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# OCCURRENCE OF *SHIGELLA* SPECIES IN RAW MILK AND KAREISH CHEESE WITH SPECIAL REFERENCE TO ITS VIRULENCE GENES

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## ABSTRACT

A total number of 100 random samples of raw milk and kareish cheese (50 for each) were collected from Assiut city farms and dairy shops. *Shigella* species were isolated, confirmed biochemically and by using Polymerase Chain Reaction (PCR). Also two virulence genes; invasive gene (invC) and plasmid- encoded virulence gene (ipaH) were identified using PCR technique. 10 isolated strains of *Shigella* isolates (37%) from dairy milk samples and 13 isolates (43%) from kareish cheese were identified as following: *S. dysenteriae, S. flexneri, S. sonnei and S. boydii.* The two virulence genes; (invC) and (ipaH) were detected in only six and four strains of the identified *Shigella species*, respectively. Four of them had both the virulence genes (isolated from milk and cheese samples). Even though conventional culture is considered the gold standard for *Shigella* detection and the PCR method is a useful tool which complements detection of foodborne pathogens such as *Shigella*. Chitosan was evaluated in this study as antibacterial substance on the identified *Shigella strains* by inoculating it in pasteurized milk, using 3 different concentrations: 0.25, 0.5 and 1% of chitosan. Chitosan reduced the inoculated *Shigella* strain mean counts with highly significant effect ($P<0.01$) at the $6^{th}$ day reached to 2.10 ±0.17, 1.00 ±0.30 and <1 log cfu /ml for 0.25, 0.5 and 1% chitosan concentrations, respectively; while at the $12^{th}$ day we noticed that chitosan concentration of 0.5% only was highly significant ($P<0.05$). Generally, the 0.5% chitosan concentration showed the highly reduction effect on the count and survival of the *Shigella* strain involved.

*Keywords:* *Shigella*, chitosan, antibacterial activities.

## INTRODUCTION

Members of the genus *Shigella*, namely *S. flexneri, S. dysenteriae, S. sonnei and S. boydii* have caused and continue to be responsible for mortality and/or morbidity in high risk populations such as children, old aged people, toddlers in day-care centers and patients in custodial institutions (Kotloff, *et al.,* 1999) .

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Virulence genes responsible for the pathogenesis of shigellosis may be located in the chromosome or on the inv plasmid borne by the organism. They are often multifactorial and coordinately regulated, and the genes tend to be clustered in the genome. Previously reported PCR-based detection methods concentrated mainly on the ipaH gene alone (Dutta, *et al.,* 2001) or on ipaH and ial genes in two separate PCR assays (Sethabutr *et al.,* 1993). As ial is found on the large inv plasmid which is prone to loss or deletions, this gene-based detection may give false negative results.

ipaH, on the other hand, is present on both the *Shigella* chromosome and on a large plasmid and hence, it is a more stable gene to detect. However, the sole presence of ipaH is not an absolute indicator of virulence as loss or deletion of the plasmid renders the bacterium noninvasive and therefore, avirulent. set1A and set1B are chromosomal genes encoding *Shigella* enterotoxin 1 (ShET1), which cause the watery phase of diarrhoea in shigellosis (Rhee, *et al.,* 2001). ial and ipaH are responsible for directing epithelial cell penetration by the bacterium and for the modification of host response to infection, respectively (Hale, 1991). However, the advent of molecular biology assays, such as the Polymerase Chain Reaction (PCR), has made bacterial detection possible without the need for bacteria isolation. PCR has become a powerful diagnostic tool for detection of microorganisms in food and clinical-samples (Lampel and Orlandi, 2002).

