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Estimation of the sensitivity of a novel technique for the detection of *E. coli* O157 in non-heat-treated milk

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ABSTRACT

Escherichia coli O157: H7 is an important pathogen that causes fatal infections. It has been the cause of several outbreaks in different areas world-wide and is associated with high mortality and morbidity. Multiple procedures were adopted to detect this serotype, but conventional culture techniques represent the gold standard. In this study we estimated peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) technique for the rapid detection of *E. coli* O157:H7. PNA-FISH technique was performed for 50 strains; 20 were *E. coli* O157 H7, 20 were *E. coli* O157 non-H7 strains, and 10 strains belonging to other *Enterobacteriaceae*. Sensitivity, specificity, and accuracy of the FISH technique were 100%, 86.67%, and 92% respectively with Confidence Interval of 95% (82.5% to 98.4%). Both PNA-FISH and conventional culture technique were used for examination of 100 non-heat-treated milk samples which were artificially contaminated with *E. coli* O157:H7. PNA-FISH sensitivity, specificity, and accuracy were 97%, 100%, and 98.5% respectively with a Confidence interval of 95% (95.7% to 99.7%). The PNA-FISH technique had approximate results to the conventional culture methods, in addition, it reduced the diagnosis time to one day.

INTRODUCTION

E. coli are a Gram-negative, rod-shaped, highly motile and facultative anaerobic bacteria. It belongs to the family *Enterobacteriaceae* and represents an ordinary inhabitant of the alimentary tract of both humans and animals. Few strains of *E. coli* can induce diarrhea and some extra-intestinal diseases **(Kubitschek 1990)**.

E. coli can be classified into 6 pathotypic groups according to the mechanism of produced disease; Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Diffuse adherent *E. coli* (DAEC), Enterohaemorrhagic *E. coli* (EHEC) and Enteroaggregative *E. coli* (EAEC) **(Tassew 2015)**.

E. coli serotype O157:H7 within the EHEC group, is the main factor of *E. coli* food poisoning outbreaks **(Karmali et al. 2010 and Segni et al. 2018)**.The major reservoir of *E. coli* O157:H7 is cattle, but the main vehicle for transmission of the organism to human is food

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(e.g meat, dairy products, and vegetables). Whereas, dairy products have been associated with many outbreaks of *E. coli* O157:H7 foodborne illness **(Trevena 1999)**. Clinical presentation of *E. coli* serotype O157:H7 infection comprises colicky abdominal pain and bloody diarrhea, as well as the fatal complication; the hemolytic uremic syndrome **(Griffin and Tauxe 1991)**. Early diagnosis of foodborne pathogens is essential to avoid outbreaks, reduce costs, and achieve ideal food safety management **(Weng et al. 2021)**. The infectious dose of the organism is very low, probably 1 to 10 ingested cells. Therefore, the detection methods must be very sensitive **(Jordan and Maher 2006)**. *E.coli* is identified by conventional culture procedures based on its inability to ferment sorbitol **(Liesegang et al. 2000)**. The gold standard technique for the recognition of *E.coli* O157:H7 is the conventional bacterial culture, a time-consuming and laborious procedure **(Kim et al. 2005)**. The conventional culture process includes bacterial cultivation, isolation, recovery, and identification procedures which require 3–7 days, which has the disadvantages of depending on personal experience **(Sugiarti and Nurhayati 2021 and You et al. 2020)**. The evolving of a quick, accurate, sensitive, and cost-effective technique for detecting foodborne pathogens has therefore been an essential achievement in food safety **(Nurmasytha et al. 2021)**. To attain the sensitivity needed to detect pathogens at low concentrations, signal amplification procedures such as amplification of enzyme-linked cascade and nucleic acid were designated and performed for pathogen recognition to meet the rising measures for food safety **(Zhao et al. 2015)**.

Fluorescence In Situ- Hybridization (FISH) is a molecular procedure extensively utilized for the identification and localization of bacteria in samples **(Amann and Fuchs 2008)**. This technique is relied on the particular binding of nucleic acid probes to target ribonucleic acids, because of their great numbers of copies inside the cells. The FISH technique was applied for the determination of *Salmonella* **(Sousa et al. 2024b)**, *E.coli* **(Sousa et al. 2024a)**, *Helicobacter pylori* **(Sousa et al. 2023)**, *Campylobacter spp.* **(Oliveira et al. 2024)**, and Coliform bacteria **(Kuo et al. 2020)**.

