Original Article

Sensitivity and Reliability of Two Antibodies in Detecting *E. coli* in Meat and Water

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Abstract

Detection of *E. coli* in water and food constitutes a major challenge. However, the outer membrane proteins of this Gram-negative bacterium represent an excellent choice for their detection and diagnosis. Two outer membrane proteins: A and C, play important roles as drug modulators and in cellular permeability for this bacterium. Here we employed the antibodies raised against these two outer membrane proteins for the development of dot-blot immunoassays to detect this bacterium in water and meat samples. This immune-based assay was not only sensitive and reliable but rapid and cheap to perform. Sensitivities and specificities were demonstrated at the lowest detection limits of just two to three cells per milliliter, while the upper limit reached 107 cells per milliliter. In conclusion, the simplicity and extremely high resolution of this colored-based immunoassay test would be of great importance for untrained food and/or water inspectors or laboratory personnel to use with immediate visual results.

Keywords: Biosensor, Immunosensor, Dot blot, PVDF sensor, *E. coli*

Introduction

Escherichia coli (*E. coli*) is a large, diverse group of bacteria, found in the environment, foods, and intestines of people and animals. Although most strains of *E. coli* are harmless, others can cause serious diseases. Over 700 strains or serotypes of *E. coli* do exist in nature, water, and foods. *E. coli* possesses quite a few virulence factors encoded on mobile genetic elements and/or plasmids or localized in pathogenicity islands. Some types of *E. coli* can cause diarrhea, while others cause urinary tract infections, respiratory illness, and pneumonia due to various virulence factors [1, 2]. These virulence factors included endotoxin (lipopolysaccharides, and polysaccharide capsule), exotoxin (Shiga toxin), invisins, adhesins, and iron acquisition factors [3-5]. The health hazards of *E. coli* and its associates had driven the development of a huge number of diagnostic and detection kits to avoid their unwanted effects. The essences on which the different kits were built can be grouped into 1) Plate counting methods, 2) gold or similar nanoparticles methods, 3) solid phase flow cytometry methods, 4) PCR-based methods and 5) immunological methods [6-8]. All these kits rely on the use of expensive instrumentation which is complicated to operate, needs time, and were relatively expensive, [9-11]. Therefore the present study is designed to explore a simple, accurate, and easy tool for the detection of *E.coli* in food samples.

Materials and Methods

Bacterial Growth Conditions

Seventeen clinical bacterial strains were collected from the medical school laboratories at Mansoura University, Egypt. Only six out of them turned out to belong to the bacterium *E. coli.* The selected isolates were grown at 37°C, 150 rpm for 10h. Two growth media were used: 1) LB broth for general purposes and 2) Tryptone Bile X-glucuronide (TBX) agar medium a selective medium for enumeration and differentiation of *E. coli* from other coliforms [12]. All the obtained *E. coli* isolates were subjected to Gram stain and biochemical tests to confirm their purity. Also, standard *E. coli* ATCC10536 was used.

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Chemicals and Buffers

Anti-*E. coli* OmpA, anti-*E. coli* OmpC primary antibodies, and anti-*E. coli* OmpA and anti-*E. coli* OmpC secondary antibodies HRP-conjugated were obtained from (MYBIOSOURCE), 3,3´,5,5´ Tetramethylbenzidine (TMB), horseradish peroxidase (HRP) substrate was obtained from Thermo-Scientific, USA 1x Transfer buffer (3g Tris, 14.4g glycine, 200ml methanol, and pH adjusted to 8), 10x TBS buffer (24.2g Tris, 80g NaCl, and adjust pH to 7.6), 1x washing buffer (TBST) (100 ml 10x TBS buffer; 1ml Tween-20 and 899 dH_2O); 5% blocking buffer (5g non-fat dried milk, 100 ml TBST, antibody dilution buffer (1g non-fat dried milk, 20 ml TBST) [13].

Protein Profiling

Six *E. coli* isolates and a single colony of *Enterobacter* sp. were grown in 10ml LB broth media at 37ºC, 150rpm for 8 h and harvested by centrifugation. The cell pellets were analyzed for the detection of the OmpA and C proteins using denatured polyacrylamide gel electrophoresis (SDS-PAGE). The wells of the SDS-PAGE were cleaned by running at 80V for 30min before loading the denatured proteins. The electrophoresis was run at 80 volts until protein samples emerged from the stacking gel, then the voltage was raised to 100 until the loading dye was close to running off the bottom of the gel [14].