Traditional antimicrobials have been utilized as preservatives to control microbial perils in the food industry. Despite the fact that these compounds, synthetic and semisynthetic, have been generally accepted, the unwanted side effects can't be ignored and don't fulfill the idea of "natural" or "healthy" food that consumers are progressively requesting. In this way, there is a requirement for new, increasingly proficient antimicrobials for use in food products to guarantee that consumers approach a safe food supply. Because of the negative effect from chemical preservatives, consideration has moved to the utilization of naturally-derived antimicrobial agents to control foodborne pathogens and preserving food. Natural antimicrobials are gotten from numerous sources, including animals as chitosan (Raybaudi-Massilia, *et al.*, 2009 and Tiwari *et al.*, 2009).

Chitosan a deacetylated products of chitin, is an adaptable food biopolymer that has discovered an assortment of utilizations in every aspect of the food sciences. Chitosan has numerous natural nutritional values for example, act as broad spectrum antimicrobial activities, antioxidant, cancer prevention agent promoting bioactivities against many chronic diseases, as hypercholesterolemia, hypertension, inflammation, immune diseases, etc., so consequently has been studied as a food preservative to improve food quality and expand the time span of usability of short-lived nourishment items (Shakeel and Saiqa, 2017). Chitosan is nontoxic and non-allergenic, so the body does not reject these compounds as foreign invaders. Biocompatibility, biodegradability and absorption properties of chitosan and its derivatives are much higher than synthetically substituted cellulose (Peter, 1995). So that in the food sciences, chitosan has an advantage over synthetic polymers, as it is considered as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (Shakeel and Saiqa, 2017).

The chitosan has been demonstrated that hydrophilicity in Gram-negative bacteria is significantly higher than in Gram-positive bacteria, making them most sensitive to chitosan. These findings are confirmed by several in vitro experiments in which Gram-negative bacteria appear to be very sensitive to chitosan, exhibiting increased morphological changes on treatment when compared to Gram-positives (Eaton *et al*., 2008; Simunek, *et al.,* 2006 and Hu and Ganzle, 2018). The polycationic behavior of chitosan in an acidic medium is the main factor contributing to its antimicrobial activity. Due to its positive surface charges under acidic conditions, chitosan interacts with anionic components on the bacteria surface: for example, negatively charged lipopolysaccharides in the outer membrane of Gram-negative bacteria, and peptidoglycan and teichoic acid in the cell walls of Gram-positive bacteria. These electrostatic interactions produce the release of most of the proteinaceous materials from the cells. This potent effect was ascribed to the electrostatic interactions between the

chitosan molecules and microbial cell membranes which led to the leakage of proteinaceous materials, consequently increasing chitosan penetration to the nucleus and binding to the DNA, thus inhibiting mRNA synthesis (Martinez *et al.*, 2010).

Three antibacterial mechanisms have been proposed: i) the ionic surface interaction resulting in wall cell leakage; ii) the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms; and iii) the formation of an external barrier, chelating metals and provoking the suppression of essential nutrients to microbial growth. It is likely that all events occur simultaneously but at different intensities. The molecular weight (MW) and the degree of acetylation (DA) are also important factors in determining such activity. In general, the lower the MW and the DA, the higher will be the effectiveness on reducing microorganism growth and multiplication (Rejane *et al.*, 2009).

The aim of this study was to investigate the presence of *Shigella* species in raw milk and kareish cheese with the application of PCR for simultaneous detection of *Shigella* invC, and ipaH virulence genes. In addition, evaluation of chitosan as antibacterial agent on *Shigella* strain in milk.

## MATERIALS AND METHOD

### 1-Sampling:
A total of one hundred random samples of raw milk and kareish cheese collected from different farms, street vendors and dairy shops located in Assiut city: 50 samples each. Samples were kept in sterile plastic bags and transported in ice box to the laboratory. These samples were aseptically opened then were analyzed for presence of *Shigella* species.

