributed to each plate. The number of cells growing in the plates were enumerated as cfu/tube. The adhesion efficiency (AMe) was determined by the % of bound cells (Cb) in total number of cells (Ct) as shown in the following equation.

$$AM_e = (C_b/C_t) \times 100$$

To investigate a suitable extraction time, the bound cells of *L. reuteri* KUB-AC5 were extracted from the mucus by standing at 4ºC for 0, 15, 60, 120 and 150 min and then vigorously mixed with vortex mixer for 1 min. The bound cells released were determined by the plate count method.

Results and Discussion

Adhesion study by PCR reaction and image analysis

In method A, only the strain KUB-AC5 was used to determine the extraction conditions. A 0.1 mg/mL sample of mucus solution was used to perform the adhesion experiment. After all the free cells and those adhering to the mucus were lysed, the DNA solution obtained was subjected to PCR reaction. Only the amplified PCR product of 259 bp from total cell was obtained as shown on Figure 1. Since no amplified PCR product from the adherent condition was detected, a higher concentration of the mucus solution of 0.5 mg/mL was applied. The same results were obtained without the amplified product (Figure 2).

The possibility of bound cells, which were not extracted into the solution, was proposed. Ouwehand (2001) has assessed the adhesion properties of probiotic strains from human intestinal mucus by a radioactive method. The mixed solution of 1% SDS and 0.1 M NaOH was used for releasing and lysis of the cells at 60ºC for 1 h. The modified method was designated method B. The results were shown in Figure 3. The amplified products from both bound and total cells appeared. However, the intensity of DNA bands from bound and total cells on 1.2% agarose gel from three replicates differed by the ratio of 1:1.1:0 and 4.5: 1:1.2, respectively, resulting in the percentage of adhesion efficiency varying between 0-45.4. It clearly showed that PCR reaction from each condition had low precision. The conventional PCR was a simple method with low cost but had several disadvantages resulting in low precision. The inhibition by substances present in the sample to be analyzed, the limitation of the small sample input, and the possibility of nonspecific binding of the primers would affect the sensitivity of this assay. Huijsdens *et al.* (2002) reported that the high sensitivity of PCR assay by Real-Time PCR showed that the detection limit for *E. coli* was 1 CFU while *Bacillus vulgatus* was 9 CFU. In addition, the average Ct value for the dilution containing 135,000 CFU of *E. coli* was 24.2, with a range of Ct values from 23.9 to 24.5, which showed the reproducibility of quantitative experiments from 5 replicates to be approximately 99%. Therefore, to apply PCR reaction for adhesion study, Real-Time PCR might be needed.

Adhesion study by microbiological method

Huijsdens *et al.* (2002) have compared the Real-Time PCR and the culture method. Their results showed that the number of CFU calculated from the fluorescent signal obtained by Real-Time PCR was 100 fold higher than the number of bacteria from the culture method. It might be that all DNA was amplified by PCR, including DNA from dead, viable and cultivatable bacteria. Therefore, to screen LAB strain with high adhesion activity, we would need to determine from viable cell. Subsequently, the basic microbiological method was studied. The adhesion procedure was performed in E-tube and 0.5 mg/mL of mucus used. Cell extraction from mucus was considered to be a critical point. A modified method of cell

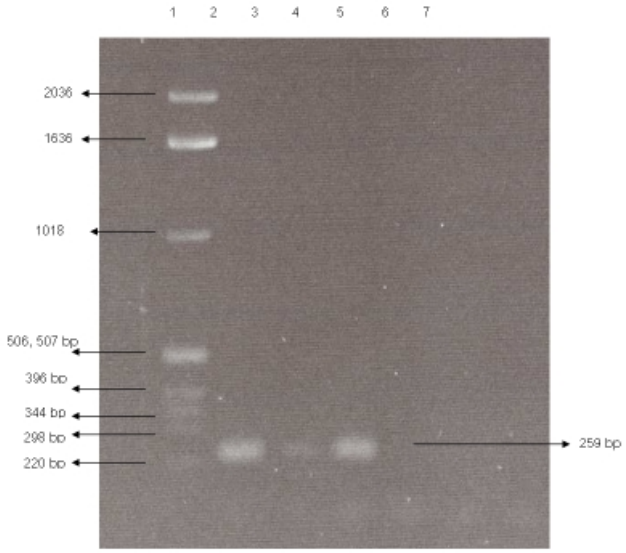

Figure 1. Amplified PCR products from various sample sources of total cells and bound cells. DNA extraction was performed by Method A and the mucus concentration of 100 µg/mL was used for adhesion experiment. Lane 1, 1 kb DNA ladder marker (Invitrogerr Co.); Lane 2-4, three replication of total cell; Lane 5-7, three replication of bound cell.

