ITALIAN JOURNAL OF PUBLIC HEALTH

FoodBioTimerAssay: a new microbiological biosensor for detection of *Escherichia coli* food contamination

Francesca Berlutti[1], Fabrizio Pantanella[1], Maria De Giusti[2], Daniela Tufi[2], Piera Valenti[1], Antonio Boccia[2]

[1]*Department of Public Health Science;2 Department of Experimental Medicine, Sapienza University of Rome, Italy*
Correspondence to: Maria De Giusti, Section of Clinical Medicine and Public Health, Dept. of Experimental Medicine, Sapienza University of Rome. Viale Regina Elena 324, 00161 Roma, Italy. E-mail: maria.degiusti@uniroma1.it

Abstract

Background: Prevention of foodborne diseases is a fundamental goal for public health and industries engaged in food preparation and distribution. The correct procedure to ensure an effective prevention of foodborne diseases consists essentially in microbiological monitoring and enumeration of indicator microorganisms of faecal contamination at critical control points along the food producing procedures. Here, we propose a new microbiological biosensor, called FoodBioTimerAssay (FBTA), for rapid and reliable detection of *Escherichia coli* as indicator of faecal contamination in food and surface samples.
Methods: A total of 122 samples were analysed using both experimental FBTA and Reference method. FBTA employs FBTM medium and counts bacteria through microbial metabolism measure: the time required for colour switch (red-to-yellow) of FBTM, due to *E. coli* metabolism, is correlated to initial bacterial concentration.
Results: FBTA results showed an overall agreement percentage with Reference method equal to 97.54%. Discrepancies concerned three samples (1 food and 2 surface samples). Moreover, the time required to perform FBTA method was 3-fold shorter than Reference one.
Conclusions: FBTA method may be considered a useful tool for detection of *E. coli* contamination in food and surface samples. Therefore, FBTA method may be successfully employed in risk analysis of foodborne diseases.

Key words: HACCP, FBTA, foodborne disease prevention, Escherichia coli detection

Introduction

"Coliform" bacteria are the most widely used microbiological indicators of faecal contamination. However, the term "coliform" indicates a group of intestinal bacteria encompassing several species as *Escherichia coli, Klebsiella pneumoniae*, and *Enterobacter aerogenes*. Taxonomical and ecological studies demonstrate that E. coli is a permanent member of intestinal flora, whereas *Citrobacter, Klebsiella* and *Enterobacter* genera can be considered as transient microbiota [1, 2].

These evidences support the recently European Directive (Directive 98/83/CE defined in Italy by Legislative Decree no. 31 issued on February 2th, 2001) that includes *E. coli, Enterococcus* spp. and *Clostridium perfringens* as indicators of faecal contamination of water for human consumption [1, 2, 3, 4], and *E. coli* as indicator of faecal contamination of foodstuffs [5, 6]. Therefore, the presence of *E. coli* in foods indicates faecal contamination and risk of foodborne disease.

Currently, prevention and control are basic concepts in public health care. Preparation and handling of safe food products and related set of kitchen utensils, requires the observance of hazard analysis and critical control point (HACCP) principles: 1- to carry out the hazard analysis; 2- to determine the critical control points (CCPs); 3- to establish the critical limits; 4- to monitor the procedures; 5- to carry out the corrective actions; 6- to verify the procedures, and 7- to establish record-keeping and documentation procedures [7].

In particular, EC Regulation 852/2004 reassesses the application of the HACCP procedure by extending it to the control of primary production and reinforces the role of Good Manufacturing Practice [7]. More recently, the Commission Regulation on the Microbiological Criteria for

Foodstuffs (EC Regulation 1441/2007) reconsiders the EC Regulation 2073/2005 [5, 6], complements the food hygiene legislation, and applies to all food businesses involved in the production and handling of food. The microbiological criteria includes as indicator for risk of foodborne disease *Listeria monocytogenes, Salmonella* spp., enterotoxin-producing *Staphylococcus aureus* and *Enterobacter sakazakii*, and as indicator of faecal contamination *E. coli* [5, 6]. Moreover, the EC Regulation 1441/2007 identifies *E. coli* as indicator of hygienic procedures defining different limits for *E. coli* load in foods and food-handling procedures [5, 6].