### 2- Isolation and identification of *Shigella* species:

Samples of 25 g or 25 ml was added to 225 ml GN broth with 3.0µg novo biocin\ml. Samples of cheese were weighed into filtered stomacher bags and then mixed with GN broth, suspension was held 10 min at room temp and shacked periodically then incubated in anaerobic jar at 42.0 °C in water bath for 20 h (Hall *et al.*, 2001). Enrichment culture suspension was streaked on XLD Agar, and then incubated for 20 h ± 4 h at 37°C ± 1°C. Characteristic colonies were picked for biochemical confirmation comprised of TSI agar with no $H_2S$, Urea agar, Methyl red, Indol production and L-Lysine Decarboxylation.

### 3-Molecular identification of *Shigella* isolates using PCR:
This part was done in Molecular Biology Lab. (accredited by EGAC, ISO17025:2017), Animal Health Research Institute, Dokki, Giza, Egypt.

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56 o C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

### 4-Molecular identification of some virulence genes in the identified *Shigella* isolates:
Oligonucleotide Primer: Primers used were supplied from Metabion (Germany) are listed in table (l).

PCR amplification: Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl

of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl

of the products was loaded in each gel slot. Generuler 100 bp DNA ladder (Fermentas, thermos, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software

**Table 1:** Primers sequences, target genes, amplicon sizes and cycling conditions.

| Target gene | Primers sequences | Amplified segment (bp) | Primary denaturation | Amplification (35 cycles) | | | Final extension | Reference |
| | | | | Secondar denaturation | Annealing | Extension | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| invC | TGC CCA GTT TCT TCA TAC GC | 875 | 94˚C 5 min. | 94˚C 30 sec. | 60˚C 40 sec. | 72˚C 45 sec. | 72˚C 10 min. | **Ojha et al. (2013)** |
| | GAA AGT AGC TCC CGA AAT GC | | | | | | | |
| ipaH | GCCGGTCAGCCACC CT CTGAGACTAC | 600 | 94˚C 5 min. | 94˚C 30 sec. | 55˚C 40 sec. | 72˚C 45 sec. | 72˚C 10 min. | **Jiménez et al. (2010)** |
| | GTTCCTTGACCGCCT TTCCGTACCGT | | | | | | | |

**5- Effect of chitosan application on survival of *Shigella spp.* in milk:**

1- Preparation of the tested strain:
Isolated and well identified *shigella* strain in this study which harboring invC and ipaH virulence genes was used *(S. flexneri).*

Bacterial dilutions were prepared as follows: *S. flexneri* strain was inoculated onto trypticase soy broth and incubated at 35º C. One milliliter of the culture was serially diluted in sterile peptone buffer. Then the suspension was adjusted to bring turbidity to 0.5 McFarland standards with a concentration of $10^7$ CFU/ml (as confirmed by the pour plate technique). From the previous diluted suspension tubes, the standard strain suspension of which 1 ml may contain approximately about $2 \times 10^5$ cfu/ ml was used directly in the experimental groups of milk (Lampel, 2001).

2- Preparation of Chitosan solutions:
Chitosan capsules (70-95% deacetylated) were purchased from Unifarma (Egypt). Chitosan solution was prepared by

dispersing it in 0.25% acetic acid solution (Aliasghari *et al.*, 2016). Concentrations of 0.25, 0.5 and 1% was prepared.

**Determination of the Minimum Inhibitory Concentration (MIC) of chitosan using agar well-diffusion method: (Balouiri *et al.*, 2016).

The prepared bacterial suspension with $1\times10^3$ cfu/ml was streaked over the entire dried surface of Muller-Hinton agar plate using sterile swab. Six mm-diameter wells were punched aseptically with a sterile cork-borer or a tip over the agar plates. Then 50 µL of the each prepared concentrations of chitosan solutions were poured into the wells. The plate was kept at a temperature of 4ºC until the materials in the wells were completely diffused into the agar, and the plates were incubated aerobically at 37ºC for 24 h. The zone of inhibition was measured using a caliper and recorded. The smallest inhibitory zone was considered as the Minimum Inhibitory Concentration.

