ANTIMICROBIAL RESISTANCE PATTERN AGAINST POULTRY SALMONELLOSIS

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خلاصہ

ایویئن سالمونیلوسس عام طور پر سالمونیلا انترک ذیلی پر جاتیوں کی اینٹرائٹائڈس کی وجہ سے ہوتا ہے جو میزبان پر جاتیوں کی ایک وسیع رینج میں اینٹرائٹس کا باعث بن سکتا ہے اور دنیا بھر میں انسانوں میں سالمونیلا کھانے سے پیدا ہونے والی انترائٹس کی اکثریت کے لیے ذمہ دار ہے۔ یہ خوراک ترقی یافتہ اور ترقی پذیر ممالک میں بنیادی تشویش کی بیماری ہے۔ مختلف قسم کے جانوروں کے ذخائر اور جانوروں اور کھانے کی مصنوعات دونوں کی وسیع تجارتی تقسیم سے بیماری کے پھیلاؤ میں مدد ملتی ہے۔ یہ بیماری سماجی و اقتصادی اثرات کے لحاظ سے صحت عامہ کے بڑے مسائل میں سے ایک ہے۔ تاہم پاکستان میں ان کے پھیلاؤ، خصوصیات اور اینٹی بائیوٹک کی حساسیت کے بارے میں بہت کم معلومات تھیں۔ موجودہ مطالعہ میں سالمونیلا انٹریٹیڈس کی خصوصیت پلازمیڈ انکوڈڈ اینٹی بائیوٹک مزاحمت اور مکمل سیل پروٹین پروفائلنگ کی بنیاد پر کی گئی تھی تاکہ متاثرہ مصنوعات کے استعمال سے وابستہ عوام کو کم کیا جا سکے۔ دس الگ تھلگوں کو چھ اینٹی بائیوٹکس کے خلاف پلاسمڈ انکوڈڈ شدہ اینٹی بائیوٹک مزاحمت کے لیے ٹیسٹ کیا گیا۔ معیاری ڈسک کے ذریعہ ان وٹرو حساسیت کے نتائج سے پتہ چلتا ہے کہ تمام الگ تھلگ امپیسلن (100٪) کے خلاف انتہائی مزاحم تھے، اس کے بعد اموکسیلن کا نمبر آتا ہے جس میں 60٪ الگ تھلگ مزاحم تھے۔ لیووفلوکساسین اور سیپروفلوکساسن (بالترتیب 90 اور 80%) کے لیے S. انٹریٹیڈس آئسولیٹس کے ذریعے انتہائی حساسیت دکھائی گئی۔ مزاحم الگ تھلگوں کی پلازمیڈ پروفائلنگ میں پلاسمڈ دکھایا گیا ہے جو ایک سیروٹائپ مخصوص وائرلینس پلاسمڈ معلوم ہوتا ہے۔ پورے سیل پروٹین کی پروفائلنگ سوڈیم ڈوڈیسائل سلفیٹ-پولیاکریلامائڈ جیل الیکٹروفورسس (SDS-PAGE) کے ذریعہ کی گئی تھی جس کے نتائج سے پتہ چلتا ہے کہ سالمونیلا انٹرٹیڈس الگ تھلگوں کے درمیان قریبی تعلق ہے اور 78.1 اور 35 کے ڈی اے بینڈ کو تمام تناؤ میں بڑے بینڈ کے طور پر دیکھا گیا ہے۔

Abstract

Salmonella enteritidis, which is mostly to blame for the bulk of Salmonella food-borne enteritis in people worldwide and may cause enteric infection in a range of host species, is the usual cause of poultry salmonellosis. This food-borne illness is a major problem in both industrialized and developing nations. The transmission of disease is facilitated by the presence of numerous animal reservoirs and the extensive commercial distribution of both livestock and food products. In terms of its effects on the socio-economic system, this disease is among the biggest public health issues. There was, however, little information available about their incidence, description, and antibiotic susceptibility in Pakistan. To lessen the public risk associated with consumption of contaminated items, *S. enteritidis* was identified in the current study using Whole cell protein profiling and plasmid encoded antibiotic resistance. Six antibiotics were used to evaluate ten isolates for plasmid-encoded antibiotic resistance. All isolates tested totally resistant to ampicillin (100%) in an in vitro susceptibility test using a typical disc, and that 60% of the isolates were resistant to amoxicillin. Levofloxacin and Ciprofloxacin were found sensitive to *S. enteritidis* i.e. 90% and 80% respectively. The plasmid profiling of resistant isolates revealed the presence of a plasmid that appears to be a serotype-specific virulence plasmid. By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), whole cell protein profiling was carried out, revealing tight relationships between *S. entertidis* isolates and the presence of the main bands 78.1 and 35 kDa in all strains.

