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Lateral flow assay strip for detection of *Escherichia coli* O157:H7

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Abstract

The use of polyclonal antibody (IgG) has recently been applied to the detection of bacteria. We developed a lateral flow assay (LFA) strip using a specific IgG in combination with colloidal gold on a nitrocellulose membrane. A conjugate, gold-anti *Escherichia coli (E. coli)* O157:H7 IgG was developed in this study for the detection of *E. coli* O157:H7 in food. The 40 nm in size of colloidal gold nanoparticles was used to conjugate the anti-*E. coli* O157:H7 IgG. The optimal concentration, 12.0 µg/ml of the anti-*E. coli* O157:H7 IgG was determined by standard curve generated in titration method. The serially diluted of *E. coli* O157:H7 was detected and clearly visualized on the LFA strip as low as 10^6 CFU/ml (result not shown). The IgG raised in rabbit have shown specific binding capacity against *E. coli* O157:H7. No other genus of bacteria, including *Salmonella typhimurium, Listeria monocytogenes* and *Campylobacter jejuni* reacted to the IgG. The LFA strip could also detect *E. coli* O157:H7 in different food samples matrices after 18 h-enrichment and this result were in accordance with the results of the polymerase chain reaction (PCR) and colony count.

Introduction

Escherichia coli (E. coli) O157:H7 infection is rarely reported in Malaysia but it does not seem to be exempted. In South East Asian countries routine screening of *E.coli* O157:H7 contamination in traded food products is important to make sure they are pathogen free and to avoid food contamination across international borders. *E. coli* O157:H7 are predominantly associated with hemorrhagic colitis and the more severe complications of hemolytic uremic syndrome (HUS) in humans (Fach *et al.,* 2003). Although human-to-human transmission of *E. coli* O157:H7 has been demonstrated, most infections have been associated with the consumption of contaminated ground beef, milk, water produce and apple juice products that have been improperly handled, stored or cooked. Cattle are consistently identified as a reservoir for this pathogen. Death can also be a result of *E. coli* O157:H7 infection. In 1996, Osaka, Japan reported 3 cases of death and about 1100 cases of infection including 100 cases of HUS caused by outbreaks of *E. coli* O157:H7 (Makino *et al*., 2012). Therefore, rapid identification will prevent ingestion of contaminated food products. *E. coli* O157:H7 may be distributed in developing countries, but it has rarely been reported (Nataro and Kaper, 1998).

Consumption of undercooked food of bovine origin according to Fernandez (2008) is the leading cause of *E. coli* O157:H7 outbreaks. Report by Sahilah *et al.* (2010) shows prevalence of *E. coli* O157:H7 in beef meat samples taken from local market was alarming to the public health concern. As more beef are consumed, Malaysians are facing higher risk of getting infected by *E. coli* O157:H7. Experiments conducted in Malaysia using antibiogram, plasmid profile and arbitrarily primed polymerase chain reaction (AP-PCR) profile (Son *et al.*, 1998) revealed the distribution of beef contaminated with *E. coli* O157:H7 was at a considerable frequency in the local retail. Sahilah (1997), Son *et al*. (1998) and Sukhumungoon *et al*. (2011) reported 76%, 36% and 23% prevalence of *E. coli* O157:H7 in beef samples purchased from retails store in Malaysia respectively (Frederick, 2011). Recently, local news paper in Malaysia (BH Online, 2015; Utusan Online, 2015) reported the Prime Minister, Datuk Seri Najib Razak, was infected with *E. coli* during his tours to flood-hit areas in early of January 2015. He has been advised to rest for a while by doctor before return to work. It is believed that he was infected by eating bacteria-infected food or drinking contaminated water.