Western Blot Analysis

E. coli OmpA and C proteins were detected by Western blot using polyclonal antibodies [15-18]. This method was initially based on the transfer of proteins resolved by SDS-PAGE to solid membrane support PVDF (polyvinylidene difluoride, Millipore, Prod. No. IPV H00010, pore size 0.45 µm) by capillary transfer method [13, 19]. Nonspecific sites on the membrane were blocked by blocking buffer for 2 h at room temperature or overnight at 4°C. Then washed off with 1x TBST buffer three times for 5 min each before adding the primary antibody at the proper dilution (anti-*E. coli* OmpA, anti-*E. coli* OmpC) a separate membrane was used for each antibody and incubated for 1h in either room temperature or overnight at 4ºC. After removal of the antibody solutions, each membrane was washed 3 times with 1x TBST buffer for 5 min. Then each membrane was incubated with the appropriate dilution of the secondary antibody (anti-*E. coli* OmpA and anti-*E. coli* OmpC HRP-conjugated) for 1 h. The solutions were discarded, and the membranes were rinsed/washed three times by 1x TBST buffer (3 x 10 min). The protein-antibody complexes were visualized by adding 50µL TMB substrate for 10-30 min in the dark.

Dot Blot Technique

Avoiding the complexity of the Western blot technique, we resorted to Dot blot analysis using PVDF membrane strips [10, 20, 21]. The PVDF membrane strips (0.45 µm) were activated by dipping into 100% Methanol for 15 sec, soaked in distilled water for 2 min, and directly equilibrated for 5 min by TBST buffer. Each of the PVDF membrane strips was positioned on a pre-wetted filter paper stack before spotting 2µl of each protein sample (*E. coli* isolates, water or food). Proteins were fixed by drying at room temperature for 90 min [22] **(**https://www.agrisera.com/en/info/dot-blot-method-description.html). Sites between protein spots (dots) were blocked for 30min at room temperature (RT), washed three times (5min each), and each strip was incubated in 10ml of diluted primary antibody (1:500 to 1: 50,000) for 1h with agitation at RT, followed by three washes (5 min each) with washing buffer. Each membrane strip was soaked in 10 ml dilution buffer containing the HRP-conjugated secondary antibody for 1h at RT before being washed with TBS-T three times (5 min. each). Visualization of reactions between OmpA or OmpC and their respective antibodies were evident after the addition of 50µL 3,3',5,5'-Tetramethylbenzidine (TMB) substrate and incubation for 1-2h at dark [23].

Specificity of OmpA and C Immunosensors

Enterobacter cloacae, a member of human's normal flora and Enterobacteriaceae family, was used to test the specificity of the OmpA and OmpC immunosensors. A cell pellet of an overnight grown colony of *Enterobacter cloacae* was dissolved in 1ml sterile H_2O and six serial dilutions were prepared for the dot blot immunoassay [24].

Determining the Limits of Detection of E. coli

An overnight culture of *E. coli* was serially diluted, and the number of colony-forming units (CFU) of bacteria in each dilution was determined and expressed by plating on TBX agar plates. All dilutions were used in dot blot assays to determine the lowest level of detection.

Effect of Physical Factors on the Efficiency of OmpA and/or OmpC PVDF Immunosensors

Time Effects

After activation of the PVDF membrane, 2 µl *E. coli* isolate was directly spotted on PVDF membrane strips, incubated for 0.5, 1, 5, 10, 30, and 60 min. The primary antibodies were added and incubated for 30 min and washed 3 times with PBS, then, 20 µl secondary antibodies were added for30 min. followed by PBS washing and finally, 20 µl TMB substrate was added in a dark room at 37ºC for 1-2 min for color visualization.

Effect of Temperature

After activation of the PVDF membrane, 2 µl *E. coli* isolate was directly spotted on PVDF membrane strips and left for air dry. The primary antibodies were added and incubated for10 min at 4, 25, 37, and 60ºC and washed 3 times with PBS. Then, 2 µl secondary antibodies were added for 10 min at 37ºC, followed by PBS washing, and finally, 5 µl TMB solution was added in a dark room at 37ºC for 1-2 min.

