

Adherence Pheno-genotypic of *Escherichia coli* O157:H7 Isolated from Beef, Feces of Cattle, Chicken and Human

I Wayan Suardana^{1,2*}, Wayan Tunas Artama³, Widya Asmara⁴, and Budi Setiadi Daryono⁵

¹Department of Veterinary Public Health, Faculty of Veterinary Medicine, Udayana University, Denpasar, Indonesia.

²Study Program of Biotechnology, The Graduate School of Universitas Gadjah Mada, Yogyakarta, Indonesia.

³Department of Biochemistry, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

⁴Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

⁵Department of Genetic, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Abstract

Generally, adherence of micro-organisms to host cells is the first step of the colonization to host surfaces. *Escherichia coli* O157:H7 can colonize to the intestine and induce attaching-effacing (AE) lesions. The capacity of inducing AE lesions is encoded by a pathogenicity island, the locus of enterocyte effacement (LEE) that contains genes involved in generation of attaching and effacing (A/E) lesions. Among which that, the *eae* gene is encoding intimin, an outer membrane protein that is responsible to intimate attachment to the intestinal epithelial cells. A total of 20 local isolates obtained from human clinically, beef, cattle, chicken, and human non-clinically were tested to adherence pheno-genotypic of *E. coli* O157:H7. The *eae* gene was identified using polymerase chain reaction with a specific primer i.e AE19 forward and AE20 reverse. To confirm phenotypic of gene, further study was performed by culturing the bacteria in vero cell, followed by Giemsa staining and Acridine Orange Fluorescent staining 3 h and 6 h after incubation, respectively. Result of study showed that there were 19 out of 20 (95%) isolates identified positive *eae* gene. Giemsa staining appeared that the bacteria with positive *eae* gene performed a cluster around of cell (localized adherence). On the other hand, the negative *eae* gene appeared as a diffuse adherence (DA). The study indicated that almost all of *E. coli* O157:H7 local isolates which was positive *eae* gene had potency to colonize to the intestine and induce attaching-effacing lesions, and also cause cytopathic effects in intestinal epithelial cell.

Keywords : *E.coli* O157:H7, *eae* gene, intimin

Introduction

Escherichia coli O157:H7 is one of the zoonotic agents that life threatening associated with food and water borne infections. Pathogen was recognized can cause several disease syndrome, including asymptomatic infection, mild diarrhea, haemorrhagic colitis,

hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura in people (Gooding and Choudary, 1997). The pathogenic capacity of bacteria resides in a number of virulence factors, including Shiga toxins (Stx1 and Stx2), enterohaemolysin and the intimin as a adherence protein (Gyles, 2007).

Generally, adherence of micro-organisms to host cells is the first step of the colonization to host surfaces (Dopfer *et al.*, 2000). *E.coli* O157:H7 can colonize to the intestine and induce attaching-effacing (AE) lesions (Cookson *et al.*, 2002). Attaching and effacing was a term

*Corresponding Author:

I Wayan Suardana

Department of Veterinary Public Health, Faculty of Veterinary Medicine, Udayana University, Denpasar-Indonesia. E-mail: iwayansuardana22@yahoo.com.

to describe an intestinal lesion (AE lesion) caused by specific strains of *E. Coli*. 'Attaching' because of the intimate attachment of the bacteria to the exposed cytoplasm membrane of the enterocyte; and 'effacing' because of the localized disappearance of the brush border microvilli (Stordeur *et al.*, 2000).

The capacity of inducing AE lesions is encoded by a pathogenicity island, the locus of enterocyte effacement (LEE) that comprises a complex of various genes (Aktan *et al.*, 2004). LEE island contains genes involved in generation of attaching and effacing (A/E) lesions, among which, that is *eae* gene encodes intimin, an outer membrane protein that is responsible to intimate attachment to the intestinal epithelial cells (Yu and Kaper, 1992). Intimin, a protein encoded by *eae* chromosomal gene, mediates adherence of attaching and effacing *E.coli* to intestinal epithelial cell (Ghanbarpour and Oswald, 2010). The bacteria can cause the forming AE lesions on the intestinal mucosa *in vivo* and on the certain tissue and cell cultures *in vitro*. The mucosal lesion is associated with stunting and fusion of villi in severe cases. Bacteria can be seen on the mucosal surface, and may often be specifically identified by immunolabelling (Wales *et al.*, 2005). Attaching and effacing *Escherichia coli* is recognised as a cause of diarrhoea and dysentery.