Recently, considerable attention has been directed towards the use of DNA-based method by using polymerase chain reaction (PCR) coupled with gene probe technology for the detection of *E. coli* O157:H7. This DNA-based method is one of the most specific and sensitive method in routine confirmatory assay for the bacterium (Suria *et al*.,

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2013). However, this approach needs to be performed by skilled and trained personnel, require the use of expensive devices, complicated protocols and need to be brought to the dedicated laboratory for the sample to be analyzed (Gharaibeh *et al.*, 2013; Singh *et al*., 2015). Thus, protein-based testing is more flexible technique compared to DNA-based testing as it provides good result stability, mobile and acceptably accurate result. Among protein analysis techniques that are simple and stable for protein detection are using one-dimensional isoelectric focusing gels, immunoblot analysis (Jeffrey and Scott, 2008) and enzyme-linked immunosorbent assay (ELISA) (Cathy and Stephen, 2013).

Demands for rapid, simple and sensitive methods for *E. coli* O157:H7 determination is high, therefore the development of rapid detection method of *E. coli* O157:H7 contamination such as lateral flow assay (LFA) strip is important especially in relation to detect food contamination, diagnose patients and prevent illness outbreaks (Yonekita *et al*., 2013). The LFA strip provides rapid detection of antigens and antibodies, simplicity of procedure, long-term stability over a wide range of environmental conditions, user-friendly format and a relatively low cost (Wang *et al*., 2010; Qi *et al*., 2011; Nian *et al*., 2012). The cost of locally produced test strip on laboratory scale was at RM10 per strip and on mass scale production was only at RM5 per strip compared to imported test strip that was at RM15 per strip.

The objective of this study was to develop a rapid and sensitive LFA strip for *E. coli* O157:H7 detection. Polyclonal antibody against *E. coli* O157:H7 (anti-*E. coli* O157:H7 IgG) will be raised in rabbit by injecting subcutaneously formalin killed *E.coli* O157:H7 (immunising antigens). Rabbits were bleed for two weeks to precipitate antibody in the serum and the antibody will be purified through affinity chromatography. Purified antibody will be conjugated with the gold nanoparticles and the conjugate complexes will be sprayed on conjugate pad on LFA strip. Colloidal gold nanoparticles are preferred choice as a label in this assay because they have been reported as easy to synthesis, visualise and conjugate with biological material with simple inexpensive laboratory preparation and stable in liquid or dried form (Singh *et al.*, 2015). The specific binding of anti-*E. coli* O157:H7 IgG to *E. coli* O157:H7 cells were employed in this study for detection of *E. coli* O157:H7. The specificity of the polyclonal antibody against *E. coli* O157:H7 will be investigated by cross reactivity studies using lateral flow assay (LFA) strip on other bacteria such as gram negative (*Salmonella typhimurium*, ST and *Listeria monocytogenes*, LM) and gram positive (*Campylobacter jejuni*, CJ). We also intended to study the food matrices favouritism of *E. coli* O157:H7 in various food matrices such as chicken, cheese and milk. We compared the result of various spike samples on LFA strip procedure with conventional PCR combined with gel electrophoresis and colony count assay. Furthermore, we also compared the sensitivity of LFA strip, PCR and colony count assay.

Materials and Methods

Bacterial strains and culture media

E. coli O157:H7 bacterial isolates was obtained from the Department of Food Science, Faculty of Food Science and Technology, UPM. Other bacterial, *Salmonella typhimurium* (ST), and *Campylobacter jejuni* (CJ) derived from the culture collection of ATCC 53648 and CL1400, respectively. *Listeria monocytogenes* (LM) was obtained from the Institute for Medical Research (IMR), L54/08B. Modified cefixime tellurite sorbitol MaConkey agar (CT-SMAC), tetrathionate broth base (TTB), fraser broth and modified charcoal cefarazone deoxycholate agar (mCCDA) was used for the isolation of *E. coli* O157:H7, ST, LM and CJ, respectively. Bacterial culture will be used for antigen preparation in lateral flow assay (LFA) and DNA isolation.

