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Isolation of *Salmonella enteritidis* Using Biochemical Tests and Diagnostic Potential of *SdfI* Amplified Gene

[1]M.H. Mirmomeni, [1]S. Naderi, [2]A. Hosseinzadeh Colagar and [3]S. Sisakhtnezhad
[1]Department of Biology, Faculty of Science, Cell and Molecular Unit,
Razi University, Kermanshah, Iran
[2]Department of Biology, Faculty of Science, Mazandaran University, Babolsar, Iran
[3]Medical Biology Research Center, Kermanshah University of Medical Science, Kermanshah, Iran

Abstract: *Salmonella enteritidis* is a food borne pathogen that affects the human health by infecting poultries. The aim of this study is to set up methods for identifying *S. enteritidis*, based on molecular properties of this bacterium. In this study, two control isolates of RTCC1623 and RTCC1624, obtained from the institute of Razi (Karaj, Iran) and 76 Kermanshah poultry derived samples were isolated using biochemical tests. Genomic DNA was extracted by universal Phenol-Chloroform method and PCR was performed via the specific primers of *SdfI-F* and *SdfI-R* of the *SdfI* (AF370707) gene. Amplified fragments of the 333 (*SdfI*) base pairs were observed in 66 of the total 76 *S. enteritidis* isolates. This study recommends that the identification of these pathogens by PCR method can be replaced with traditional bacteriological techniques. The PCR method is a rapid approach for recognizing and identifying the *S. enteritidis* infections in chicken products.

Key words: *Salmonella enteritidis*, *SdfI* gene, PCR, biochemical test

INTRODUCTION

Despite global improvements in public health facilities, bacterial infections still remain an important public health problem worldwide. *Salmonella* are enteric bacteria and one of the most important food-borne pathogens (Weigel *et al*., 2004; Mizumoto *et al*., 2005). The disease caused by members of *Salmonella* is called salmonellosis. *Salmonella* have several subspecies. *Salmonella enterica* subspecies *enterica* (subspecies I) is responsible for 99.5% of the infections in human and animals (Pignato *et al*., 1998). Most of the infections are zoonotic in origin (Yan *et al*., 2003). *Salmonella enteritidis* is a member of this subspecies, which was emerged as a pathogen of poultry in the mid 1970s, but later become an important human pathogen. *S. enteritidis* is a major cause of human food-borne illness and is the most frequent serovar detection in outbreaks of human salmonellosis (Darwin and Miller, 1999; Ochoa-Reparaz *et al*., 2004). Human infections showed a dramatic increase since, the 1980s and are the most commonly isolated serotype in many countries (Sakai and Chalermchaikit, 1996; Rabsch *et al*., 2001). Recently, it has been identified that the main reservoir of *S. enteritidis* is poultry and the bacterium is usually transmitted though the consumption of eggs and poultry meat (Cogan *et al*., 2004; Nayak *et al*., 2004; Grijspeerdt *et al*., 2005; Sadeyen *et al*., 2006). Moreover, the increased consumption of fast food of animal products and the international food trade between countries have also played an important role in spreading *S. enteritidis* (Landeras *et al*., 1998). *S. enteritidis* can also, spread via the environment through fecal contamination by humans and animals (Okafo *et al*., 2003). Due to the high economic losses, as well as public health problems, the detection and eradication of *S. enteritidis* from poultry farms is of primary importance. Conventional detection methods for *Salmonella* are based on cultures using enrichment in nonselective and selective media and characterization of suspicious clones by biochemical and serological tests. These methods are time-consuming and require at least 72 h to obtain negative or positive results. Furthermore, these methods can be applied only in specific fields and have limitations in detection (Yu and Kaper, 1992; Van Der Zee, 1994). Recently, progress in molecular biology techniques has created opportunities of developing new methods. Among these, the PCR has successfully been applied for detection of pathogenic bacteria using a member of largest sequences (Drahovska *et al*., 2001; Pan and Liu, 2002; Malkawi and Gharaibeh, 2004; Mirmomeni *et al*., 2008). The use of PCR is one of the most promising approaches for the detection of *Salmonella* sp. Detection of *Salmonella* by PCR based on the enzymatic application of a pre-selected DNA is a

Corresponding Author: M.H. Mirmomeni, Department of Biology, Faculty of Science, Cell and Molecular Unit, Razi University, Kermanshah, Iran

highly sensitive, specific and rapid test for analysis without a negative effect on the analytical parameters. Combination of the PCR with a nonselective and selective step has improved the sensitivity of the assay and assisted in diluting out PCR inhibitory substance (Mirmomeni *et al*., 2007).

