

Detection of molecule adhesion sub-unit pili 48 kDa *Salmonella Typhi* by immunochemistry method using sera patients suffering from typhoid fever

Sumarno^{1*}, Uun Yanuhar², Sri Winarsih¹, Samsul Islam¹ and Sanarto Santoso¹

¹Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya, Malang Indonesia

²Department of Fishery Faculty of Agriculture, University of Brawijaya, Malang Indonesia

ABSTRACT

The typhoid fever is an infectious disease and is caused primarily by the *Salmonella Typhi* (*S. Typhi*) which is still endemic in the developing country including Indonesia. Pilus bacteria have a role in the pathogenesis of typhoid fever by attaching itself on the receptor cell in the cell host. Mice enterocyte can be used as a receptor cell for molecule adhesion. The aim of this study is to verify that the protein sub-unit pili *S. Typhi* are a molecule hemagglutinin and has a molecule adhesion so it can adhere to the enterocyte of a mouse. The protein sub-unit pili *S. Typhi* was isolated by pili bacteria cutter. The detection of protein sub-unit pili bacteria *S. Typhi* as a protein hemagglutinin was done using the erythrocyte of a mouse. Isolated mouse enterocyte was used as a cell receptor of protein hemagglutinin. Adhere protein hemagglutinin sub-pili bacteria *S. Typhi* which adheres to enterocyte as an antigen could react with sera patient suffering from typhoid fever. Antigen antibody reaction was detected by immunochemistry method by using sera patient suffering from typhoid fever. The results showed that the protein sub-unit pili bacteria *S. Typhi* had a molecular weight of 48 kDa in the tip of its pili. It functioned as protein hemagglutinin. The attachment of a protein hemagglutinin on enterocyte was predicted from the result analysis of immunochemistry method. The protein hemagglutinin as antigen could react with an antibody of sera patient suffering from typhoid fever compared not suffering from typhoid. The conclusion of the study is that the protein sub-unit pili 48 kDa is a molecule adhesion of *S. Typhi* bacteria.

Keywords: *S. typhi*, sub-unit pili, immunochemistry, adhesion

INTRODUCTION

Typhoid fever is an infectious disease primarily due to *S. Typhi*. In the first step of the pathogenesis of typhoid fever disease, bacteria *S. Typhi* adheres to a receptor host cell. All adherence Gram negative rod bacteria use pili bacteria [1].

Gram negative rod bacteria *Vibrio cholerae* (*V. cholerae*) can adhere on mouse enterocyte. In our preliminary study the adherence of bacteria *V. cholerae* used a protein in the tip of pili and in an outer membrane of protein of cell wall. A molecular weight of adhesion was 38 kDa in the tip of pili and 76 kDa in the outer membrane of bacteria *V. cholerae*. The method for identifying its molecule adhesion was by using adhesion index (2).

The same method was also done by Nagayama to determine molecule adhesion of *V. parahaemolyticus* [3]. To confirm a molecule adhesion was time consuming and needs many stage [4].

Electron microscope can also detect the adherence of A 20-kD pilus protein with haemagglutination and intestinal adherence properties is expressed by clinical isolate of non-O1 *Vibrio cholerae* [4].

This study developed a new method for isolating a molecule adhesion in a tip of bacteria *S. Typhi* and the confirmation of the molecule hemagglutinin was molecule adhesion by using immunochemistry method.

MATERIALS AND METHODS

Bacteria *S. Typhi* came from a patient suffering from febrile illness in the General Hospital of the Faculty of Medicine, University of Brawijaya, Malang Indonesia.

Bacterial culture

The TCG media was used to enhance pili production of *V. cholera* according to Ehara method. The content of TCG media were 0,02% thioproline, 0,3% NaHCO₃, 0,1% mono sodium l-glutamat, 1% bacto tryptone, 0,2% yeast extract, 0,5% NaCl, 2% bacto agar and 1 mM β-amino ethyl ether -N,N,N,"n", -tetra acetic acid (EGTA) [5].