Effect of Salt Concentration

E. coli strain no. 4 was serially diluted in a 9 ml NaCl solution of different concentrations (0.1, 0.6, and 1M) from

10^{-1} to 10^{-7} for each concentration, then tested using the fabricated dot blot immunoassay.

Effect of pH

To get the greatest indication response, different pH ranges (6.2, 6.6, 7.0, 7.4, and 7.8) were selected to be tested on the PVDF immunosensor [25].

Application of the Constructed PVDF Immunosensor for Examining Meat Products at Local Markets

The constructed PVDF immunosensors were directly pressed against the surface of different food samples (frozen beef, frozen chicken, raw beef, and raw chicken) for different periods (5, 10, 15, and 20 min.). The membranes were incubated with the primary antibodies for 10 min. at 37ºC, washed 3 times with buffer, followed by incubation with HRP-conjugated secondary antibodies at 37º C for 10 min. After washing 3 times with buffer, the substrate was added in a dark room at 37ºC for 1-2 min for color development.

Image's Analysis

Analyses of PVDF immunosensors images were performed using the public domain or the open platform ImageJ program developed at the National Institutes of Health (NIH), USA. The digitization of each dot/spot resulted in an Integrated Density Value (IntDen) of that dot proportional to the number of *E. coli* cells spotted**.**

Results and Discussion

All six *E. coli* isolates used during this study were grown on the selective medium, TBX, and McConkey agar. They produced deep green blue colonies and red colonies, respectively which conforming the purity of the isolates. The uniformity of all isolates was further ensured by the protein banding patterns and the MALDI-TOF-MS analyses**.** The m/z values of all isolates were the same, but the heights of the spectra were variable. Further data analyses showed the molecular weight of total cellular proteins of the five local isolates and the standard *E. coli* ATCC10536 with the concentration that can be inferred from the area under the peak.

Western Blot Detection

The Western blot technique of *E. coli* OmpA and OmpC **(Figure 1)** showed the single bands of molecular weight 35.177 KDa 40.4KDa representing *E. coli* OmpA and OmpC respectively. While dot blot assay analyses of the interaction between *E. coli* dilutions spotted on the PVDF membranes and Rabbit raised anti-*E. coli* OmpA and anti-*E. coli* OmpC showed direct proportional to the *E. coli* counts per spot. The color intensities illustrated in the presented quantitative dot blot (QDB) curves confirmed the quantitative correlation between the *E. coli* number/spot and the integrated intensity of color. The PVDF membrane data were followed by its QDB analysis to make it easy to evaluate the accuracy and sensitivity of the constructed membrane biosensors. The results of *Enterobacter* sp. were used as a negative control, since it lacks the exact domain of the outer membrane proteins A and/or C used to raise the antibodies used in this study. The MALDI analyses confirmed the identification of the six isolates as *E. coli* as suggested by the databases of the manufacturer of the instrument: Bruker, Germany.