A synthetic DNA analog, termed peptide nucleic acid (PNA), which has the hybridizing ability with complementary nucleic acid goal, was developed and performed FISH technique simple and effective **(Wilks and Keevil 2006)**. PNA-FISH technique was successfully used to detect multiple pathogenic microorganisms in clinical specimens and food industry **(Søgaard et al. 2005)**.

This work aimed to apply a PNA-FISH technique for the detection of *E. coli* O157:H7 in non-heat-treated milk specimens and to compare its performance with that of the conventional culture technique.

MATERIALS AND METHODS

This work was conducted between March 2023 to November 2023 in the Bacteriology Department, Animal Health Research Institute, Sohag branch and Centre of Biotechnology at Alexandria University.

Bacterial growth and culture media:

This study included 20 *E. coli* O157:H7 strains, 20 *E. coli* non-O157:H7 strains, and 10 other strains belong to *Enterobacteriaceae* family. They were obtained from the Centre of Biotechnology at Alexandria University. All strains were kept on tryptic soy slant agar (Table 1).

Peptide nucleic acid (PNA) probe layout:

To identify prospect beneficial oligonucleotides to utilize as probes, 16S and 23S rRNA gene sequences accessible at the Centre for Biotechnology Information were selected. Using the ClustalW program from the European Bioinformatics Institute, sequence alignment was assigned to select potential target regions. This design based on a conserved region within 23S rRNA of *E. coli* O157:H7 isolates.

The persuading probe sequence was chosen: 5'CAA CAC ACA GTG TC-3'. This sequence hybridizes between sites 1169 and 1183 of *E. coli* O157:H7 strain TW14359 (accession number CP_001368); hence, it was termed EcoPNA1169.

Theoretical evaluation of PNA probe implementation:

Following probe design, its theoretical sensitivity and specificity were determined through performance evaluation. These parameters were evaluated using the Probe Check software, available through the ARB Silva database. The theoretical evaluation has used only high-quality sequences of at least 1,900 base pairs, along with *E. coli* strains of the chosen serotype. The probe was then tested versus both large and small ribosomal subunit databases, ([LSU]; 23/28S) and ([SSU]; 16/18S) respectively.

Hybridization procedure:
The study used hybridization temperatures from 53 to 61°C and investigated numerous hybridization times of 30, 45, 60, and 90 minutes, utilizing 80% ethanol for fixation. A standard technique was applied as each strain smear was immersed with in paraformaldehyde 4% (wt/vol), persuaded by ethanol 50% (vol/vol) for ten minutes each before air-drying.
Each smear received twenty microliter of a hybridization solution comprising a mixture of dextran sulfate 10% (wt/vol), ten millimeters sodium chloride, sodium pyrophosphate 0.1% (wt/vol), formamide 30% (vol/vol), Ficoll 0.2% (wt/vol), five millimeters disodium EDTA, fifty millimeters Tris-HCl (pH 7.5), 0.1% Triton X-100 (vol/vol) and two hundreds nanometers EcoPNA1169 probe.
Specimens were protected by coverslips, placed in humid containers, and incubated for forty five minutes at 59°C. Afterward, the coverslips have been taken off and the slides have been put into a pre-heated (59°C) washing sol. comprising fifteen millimeters sodium chloride, 1% Triton X-100, and five millimeters Tris base (pH 10). Washing was conducted at 59°C for thirty minutes, and the slides were kept for air dryness. The slides were kept in darkness for 24 hours before being examined under a microscope. The experimental sensitivity and specificity of the probe were assessed **(Almeida et al. 2013)**.

Selection of the milk samples for inclusion in the study:
Non-heat-treated milk samples were subjected to conventional culture methods to select 100 negative samples for *E. coli* O157:H7 serotype following ISO 16654:2001 **(Tozzoli et al. 2019)**.