*Corresponding Author: Sumarno, Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya, Malang, East Java of Indonesia. Email: sumarno_prof@yahoo.com, sumarno_prof@ub.ac.id

Isolation of protein pili

Cultured bacteria was collected from the TCG media in 20 roux bottles. Pili bacteria was isolated according to our method by using a pili bacteria cutter. The pili bacteria cutter was developed from a modified omni mixer. The differences between the cutter and omni mixer are their knife, speed and time adjustment. Pili bacteria cutter doesn't have a knife because it has been replaced by a cylindrical metal which can rotate. The tip of cylindrical metal has a rough, sharp surface. The sharp surface is composed of flat pins. The cylinder metal is rotated and adjusted in terms of time and speed. Therefore the pili bacteria is cut in this way.

The collection of bacteria from 20 roux bottles media were re-suspended by trichloroacetic acid until the concentration reached 3%. Suspension was shaken thoroughly for 30 seconds and stood at room temperature for 1 hour. The pellet was collected by centrifugation of suspension with a speed of 6,000 rpm, for 30 minutes and temperature of 4° C. Three grams of the pellet was re-suspended by 6 ml of PBS pH 7.4. The suspended bacteria were placed into the tube of the pili bacteria cutter.

After that the pili bacteria cutter was adjusted for first cutting in speed of 5000 rpm, time in 30 second and temperature at 4° C. The supernatant contains rich pili protein of the bacteria separated from the pellet and stored in 4° C. The collection of supernatant of the second to third cutting was isolated from the pellet with the same procedure with the first cutting. The isolation of the pili bacteria was continued to the fourth to seventh cuttings by centrifugation of a suspension of the cutting pili with a speed of 12,000 rpm, for 30 minutes and temperature of 4° C. The collection of pili continued because otherwise there was a difference between the first cuttings only in adjustment speed of 10000 rpm, within 60 seconds of pili bacteria cutter.

Hemagglutination method

Hemagglutination test was performed as described by Hanne and Finkelstein [6]. The V micro titer plate was added by 50 ul PBS in every well. Every sample of the protein of sub-unit pili *S. typhi* 50 ul was made in a serial dilution and was added into the well in a row of the V micro plate wells. Then 50 ul of mice erythrocyte 50 ul concentration were added in every well. After that the micro titer was placed in a maximal adjustment of rotator plate for one minute. Lastly the micro titer plate was put at room temperature.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli [7]. The samples were boiled for 5 min in a final sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue with 5% (vol/vol) β -mercaptoethanol prior to electrophoresis through 5% stacking and 15% separating gels.

Isolation of mouse enterocyte

The isolation of an enterocyte was performed by Weisler as was used by Nagayama [3]. Two Balb/c mice three months old were killed using chloroform. The mouse abdomen was opened by scissors and the intestine was taken out. The intestine was separated from the omentum and was cut 5 cm each. Five pieces of intestine cut were collected from every mouse. Then every piece of cut intestine was cut longitudinally the content of the intestine cut was washed with PBS pH 7.4 solution with dithiothreitol 1mM. The intestine cut was put into solution 1,5 mM KCl, 9,6 mM NaCl, 2,7 mM Na-citrat, 8 mM KH₂PO₄ dan 5,6 mM Na₂HPO₄ pH 7,3. After that it was placed into water incubator with a temperature of 37° C and was slowly oscillated for 30 minutes. The solution was changed with PBS pH 7,4, EDTA 1,5 mM and dithiothreitol 0,5 mM pH 7,4. The intestine cut in the solution were strongly oscillated for 20 minutes, with a temperature of 37° C. The intestine cut was washed with PBS pH 7,4 by doing centrifugation of 1000 rpm for 5 minutes, temperature 4° C. After being washed with PBS the intestine cut was re-suspended with PBS pH 7,4. The result was shaken slowly, then a part of the white suspension rich in enterocyte cells was separated into eppendorf.

Immunocytochemistry to detect molecule adhesion

The research developed a new method to detect molecule adhesion on receptor cell (enterocyte of mouse). The concentration of enterocyte was calculated until an amount enough to examine under a microscope. Every field of examination in microscope was adjusted to find 8 until 10 enterocytes with magnification 1000 times. Eppendorf tube which contained 500 ul and enough concentration of the enterocyte was added with 500 ul protein hemagglutinin of the sample. The source of a sample came from protein hemagglutination which had the highest titer. Then eppendorf tube was placed in a shaker water bath temperature 37° C for one hour. For washing protein which did not adhere to enterocyte, the sample tube was centrifuged at 1000 rpm temperature 4° C for 15 minutes with PBS three times. The final volume of eppendorf was adjusted into 500 ul. 20 ul sample from eppendorf was placed on glass object to dry in room temperature.