3-Effect of chitosan concentrations in pasteurized milk:

Pasteurized milk samples were purchased from dairy shop and then pasteurized again in the laboratory, to carry out this work, milk is commonly heated to provide stability during storage and assure microbiological safety to consumers. We have evaluated the microbial status and pH of pasteurized milk stored at refrigerator temperature. Also, all the pasteurized milk samples showed negative phosphatase test.

After cooling, the sample was divided into 5 treatment groups; One ml of the previously prepared *S. flexneri* suspension mixed with 100 ml of pasteurized milk and divided into suitable sterile jars except the fifth group was free from strain suspension and chitosan as a negative control. The first group was positive control jars without chitosan; the 2nd, 3rd and fourth groups were mixed by different chitosan concentrations 0.25, 0.5 and 1%, and then all jars stored at 4°c, examined for the count of *shigella* every three days until the end of experiment when the spoilage of the positive control group detected by clot on boiling test.

Statistical Analysis:

The statistical analysis was performed using programs GraphPad Prism 5.04 (GraphPad, Inc., San Diego, USA) and Statistical 12.0 (Dell, Inc., Tulsa, USA) after transforming of data to $\log_{10}$ values. The bacterial count represented by mean ± SD (standard deviation). The means were separated using ANOVA and LSD except for the 9th day where independent T-test was used by the Microsoft Excel Spreadsheet.

## RESULTS

**Table1**: Incidence of *isolated Shigella species* in the examined samples (n=100):

| Types of examined samples | Number of examined samples | Number of Positive samples | Isolated strains | |
|---|---|---|---|---|
| | | | No. | % |
| **Raw milk** | 50 | 27 | 10 | 37 |
| **Kareish cheese** | 50 | 30 | 13 | 43 |
| **Total** | 100 | 57 | 23 | 40.4 |

**Table 2**: Frequency distribution of the isolated *Shigella* species in the positive samples biochemically.

| Types of examined samples | Number of isolated strains | The isolated *Shigella species* | | | |
|---|---|---|---|---|---|
| | | *S. dysenteriae* | *S. flexneri* | *S. sonnei* | *S. boydii* |
| **Raw milk** | 10 | 1 | 3 | 6 | 0 |
| **Kareish cheese** | 13 | 2 | 5 | 5 | 1 |
| **Total** | 23 | 3 | 8 | 11 | 1 |

**Table 3:** The occurrence of virulence *Shigella species* using PCR technique.

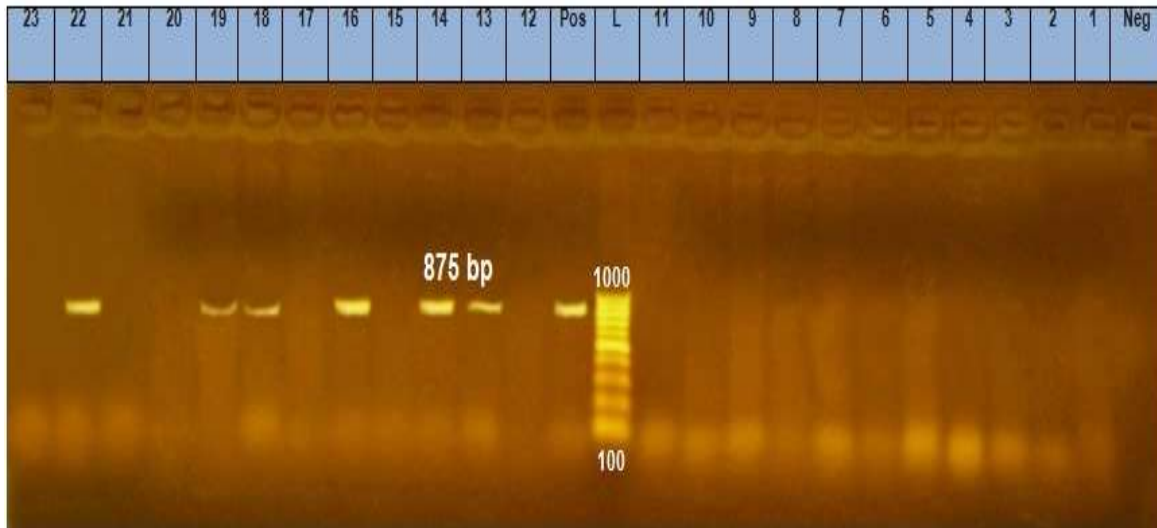| Examined samples | Identified strains by PCR | invC gene | ipaH gene | Both genes |
|---|---|---|---|---|
| Raw Milk | 10 | 2 | 2 | 2 |
| Kareish cheese | 13 | 4 | 2 | 2 |