Inoculation of milk samples with *E. coli* O157:H7 serotype:
A loopful of *E. coli* O157:H7 - CECT 4267 was added to twenty millimeters of tryptic soy broth (TSB) and incubated in an orbital incubator at 37°C and 120 rpm for 18 hours. Bacterial suspensions were diluted in a phosphate-buffered saline solution (PBS) and set to a concentration of 0.5 McFarland standards matching to nearly 1 $\times 10^8$ cells/ml. These were. The cell concentrations were further diluted in PBS to gain 1×10^2 CFU/ml asserted by plating on tryptic soy agar.
Twenty-five milliliters obtained from each milk sample were mixed with 225 ml of modified tryptic soy broth supplemented with novobiocin.
The diluted milk specimens were then artificially inoculated with *E. coli* O157:H7 strain at conc. of ten CFU per 25 ml milk and one CFU per 25 ml milk, then incubated in an orbital incubator at120 rpm, 37°C and for18 hours.

Detection of *E. coli* O157:H7 serotype in examined milk samples by conventional culture technique:
The detection of *E. coli* O157:H7 serotype by application of culture-based methods that done following ISO 16654:2001 **(Tozzoli et al. 2019)**.
A loopful from the artificially contaminated milk sample was inoculated into Eosin Methylene Blue (EMB) and sorbitol MacConkey's agar (SMA) and then incubated at 40°C for one day according to **Collee et al. (1996)**.
Typical colonies of *E. coli* O157:H7 produce large circular blue-black colonies with a green metallic sheen on EMB agar but are colorless ones on Sorbitol MacConkey agar **(Harrigan and McCance 2014)**.
Microscopic examination: the direct film was prepared from pure culture and stained by Gram stain to observe the morphological characteristics of *E. coli* O157:H7 strain which is Gram-negative short rods bacilli, that occur singly or in pairs **(Cheesbrough 2006)**.

Biochemical Confirmation of *E. coli* O157:H7

serotype; a single colony of a pure culture from SMA were streaked onto nutrient agar plate then incubated at 37°c for 24 hrs. Catalase, Oxidase, Methyl red, Voges-Proskauer (VP), Indole, and Citrate tests were carried out to confirm the existence of *E. coli* O157:H7 in the test specimens **(Cerqueira et al. 2008)**.
Serological identification of *E. coli* O157:H7 serotype was performed; presumptive *E. coli* O157:H7 isolates were subjected to latex agglutination test **(Kok et al. 1996)**.

Detection of *E. coli* O157:H7 isolates in milk specimens by PNA-FISH:
Fifteen µl of each artificially contaminated milk sample were added to 15µl of 1% Triton X-100 sol. And poured directly on the microscope slides. All specimens were dried for five minutes at 59°C, and then hybridization was carried out **(Almeida et al. 2013)**.
Following mounting with a drop of non-fluorescent immersion oil (Merck, USA), the smears were examined using an Olympus BX51 epifluorescence microscope (Olympus Portugal SA, Porto, Portugal). Detection of the Alexa Fluor 594-labeled EcoPNA1169 probe was achieved with a filter set (excitation 530-550 nm, barrier 570 nm, emission >591 nm). The absence of auto-fluorescence was confirmed using other filter sets.
For each sample, a negative control was applied simultaneously persuaded all described steps, but no probe was involved through the hybridization process.
The Olympus CellB software (Olympus Portugal) was used to capture all images at ×1000 magnification degree.

Statistical assessment:
The correlation between FISH and conventional culture method (Correlation coefficient r) was determined. Receiver operating characteristic (ROC) curve assessment, specificity, sensitivity, test agreement, 95% confidence intervals, the accuracy and Youden index were measured via a statistical software program (MedCalc Windows, version 22.0.18, MedCalc Software, Mariakerke, Belgium, https://www.medcalc.org).
Statistical significance was supposed at $P<0.05$. Inter-rater agreement was calculated by Weighted Kappa (K), interpreted as pursues: poor; < 0.20, fair; 0.21–0.40, moderate; 0.41–0.60, good; 0.61–0.80, and very good; 0.81–1.00 **(Altman 1990)**.