Methanol solution was used to fix the sample on the glass object two times. Every 5 minutes the sample was washed with H₂O 3 times. Then H₂O₂ 3% was dropped on the surface of glass object and was incubated for 10 minutes in room temperature. After that the sample was washed with PBS pH 7.4. For blocking nonspecific protein was used the NGS 1% was used for one hour. Blocking solution was avoided by filter paper.

The sample was given primary antibodies (sera patient) and was incubated at room temperature 4° C for one night. After incubation period had finished the sample was cleaned up by PBS pH 7.4 three times every 5 minutes. After that secondary antibody alkali labeled phosphatase was dropped on it and was incubated at room temperature for 60 minutes. The sample was washed with pH 7.4, three times every 5 minutes and then SA-HRP was dropped on it and was incubated for 40 minutes. Final washing used the PBS pH 7.4 three times every 5 minutes. Floated with DAB at 3 minute, washed with H2O three times every 5 minutes. A counter stain used methylene green, washed with tap water after that was dried at room temperature and was ready for examination.

RESULTS AND DISCUSSION

We have already designed pili bacteria cutter which was used for the tool isolation of pili bacteria as seen in figure 1 [2].

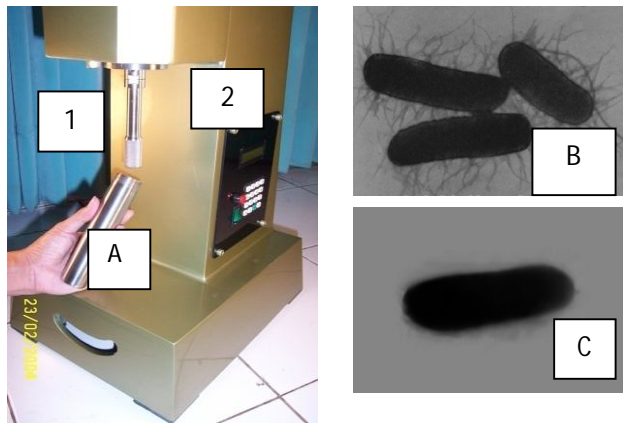


Figure 1: The result of separation pili from the whole bacteria using *V. cholerae*.

- A. Pili bacteria cutter
 1. The knife of pili bacteria cutter
 2. The tombol for adjusting the time and velocity of rotate
- B. Whole ceel bacteria with the pili
- C. Cell bacteria without pili after cutting seven steps

The cutter of pili made from stainless steel stick which has 11 cm long and diameter 18 mm. The surface of the tip of stick has a long 3.5 cm and diameter 22 mm was cartel led 1 mm deep. Where as the surface of this part has the smooth and sharp protrude, the protrude has a function as knife. Opposite of the tip of stick was fixed to the motor apparatus so the stick can move around. Three grams of the pellet was re-suspended using 6 ml of PBS enough and can put it in. The pili bacteria cutter is reproducible and maybe is accepted as a tool for bacterial pili collection [2].

Profile protein of pili sub-unit *S. typhi* as a result of pili bacteria cutter was shown in Figure 2.

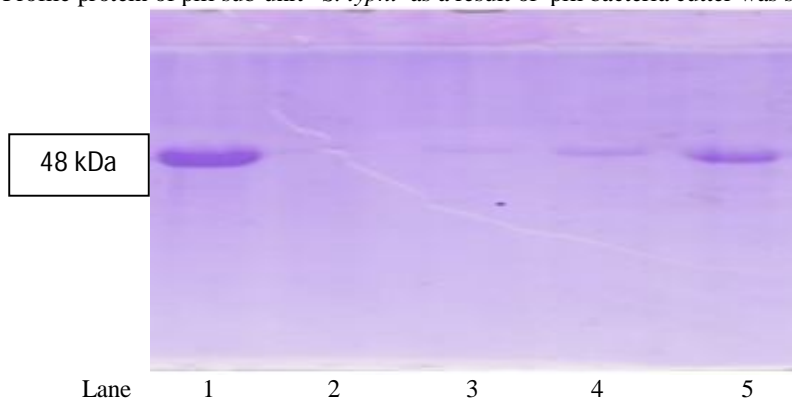


Figure 2: The profile of SDS-PAGE protein sub-unit pili *S typhi* cut by pili bacteria cutter