The bacterial adherence test *in vitro* has been usually proved by Giemsa and May-Grünwald staining (Aidar-Ugrinovich *et al.*, 2007), or by acridine orange fluorescent staining which binds the nucleic acids of bacteria and stain them with orange. Acridine orange, a fluorochrome stain, is potentially superior to the Gram staining in the direct microscopic examination because it gives striking differential staining between bacteria and background cells and debris (Lauer *et al.*, 1981).

The aim of study was to determine relationships between genotypic profile by analysis of *eae* gene and phenotypic feature of adherence by Giemsa and Acridin-Orange fluorescent staining of *E.coli* O157:H7 local isolates isolated from beef, feces of cattle, chicken, and human, therefore it can

contribute to knowledge of the epidemiology and pathogenic capacity of this pathogen.

Materials and Methods

E.coli O157:H7 isolates

A total of 20 *E.coli* O157:H7 isolates obtained from beef, feces of cattle, chicken, and human in Bali were performed in Table 1. *E.coli* O157:H7 ATCC 43894 obtained by from BBVET

Table 1. *E.coli* O157:H7 isolates in this study

| No | Isolates code | Source |
|-----|---------------|------------------------------------|
| 1. | ATCC 43894* | Human Feces diarrhea |
| 2. | KL-52(7) | Human with kidney failure symptoms |
| 3. | KL-87(7) | Human with kidney failure symptoms |
| 4. | KL-30(4) | Human with kidney failure symptoms |
| 5. | KL-45(1) | Human with kidney failure symptoms |
| 6. | KL-48(2) | Human with kidney failure symptoms |
| 7. | KL-85(1) | Human with kidney failure symptoms |
| 8. | KL-83(5) | Human with kidney failure symptoms |
| 9. | KL-24(5) | Human with kidney failure symptoms |
| 10. | KL-68(1) | Human with kidney failure symptoms |
| 11. | KL-106(3) | Human with kidney failure symptoms |
| 12. | KL-55(6) | Human with kidney failure symptoms |
| 13. | MK-35 | Chicken feces |
| 14. | MK-19/8(4) | Chicken feces |
| 15. | M-14(4) | Human feces with non symptoms |
| 16. | M-17(1) | Human feces with non symptoms |
| 17. | DS-21(4) | Beef |
| 18. | DS-16(2) | Beef |
| 19. | SM-25(1) | Cattle feces |
| 20. | SM-7(1) | Cattle feces |

*Strain reference

Bogor was used as a reference strain. All strains were maintained in nutrient agar slants.

Isolation of DNA templates

All *E. coli* isolates and reference strains were grown in LB agar at 37°C over night. Isolation of DNA template was performed by commercial kit (Qiagen 51304). A total of 5 ml *E. coli* O157:H7 culture aliquots were centrifuged at 7,500 rpm for 10 min, and the supernatant was discarded. The cell pellet was added by 180 µl of buffer ATL. Add 20 µl of proteinase K prior of added 200 µl AL buffer. Mix immediately by vortexing for 15 s, incubate at 56°C for 10 min, then add 200 µl ethanol (96-100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, remove drops from the inside of the lid. Carefully apply the mixture to QIAamp Mini Spin Column, and centrifuge at 8000 rpm for 1 min, and discard the tube containing the filtrate. Add 500 µl buffer AW1 then centrifuge at 8000 rpm for 1 min and discard the filtrate. Add 500 µl of buffer AW2, then centrifuge at full speed (14,000 rpm) for 3 min. Place the column in a clean 1,5 ml microcentrifuge tube, add 50 µl buffer AE, incubate at room temperature (15-25°C) for 1 min, and then centrifuge at 8000 rpm for 1 min.

PCR Amplification of *eae* gene

The *eae* gene of *E. coli* O157:H7 was amplified using a specific primers according to Moon *et al.*, (2004) as follows: forward primer 5'- CAG GTC GTC GTG TCT GCT AAA-3' and reverse primer 5'-TCA GCG TGG TTG GAT CAA CCT-3'. Primers were diluted at a concentration 25 pmol/µl. To amplify, it was used Platinum PCR SuperMix (Invitrogen) and amplified using *thermocycler* (TC25/H) with condition: initial denaturation at 94°C for 7 min, 36 cycles of 94°C for 1 min, 50°C for 35 s, 72°C for 2 min, and the final extension step at 72°C for 5 min. Amplification products were analyzed by electrophoresis in 2% agarose gels which was stained with ethidium bromide.