RESULTS

To assess the probe's experimental sensitivity and specificity, the PNA-FISH technique was carried out on 50 strains (Table 1); twenty strains were *E. coli* O157 H7 serotype, other 20 strains were *E. coli* O157 non-H7 and ten strains were belonged to other *Enterobacteriaceae*. Results showed that the hybridization only takes place with *E. coli* O157:H7. On the other side, two *E. coli* O55 strains (*E. coli* CECT 730, *E. coli* CECT 731) and two O157 non-H7 strains (negative for H7 antigen0; (*E. coli* CCC-26-12, *E. coli* CCC-18-12) were detected. All examined *Enterobacteriaceae* strains other than *E coli* were not detected by the FISH method (Table 1); therefore, accuracy, specificity, and sensitivity of the FISH method were 92%, 86.67%, and 100% respectively with Confidence Interval of 95% (82.5% to 98.4%) (Table 2) (Figure 1).
PNA-FISH method probe was tested in one hundred non-heat-treated milk samples artificially contaminated with a strain *E. coli* O157:H7 CECT 4267 by conc. of ten CFU/25 ml milk and one CFU/25 ml milk then examined on epifluorescence microscope (Figure 2)
PNA-FISH results were compared with conventional culture method as a gold standard to determine the accuracy, specificity, and sensitivity values.
The positive samples by the conventional culture method were 100 (100%) while FISH detected 97 (97%) samples and failed to detect three samples (Table 3).
Relied on these data, PNA-FISH accuracy, specificity, and sensitivity were 98.5%, 100% and 97% respectively with a Confidence interval of 95% (95.7% to 99.7%) (Table 4) (Figure 3).
Weighted Kappa 0.97 indicated that there was a very good agreement between the conventional culture method and the FISH technique.

Table 1. Fluorescence In Situ Hybridization test on examined bacterial strains and species.

Strains and species	Number	Details	FISH	
			Positive	Negative
E. coli O157:H7	20	*E. coli* CECT 4267, *E. coli* CECT 4782, *E. coli* CECT 4783, *E. coli* CECT 5947, *E. coli* NCTC 12900, *E. coli* CCC-1-12, *E. coli* CCC-5-12, *E. coli* CCC-7-12, *E. coli* CCC-10-12, *E. coli* CCC-11-12, *E. coli* CCC-12-12, *E. coli* CCC-13-12, *E. coli* CCC-14-12, *E. coli* CCC-15-12, *E. coli* CCC-16-12, *E. coli* CCC-17-12, *E. coli* CCC-6 -12, *E. coli* CCC-23-12, *E. coli* CCC-24-12, *E. coli* CCC-25-12.	20 (100%)	---------
E. coli O157: not H7	20	*E. coli* CECT 352, *E. coli* CECT 504, *E. coli* CECT 533, *E. coli* CECT 727, *E. coli* CECT 730, *E. coli* CECT 731, *E. coli* CECT 736, *E. coli* CECT 740, *E. coli* CECT 744, *E. coli* CECT 832, *E. coli* CECT 4537, *E. coli* CECT 4555, *E. coli* CCC-18-12, *E. coli* CCC-26-12, *E. coli* CECT 352, *E. coli* CECT 504, *E. coli* CECT 533, *E. coli* CECT 730, *E. coli* CECT 736, *E. coli* CECT 740.	4 (20%)	16 (80%)
Other *Enterobacteriaceae*	10	*Shigella boydi* ATCC9207, *Salmonella* Typhimurium NCTC12416, *Salmonella* Typhi SGSC3036, *Klebsiella Pneumonae* ATCC11296, *Shigella Sonnei* ATCC25931, *Klebsiella Oxytoca* ATCC 13182, *Citrobacter freundii*, *Citrobacter Koseri*, *Enterobacter helveticus*, *Enterobacter Cloacae*.	----------	10 (100%)

Table 2. Statistical analysis of FISH on examined bacterial strains and species.

Statistical parameter	Mean	Rang
Area under the ROC curve (AUC)	0.933	0.825 to 0.984
95% Confidence interval	95	82.5 to 98.4
Youden index J	0.8667	0.8315 to 1
Sensitivity	100	83.157% to 100.000%
Specificity	86.67	69.278% to 96.245%
Accuracy	92	80.766% to 97.777%

Table 3. Comparison between FISH test and conventional culture method for detection of *E coli* O157:H7 in artificially contaminated milk samples.

Evaluation method	Positive samples	Negative samples
The gold standard (conventional culture)	100 (100%)	---------
FISH	97 (97%)	3 (3%)

Table 4. Statistical analysis of FISH in comparison to conventional culture method.