1. Protein subunit pili *S. typhi* first cutting (10,000X/minute for 60 second)
2. Protein subunit pili *S. typhi* fifth cutting(10,000X/minute for 30 second)
3. Protein subunit pili *S. typhi* fourth cutting (10,000X/minute for 30 second)
4. Protein subunit pili *S. typhi* third cutting (10,000X/minute for 30 second)
5. Protein subunit pili *S. typhi* second cutting (10,000X/minute for 30 second)

The result was shown as in figure 1, protein sub-unit pili *S. typhi* had a molecular weight of 48 kDa. Its protein was prominent in the first cutting and gradually it became smaller in the later cuttings.

Generally the protein sub-unit pili bacteria are a molecule hemagglutinin. Hemagglutination assay was done to confirm hemagglutination activity and the result was shown in Figure 3.

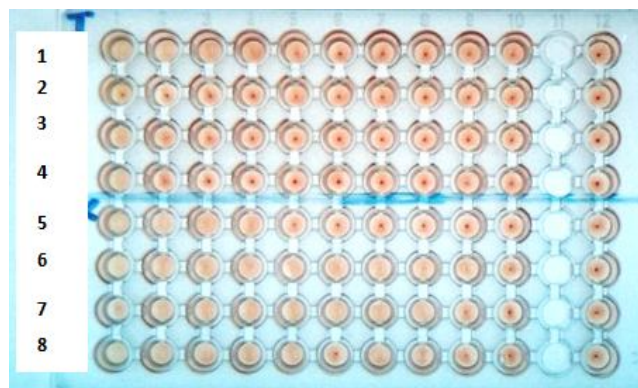
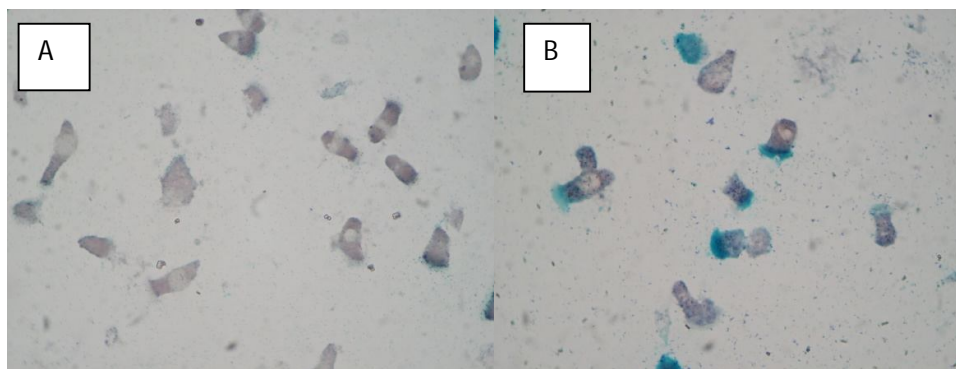


Figure 3: The result of hemagglutination assay of protein sub-unit pili *S. Typhi*

1. Whole *S. Typhi* bacteria
2. Protein subunit pili *S. typhi* fifth cutting
3. Protein subunit pili *S. typhi* fourth cutting
4. Protein subunit pili *S. typhi* fourth cutting
5. Protein subunit pili *S. typhi* third cutting
6. Protein subunit pili *S. typhi* first cutting
7. Protein subunit pili *S. typhi* first cutting
8. Protein subunit pili *S. typhi* second cutting

Figure 3 shows that protein sub-unit pili first cutting had the highest titer. Protein sub-unit pili first cutting was used in adherence assay on mice enterocyte.

The results of immunocytochemistry to detect antigen antibody reaction can be shown as in Figure 3.