Bacterial adherence and invasion assay on vero cell culture

The methods of Aidar-Ugrinovich *et al.* (2007) with modification were used. Vero cell with 1 x 10⁴ cells/well were grown to subconfluence in 24-well tissue culture plates containing a sterile circular coverslip and 500 µl of medium M199 supplemented with 10% fetal calf serum, 2% penicillin-streptomycin, and 0,5% fungizone. Monolayers on the coverslips were washed 3 times with medium M199, and were incubated in duplo with 1 x 10⁶ bacteria/well. After incubation for 3 h, cells with the coverslips were washed 3 times with medium M199, to remove non-adherent organisms. Afterwards the coverslips were fixed with metahol and then stained with Giemsa, followed by observation with light microscopy (400x).

Acridine orange staining of *E. coli* adhering vero cells

The preparation of cells and bacterial adherence assays were described as above. After incubation 6 h, the media was aspirated from each well and the cover slips were washed 3 times with medium M199. The cell were fixed for 10 min with 3% formaldehyde and then washed (3 x) with PBS (pH 7,2) steril. Cells were permeabilised by incubation with 0,1% Triton X-100 (Sigma) in PBS. The Triton was removed by washing 3 x with PBS, and the cells were covered with 15 µl PBS containing ethium and acrydin orange 0.05%. The cover slips were mounted on glass slide, and the slides were examined by fluorescence microscopy.

Results and Discussion

Adherence genotypic of *E. coli* O157:H7

Adherence genotypic by ampification of *eae* gene used a specific primers according to Moon *et al.*, (2004). The total length of target *eae* gene that should be amplified is 3106 bp, but the primers were not designed from the beginning and the end of the sequence, so the fragment length that can be amplified is only 1087 bp. The result of DNA amplification using specific primers as seen in Figure 1.

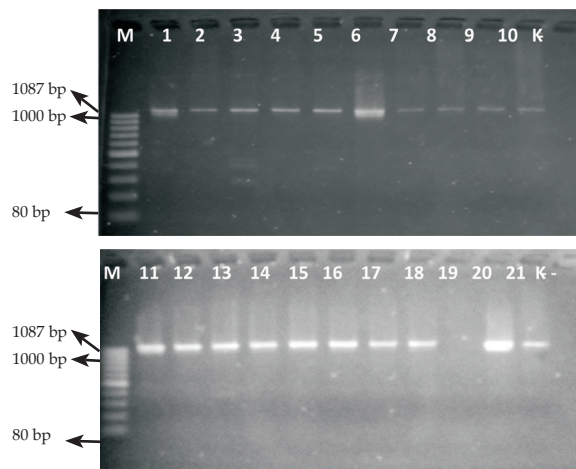


Figure 1. Amplification of *eae* gene using specific primers AE19 forward and AE20 reverse on 2% agarose gel. Lane 1, positive control ATCC 43894; lane 2 to 13 were isolates origin human feces clinically i.e; line 2: KL52(7); line 3: KL87(7); line 4: KL30(4); line 5: KL45(1); line 6: KL48(2); line 7: KL85(1); line 8: KL83(5); line 9: KL24(5), and line 10: KL68(1), line 11: positive control ATCC 43894; line 12: KL106(3); line 13: KL55(6); line 14 to 15 were isolates origin chicken feces i.e; line 14: MK35; line 15: MK19/8(4); line 16 to 17 were isolates origin human feces non-clinically i.e; line 16: M14(4); line 17: M17(1); line 18 to 19 were isolates origin beef i.e; line 18: DS21(4); line 19: DS16(2); line 20 to 21 were isolates origin cattle feces i.e; line 20: SM25(1), and line 21: SM7(1); M: 100 bp DNA ladder (Microzone Ltd), K: negative control (no template).