Therefore, *E. coli* plays a pivotal role to indicate the need for performing corrective hygienic actions at CCPs to fit microbiological criteria of food safety as well manufacturing, handling and distribution processes.

The EC Regulation 1441/2007 indicates also the standard culture methods to count and identify *E. coli* [8], however, a rapid method should be developed and included in routine CCPs screening to conform to EC Regulations 852/2004 and 1441/2007 [6, 7].

Moreover, many bacterial species are able to grow, survive and persist in foods as well as to adhere to catering surfaces and utensils in biofilm form. [9, 10] As bacterial biofilm shows high resistance to disinfectants and biocides [11, 12] it may be a cause of foodborne infections and diseases in humans [13, 14, 15, 16].

Standardized enumeration of bacterial population is based on colony forming unit (CFU) method. However, CFU method is not satisfactory to quantify bacteria in biofilm or adherent onto surfaces [17]. To overcome the problem of CFU count unreliability, several attempts have been carried out to enumerate bacterial populations in biofilm through indirect method as those based on ATP detection and on the visible blue-to-red transformations of achromatic polymer [17, 18]. The standard method based on the determination of the most probable number (MPN) [5, 6] could overcame the problem of counting bacteria in biofilm. However, the MPN method cannot be applied to count bacteria on surfaces, in addition it is labour and time consuming. Therefore, the development of microbiological methods allowing rapid and reliable detection of bacteria in biofilm could be highly desirable to evaluate the bacterial contamination of food and surfaces.

Here, we propose a new microbiological method for rapid enumeration of *E. coli* as biological indicator of faecal contamination of food and surfaces. The new method called *FoodBioTimerAssay*(FBTA) has been applied to analyse food and surfaces samples showing an overall 97.5% agreement with Reference method.

Materials and Methods

Samples

A total of 122 foods and surfaces samples were collected at Umberto I Hospital kitchens in Rome in the period from December 2006 to July 2007 and analysed for *E. coli* contamination.

Samples, including 82 raw and cooked foods (65

Table 1. Food samples.

Samples		Number
Raw	vegetables	38
	minced	4
	beef	3
	chicken	1
	fish	2
	milk products	8
	milk	1
	eggs	2
	bread	2
	spices	2
	egg dry pasta	1
	dry pasta	1
Cooked	beef	5
	minced	4
	chicken	2
	vegetables	5
	Potato in oil	1
	Total	**82**

raw and 17cooked foods) (Table 1) and 40 surface samples (Table 2) were processed within 1 hours from the collection.

Table 2. Surface samples.

Specimens	Number
Chopping board	9
Plan steel	7
Tools	7
Hands	4
Vegetables bath	2
Food packaging film	4
Milk bath	1
Dish balance	1
Inside fridge	4
Sink board	1
Total	**40**

Strain and culture media

Escherichia coli MG1655 ATCC47076 was maintained in Trypticase soy broth (Difco Laboratories, MD) with glycerol (25%) at -80 °C and checked for purity on Columbia CNA agar (Difco Laboratories, MD, USA) with 5% red sheep cells before use. Mac Conkey agar n.3 - MK3 (Oxoid Ltd, Basingstoke, UK), prepared following manufacturer's instruction, was used for bacterial count, following the colony forming unit (CFU) method. In brief, 10 µl of proper dilutions of *E. coli* MG1655 cultures were plated on to MK3 plates. Each dilution was plated in triplicate. After incubation at 37°C, the number of CFUs was determined.

Evaluation of faecal contamination

The presence of *E. coli* as indicator of faecal contamination in collected samples was detected using both Reference [8, 19] and FoodBioTimer Assay methods [17].