**Photo 1:** The amplified **invC** gene of *Shigella* recovered from milk and kareish cheese samples.

Lane L: Molecular marker; Lane pos: Positive control; Lane Neg: Negative control; Lanes **15, 17, 20, 21, 23** negative isolates for invC gene; Lane **13, 14, 16, 18, 19, 22**: positive isolates for invC gene
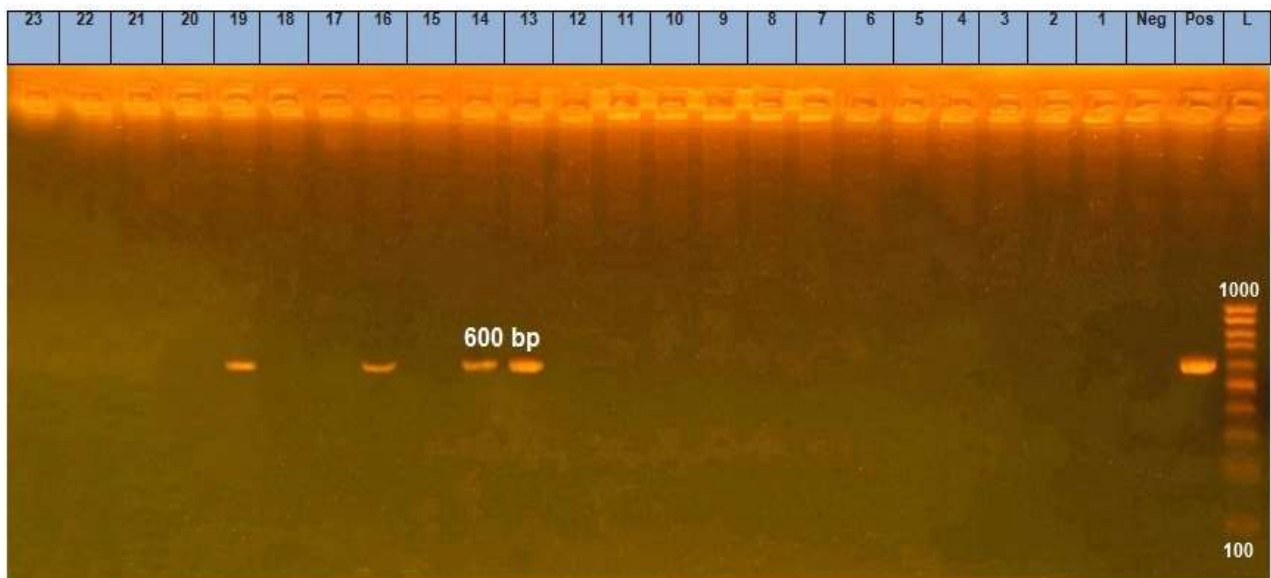


**Photo 2:** The amplified **ipaH** gene of *Shigella* recovered from milk and kareish cheese samples.

Lane L: Molecular marker; Lane pos: Positive control; Lane Neg: Negative control; Lanes **15, 17, 18, 20, 21, 22, 23** negative isolates for **ipaH** gene; Lane **13, 14, 16, 19**: positive isolates for **ipaH** gene.