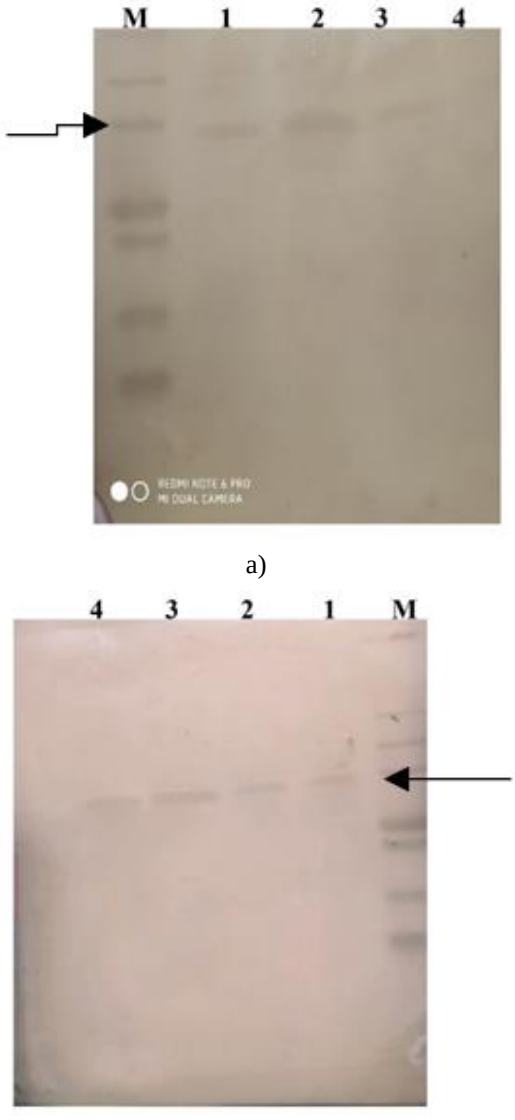

Figure 1. western blot analysis of *E. coli* ompA and ompC. (a) revealed *E. coli* ompA of molecular at molecular weight 55.3 KDa and (b) revealed *E. coli* ompC of molecular weight 40.4KDa.

Dot Blot Blotting and Detection

The dot blot technique was carried out to determine the sensitivity of the immunoassay, compare the sensitivity of the two primary antibodies (A+C) and detect the specificity of the immunoassay against the bacterium (*Enterobacter* sp.). The specificity of dot blot assay against a different enteric bacterium (*Enterobacter sp.)*, which lacks OmpA or C confirmed its specificity and inability to cross-react**.** The

reaction was influenced by the time that showed a directly proportional relationship and intensity of blue color developed **(Figures 2)** below, either dot blot on PVDF membranes or solid-state glass slide sensors.

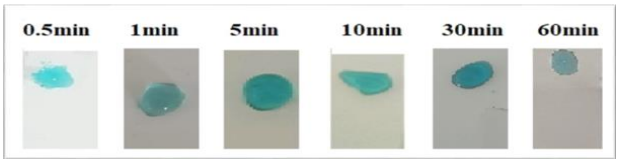

Figure 2. Effect of time (0.5, 1, 5, 10, 30, and 60 min) on PVDF immunosensor using an anti-OmpA antibody. The results indicated that the intensity of color increased with increasing incubation time.

The appearance of blue color indicates a positive result while the absence of the color indicates a negative result. The intensity of the color corresponds to the concentration, or the number of *E. coli* cells spotted. Moreover, the negative results of *Enterobacter* sp. with the two antibodies support the specificity and sensitivity of our protocol.

Detection Limits of the Dot blot Immunosensors

The total count of each serial dilution for each of the six *E. coli* isolates is shown in **Table 1**. The color intensities of each spot on the PVDF immunosensors were digitized and converted into a number as shown in **Table 2**. These intensities were used to calculate the CFU/ml for each spot. The results of the total *E. coli* count are in **Table 3** indicates the detection limits that ranged from three CFU/ml to $2*10^7$ CFU/ml.

Table 1. Total *E. coli* (Ec) count per dilution used in the dot-blot assays

Ecoli No.	Dil.	CFU/mL	Ecoli No.	Dil.	CFU/mL	Ecoli No.	Dil.	CFU/mL
	S*	$16*10^6$		S	$13*10^6$		S	$2*10^7$
	10^{-1}	1910		10^{-1}	1000		10^{-1}	1100
	10^{-2}	1480		10^{-2}	900		10^{-2}	915
4	10^{-3}	920	10	10^{-3}	779	14	10^{-3}	475
	10^{-4}	250		10^{-4}	489		10^{-4}	334
	10^{-5}	60		10^{-5}	130		10^{-5}	200
	10^{-6}	20		10^{-6}	30		10^{-6}	10
	S	10^7		S	$58*10^5$		S	$11*10^6$
	10^{-1}	1010		10^{-1}	800		10^{-1}	990
	10^{-2}	900		10^{-2}	600		10^{-2}	888
9	10^{-3}	802	11	10^{-3}	320	17	10^{-3}	692
	10^{-4}	309		10^{-4}	150		10^{-4}	340
	10^{-5}	100		10^{-5}	48		10^{-5}	110
	10^{-6}	15		10^{-6}	3		10^{-6}	23