Preparation of polyclonal antibody (IgG) against E. coli *O157:H7*

The concentration of *E. coli* O157:H7 has been determined at 600 nm using spectrophotometer with absorbance 2.3 for 10^9 CFU/ml. The *E. coli* O157:H7 was then formalin killed in 0.2 % formalin solution stirred for 18 h to generate an immune response for immunisations. The preparation was used as immunising antigens and injected subcutaneously into rabbits. Rabbits were bleed for antibody titer determination in two weeks after each boost. Blood was collected 30 days after the first immunisation. Antibody in serum samples was precipitated with saturated ammonium sulphate, centrifuged, dialyzed and eluted through a protein A sepharose affinity chromatography (AKTAprime, GE Healthcare). Fractions giving the highest absorbance reading for protein at A280 nm were collected and freeze dried for long term storage.

Preparation of gold–polyclonal antibody (IgG) conjugate

Colloidal gold sizes of 40 nm were purchased from Kestrel BioSciences (Thailand). pH of colloidal gold was adjusted to 8.0 with 0.2 M potassium

carbonate (K_2CO_3) and 0.1 N hydrogen chloride (HCl). To saturate the gold particles, 86.9 µl of anti-*E. coli* O157:H7 IgG solution (12 µg/mL) was added to 20 ml of the pH-adjusted colloid gold solution. Bovine serum albumin (BSA) was filtered through 0.45 µm filter to make sure it was <45 nm in sizes. The purpose is to block the unconjugated surfaces of colloidal gold that is 40 nm in sizes. The mixture was gently mixed for 10 min, blocked with 2 ml of 10 % filtered BSA for 30 min and centrifuged at 11,000 g for 10 min. After centrifugation, 10 µl of the gold-IgG conjugate pellets were resuspended in 990 µl passive diluents buffer (PDB) containing 1 % BSA, 2 mM sodium tetraborate and 0.005 % sodium azide. PDB pH was adjusted to 8.2 with 1 N sodium hydroxide (NaOH) and 1 N HCl. Optical density (OD) of gold-IgG conjugate suspension in PDB was measured at 540 nm (labelled as OD^0) using spectrophotometer with PDB as a blank. Total volume of pelleted gold-IgG conjugate was measured and labelled as V^0. The volume of PDB (V') required to make of working gold conjugate suspension with OD 10 (OD') was determined using a formula as follow:

$$OD^0V^0 = OD'V'$$

Resulted conjugate was filtered through 0.45 µm filter to clean the conjugate from any of free antibody and BSA and stored at 4°C. Prior to use for coating the resulted conjugate solution was mixed with 10 % sucrose and 5 % trehalose and must be coated on conjugate pad within 1 week only.

Development of the lateral flow assay (LFA) strip

Development of lateral flow technology involved some basic components such as backing card (holding all pads), conjugate pad, sample pad, test pad and absorbent pad. The gold-polyclonal conjugate in PDB was coated on conjugate pad (Whatman GF33, Kestrel BioSciences) manually using micropipette and dry at 37°C for 2 h. To prepare 1 mg/ml of control line (C), 250 µl of 2 mg/ml goat anti-rabbit IgG (Pierce) was added onto 250 µl of 0.02 M phosphate buffered (PB). Goat anti-rabbit IgG were then applied on test pad (Milipore) manually and dry at 37°C for 2 h. For test line (T), 2 mg/ml of commercial rabbit anti-*E. coli* O157:H7 IgG in 0.02 M PB with 1 % sucrose was used. Anti-*E. coli* O157:H7 IgG was applied on test pad manually and dry at 37°C for 2 h.