Statistical parameter	Mean	Rang
Area under the ROC curve (AUC)	0.985	0.957 to 0.997
95% Confidence interval	95	95.7 to 99.7
Youden index J	0.97	0.9148 to 1
Sensitivity	97	91.482% to 99.377%
Specificity	100	96.378% to 100.000%
Accuracy	98.5	95.679% to 99.690%
Weighted Kappa[a]	0.97	0.93632 to 1
Correlation coefficient r	0.9704	0.9611 to 0.9776

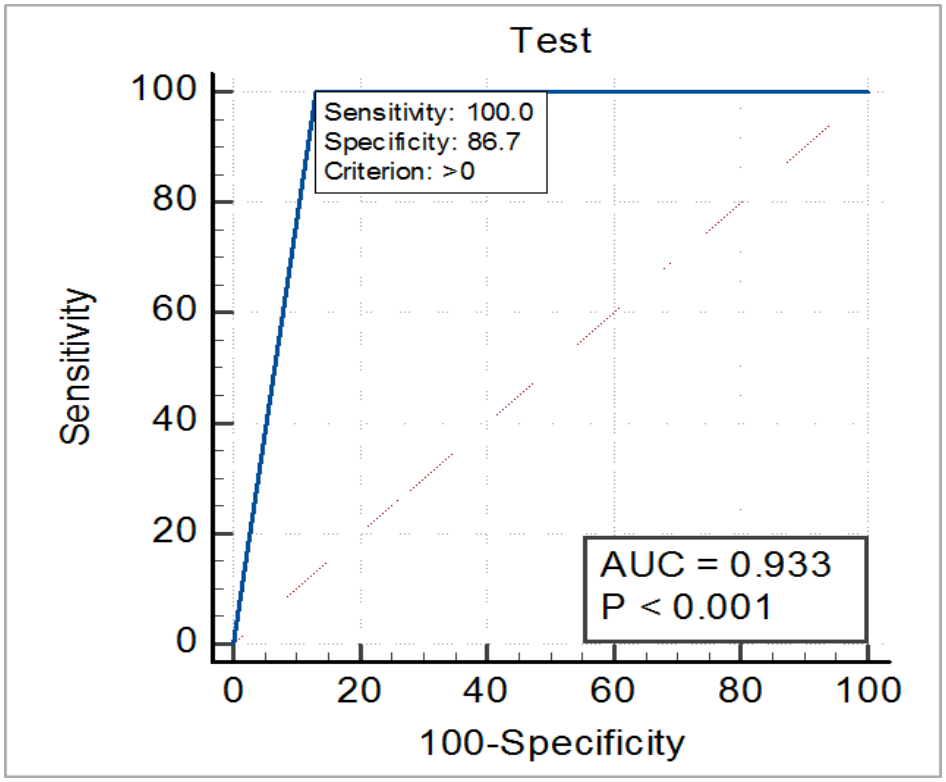

Figure 1. Sensitivity, specificity, and AUC of FISH on examined bacterial strains and species.