Based on Figure 1, the amplification using specific primer i.e AE19 forward and AE20 reverse, showed that the band as an amplification product of the *eae* gene was 1087 bp, and almost all of the isolates except isolate no.9 were detected positive. The positive gene has been reported 95% when isolating VTEC from ill humans (Bockemuhl and Karch, 1996 in Muffling *et al.*, 2007), and 57% from bovine isolates (Blanco *et al.*, 2003). The presence of *eae* gene indicated that the local isolates of *E.coli* O157:H7 that were origin from various source i.e: from human clinically, beef, cattle, chicken, and human non-clinically had an adhesion molecule that was termed as intimin. Ghanbarpour and Oswald, (2010) described that intimin as a protein encoded by *eae* chromosomal gene mediates adherence of attaching and effacing *E.coli* to intestinal epithelial cell. Intimin protein is necessary

for the development of the mature AE lesion (Wales *et al.*, 2005).

Phenotypic of adherence test giemsa staining

Based on the *eae* gene that is amplified as seen in Figure 1, further study was performed in order to know the adherence characteristic of *E. coli* O157:H7 local isolate in vero cell culture after 3 h of incubation as seen in Figure 2.

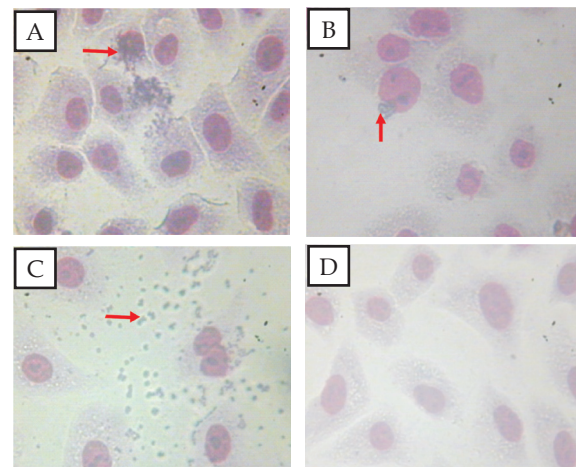


Figure 2. Adherence Characteristic of *E. coli* O157:H7 Local Isolates in Vero Cell Culture after 3 h of Incubation. (A): positive control ATCC 43894; (B) local isolates origin from human feces clinically i.e; KL55(6); (C): local isolates origin from beef i.e DS16(2); (D): negative control (no bacteria culture)

Figure 2, showed that a cluster bacterial (localized adherence) appeared in around of cell i.e for control isolate ATCC 43894 and local isolate KL 55(6), on the contrary, local isolate DS16(2) appeared diffuse adherence (DA) around of cell. On the other hand, no bacterial antigen has shown in negative control (vero cell without bacterial culture). According to Nataro *et al.*, (1987) the adherence patterns were classified as localized adherence (LA), if the bacteria adhere to the cell surface as tight cluster; localized-like adherence (LAL), if the bacteria adhere to the cell surface forming loose clusters; aggregative adherence (AA), if the bacteria adhere to the cell surface and to the cover slip in a stacked brick pattern; diffuse adherence (DA), if the bacteria adhere

diffusely to the cell surface, and undetermined adherence (UND), if bacterial adherence could not be classified in one of these defined patterns. Result of study verified that the *eae* gene has been shown to be necessary for attaching and effacing cavity. Ghanbarpour and Oswald (2010) intimin as a protein which is encoded by *eae* chromosomal gene, mediates adherence of attaching and effacing of *E. coli* to intestinal epithelial cell. Numerous investigators have been underlined the strong association between the carriage of *eae* gene and the capacity of STEC causing severe human disease, especially *haemolytic uremic syndrome/HUS* (Blanco *et al.*, 2004). This important virulence gene was detected in 19 out of 20 local isolates (95%), and gave evidence that local isolates of *E. coli* O157:H7 were had potency to induce attaching and effacing lesion.

Acridine orange fluorescent staining

Acridine orange (OA) is a fluorochrome which binds to nucleic acid of eukaryocytes as well as prokaryocytes. Using OA stain and fluorescent microscopy, it showed that bacteria were specifically stained red-orange, while mammalian cellular elements and background debris were retained an apple green to yellow color (Greenfield *et al.*, 1985). Further study in order to confirm the adherence characteristic of *E. coli* O157:H7 local isolate in vero cell culture after 6 h of incubation after staining with acridine orange as seen in Figure 3.