S* undiluted overnight culture. Dil. Dilution factor, CFU/mL: Colony Forming Unit/mL

Table 2. The local isolates grouped after the MALDI-TOF-MS analysis

Ec. No.	Isolate Name	Score	NCBI Identifier
4	*Escherichia coli* DSM 1103_QC DSM	1.84	562
Std	*Escherichia coli* DSM 1103_QC DSM	1.93	562
9	*Escherichia coli* DSM 1576 DSM	1.82	562
10	*Escherichia coli* MB11464_1 CHB	2.41	562
14	*Escherichia coli* DSM 1103_QC DSM	2.29	562
17	*Escherichia coli* DSM 682 DSM	1.9	562

Table 3. Limits of detection of *E. coli* using PVDF-based immunosenors

E. coli Isolate number	Bacterial Dilution	CFU/ml	IntDen of anti-OmpA-based sensor	IntDen of anti-OmpC-based sensor
	S	$16*10^6$	261264	93357
	10^{-1}	19100	62264	58673
	10^{-2}	1480	9312	17881
4	10^{-3}	920	8067	10494
	10^{-4}	250	4168	9828
	10^{-5}	60	2253	5553
	10^{-6}	10	841	2239
	S	10^7	53166	73241
	10^{-1}	1010	30034	37237
	10^{-2}	900	14000	14603
9	10^{-3}	802	8043	8528
	10^{-4}	309	5324	6695
	10^{-5}	100	3379	4720
	10^{-6}	15	1166	2914
	S	$13*10^6$	79182	85282
	10^{-1}	1000	25986	47034
	10^{-2}	900	17414	21963
10	10^{-3}	779	10544	17837
	10^{-4}	489	4328	10416
	10^{-5}	130	3000	5027
	10^{-6}	10	1500	2058
	S	$58*10^5$	64155	65518
11	10^{-1}	800	18884	20692

	10^{-2}	600	8166	13509
	10^{-3}	320	7241	9149
	10^{-4}	150	4153	7581
	10^{-5}	48	2900	3326
	10^{-6}	3	1120	1250
	S	$2*10^{7}$	44971	96889
	10^{-1}	1100	25986	47144
	10^{-2}	915	18724	19971
14	10^{-3}	475	9342	10127
	10^{-4}	334	6826	5829
	10^{-5}	200	4614	3270
	10^{-6}	10	1620	2008
	S	$11*10^{6}$	99249	95229
	10^{-1}	990	44833	45381
	10^{-2}	888	14555	27795
17	10^{-3}	692	10998	12741
	10^{-4}	340	8650	7624
	10^{-5}	110	5339	5388
	10^{-6}	9	3427	2262

E. coli Isolates

The curves of sensitivity (blue color intensity) were directly proportional to the concentration of the *E. coli* cells number. The disappearance of the blue color is considered the cut value of detection or sensitivity limit. Moreover, the colored spots were digitized, and the intensity of color was quantified using the Image J computer program.

Temperature Effects on the Efficiency of the Biosensor

The impact of incubation temperature was checked in the range of (4, 25, 37, and 60°C) by both dot blot analysis and glass immunosensor. It was found that the maximum color intensity materialized and optimized at 37°C. Overall the intensity of the color (IntDen) increased with time, peaked at 37, and dropped at 60°C; as follows (48455; 92109; 267897; 51252 for the anti-OmpA-based sensor) and (49604; 75146; 121677; 50000, for the anti-OmpC-based sensor).

Salt concentrations Effects on the Efficiency of the Biosensor

The different NaCl concentrations (0.1, 0.6, and 1M) were intended to simulate the concentration of seawater (6M) and two other concentrations were included to study their effect on the quality of data obtained by our constructed sensors. E. coli strain no. 4 was serially diluted in 9 ml NaCl soln. of different concentrations (0.1, 0.6, and 1M), sterile distilled water was used as a control. The results indicated that the intensity of color decreased as the concentrations of NaCl increased **(Figure 3)**. Moreover, the salt concentration did not affect the color clarity.