**Table 4**: Effect of different concentrations of chitosan on the survival of *Shigella* strain in the pasteurized milk samples stored at 4 ºC

| Chitosan concentration | Zero time | 1st day | 3rd day | 6th day | 9th day | 12th day |
|---|---|---|---|---|---|---|
| Positive control | 3.21 [a] ±0.34 | 3.69 [a] ±0.09 | 5.33 [a] ±0.33 | 5.04 [a] ±0.17 | 5.04 [a] ±0.24 | 3.85 [a] ±0.22 |
| Conc. 0.25% | 3.30 ±0.30 | 3.31 ±0.17 | 3.78 [b] ±0.26 | 2.10 [b] ±0.17 | 1.30 [b] ±0.30 | 1.46 [b] ±0.15 |
| Conc. 0.5% | 3.56 ±0.24 | 3.24 ±0.47 | 1.20 [c] ±0.17 | 1.00 [c] ±0.30 | <1 | <1 |
| Conc. 1% | 3.86 [a] ±0.44 | 3.17 [a] ±0.23 | 1.36 [c] ±0.32 | <1 | <1 | 1.40 [b] ±0.17 |

(Mean count log cfu/ml ± SD* of three replications)

* Standard deviation

**<1 was excluded from statistical analysis, only positive count values were included

There are significance differences (*P<0.05*) between mean having different letters in the same column (same day)
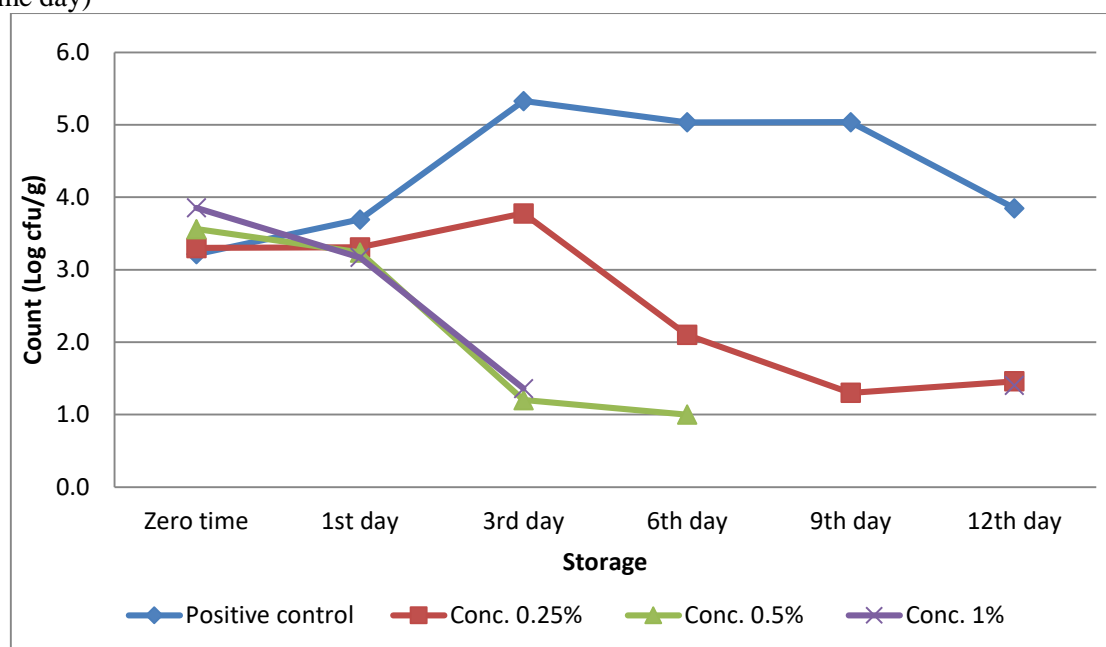


**Fig. (1):** Antibacterial effect of chitosan on *Shigella strain* inoculated in pasteurized milk

## DISCUSSION

As shown in Table 1, 37% of milk and 43% of kareish cheese samples were contaminated by suspected *Shigella* species when applied the conventional biochemical tests and after PCR confirmation. The occurrence of virulence *Shigella* species; was in two milk samples while four of kareish cheese samples were positive for virulence *Shigella* of the invC gene. About the ipaH gene, two samples of each milk and cheese possess the gene. Otherwise, four of them had both the virulence genes as shown in Table 3.