Figure 3 has been showed the formation of polykaryocytes in ATCC 43894 and KL55(6) isolates within 6 h after inoculation. Albrecht *et al.* (1996) reported that polykaryocytes are a larger cytoplasmic masses that contain many nuclei and are usually produced by fusion of infected cells. Polykaryocytes usually are used as a marker of cytopathic effects. According to Konishi *et al.*, (2008), the formation of polykaryocytes in vero cell depend on the pH of vero cell. Polykaryocytes were detected under pH 7 as early as 9 h after infection. Erez *et al.*, (2009) reported that polykaryocytes /

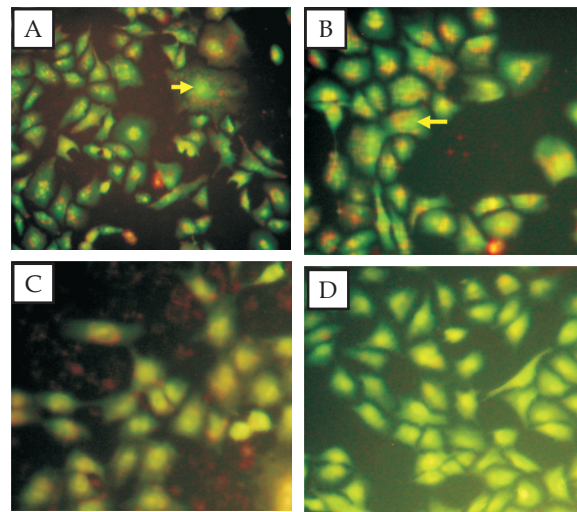


Figure 3. Adherence Characteristic of *E. coli* O157:H7 Local Isolates in Vero Cell Culture after 6 h of Incubation. (A): positive control ATCC 43894; (B) local isolate origin from human feces clinically i.e; KL55(6); (C): local isolate origin from beef i.e DS16(2); (D): negative control (no bacteria culture).

syncytia formation is largely separated into two major routes: One is induced by large number of agent particles which are present in the medium, attach the membrane and thus induce fusion. The other results from high amount of intracellular agent particles which induce fusion.

In conclusion, there were 19 out of 20 (95%) local isolates identified positive *eae* gene. Giemsa staining appeared that the bacteria which were positive *eae* gene performed a cluster around of cell (localized adherence). On the other hand, the negative *eae* gene appeared as a diffuse adherence (DA). The study indicated that almost all of *E. coli* O157:H7 local isolates which were positive *eae* gene had potency to colonize to the intestine and induce attaching-effacing lesions and also cause cytopathic effects in intestinal epithelial cell.

Acknowledgment

We thank to Mr. Prof. Supar, APU from BBVET Bogor for his giving a *E. coli* O157:H7 ATCC43894 standard, and Mrs. Istini as a technician at Integrated Testing and Researching Laboratory, Universitas Gadjah

Mada for her participation on the study and giving a vero cell that was used.