The aim of this study is to compare the conventional and molecular diagnosis techniques and determine methods for identifying *S. enteritidis*, based on molecular properties of *S. enteritidis*.

MATERIALS AND METHODS

Bacterial strains and growth condition: The bacterial strain (RTCC1623 and RTCC1624), which were used in this study were prepared from the Razi Institute (Karaj, Iran). In order to detect *S. enteritidis*, 380 food samples were prepared from poultry products in Kermanshah Province of Iran (Table 1).

The reference isolates was cultured in Luria-Bertani (LB) medium at 37°C and then in *Salmonella Shigella* agar (SS agar). In this study, the obtained clones were cultured in Triple Sugar Iron (TSI) agar and stocked at 4°C for 3 months.

In order to isolate different *S. enteritidis* strains, isolated samples from poultry products were cultured in enrichment selenit F broth at 37°C for 12 h. At the next step, cultured bacteria in this broth were transmitted to specific media such as SS agar and MacConky agar at 37°C for 18-24 h. Subsequently, one clone from cultured bacteria in this media was again cultured in SS agar at the previously mentioned condition.

Biochemical tests: After isolation of clone from SS agar, in order to detect *Salmonella* strains, positive differential and selective biochemical tests such as H_2S production, gas production from glucose, citrate consumption and carbohydrate fermentation (including manitol, dolsitol, sorbitol, arabinose and glucose) and negative biochemical tests such as urease test, indole production and carbohydrate fermentation (including sucrose and lactose) were used. For this aim, bacteria were cultured in the mentioned media at 37°C for 18-24 h and hence, their positive or negative states were distinguished according to phenotypic changes.

DNA extraction: The bacterial chromosomal DNA was extracted by the phenolchloroform method presented by Jaufeerally-Fakim and Dookun (1999). The concentration of DNA was determined using spectrophotometer.

PCR: PCR amplification was carried out for *sdfI* gene in 50 μL reaction mixture using designed *SdfI-F* (5`-TGTGTTTTATCTGATGCAAGAGG-3`) and *SdfI-R* (5`-CGTTCTTCTGGTACTTACGATGAC-3`) primers with oligo 5 software. For PCR, a volume of 5 μL of DNA template solution was added to 45 μL reaction mixture containing ddH_2O, 10X PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP, forward and reverses primers and Taq polymerase enzyme. Amplification was carried out with temperature program consisting of initial denaturation (3 min at 94°C), 30 amplification cycles (1 min at 94°C, 80 sec at 52°C and 1 min at 72°C) and the final extension (10 min at 72°C). The amplification products were visualized by staining with ethidium bromide, after electrophoresis at 0.8% agarose gel (Sambrook *et al*., 2001).

Table 1: The biochemical tests and PCR results

		Biochemical tests results		PCR results	
Isolate source	Total no.	Positive	(%)	PCR^+	(%)
Hen meat	75	11	15	10	90
Hen liver	105	20	20	18	90
Spleen	90	15	17	11	73
Egg	60	25	08	05	100
Chicken fecal	50	05	50	22	88
Total sample	380	76	20	66	87

RESULTS AND DISCUSSION

The poultry samples, which were isolated from poultry products were cultured in selenit F broth and enriched. Then, enrichment bacteria were cultured in SS agar and MacConky agar. Results show that due to *Salmonella*, clones in this growth culture can not carry out lactose fermentation, thus, these clones were bleached and occasionally fogged. In the center of some of the clones, blacked points were spotted. After collecting poultry products and culture in a specific media, *S. enteritidis* strains were isolated using positive and negative biochemical tests (Table 1), thus, *Shigella* isolates were cancelled.

S. enteritidis can ferment the carbohydrates such as glucose, manitol, surbitol and arabinos. Due to fermentation process and acid production, basal color of this medium change from violet (red) to yellow. The *S. enteritidis* growth in citrate medium cause color changes from green to blue and occasionally is without color change. Moreover, its growth in TSI causes production of yellow and red color in deep and surface of medium, respectively. At the end of some of the tubes gas was produced by the bacteria. In some clones, surface of clones were blacked, which indicated that H_2S was produced by the bacterial cells. The growth of bacterial clones along with the inoculation course in SIM after 24 h indicated that bacterial cells were movable and the test was positive (Fig. 1). For investigating, the gas production Durham tubes were used in glucose culture. The air bulb collection in this tube indicated that bacterial

Fig 1: Differential and selective media used for the detection of *Salmonella enteritidis* in poultry products. The tubes are before inoculation media. A: lysine, B: phenylalanine, C: SIM, D: TSI, E: urea, F: cimon citrate, G: MR and H: VP

cells produced gas. LIA medium was used for several purposes including lysine decarboxilation, glucose fermentation and H_2S production. Lysine decarboxilation with bacterial enzymes cause alkaline in the end of medium, moreover glucose fermentation in this area cause acid production and generally in the end of the medium was neutralize and with no color change state. Because of H_2S production, the ends of tubes were blacked.