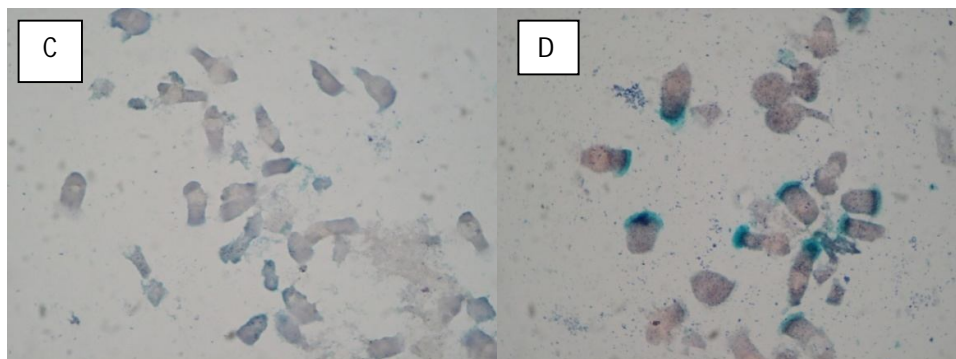


Figure 4: Antigen antibody reaction was detected using the immunocytochemistry method

- A. and C. Sample sera patient suffering from typhoid fever
- B. Sample sera patient not suffering from typhoid fever
- D. Sample sera patient suffering from typhoid fever without secondary antibody

The green color on the enterocytes were caused by methylen green as counter stain. The molecule hemagglutinins attached to the surface of the enterocytes which constituted a villi. In the part of enterocytes other than villi green color was not found and it is only dominated by brown color. This observations were prominent in the sample A and C.

The first step pathogenesis of diseases were caused by bacteria including *S. Typhi* is adhere on a receptor host cell. Almost all adherence of bacterial Gram negative rod use pili. So to the clarification of the first step we have to isolate the pili.

We have already designed pili bacteria cutter which was used for the tool isolation of pili bacterias as seen in figure 1. The principle of difference our tool between others was usually used is in the knife. Others tool were usually used for isolation pili of *V. cholerae* has four knives which make a cross each other. The cutter of pili made from stainless steel stick which have 11 cm long and diameter 18 mm. The tip of stick has a long 3.5 cm and diameter 22 mm and the it surface was cartel led 1 mm deep. So the surface of this part has the smooth and sharp protrudes and has a function as knife. Opposite of the tip one, the tip was fixed to the motor apparatus so the stick can rotates. The tube for collecting the sample is smaller than the omni mixer. Three grams of the pellet was resuspended using 6 ml of PBS enough and can put it in. The pili bacteria cutter is reproducible and maybe is accepted as a tool for bacterial pili collection beside omni mixer [2].

The results of the first cutting to the fifth cuttings of the profile proteins a sub-unit pili *S. Typhi* in figure 2 are consistent. Proteins with molecular weight of 48 kDa are dominant although their thickness is not similar. They become smaller along the cuttings. The proteins of 48 kDa are the thickest in all the cuttings. It can also be concluded maybe that the localization of the 48 kDa is in the tip of the pili *S. Typhi* and it may have a function as a molecular adhesion.

In our preliminary study the molecule hemagglutinin in the tip of the pili *V. cholerae* has a molecular weight of 38 kDa and has a function as a molecular adhesion. Naturally in the outer membrane bacteria *V. cholerae* have a protein dimer molecular form weight of 76 kDa. Protein weight of 38 kDa is a monomer form of protein weight of 76 kDa in the outer membrane of *V. cholerae*. The protein weight of the 38 kDa in the tip of the pili *V. cholerae* is identical with the monomer form of 38 kDa in the outer membrane of protein. Combinations of protein sub-unit pili 37.8 kda *V. cholerae* with sub-unit B CT *V. cholerae* can protect come out of the solution in the intestinal mice (2). Sperandio also found the protein of 38 kDa in the outer membrane of *V. cholerae* [8].

Figure 3 is the result of hemagglutination of protein sub-unit pili *S. Typhi* and is found in the first cutting and the titer of hemagglutination is the highest. The result is not so different with the profile of hemagglutination of sub-unit protein pili *V. cholerae* which was isolated by pili bacteria cutter (2).

To confirm that the protein hemagglutinin sub-unit pili *S. typhi* is a molecule adhesion can be seen in Figure 3. The Weisler method was reproducible to separate the enterocyte of each other from the surface of mucous intestinal tract. The separation of enterocyte is to make observation easier as a receptor side of the molecule adhesion.

Hep-2 cell was used to detect adherence assay diarrheagenic *Escherichiae coli* and *Klebsiella pneumoniae*. Preparation of Hep-2 cell is time consuming and more expensive compared to isolation of enterocyte using Weisler method [9] [10] [11].