References

- Aidar-Ugrinovich, L., Blanco, J., Blanco, M., Blanco, J.E., Leomil, L., Dahbi, G., Mora, A., Onuma, D.L., Silveira, W.D., and Pestana de Castro, A.F. 2007. Serotypes, Virulence Genes, and Intimin Types of Shiga Toxin-Producing *Escherichia coli* (STEC) and Enteropathogenic *E. coli* (EPEC) Isolated from Calves in Sao Paulo, Brazil. *Int. Jour. Food Microbiol.* **115**, 297-306.
- Aktan, I., Sprigings, K.A., La Ragione, R.M., Faulkner, L.M., Paiba, G.A. and Woodward, M.J. 2004. Characterisation of Attaching-Effacing *Escherichia coli* Isolated from Animals at Slaughter in England and Wales. *Vet. Microbiol.*, **102**, 43-53.
- Albrecht, T., Fons, M., Boldogh, I. And Rabson, A.S. 1996. Effect on Cells. *Medical Microbiology*, 4thEd. Edited by Samuel Baron. <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mmed>. Search 10-31-2010.
- Blanco, J., Blanco, M., Blanco, J.E., Mora, A., Gonzales, E.A., Bernardez, M.I., Alonso, M.P., Coira, A., Rodriguez, A., Rey, J., Alonso, J.M., Usera, M.A., 2003. Verotoxin-producing *Escherichia coli* in Spain: Prevalence, Serotypes, and Virulence Genes of O157:H7 and non-O157 VTEC in Ruminants, Raw Beef, and Humans. *Exp. Biol. Med.* (Maywood) **228**, 345-351.
- Blanco, J.E., Blanco, M., Alonso, M.P., Mora, A., Dahbi, G., Coira, M.A. and Blanco, J. 2004. Serotypes, Virulence Genes and Intimin Types of Shiga Toxin (Verotoxin)-Producing *Escherichia coli* Isolates from Human Patients: Prevalence in Lugo (Spain) from 1992 through 1999. *J.Clin. Microbiol.* **42**, 311-319.
- Cookson, A.L., Wales, A.D., Roe, J.M., Heyes, C.M., Pearson, G.R. and Woodward, M.J. 2002. Variatiaon in Persistence of *Escherichia coli* O157:H7 in Experimentally Inoculated 6-week Old Conventional Lambs. *J.Med.Microbiol.* **51**,1032-1040.
- Dopter, D., Almeida, R.A., Lam, T.J., Nederbragt, H., Oliver, S.P. and Gaastra, W. 2000. Adhesion and Invasion of *Escherichia coli* from Single and Reccurent Clinical Cases of Bovine Mastitis in Vitro. *Vet. Microbiol.*, **74**, 331-343.
- Erez, N., Paran, N., Maik-Rachline, G., Politi, B., Israely, T., Schnider, P., Fuchs, P., Melamed, S. and Lustig, S. 2009. Induction of Cell-cell Fussion by EctromeliaVirus is not Inhibited by Its Fusion Inhibitory Complex. *Vir. Jour.*, **6**, 1-13.
- Ghanbarpour, R. and Oswald, E.2010. Phylogenetic Distribution of Virulence Genes in *Escherichia coli* Isolated from Bovine Mastitis in Iran. *Res. Vet. Sci.*, **88**, 6-10.
- Gooding, C.M. and Choundary, P.V. 1997. Rapid and Sensitive Immunomagnetic Separation-Polymerase Chain Reaction Method for The Detection of *Escherichia coli* O157:H7 in Raw Milk and Ice-Cream. *J.Dairy Res.*, **64**, 87-93.
- Greenfield, M.D., Clausen, C.R. and Smith, A.L. 1985. The Use of Acridine Orange as a Rapid Method for the Quantitation of Bacterimia in Laboratory Animals. *Infection.* **13**, 137-139.
- Gyles, C.L. 2007. Shiga Toxin-Producing *Escherichia coli*: in Overview. *J.Anim. Sci.* **85**,45-62.
- Konishi, E., Yagawa, K. and Yamanaka, A. 2008. Vero Cells Infected with Vaccinia Viruses Expressing *Japanese Encephalitis Virus* Envelope Protein Induce Polykaryocyte Formation under Neutral Conditions. *Jpn. J. Infect.Dis.* **61**, 410-411.
- Lauer, B.A., Barth Reller, L. and Mirrett, S. 1981. Comparison of Acridine Orange and Gram Stains for Detection of

- Microorganisms in Cerebrospinal Fluid and Other Clinical Specimens. *Jour. Clin. Microbiol.*, **14**, 201-205.
- Moon, G.S., Kim, W.J. and Shin, W.S. 2004. Optimization of Rapid Detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by PCR and Amplification to Field Test. *J. Food Prot.* **8**, 1634-1640.
- Muffling, T., Smaijlovic, M., Nowak, B., Sammet, K., Bulte, M. And Klein, G. 2007. Preliminary Study of Certain Serotypes, Genetic and Antimicrobial Resistance Profiles of Verotoxigenic *Escherichia coli* (VTEC) Isolated in Bosnia and Germany from Cattle or Pigs and Their Products. *Int. Jour. Food Microbiol.*, **117**, 185-191.
- Nataro, J.P., Kaper, J.B., Robins-Browne, R., Prado V., Vial, P. and Levine, M.M. 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr. Infect. Dis. J.*, **6**, 829-831.
- Stordeur, P., China, B., Charlier, G., Roels, S. and Mainil, J. 2000. Clinical Signs, Reproduction of Attaching/Effacing Lesions, and Enterocyte Invasion after Oral Inoculation of an O118 Enterohaemorrhagic *Escherichia coli* in Neonatal Calves. *Microbes and Infection.* **2**, 17-24.
- Wales, A.D., Woodward, M.J. and Pearson, G.R. 2005. Attching-Effaching Bacteria in Animals. *J.Comp.Path.* **132**, 1-26.
- Yu, J. and Kaper, J.B., 1992. Cloning and Characterization of The *eae* gene of Enterohaemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* **6**, 411-417.