At the next step, to distinguish the negative or positive state of the bacteria, cells were cultured in the urease medium for 24 h and results show no change in this medium. After addition of covax indicator to SIM, if indole is produced, the surface of medium should be in purple-red color. However, there no color change was seen. Although, *S. enteritidis* could ferment most carbohydrates, it could not ferment lactose and sucrose. These results were endorsed by the no color change in the carbohydrate enriched medium. MR-VP medium and MR-VP broth (Methyl Red-Voges Proskauer medium/broth, also known as Buffered Peptone-Glucose broth) are used for the differentiation of bacteria by means of the methyl red and Voges-Proskauer reactions. MR-VP medium/broth was developed to enable both the MR and the VP tests to be performed in the same medium, although in different tubes or on aliquots from the same tube. MR test shows that *S. enteritidis* ferment glucose by producing acid, which was made visible with the addition of methyl red. This acid gave a pH below 4.4, which means methyl red turns to red. VP test can detect

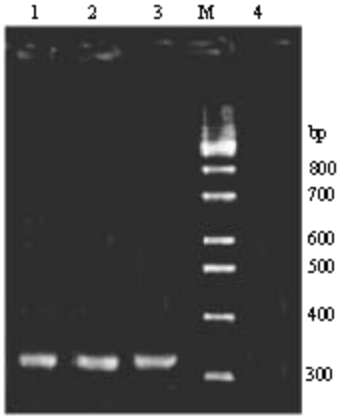

Fig. 2: The PCR amplification of *SdfI* gene using *SdfI-F* and *SdfI-R* primers. Lane 1: Control PCR product (333 bp). Lane 2 and 3: PCR products for poultry product samples. M: 1 kb marker and Lane 4: Negative control

acetoin and 2,3-butanediol was produced due to the fermentation of glucose. Under the alkaline conditions, these two compounds oxidize themselves to diacethyl. Diacethyl reacts with creatine (a guanidine derivative) to a red or with α-naphtol to a violet compound. VP test results showed that *S. enteritidis* can not ferment glucose to acetoin and 2,3-butanediol and thus can not grow in this medium. The study of isolated bacteria from poultry products using biochemical test is the first phase in bacterial identification. When biochemical tests were carried out, 76 positive *S. enteritidis* isolates were isolated from different poultry products (Table 1), which were 20% of the collected samples.

At the next step, PCR technique was used to make sure that these isolated bacteria are *S. enteritidis*. The use of PCR is one of the most sensitive, specific and rapid tests for analysis without a negative effect on the analytical parameters. Thus, PCR method was used for rapid detection of *Salmonella* genius and its isolates. *SdfI* is one of the *S. enteritidis* genes, which are well candidates for the detection of *S. enteritidis* by PCR method. During the 30 cycles, the PCR amplification for *SdfI* gene was performed using *SdfI* primers and then amplification of *SdfI* was checked by electrophoresis on the 0.8% agarose gel. The results of electrophoresis showed that a specific band of PCR product was observed at the position corresponding to the expected size of DNA amplification products about 333 bp for *SdfI* gene (Fig. 2). The PCR amplification was carried out for all isolates and control. The PCR results showed that from 72 isolates, which were identified by biochemical tests, 66 isolates had 333 bp fragment. Thus, by using PCR method, 13% of mistakes made during the biochemical test were prevented.

The isolates detection by plasmid profiling was carried out in the researchers previous study and also other researcher's studies of Pohl *et al.* (1991), Rychlik *et al.* (1993), Dorn *et al.* (1993) and Brown *et al.* (1994). The results of these studies indicated that isolate identification by studying plasmid profiling can be used for bacteria, which have several molecular plasmids, but this method can not be used for bacterial isolates, which have a single plasmid. Thus, these results showed that plasmid profiling method was not a suitable method for the identification of *S. enteritidis* isolates.

CONCLUSION

The present study indicates that biochemical tests can not be considered as rapid and sensitive methods for typing of *S. enteritidis* and PCR method is the suitable method for typing of *S. enteritidis* in the food clinical, samples.

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