Cross reactivity studies with LFA strip

For cross reactivity studies with lateral flow assay (LFA) strip 1 ml of selective enriched samples (*E. coli* O157:H7, ST, LM and CJ) was transferred to 1.5 ml microcentrifuge tube. Samples were centrifuged at 10,000 g for 5 min to pellet it. Supernatant was discarded and 100 µl lysis buffer (pH 8.0 adjusted with 1 N HCl) was added. Lysis buffer containing 0.303 % tris base pentasodium, 1% tripolyphosphate, 0.1% sodium azide, 1 ml/L trition X405, 0.0904% EDTA and 0.5% sodium casein. Sample was resuspended in lysis buffer by sucking it in and out using pipette. 3 drops (or 100 µl) of resuspended sample was placed into the sample well. Wait 3 minutes for the sample to be absorbed. Results must be read within 15 minutes. Results obtained after 15 minutes are invalid because the colour intensity of the lines are sensitive to surrounding temperature and humidity. The result of LFA strip that are exposed for more than 15 minutes is invalid as temperature and humidity influence the strip's reaction. We consider the 15 minutes duration is enough for the sample solution to pass through the viewing window test and to complete the test reaction time of 3 minutes. Lysis buffer was used as negative control. In positive result, ruby red line will appear at the control line (C) and test line (T). For negative result, ruby red line will appear only at C line.

Preparation of spike samples for comparative studies

Glycerol stock of *E. coli* O157:H7 were subcultured in tryptic soy broth (TSB) and incubated overnight at 37°C. On the next day a serial dilution of overnight culture was prepared and plated on nutrient agar (NA) in triplicate for colony count. To make a concentration of 10^1 *E. coli* O157:H7 CFU/25 g to be spiked into the samples (chicken, milk and cheese), 25 µl of 10^5 CFU/ml *E. coli* O157:H7 stock was added to 225 ml modified trypton soy broth (mTSB) plus 1.0 µg/ml novobiocin (n). Inoculated mTSB + n was then added to the filter stomacher bag, which were thoroughly hand massaged and then stomached at 190 rpm for 30 s and incubated overnight at 37°C. On the next day, the overnight spiked culture of *E. coli* O157:H7 was then serially diluted and plated (100 µl) in triplicate onto nutrient agar (NA) for enumeration of inoculums (colony count). One ml of overnight culture of each spiked samples was transferred onto a microcentrifuge tube for DNA extraction and lateral flow assay (LFA). The extracted DNA was used as template in polymerase chain reaction (PCR).

LFA strip test of spike sample matrices

For comparative studies with LFA strip 1 ml of mTSB + n culture grown overnight for all the spiked samples was transferred to 1.5 ml microcentrifuge tube. Samples were prepared follow the steps as in sample preparation in cross reactivity studies with LFA strip described before.

DNA extraction of spike sample matrices

Total DNA was isolated from 1 ml of mTSB + n culture grown overnight for all the spiked samples used in the study. DNA was extracted with a DNA isolation kit (Qiagen, Germany) according to the manufacturer's instructions. The genomic DNA was checked for the concentration and purity using spectrophotometer (Shimadzu 1601, Japan).

PCR conditions of spike sample matrices

The PCR mixtures were prepared with slight modification according to the manufacturer's instructions (Transgene). The primer sequences used in the PCR are as follows:

Forward: 5'-TCTTTCCTCTGCGGTCCTA-3'
Reverse: 5'-CAGGTGAAGGTGGAATGGT-3'

In general, PCR mixtures contained 12.5 µl of 2X EasyTaq SuperMix, 5 µl of template DNA and 0.4 µM of each forward and reverse rfbE primers. DNA amplification was carried out in a Bioresearch thermalcycler using an initial denaturation step at 95°C for 2 min, followed by 34 cycles of amplification with denaturation at 95°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s, ending with final extension at 72°C for 4 min. The fragments were separated on 1.8% agarose gels, followed by ethidium bromide staining and photograph under UV light. DNA molecular size standards (100 bp gene ruller, Fermentas) were included in each agarose gel electrophoresis run.