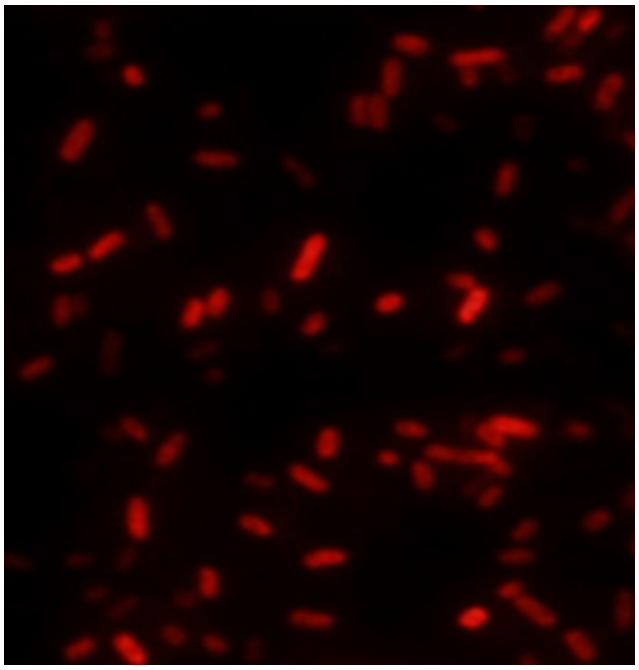

Figure 2: PN A-FISH result for non-heat-treated milk samples artificially contaminated with 10 CFU/25 g of *E. coli* O157:H7 CECT 4267.
Visualization of the *E. coli* O157:H7 microscopic field in the red channel, appeared as red fluorescent on black background by Olympus BX51 epifluorescence microscope with filter sensitive to the Alexa Fluor 594 molecule attached to the EcoPNA1169 probe.

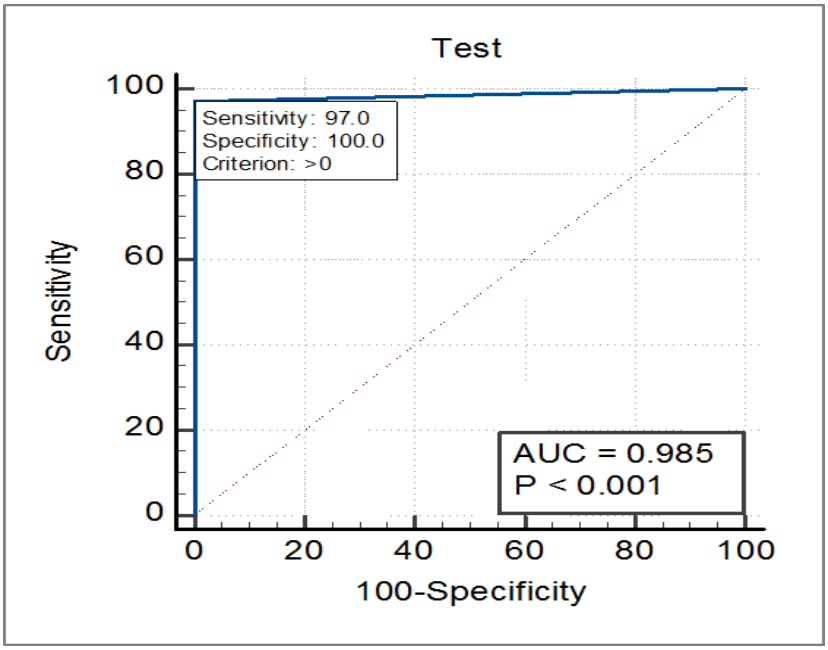

Figure 3. Sensitivity, specificity, and AUC of FISH in comparison to conventional culture method.

DISCUSSION

PNA-FISH is known as a reliable microbial detection and identification technique **(Cerqueira et al. 2008)**. PNA-FISH purposes rRNA sequences, which are universal phylogenetic markers. EcoPNA1169 is combined with a probe aiming a particular sequence of the EHEC group. It can assess the wanted recognition limit of one CFU **(Spano et al. 2005)**.
PNA-FISH has safety, high stability, high sensitivity, cell visualization capacity, numerous color labeling capacity and short detection time **(Wagner and Haider 2012)**.

Oliveira et al. (2024) reported that PNA-FISH is a promising alternate for detection *Campylobacter spp.* in food specimens. The PNA-FISH technique exhibited specificity and sensitivity values of 96.9% and 92.0%, respectively, compared with the conventional culture method.
The FISH had a short determination time and a high accuracy in the identification of coliform in domestic wastewater and simulated water specimens **(Kuo et al. 2020)**.

Almeida et al. (2010) used a peptide nucleic acid (PNA) probe as FISH technique for the rapid detection of *Salmonella spp*. The probe's theoretical sensitivity and specificity were 100%. They recommended that this procedure be used as an alternative to culture-based techniques.
In the present study, FISH represented a quick, precise technique for the recognition of *E. coli* O157:H7. The accuracy, specificity, and sensitivity of the FISH method were 92%, 86.67%, and 100% respectively with Confidence Interval of 95% (82.5% to 98.4%).