Reference methods

E. coli detection in foods was performed according to ISO 16649-2 guideline [8]. Briefly, 10g of food samples were homogenized and suspended in 90 ml of Buffered Peptone water according to ISO 6887-1 [19]. A 1 ml-volume was then analysed for *E. coli* recovery using MK3 plates. *E. coli* detection in surface samples was performed using the RODAC plates [20, 21] filled with about 17 ml of MK3 medium (Oxoid). RODAC plates were placed on selected surfaces and pressed with dedicated RODAC Weight (International PBI S.p.A., Milan, Italy) for 30 seconds. All plates were incubated for 48-72 h at 37°C.

FoodBioTimer Assay (FBTA)

FoodBioTimer Assay (FBTA) is based on a previously described method employed to count adherent bacteria on abiotic surfaces [17]. The present FBTA employs FoodBioTimer Medium (FBTM) that was prepared as follows: Hearth Infusion broth (HI) 2 g/l (OXOID Ltd., Basingstoke, UK), glucose 10 g/l, colic acid 10g/l (Sigma Chemical Co.), Na_2HPO_4 1.5 g/l, phenol red 25 mg/l, and distilled water to 1000 ml. After sterilization at 121 °C for 15 minutes, pH was checked and adjusted at 7.2 ± 0.1. The final medium appeared clear and red. To evaluate faecal contamination of food samples, FBTM was prepared at two-fold concentration. FBTA measures microbial metabolism: the time required for colour switch (red-to-yellow) (Figure 1) of FBTM due to *E. coli* metabolism is correlated to initial bacterial concentration. Therefore, the time required for colour switch determines the number of bacteria present in a sample at time 0 through a correlation line. To draw the correlation line specific for *E. coli*, 0.2 ml of overnight broth cultures were mixed with 1.8 ml of FBTM. Serial twofold dilutions in 1 ml of FBTM were performed in 24-well plates (BD, Italy) and incubated at 44°C. Simultaneously bacterial dilutions were counted using colony forming unit (CFU) method. The colour of the inoculated FBTM was checked at regular time intervals. For each two-fold dilution, the time required for colour switch of FBTM was recorded and plotted versus the log10 of CFUs.

To count *E. coli* in food samples by FBTA, 10 ml of suspended food sample (see above) were used to inoculate 10 ml of FBTM. To count *E. coli* on surface samples, RODAC plates filled with 1.5% agar were pressed for 30 seconds on surface areas and then immersed in 50 ml of FBTM. The time needed for colour switch of FBTM was monitored and used to count *E. coli* by correlation line.

Figure 1. Correlation line relating the time for colour switch of FoodBioTimer Medium (FBTM) and the Log10 of *Escherichia coli* MG1655 number determined by colony forming unit method. In the insert, the red-to-yellow colour switch of FBTM is depicted.