Colony count of spike sample matrices

To get single colony of *E. coli* O157:H7, 100 µl of the mTSB + n culture grown overnight for all the spiked samples used in the study was plated in triplicate on nutrient agar (NA) and incubated overnight at 37°C. The CFU/ml of culture was calculated using a formula as follow:

$$C = N_{CFU} \times DF \times 10^1$$

C = Number of CFU per milliliter
N_{CFU} = Colony number
DF = Dilution factor
10^1 = Volume of sample plated

Results and Discussion

Cross reactivity studies

The developed lateral flow assay (LFA) strip showed specificity for *Escherichia coli* O157:H7 cells. The specificity of the assay was investigated in relation to other bacteria such as gram's negative (*Salmonella typhimurium*, ST and *Listeria monocytogenes*, LM) and gram positive (*Campylobacter jejuni*, CJ) which is the most common bacterial contamination in food samples. Cross reactivity studies using developed LFA strip showed obvious ruby red line on C and T Line for *E. coli* O15:H7 sample tested and no band appear on T Line for negative control (lysis buffer). There was no band appeared on T line for ST, LM and CJ samples tested. These showed that there was no cross-reactivity in the developed LFA strip when tested with enterobacteria sp. (Figure 1). In this study we manually developed C and T line, using micropipette to dispense a small volume (0.3 µl and 0.4 µl) of antibodies (goat anti-rabbit IgG and commercial rabbit-anti *E. coli* O157:H7 IgG). One of the factors that should be controlled properly in manually developing C and T line using micropipette is to apply consistent pressure on the test pad. The pressure applied by the end of micropipette tip that was hand handled by the operator may not evenly distribute and will form a ditch across the surface of test pad. This could contributes to false positive results, due to the gold-anti *E. coli*-O157:H7 IgG conjugate will easily trapped onto the ditch while in lateral flow moving when sample was applied in the test. In developing LFA strip it is also important to develop a uniform size and width of the C and T line. By developing the C and T line manually we found that it was difficult to develop a uniform size and width of the lines compared with when using automated dispensing system. Developing neat line manually will require more effort and time. Proper and sterile antigen preparation and the right usage of isolation media are also important as to make sure the prepared antigen for the test is free from other contaminants. The sensitivity of developed LFA strip was as low as 1 x 10^6 CFU/ml (result not shown).

Result of comparative studies of spike sample matrices by LFA strip

To simulate the actual procedure when using the LFA strip on real samples in future, we have spiked several food matrices with *E. coli* O157:H7. Among the food matrices that was used in this study were chicken, cheese and milk. This is also intended to look in which food matrices the *E. coli* O157:H7 was most favoured. The LFA strip result for spiked matrices showed that the ruby red line appears on C and T line for three spiked matrices for chicken, cheese and milk (Figure 2). The result were verified with no band appears on T line for three control unspiked matrices for chicken, cheese and milk. There was obvious ruby red line appeared on T line

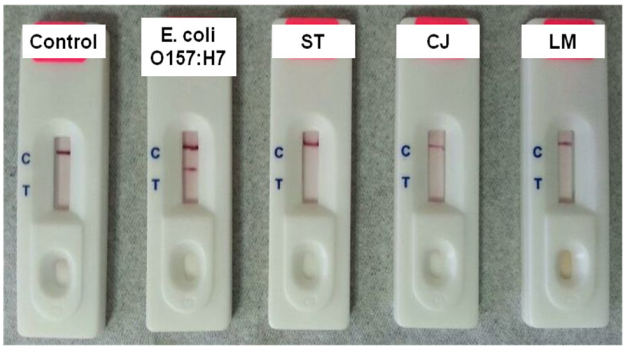

Figure 1. Relative response of anti-*E. coli* O157:H7 IgG with gram negative bacteria (*Salmonella typhimurium,* ST and *Listeria monocytogenes*, LM) and gram positive bacteria (*Campylobacter jejuni,* CJ) on LFA strip