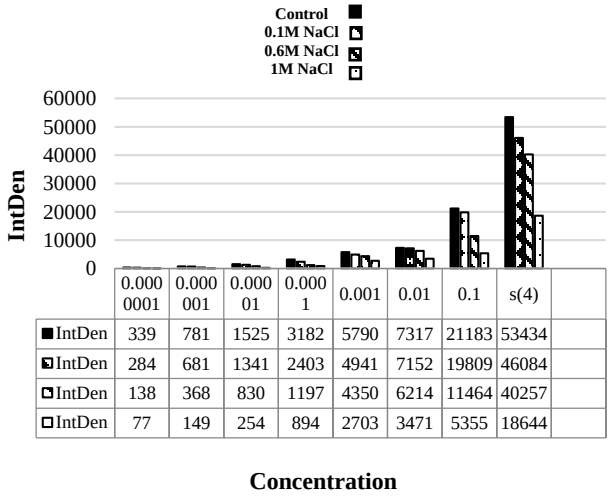

Figure 3. The curve of different NaCl concentrations effect on the efficiency of the anti-OmpA-based immunosensor. As the NaCl concentration increased the color intensity decreased.

Effect of pH on Immunosensor Efficiency

The intensity of color firstly increased with the increase of pH (6.2– 7.4) but decreased with any further increase in pH value (7.4–7.8). The maximum change of intensity occurred at a pH of 7.4 as shown **(Figure 4)**.

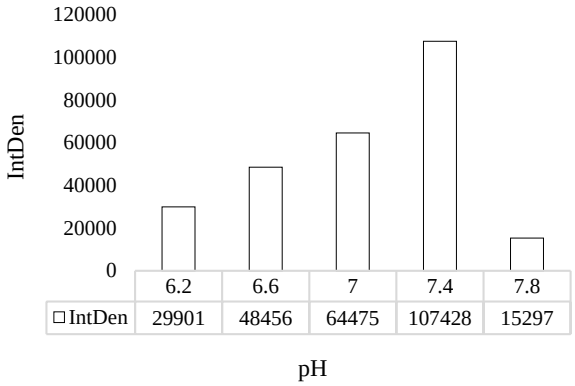

Figure 4. The curve of the effect of different pH values (6.2, 6.6, 7, 7.4. and 7.8) on the performance of anti-OmpA-based glass immunosensor. The highest color intensity occurred at pH 7.4.

Considering preciousness of human health and reviewing a recent World Health Organization [26] report on the widespread diarrheal disease among children under five years of age which contains horrible numbers about the infection (1.7 billion) and rate of death (525,000) related mainly to consumption of contaminated food or drinking water. The vulnerability of weaned infants to diarrheal illnesses when introduced to fluids and solid foods is so great that extreme care should be exercised to avoid mortality [27-29]. Therefore, the development of a highly sensitive, accurate, and reliable detection system for pathogens was a

cornerstone for the efforts of many scientists and industry investments. This focus has attracted our attention and derived us to develop a fast, cheap, and reliable method for detecting pathogenic agents in food and water and make it accessible for interested parties [30].

Here we detailed the fashioning of a fast, simple, highly sensitive, and reliable immunosensor for the detection of *E. coli* strains in food and different types of water. Moreover, the immediate onsite visual result (in less than an hour) makes this sensor attractive to untrained users and speeds up the decision-making process about the fate of the examined samples. Targeting *E. coli* for detection was deliberate because of its involvement in causing diseases and its function as a universal indicator of fecal contamination [31]. Despite the availability of a large number of detection kits for this bacterium, the complication in their construction and uses necessitates the development of simple, sensitive, reliable, and cheap methods instead. The electrophoresis result showed that there were proteins with 55.3 KDa and 40.4 KDa of molecular weight which is the protein A and C in the *E. coli* outer membrane. These proteins were the protein target for this immunological assay [32].

The dot blot technique is a technique for detecting, analyzing, and identifying proteins, like the western blot technique but differs in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane or paper substrate [23, 33]. When successfully developed, the dot blot assay for *E. coli* detection in food and water is both economical and time-saving. The assay requires less than 2h to be performed compared to days consumed in other traditional and widely used methods.