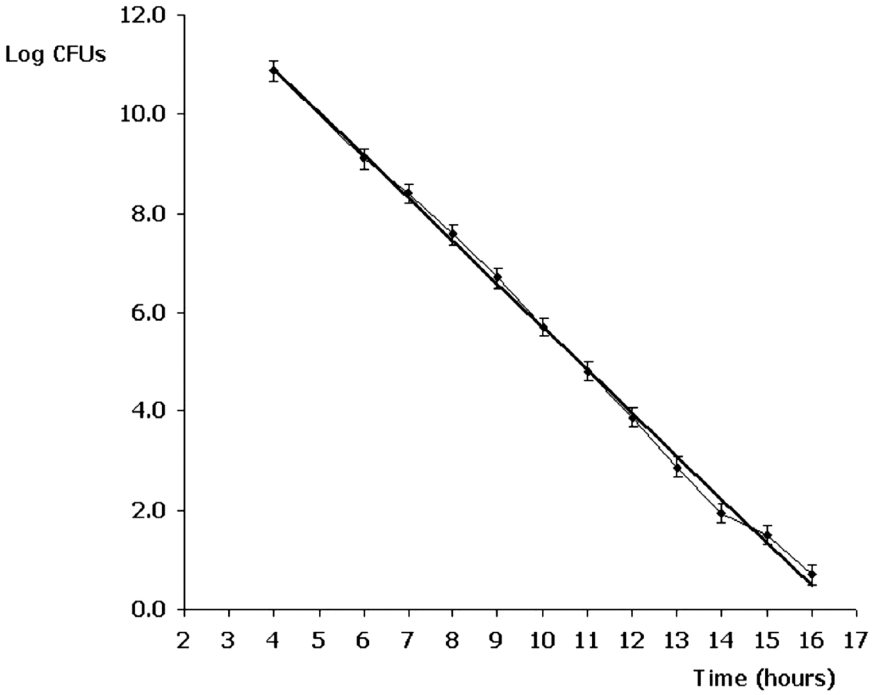


Table 3. Count of *Escherichia coli* MG1655 in experimentally contaminated meat.

Dilution of *E. coli* MG1655 culture [a]	Count of *E. coli* MG1655 [b]		
	FBTA[c]		RM
	Time for FBTM switch	CFUs/g	CFUs/g
None	2h 40min	$6*10^7$	$5.6*10^7$
1:10	3h 30min	$6*10^6$	$8*10^6$
$1:10^2$	5h 5min	$5.8*10^5$	$7.5*10^5$
$1:10^3$	6h 10min	$4.8*10^4$	$4.5*10^4$
$1:10^4$	7h 20min	$4.8*10^3$	$3*10^3$
$1:10^5$	14h 20min	$4.1*10^2$	$3*10^2$
$1:10^6$	14h 40min	50	55
$1:10^7$	15 h 0 min	25	16
$1:10^8$	NS	0	3
Uncontaminated meat	NS	0	NG

Legend: [a] dilution rate of *E. coli* MG1655 overnight broth culture used for experimental contamination of meat samples; [b] CFU/ml of *E. coli* MG1655 in experimentally contaminated ground meat counted by FoodBioTimer Assay (FBTM) and Reference method (RM); [c] : counts of *E. coli* MG1655 were performed using the correlation line (that correlates FBTM time of colour

Experimental contamination of meat

Experimental contamination of meat was carried out using *E. coli* MG1655. A 10 ml-volume of overnight HI broth culture was used to prepare serial ten-fold dilutions. Each dilution was used to inoculate meat samples prepared as follows: 10 g of uncooked ground meat were mixed with 90 ml of sterile Buffered peptone water (Oxoid). After shaking for 30 minutes at room temperature, the mixture was divided in 9 ml-aliquots. Each aliquot was inoculated with 1 ml of the serial ten-fold dilutions of *E. coli* MG1655 prepared as above described. Volumes of 0.1 ml of each contaminated meat aliquot were counted by CFU method and used to inoculate 0.9 ml of FBTM. The colour of the inoculated FBTM was checked at regular time intervals. The time required for colour switch of FBTM was used to determine the number of bacteria in the mixtures through the correlation line.

Statistics

Independent experiments were repeated at least five times to obtain mean values and standard deviations. Correlation line was obtained by linear regression analysis, and linear correlation coefficients were calculated from the equation:

$r = (n\Sigma xy - \Sigma x\Sigma y)/(\text{sqrt}((n\Sigma x^2 - (\Sigma x)^2)\ (n\Sigma y^2 - (\Sigma y)^2)))$.

Results

FoodBioTimer Assay: correlation line

To count bacteria by FBTA, a correlation line was drawn for *E. coli* MG1655. Tubes containing 1 ml of FBTM were inoculated with *E. coli* serial two-fold dilutions (Time 0). After inoculation, the colour of FBTM remained unchanged for a lag time, whose length was inversely related to the number of bacteria present in the medium at Time 0. After lag time, FBTM colour switched from red to yellow (Figure 1). The time (t*), required for colour switch was plotted versus the $\log_{10}$ of CFUs of bacteria present in the medium at Time 0. The equation and the linear correlation coefficient describing the correlation line was calculated on the whole data set and was: $y=-0.8723x + 14.428$, $r^2 = 0.9970$. The sensitivity of the FBTA method was equal to 10 CFUs.