The results of protein hemagglutinin sub-unit pili *S. Typhi* which had molecular weight of 48 kDa can attach on enterocyte in Figure 2 as a molecule adhesion were indirect experiment. We only see the attachment

of molecule adhesion on enterocyte only from a kind of color on villi enterocyte. In this phenomenon the function of the molecule adhesion on villi enterocyte is as the antigen. Green color can be interpreted that there is no match antigen antibody reaction. If the molecule adhesion as an antigen does not join with the antibody in the patient serum, a methylene green replace antibody and attach to the antigen. Attachment of methylene green to antigen will develop green color. On the contrary if antibody in the patient serum suffering from typhoid fever attaches to the antigen on enterocyte, the methylene green can't replace it and the antigen green color is not visualized. From the result of the experiments the brown color can be seen in the villi of the enterocyte.

The visualization of bacteria attachment to a receptor on a cell host usually uses a light microscope. If a result of bacteria attachment is not clear may be electron microscopic examination can replace the visualization.

To confirm that the substance bacteria have a function as molecule adhesion an adhesion index is usually used. Adhesion index is a mean of amount of bacteria attaches to the cell host [6].

CONCLUSION

The result of this study verified that protein hemagglutinin sub-unit pili *S. typhi* which had a molecular weight of 48 kDa was molecule adhesion on mouse enterocyte. The method of immunocytochemistry can be use to confirm attachment of molecule adhesion to receptor. The usefulness of the method of the molecule adhesion sub-unit pili 48 kDa *S. typhi* is detected by immunocytochemistry method in the diagnosis of typhoid fever is a going research.

REFERENCES

1. Soto, G.E. and Hultgren, S.J. 1999. *Minireview* Bacterial adhesins: Common themes and variation in architecture and assembly. *Infect. Immun.* 181: 1059-107
2. Sumarno, Susanto A, Gatoet I and Winarsih S. 2011. Combinations of protein sub-unit pili 37.8 kda *V. cholerae* with sub-unit B Cholera Toxin *V. cholerae* can protect come out of the solution in the intestinal mice. *J.Pharm.Biomed. Sci.* 1:154-160.
3. Nagayama, K., Oguchi, T., Arita, M. and Honda, T. 1995. Purification and characterization of cell-associated hemagglutinin *Vibrio parahaemolyticus*. *Infect. Immun.* 63:1987-1992.
4. Sengupta, T.K., Sengupta, D.K. and Ghose, A.C. 1993. A 20-kD pilus protein with haemagglutination and intestinal adherence properties expressed by clinical isolat of non-O1 *Vibrio cholerae*. *FEMS Microb. Lett.* 112,237-242.
5. Ehara, M., Ishibasi, M., Watanabe, S., Iwanaga, M., Shimotori, S. and Naito, T. 1986. Fimbriae of *Vibrio cholerae* O1: Observasion of fimbriae on the organisms adherent to the intestinal epithelium and development of a new medium to enhance fimbriae production. *Trop. Med.* 28:21-23.
6. Hanne, L.F. and Findkelstein, R.A. 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. *Infect. Immun.* 36:209-214..
7. Laemli, UK. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*: 680-686
8. Sperandio, V., Giron, J.A., Siveira, W.D. and Kaper, J.B. 1995. The Omp U membrane protein, potensial adhereance factor of *Vibrio cholerae*. *Infect. Immun.* 63: 4433-4438.
9. Barret, T.J., Cameron, D.N., and Wachsmuth, I.K., 1986. Localization of determinant for Hep-2 adherence by enteropathogenic *Escherichia coli*. *Infect. Immun.* 52:334-336.
10. Levine, M.M., Prado V., Robin-Brawne, R., Lior, H., Kaper, B.J., Mosely, S.L., Gicquelais, R., Nataro, J.P., Vial, P. and Tall, B. 1988. Use of DNA probes and Hep-2 cell adherence assay to detect diarrheagenic *Escherichiae coli*. *J.Infect. Dis.* 158:224-228,
11. Di- Martino P, D., Bertin, Y., Gिरardeau, J., Livrelli, P., Joly, B. and Darfeuld-Michaud, A. 1995. Moleculer characterization properties of CF29K, an adhesin of *Klebsiella pneumoniae* strain involved in nosokomial infection. *Infect. Immun.* 63:4336-4344.