This study found that FISH is highly sensitive for the determination of *E. coli* O157: H7. On the other side, two *E. coli* O55 isolates and two O157 non-H7 strains (-ve for H7 antigen) were recognized. The match with two O157 non-H7 strains points that the technique is specific for O157 regardless the H7 and toxin existence. This is a merit for the recognition of other EHEC as *E. coli* O157 –ve H7 strains **(Alpers et al. 2009)**.

The match of 2 *E. coli* O55:H7 serotype isolates may be a weak point. Similarly, enzyme-linked immunosorbent assay (ELISA) and PCR techniques designed to identify O157 strains have also shown cross-hybridization with O55 strains **(Arthur et al. 2005)**. This may occur because O55:H7 is the serotype most closely linked to O157:H7 **(Zhou et al. 2010)**. Both serotypes cause diarrhea via an adherence-effacement mechanism due to pres-

ence of the locus of enterocyte effacement (LEE) island (Garmendia et al. 2005). *E. coli* O157:H7 is thought to be originated from *E. coli* O55:H7 that, through evolution, gained bacteriophage encoding Stx1 and/or Stx2 toxins **(Nataro and Kaper 1998)**.

In this study, the PNA-FISH method probe was examined in one hundred non-heat-treated milk specimens artificially inoculated with *E. coli* O157:H7-CECT4267 in comparison to the conventional culture method with a concentration of ten CFU/ twenty five ml milk and one CFU/ twenty five ml milk.

Conventional culture technique detected *E. coli* O157:H7 in milk specimens with a percentage of 100% while FISH detected 97 (97%) samples and failed to detect three samples.
PNA-FISH accuracy, specificity, and sensitivity were 98.5%, 100% and 97% respectively with a Confidence interval of 95% (95.7% to 99.7%). Also, there was a very good agreement between the conventional culture method and the FISH technique.

Although conventional culture technique is more precise for the recognition of *E. coli* O157:H7, it is a time-consuming method, which often extends to 3–7 days, requiring professional staff and a complicated operation process to detect the pathogen.
FISH technique can detect E*. coli* O157:H7 in 1 day, so it is recommended to be used as an alternative to culture-based techniques.

The pre-enrichment is a critical step in different microbial recognition techniques, primarily due to the low count of desired bacteria, a high level of other competing microorganisms, and the limitations of the detection technique **(Vimont et al. 2006)**. It is crucial to carefully optimize this step to attain high sensitivity values.
The enrichment step is usually recommended because of *E. coli* O157:H7 usually exist in low contamination levels in the food samples **(Garmendia et al. 2005)**. This step can be done using many kinds of culture media, ranged from complex enrich media (such as BPW or TSB) to just selective media, such as *E. coli* (EC) broth or Gram-negative (GN) broth **(Vimont et al. 2006)**.

TSB is known as the most utilized enrichment broth. Other additives; antibiotics as novobiocin (the most commonly used), cefsulodin, cefixime, and vancomycin, in addition to other selective materials e.g., bile salts which used to prohibit the non-*Enterobacteriaceae* strains **(Vimont et al. 2006)**.

The pre-enrichment step allows FISH to recognize *E. coli* O157:H7 with the determination limit of one CFU/ twenty five millimeters milk **(Oliveira et al. 2024)**.

Concerning the enrichment temperature, it is obvious that the incubation temperature is not linked to the type of serogroup **(Vimont et al. 2006)**. However, some authors demonstrated that O157:H7 strains typically have an ideal temperature of approximately 40°C. This implies that adjusting temperature can control the existing microorganisms and support the growth of *E. coli* O157. In reality, the ISO 16654:2001 advised a pre-enrichment step at 41.5°C for mTSBN O157 determination in food samples **(Tozzoli et al. 2019)**.

CONCLUSION

EcoPNA1169 is greatly sensitive and specific to *E. coli* O157:H7 serotype isolates; whilst, some cross-hybridization can take place with the closely linked O55:H7 serotype. PNA-FISH method can detect pathogen with concentration of one CFU per twenty five ml of non- heat-treated milk specimens. Utilizing the PNA-FISH technique can reduce detection time by a minimum of 3 days in recognition of *E. coli* O157:H7 paralleled to the conventional method. Finally, a comparison of data to those of the conventional culture procedure has revealed high sensitivity, specificity and accuracy 97%, 100%, and 98.5% respectively with a Confidence interval of 95% (95.7% to 99.7%).

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