Experimental contamination

To validate the FBTA method for faecal contamination of foods, samples of ground meat were contaminated with serial dilutions of *E. coli* MG1655 culture. The number of *E. coli* MG1655 in meat samples was then determined using both Reference and FBTA methods. Results obtained using FBTA method agreed at 100% with those obtained using Reference method (Table 3). Moreover, FBTA allowed to count bacterial contamination more rapidly than Reference method. In particular, using FBTA, the time required for *E. coli* MG1655 counts was inversely related to bacterial load being shorter in the presence of higher bacterial concentration and ranged between 2 hours and 40 min and 15 hours to count $\sqrt{6x10^7}$ and 25 CFUs, respectively. Conversely, using Reference method, the time required for bacterial count was independent on bacterial load being of about 24 hours for each contaminated meat sample.

Detection of E. coli in food and surface samples

All food and surface samples were examined for *E. coli* contamination using both Reference and FBTA methods.

Of the 122 samples examined for *E. coli* presence, 110 were negative (90.16%) and 9 positive (7.37%) for both methods. Discrepancies concerned only three samples (2.46%) . Reference method gave positive results for two surface samples and FBTA gave positive result for one food sample (Table 4). The overall agreement percentage between two methods was of 97.54%. The time required to reach the diagnosis of *E. coli* contamination using FBTA was compared to that of Reference method (Table 5). Concerning negative samples, 5328 and 1776 hours were needed for Reference and FBTA method, respectively (i.e.: 48 and 16 hours per sample, respectively). Moreover, in Table 4 the time needed to analyse positive samples is reported. In particular, FBTA required a 3.7-fold shorter time than Reference method. The trend of promptness in diagnosis of *E. coli* faecal contamination using Reference and FBTA method is showed in Figure 2.

Discussion

Foodborne diseases are widespread and represent an increasing public health problem involving up to 30% of population each year in industrialized countries [22]. Preliminary FoodNet data show that the estimated incidence of infections caused by several enteric pathogens did not change significantly compared with 2004-2006. However, none of the targets for 2010 national health objectives were reached [23].

Moreover, the food industries and factories, in response to the market demand, have developed new processing and packaging technologies, such as precooked products and vacuum packaging, which may introduce further environments useful for biofilm growth and persistence.

Table 4. Positive food and surface samples.

	RM		FBTM	
Sample	CFUs/gr	Time* (hours)	CFUs/gr	Time* (hours)
Ground meat	1000	48	900	14
Ground meat	550	48	1000	13
Ground meat	1120	48	2000	12.7
Cheese	14	48	20	15
Fresh cow's milk cheese	1500	48	2500	12.5
Fresh cow's milk cheese	0	48	750	13.5
Spices	4600	72	7000	12
Chicken	140	72	175	14
Vegetable	1333	48	1400	13
Vegetable	40	48	100	16
Kitchen tool	3	48	0	16
Hands	1	48	0	16

Legend: RM: Reference Method; FBTA: FoodBioTimerAssay;

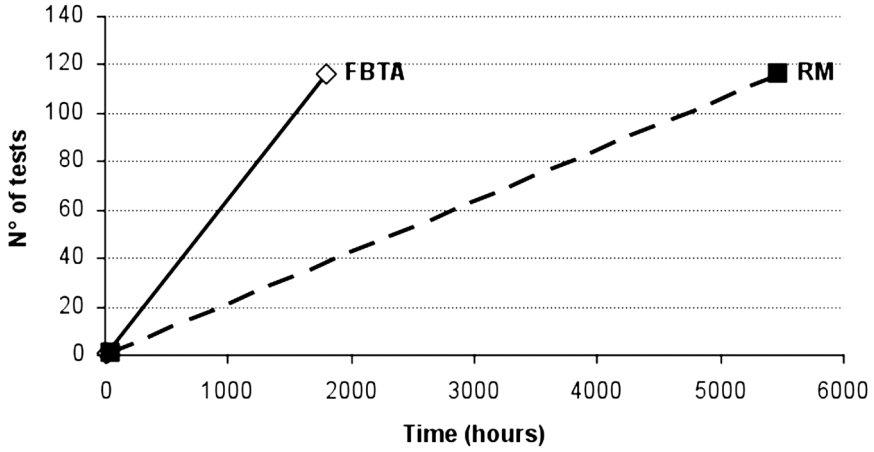
* Time required for diagnosis.