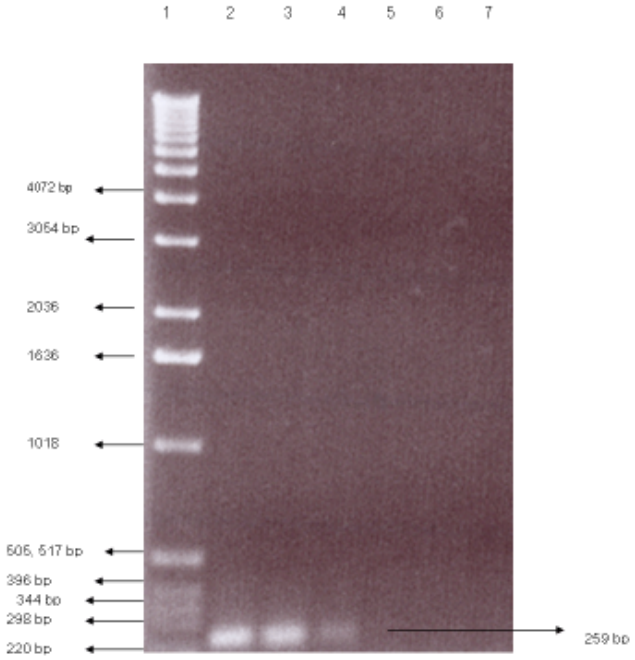

Figure 2. Amplified PCR products from various sample sources of total cells and bound cells. DNA extraction was performed by Method A and the mucus concentration of 500 µg/mL was used for adhesion experiment. Lane 1, 1 kb DNA ladder marker (Invitrogerr Co.); Lane 2-4, three replication of total cell; Lane 5-7, three replication of bound cell.

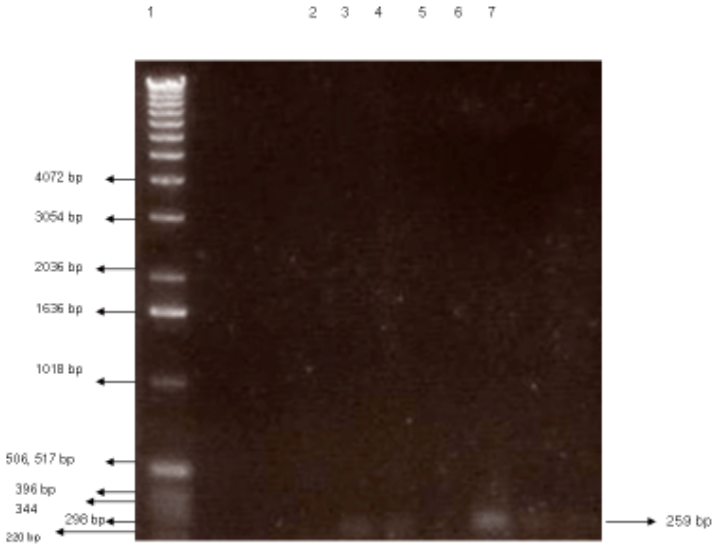

Figure 3. Amplified PCR products from various sample sources of total cells and bound cells. DNA extraction was performed by Method B and the mucus concentration of 100 μg/mL was used for adhesion experiment. Lane 1, 1 kb DNA ladder marker (Invitrogerr Co.); Lane 2-4, three replication of bound cells; Lane 5-7, three replication of total cells.

extraction was proposed. The mucus with cell adherence was dissolved in saline buffer at 4ºC for various time intervals of 0, 15, 60, 120 and 150 min. The results are shown in Figure 4. The maximum cell number was obtained after 120 min incubation.