The genus *Shigella* belongs to the family *Enterobacteriaceae* and consists of four species; each of the species, with the exception of *S. sonnei*, is subdivided by serotype. Batt (1997) and Wachsmuth and Morris (1989) indicated that by means of human transmission, *Shigella* can contaminate several kinds of foods, included raw milk and some dairy products.

In this study, the number of identified *Shigella* strains were 23; distributed as showen in Table 2, one strain of *Shigella dysenteriae*, three strains of *Shigella flexneri* and six strains of *Shigella sonnei* but *Shigella boydii* could not be detected in the examined milk samples. The number of *S. dysenteriae* and *S. boydii* were two and one, respectively. Otherwise, both *S. flexneri and S. sonnei* were five in the tested cheese. The culture-based techniques were used as the gold standard for the detection of *Shigella* spp. in various samples, but the conventional procedures required multiple subculture steps, biochemical and serological confirmation, which took about 7 days, and were time-consuming and laborious (Mokhtari *et al.,* 2012). Virulent Shigella organisms cause the human illness known as bacillary dysentery, (shigellosis) causes mild diarrhea, fever, abdominal cramps and sever fluid loss. Hale *et al.* (1983) who was first described *S. flexneri*. It was established that the loss of the virulence plasmid results in a virulent strains and that the genes implicated in virulent functions are localized not only in the virulence plasmid but also in the chromosome. Uchiya *et al.* (1995) said that the virulence plasmid of *S. flexneri* has been implicated in invasion and intercellular spreading. In recent years, molecular technologies, such as PCR and real-time PCR assays, have been successfully applied to detect *Shigella* spp. PCR-based technologies have been successfully developed to detect *Shigella* from various food products and environment samples (Villalobo and Torres, 1998; Lin *et al.*, 2010; Jiménez *et al.*, 2010 and Law *et al.*, 2014).

From Photo1 and Photo 2, it was found that of 23 identified *Shigella* strains only six harbour the invasive gene (invC) virulence gene but only four strains had chromosomal and plasmid-encoded virulence gene (ipaH) that involved in this study. This is in agreement with Mokhtari *et al.* (2012). The ipaH gene, coding an invasion-associated plasmid antigen, was present in multiple copies in both the chromosomes and the plasmids of all *Shigella* species, which could be selected as mark gene for detection of all *Shigella* strain (Vu *et al.,* 2004). The percentage of *Shigella* isolates and ipaH & invC virulence genes demonstrated a marked pattern of seasonality, increasing in summer and related to the environment contamination. On the other hand, *Shigella* is considered a fastidious pathogen for bacteriological isolation, which in the context of indigenous micro flora and other substances makes detection less feasible (Jiménez *et al.*, 2010).

PCR is considered a fast, highly sensitive and specific assay that quickly amplifies specific sequences of the target DNA from bacterial pathogens such as *Shigella spp*. The target DNA is a specific sequence from a gene related to virulence mechanisms.

Epidemiological studies on *Shigella* have established that 10 cells are sufficient to be an infective dose according to ISO 6579 (2002). Legislation in many countries requires the absence of *Shigella* in 25-g amounts of foods, Egyptian Standards Requires complete absence of *Shigella* in 25 g sample (ESS, 2005), this proved that *Shigella* species of public health significant and thus milk and milk products should be free from *Shigella* species.