In this study, we fabricated a dot blot immunosensor targeting the OmpA and OmpC of *E. coli* for detection. A combination of specific antibodies raised against these two outer membrane proteins and their respective HRP-conjugated secondary antibodies were used. The positivity (blue) and/or negativity (colorless) of detection were visualized by color changes of TMB (3,3´,5,5´ Tetramethylbenzidine). The results suggested that OmpA was a better choice than anti-*E. coli* OmpC; as the color formation required less time to develop and the intensity was higher. Moreover, almost 1000 copies of the OmpA naturally exist in the outer membrane of *E. coli*. PVDF immunosensor was applied for the detection of *E. coli* in raw fresh and frozen samples of beef and chicken obtained from local markets. All examined samples gave positive results (blue color) due to the presence of E. coli on meat samples.

Glass immunosensors built using either anti-OmpA or anti-OmpC antibodies were very useful in the detection of *E. coli* contaminants in all samples examined, including *E. coli* cells [34, 35]. The six clinical *E. coli* isolates were examined using our indirect immunosensor. The appearance of blue color indicated that is a positive result as shown from.

This result was in agreement with published reports by Jiang *et al*., 2013 who stated that the specificity of antigen in all immunosensor devices is based on the antibody used. Moreover, a linear relationship was drawn between the intensity of the developed color by the current immunosensor and the number of *E. coli* that exist in the tested samples as reported by several authors [36, 37].

The specificity of dot blot assay against a different enteric bacterium (*Enterobacter sp.)*, which lacks OmpA or C confirmed its specificity and inability to cross-react [38, 39]. The present study showed that the reaction was influenced by time as there was a directly proportional relationship and intensity of blue color developed, either dot blot on PVDF membranes or solid-state glass slide sensors. The quantitative color intensities increased with the length of reaction duration from 0.5 to 60 minutes (0.5, 1, 5, 10, 30, and 60 min). In practice, the lowest time (0.5 min.) is a big advantage to the untrained user because it saves visualization time.

Incubation temperature was a vital factor affecting the immunological reaction. It was found that the maximum color intensity materialized and optimized at 37°C. Overall the intensity of the color (IntDen) increased with time, peaked at 37, and dropped at 60°C.

Regarding NaCl concentration, the results of the present study indicated that the intensity of color decreased as the concentrations of NaCl increased. Moreover, the salt concentration did not affect the color clarity. Regarding the Effect of pH on immunosensor efficiency, the study showed The intensity of color firstly increased with the increase of pH (6.2– 7.4) but decreased with any further increase in pH value (7.4–7.8). Many studies have shown that pH values have great effects on the performance of immunosensor.

Overall, the report proves to be an effective tool that players can use to gain a competitive edge over their competitors and ensure lasting success in the global *E. Coli.* Diagnostic Testing market. All the findings, data, and information provided in the report are validated and revalidated with the help of trustworthy sources. The analysts who authored the report took a unique and industry-best research and analysis approach for an in-depth study of the global *E. Coli* diagnostic testing market.

Although our test and the litmus Dip test developed by Gunda, *et al*. (2017) share the immediate visibility and simplicity, we think the high degree of sensitivity of our immune sensor is more advantageous than it [40]. This is due to the nature of OmpA and C which are integral membrane proteins of the *E. coli* cells, while the litmus Dip test does not ensure a high degree of sensitivity [41].

Two of these proteins, the ompA, and ompC, were chosen by us to develop a simplified and highly sensitive method for the detection of *E. coli* in water and meats. The differences in

spectra suggested different rates of expression of the cellular proteins.

Conclusion

We successfully constructed two types of immunosensors using either membrane (PVDF) or glass supports and antibodies against OmpA and OmpC. The construct sensors proved to be rapid, sensitive, and accurate in detecting pathogenic E. coli regardless of the nature of the examined samples; water, and meat. The constructed biosensors demonstrated high specificity to E. coli with LOD of 2 CFU/ml for the glass immunosensor and 3 CFU/ml for the dot blot immunosensor (PVDF immunosensor). The biosensors constructed were optimized to work best at 37°C, 60 minutes, pH 7.4, and up to 1M. Our sensors were designed to be used by non-specialized people and the results will be visualized quickly and can be delivered to the decision makers to accept and/ or reject goods for importing or local market monitoring.

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CONFLICT OF INTEREST: None

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ETHICS STATEMENT: The study was approved by the Deanship of Scientific Research at Umm Al-Qura University.

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