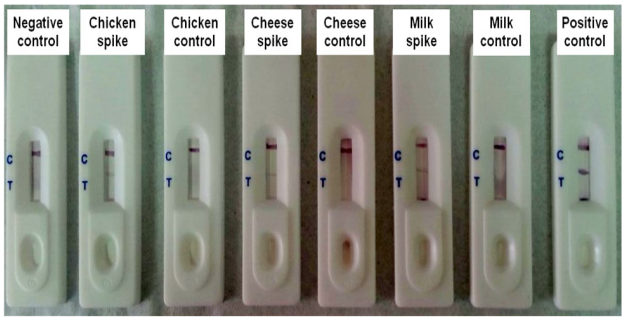

Figure 2. Comparative studies for food matrices favouritism of *E. coli* O157:H7 in chicken, cheese and milk samples using LFA strip

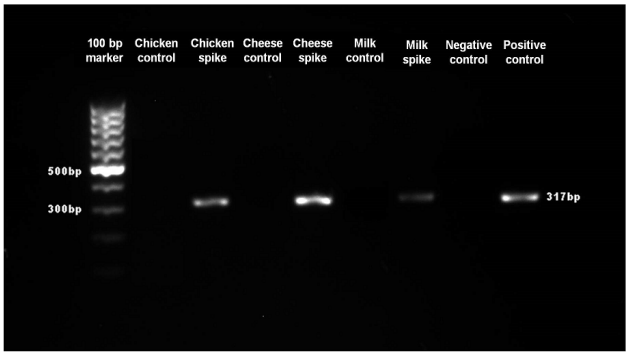

Figure 3. Comparative studies for food matrices favouritism of *E. coli* O157:H7 in chicken, cheese and milk samples by polymerase chain reaction (PCR)

of positive control (*E. coli* O157:H7 culture) showed that the LFA strips are sensitive enough for detection of *E. coli* O157:H7. The ruby red line on T line for three spiked matrices were not intense as in positive control although their colony count (1 x 10^9 CFU/ml vs. 1 x 10^8 CFU/ml and 1 x 10^7 CFU/ml) was not significantly different. It might be due to the presence of inhibitors in the food matrices that inhibit the interaction between *E. coli* O157:H7 (antigen) with the gold-anti *E. coli* O157:H7 conjugate when samples were applied on sample pad. Rossen *et al.* (1992) have reported that there is presence of inhibitors in food protein preparation solutions which may influence the effectiveness of the LFA strip and reducing its sensitivity. The sensitivity of *E. coli* O157:H7 by LFA strip in food matrices sample were lower than that of *E. coli* O157:H7 culture supernatants. These results were in accordance with findings by Nakasone *et al.* (2007) as samples may contain various components such as carbohydrates, lipids and detergent that may interfere with antigen-antibody interaction. The sample preparation procedure was confirmed sterile with no ruby red line appears on T line for negative control (lysis buffer).

However direct detection of *E. coli* O157:H7 from food matrices are challenging because developed LFA strip still need enrichment steps in food sample preparation. The enrichment step was necessary to increases bacteria copy numbers and thus will also dilute inhibitory substances. Gordillo *et al.* (2011) have reported that high level presence of other microorganisms in foods, including different meat products, may mask small numbers of potentially hazardous *E. coli* O157:H7 and make a *E. coli* O157:H7 discovery is often difficult. Therefore, an initial selective enrichment step is necessary to minimize competitor microorganisms and to increase the number of target bacteria cells and the sensitivity of the LFA strip in inoculated food samples. Direct soaked of LFA strip into sample solution without obtaining clear supernatant by centrifugation will lead to false negative result in detecting target antigen (Nakasone *et al.*, 2007). Our previous study (Suria *et al*., 2013) shows that the minimum concentrations of *E. coli* O157:H7 detectable by PCR were 2.8 x 10^3 CFU/ml and by LFA strip developed in this study were 1 x 10^6 CFU/ml (result not shown). Although LFA strip was not as sensitive as PCR method which is very sensitive that could detect a minimum of 100 ng of DNA or single copy of gene but LFA strip is simple, user friendly and adequately sensitive. Compared to PCR that require skilled and trained personnel, laborious and dedicated laboratory (Gharaibeh *et al*., 2013; Singh *et al*., 2015).