Regarding Table 4 and Fig 1, chitosan reduced the inoculated *Shigella* strain mean counts with highly significant effect at $6^{th}$ day reached to 2.10 ±0.17, 1.00 ±0.30 and <1 log cfu/ml for 0.25, 0.5 and 1% chitosan concentrations, respectively. Moreover, the mean count of positive control samples was 5.04 ±0.17on the same day comparing zero day and increased progressively during storage and reached to 1.30 ±0.30 at $9^{th}$ day for 0.25% chitosan and < 1 log cfu/ml for both 0.5 and 1%. While at $12^{th}$ day chitosan concentration of 0.5% only was of highly significant, too and the other concentrations

of chitosan 0.25 and 1% gave nearly the same mean counts (1.46 ±0.15 and 1.40 ±0.17 log log cfu/ml) and of moderate significance. Generally, the 0.5% chitosan concentration showed the highly reduction effect on the count and survival of the *Shigella* strain involved. The decrease on antibacterial activity as the concentration increases can be discussed in terms of the special arrangement of the polymer concentration yields a better molecular distribution in the solvent with a relatively small number interaction between the neighboring chains, so the charged sites available for external coupling are maximized (Halabalova *et al.*, 2011 and Goy *et al.*, 2016).

The disparity in lethality of chitosan shown among different reports may be attributed to the variation in chitosan property, food matrix and approaches of chitosan application. Chitosan also exerts other beneficial effects on food quality and maintaining freshness and sensory attributes. This study demonstrated the existence of *Shigella* in milk and kareish cheese and dispersion of different virulence genes among these isolates. Both PCR technique and conventional culture showed good analytical and diagnostic accuracy, all *Shigella* strains were positive by both methods. The molecular method showed a higher diagnostic sensitivity and a faster result as compared to the conventional culture.

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## مدي تواجد ميكروبات الشيجلا في اللبن الخام والجبن القريش مع الاشارة الي جينات الضراوة

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تم جمع ١٠٠ عينة من الحليب الخام والجبن القريش من مزارع مدينة أسيوط ومحلات الألبان. ثم تم عزل ميكروب الشيجلا وتصنيفها باستخدام الاختبارات البيوكيميائية و تفاعل البلمرة المتسلسل حيث عزلت ١٠ عترات (٣٧٪) من عينات حليب الألبان و ١٣ عترة (٤٣٪) من عينات الجبن القريش التي تم فحصها. تم تصنيف الشيجلا المعزولة علي النحو الاتي: شيجلا ديسينتراي وشيجلا فليسكنيري و شيجلا سوني و شيجلا بويدي. كما تم التعرف علي نوعين من جينات الضراوة في ٦ معزولات فقط من ميكروب الشيجلا وهي: جين(invC) وجين(ipaH) باستخدام تفاعل البلمرة المتسلسل . تم تقييم الشيتوزان في هذه الدراسة كمادة مضادة للجراثيم المحددة على عترات الشيجلا المحددة عن طريق اضافتها الي الحليب المبستر، وذلك باستخدام ٣ تركيزات مختلفة: ٠,٢٥ ، ٠,٥ و ١ ٪ من الشيتوزان. وجد أن الشيتوزان قلل من تعداد سلالة الشيجلا الملقحة بتأثير معنوى في اليوم السادس ووصل إلى ٢,١٠ ± ٠,١٧،١,٠٠ ± ٠,٣٠ و >١ml / cfu باستخدام ٠,٢٥ و ٠,٥ و ١ ٪ من تركيزات الشيتوزان ، على التوالي ؛ بينما لاحظنا في اليوم الثاني عشر أن تركيز الشيتوزان بنسبة ٠,٥ ٪ فقط كانت ذات تاثير كبير للغاية، وكذلك تركيزات الشيتوزان الأخرى ٠,٢٥ و ١ ٪ أعطت تقريبا نفس متوسط التعدادات (١,٤٦ ± ٠,١٥ و١,٤٠ ± cfu ٠,١٧، / مل) وذات تاثير معتدل عمومًا ، أظهر تركيز الشيتوزان بنسبة ٠,٥٪ تأثير الخفض الشديد على عدد وبقاء سلالة الشيجلا المستخدمة بالتجربة.