Incubation of 2 h at 4ºC for cell suspension might or might not affect the growth or death of cells. Therefore, comparison of the cell number of AC5 at 0 h and 2 h at 4ºC was performed. The results showed no change during incubation at 4ºC, as shown in Figure 5. Therefore, a method for the preparation of cell adherence to mucus was proposed, i.e. by incubating the cell suspension at 4ºC for 2 h and mixing with a vortex mixer for 1 min.

Adhesion of LAB and pathogen strains by microbiological method

The modified microbiological method was used to measure the adhesion ability of 5 different strains of LAB and 4 strains of *E. coli* and *Salmonella* sp. The results showed that adhesion activity from different strains varied from 0.89 - 23.1%, as shown in Table 1. Both *L. reuteri* KUB-AC5 and *L. acidophillus* KV-1 exhibited high activities of 21.6 and 23.1% while *L. reuteri* KUB-AC16 had low activity of 0.89%. In addition, these two active strains were also able to adhere to the mucus cell 2-8 times stronger than *E. coli* and *Salmonella* sp. Both strains would be candidates as probiotics to be applied to the animal in the future.

Conclusion

Between the two methods for the determination of adhering activity, i.e. PCR reaction and microbiological methods, the conventional PCR method appeared to have low consistency as a DNA extraction technique and PCR reaction. To determine the adhesion activity from viable cells, a simple microbiological method with optimum extraction conditions of 2 h at 4ºC was proposed. Five different strains of LAB and 4 strains of *E. coli* and *Salmonella* sp. were tested using the

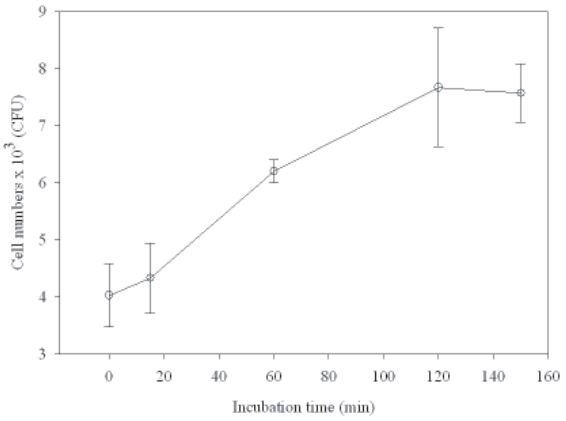

Figure 4. Amount of bound cells extracted after incubation for different lengths of time at 4ºC.

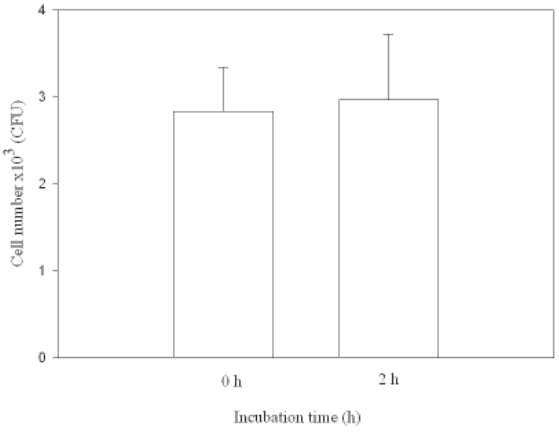

Figure 5. Number of *Lactobacillus reuteri* KUB-AC5 in saline solution after incubation for 0 and 2 hours.

Table 1. Adhesion efficiency of various bacterial strains by microbiological method.

Bacterial strain	% Adhesion
Lactobacillus reuteri KUB-AC5	21.58±4.28
L. reuteri KUB-AC16	0.89±0.68
L. reuteri KUB-AC20	5.78±1.47
L. salivarius KUB-AC21	1.18±0.52
L. acidophilus KV-1	23.1±8.45
E. coli E010	10.64±0.97
Salmonella sp. S003	10.61±1.97
E. coli ATCC8739	4.44±0.3
Salmonella typhimurium ATCC13311	3.28±0.45

modified microbiological method. The results showed that both *L. reuteri* KUB-AC5 and *L. acidophillus* KV-1 exhibited higher activities than the other LAB or *E. coli* or *Salmonella* sp.

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