Result of comparative studies of spike sample matrices by PCR

Identification of *E. coli* O157:H7 isolates by PCR using primers pairs for *rfbE* genes confirmed to amplify a 317 bp fragment (Figure 3). The specific presence of *rfbE* gene in *E. coli* O157:H7 spiked samples validate the results from LFA strip for *E. coli* O157:H7 detection. There was no band appears in control unspiked chicken, cheese and milk matrices. These results showed that the preparation of thus matrices and spiked procedure are sterile. There was also no band appears in negative control (PCR mixture without template) which showed that the PCR

Table 1. Comparative studies by colony count of *E. coli* O157:H7 in three different food matrices

Samples		Colony count (CFU/ml)
Chicken	Spike	1.83×10^{8}
	Control	No growth
Cheese	Spike	2.49×10^{8}
	Control	No growth
Milk	Spike	2.07×10^{7}
	Control	No growth

E. coli O157:H7 most favours on cheese matrices followed by chicken matrices and milk matrices.

preparation mixture are sterile. For band that appears in positive control (pure DNA of *E. coli* O157:H7) showed that the PCR conditions are optimized for amplification of rfbE gene in identification of *E. coli* O157:H7. The intensity of the bands was also in accordance with the results in colony count methods which the band of cheese spiked matrices shows the most intense band followed by the bands of chicken and milk spiked matrices. Although DNA-based (PCR) method is more sensitive and specific compared with lateral flow assay (LFA) strip but it requires skilled and trained personnel, laborious and dedicated laboratory (Gharaibeh *et al*., 2013; Singh *et al.*, 2015). Such protocols however are not suitable for routine analysis where speed and sensitivity are critical. The developed LFA strip in this study is rapid, on-site, user friendly, simple and cost effective.

Result of comparative studies of spike sample matrices by colony count

Colony count on spiked sample matrices showed that *E. coli* O157:H7 most favours on cheese matrices with growth of 2.49×10^{8} CFU/ml followed by chicken matrices with growth of 1.83×10^{8} CFU/ml (Table 1). Milk matrices showed the last favours of growth matrices for *E. coli* O157:H7 with growth of 2.07×10^{7} CFU/ml. The colony count results were in accordance with LFA strip and PCR results. There is no colony growths on nutrient agar for control of all three food matrices shows that the procedure of food matrices spiking was sterile. For screening purposes, selective plating methods are ineffective to employ on sites as it is relatively time-consuming, required 12 - 24 h for optimal growth. Although it is quantitative but potentially inaccurate (Gharaibeh *et al*., 2013). Previously we have successfully developed multiplex PCR method for detection of *E. coli* O157:H7 (Suria *et al.*, 2013). But as we realised DNA-based method are not suitable for on-site screening purpose we have come with LFA strip as presented in this study.

Table 2. The advantages and disadvantages of comparative studies by three different methods

	LFA strip	PCR	Colony count
Time consume	2 - 3 h	4 - 6 h	2 days
Cancer agent	Non-carcinogenic	Carcinogenic	Non-carcinogenic
Workload level	Simple, user friendly	Skilled personnel	Laborious
Type of data	Qualitative	Qualitative	Quantitative

The advantages and disadvantages between the three methods used in comparative studies were summarized in Table 2.

Conclusion

A rapid and specific method for detection of *E. coli* O157:H7 was developed. When LFA strip for *E. coli* O157:H7 are tested with others enterobacteriaceae it can differentiate between them and test results can be obtained within 15 minutes after enrichment step. The method is rapid, on-site, simple, user-friendly and specific without special equipment. The method potentially has a range of practical applications especially for screening proposes. Further refinement of the LFA assay will hopefully allow for increased detection sensitivity to increase its usefulness on-site.

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