Table 5. Total time required for diagnosis of *Escherichia coli* contamination of 122 samples.

	RM		FBTA	
Samples	Hours	samples n°	Hours	samples n°
Positive	576.0	11	167.7	10
Negative	5328.0	111	1792.0	112
Total	5904.0	122	1959.7	122

Legend: RM: Reference method; FBTA: FoodBioTimerAssay;

Figure 2. Trend of promptness in diagnosis of faecal contamination using Reference (RM) and FoodBioTimerAssay (FBTA) methods.



As consequence, the putative presence of pathogenic bacteria should be constantly monitored to employ reliable, rapid, and easy-to-use methods. The HACCP protocol is designed to monitor and check each step of food processing. However, standard microbiological methods used to control the putative presence of pathogenic bacteria require a long period to be performed (up to 72 hours) and many materials. Moreover, a small amount of food samples can be analysed (about 0.1 g) that may not be representative of the actual bacterial contamination of food. Therefore, the development of rapid, reliable, sensitive and easy-to-use method for counting bacteria in food samples should be desirable.

Here, we present a new microbiological method that allows to count *E. coli* both in foods and on surfaces. The method called FoodBioTimerAssay (FBTA) based on a previously described protocol [17], can be usefully applied to directly enumerate *E. coli* without any manipulation of sample. The FBTA method counts *E. coli* by a correlation line relating the time for colour switch of the specific FBTM medium and the initial bacterial concentration.

FBTA is an easy-to-perform, reliable and rapid method and it does not require sophisticated apparatus. As matter of fact, to employ FBTA method it is necessary only to draw an *E. coli*-specific correlation line [17]. The results of the evaluation of FBTA in *E. coli* recovery from 122 food and surface samples are compared with those of Reference method and show High overall agreement percentage (97.54%) as identical results were obtained in 119 out 122 samples. Discordant results concerned three samples (1 food, 2 surfaces;Table 4). Concerning food sample, it should be underlined that FBTA allows to analyse a 10-fold greater amount of food than Reference method do. Therefore, it is conceivable that FBTA analyses a more representative amount of samples giving rise to a more reliable result than Reference method. As matter of fact, FBTA counts a greater *E. coli* number in 8 out 9 positive food samples than Reference method. Concerning surface samples, the discrepancies could depend on fact that samples were collected in nearby surfaces that may be differently contaminated.

The time required to achieve faecal contamination diagnosis for all samples was 3-fold shorter using FBTA than Reference method. The trend of promptness in diagnosis (Figure 2) clearly shows that FBTA may be considered very effective for HACCP application, as corrective actions at CCPs can be quickly taken. Actually, using FBTA method, *E. coli* contamination can be detected in few hours and, in particular, the time will be shorter in the presence of higher than lower *E. coli* contamination. In conclusion, FBTA may be considered a useful tool for risk analysis of foodborne disease due to *E. coli* contamination of many food categories and surfaces. Moreover, FBTA method can be employed for the screening of food samples and for the determination of products safety according to the recent Microbiological criteria for foodstuffs [6] and to track food products according to Regulation 178/2002 [24].

References

1) Edberg SC, Rice EW, Karlin RJ, Allen MJ. Escherichia coli: the best biological drinking water indicator for public health protection. Symp Ser Soc Appl Microbiol 2000;88:106S–116S.
2) Leclerc H, Mossel DA, Edberg SC, Struijk CB. Advances in the bacteriology of the coliform group: their suitability as markers

of microbial water safety. Ann Rev Microbiol 2001;55:201–34.
3) Directive 98/83/CE on the quality of water intended for human consumption. Official Journal of the European Union 5.12.1998 L330/32.
4) Legislative Decree 31/2001 (transposition of European Directive 98/83/EC) on the quality water intended for human consumption. Official Italian Journal No 52, 3 march 2001.
5) Regulation (EC) n. 2073/2005 of the European Parliament and of the Council of 15 november 2005 on microbiological criteria of foodstaffs. Official Journal of the European Union 22.12.2005 L338/1.
6) Regulation (EC) n. 1441/2007 modified of EC Regulation 2073/2005 of the European Parliament and of the Council of 5 december 2007 on microbiological criteria of foodstaffs. Official Journal of the European Union 7.12.2007 L322/12.
7) Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. Official Journal of the European Union 30.04.2004 L13971.
8) International Organization for Standardization (ISO). Microbiology of food and animal feeling stuffs. Horizontal method for the enumeration of β- glucuronidase-positive Escherichia coli. Part.2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl β –D-glucuronide. ISO 16649-2:2001.
9) Wilks SA, Michels H, Keevil CW. The survival of Escherichia coli O157 on a range of metal surfaces. Int J Food Microbiol 2005;105:445-54.
10) Wilks SA, Michels H, Keevil CW. Survival of Listeria monocytogenes Scott A on metal surfaces: implications for cross-contamination. Int J Food Microbiol 2006;111:93-8.
11) Ntsama-Essomba C, Bouttier S, Ramaldes M, Dubois-Brissonnet F. Resistance of Escherichia coli growing as biofilms to disinfectants. Vet Res 1997;28(4):353-63.
12) Byun MW, Kim JH, Kim DH, Kim HJ, Jo C. Effects of irradiation and sodium hypochlorite on the micro-organisms attached to a commercial food container. Food Microbiology 2007; 24:544-8.
13) Brown CA, Wang B, Oh JH. Antimicrobial activity of lactoferrin against foodborne pathogenic bacteria incorporated into edible chitosan film. J Food Prot 2008;71(2):319-24.
14) Griffiths MW. How novel methods can help discover more information about foodborne pathogens. Can J Infect Dis 2000;11(3):142-53.
15) Gandhi M, Chikindas ML. Listeria: A foodborne pathogen that knows how to survive. Int J Food Microbiol 2007;113(1):1-15.
16) Oliver SP, Jayarao BM, Almeida RA. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathog Dis 2005; 2(2):115-29.
17) Berlutti F, Rosso F, Bosso P, et al. Quantitative evaluation of bacteria adherent to polyelectrolyte hema-based hydrogels. J Biomed Mater Res A 2003; 67(1):18-25.
18) Silbert L, Shlush BI, Israel E, Porgador A, Kolusheva S, Jelinek R. Rapid chromatic detection of bacteria by use of a new biomimetic polymer sensor. Appl Environ Microbiol 2006; 72(11):7339-44.
19) International Organization for Standardization (ISO). Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. ISO 6887-1: 2000.
20) Rohde PA. A new culture plate-its applications. Bull Parameter Drug Assoc 1963;17:11-3.
21) Brummer B. Influence of possible disinfectant trasfer on Staphylococcus aureus plate counts after Agar Contact Sampling. Appl Environ Microbiol 1976;32(1):80-4.
22) World Health Organization. Foodborne Disease Surveillance. Available from http://www.who.int/foodborne_disease/en [Accessed May 22, 2008].
23) Centers for Disease Control and Prevention (CDC). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food–10 states, 2007. MMWR Morb Mortal Wkly Rep 2008; 57(14):366-70.
24) Regulation (EC) N. 178/2002 of the European Parliament and of the Council of 28 January 2002 on laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal of the European Union 1.02.2002 L31/1.