Keywords: Salmonella, PCR, plasmid, antibiotic, poultry

Introduction

Salmonelosis is one of the most significant bacterial infections in poultry, This infection is brought on by a range of Salmonella species and results in significant economic losses due to death and decreased output. The majority of Salmonella food-borne enteritis in people worldwide is caused by avian *S. enterica* serovar *enterica*/*entertidis*, which may infect a variety of hosts and cause enteritis in humans (Enga *et al.*, 2015; Al-ledani *et al.*, 2014). According to the World Health Organization (WHO), non-typhoid salmonellosis causes 1.3 billion instances of acute gastroenteritis or diarrhea each year, resulting in 3 million fatalities. Each year, the United Kingdom reports about 30000 instances of human salmonellosis, many of which are linked to eating tainted chicken meat or eggs. Similarly, salmonella infection still poses a severe health risk in nations

throughout Asia and Africa (United Nation, 2019; Maripandi *et al.,* 2010). *S. enteritidis* was fairly common in poultry meat, eggs, and other items obtained from poultry in Pakistan. Although research on the frequency of salmonella serotypes in chicken meat and eggs and their antibiograms are available (Obe *et al.,* 2020).

Controlling *S. enterica* infections in poultry birds is crucial for lowering the danger to the public health posed by consuming contaminated products. Salmonella is a significant food-borne illness cause, hence several typing techniques, including to understanding the epidemiology of infection and locating the contaminated source have both been accomplished using phenotypic and genotypic methods (Ferrari *et al.,* 2019; Fei *et al.,* 2018; Tang *et al.,* 2019). These tools include customary typing approaches i.e. serological and phage typing or antibiotic resistance arrays (Akhtar *et al.,* 2010). Molecular genetic methods like electrophoretic separation of whole cells and external protein membranes, or lipids, by SDS-PAGE, plasmid characterization, amplifying DNA finger printing, pulsed field gel electrophoresis (PFGE), and random amplified a polymorphic DNA analysis (RADP-PCR), are currently supporting the characterization of bacterial macromolecules using RFLP and RADP-PCR (Chu *et al.,* 2009).

In fact, microbiological resistance is the loss of a bacterium's sensitivity to the antibiotics to which it was first receptive (Okamoto *et al.,* 2009). Salmonellosis treated using antibiotics or other chemotherapeutic medications usually resulted in the creation of resistant strains because of the transmission of drug resistance by episomal transfer of resistance components, posing a major risk to public health (Alba *et al.,* 2020; Prakash *et al.,* 2005). This resistance can be acquired by chromosomal DNA mutations or by plasmids and transposons acquiring extra-chromosomal genetic material. Salmonella's increasing resistance to antibiotics has sparked worries that the extensive use of antibiotics in animal agriculture may encourage the creation of resistant bacteria or resistance genes that can transmit to germs that infect people (Carfora *et al.,* 2018; Okamoto *et al.,* 2009). It has been discovered that several Salmonella strains have plasmids of different size, such as a virulence plasmid, that is around 50 and 100 kb in size (Rychlik *et al.,* 2006). There are not many low molecular weight plasmids, which are thought to be a found in 10% of the Salmonella wild strains. However, the majority of plasmid research focused on the prospect of using plasmid profiling as instruments for molecularly classifying Salmonella species (Chu *et al.,* 2009).

In fact, over the past ten years, the usage of bacterial protein profiling by SDS-PAGE has increased. This approach is appropriate for bacterial categorization, identification, typing, and comparative investigations. It can be used to investigate microorganisms that are difficult to classify using conventional typing techniques. In taxonomic analyses, protein profile analysis is crucial since it allows for a comparison of various bacterial strains (Kustos *et al.,* 1998).

Therefore, the objective of the current study was to evaluate the whole cell protein profile and plasmid-encoded antibiotic resistance of poultry Salmonellosis.

Materials and Methods

Inoculation of Culture

Characterized ten cultures of Avian *Salmonella enteritidis* were acquired from WTO Quality Control Laboratory, University of Veterinary & Animal Sciences (UVAS), Lahore.

However, the pre-enrichment media for the bacterial culture's growth was buffered peptone water with the pH of 8.4. The peptone water with a buffer was prepared by weighing out each component, dissolving it in the distilled water, and then pouring 5 mL of the resulting mixture into 10 test tubes. These test tubes were subsequently sterilized for 15 minutes at 121°C. Ten test tubes were afterwards injected using a platinum loop with colonies from each isolate and cultured at 37°C for an overnight period after being allowed to cool at room temperature (25ºC). Additionally, tetrathionate broth was applied to the culture's enrichment media

Testing for antimicrobial susceptibility

The following antimicrobial discs i.e. amoxicillin and ciprofloxacin, chloramphenicol, gentamicin, ampicillin, and levofloxacin were used to assess the susceptibility of the isolates. All the discs were impregnated under sterile condition on nutrient agar plates, streaked with respective bacterial culture. Agar plates were examined to earn antibiotic susceptibility and resistance after being incubated in their whole for 24 hours at 37°C (Lynne *et al.,* 2009).

Bacterial Harvesting

S. enteritdis colonies were selected for inoculation into peptone-buffered solution against plasmid and protein profiling after being tested for antibiotic resistance. Centrifugation was used to separate the bacterial culture at 4000 g´ for 10 minutes under controlled temperature at 4°C.

Profiling of Plasmid

The alkaline technique was employed to extract the plasmid DNA as described by Chu *et al.,* (2009). In buffered peptone water, bacteria were cultivated before being centrifuged for 30 minutes at 18000 g´. The tubes were put in a rack vertically for 5-10 seconds after the supernatant was decanted, and further 100µl of suspension solution was added into each culture tube.

However, 200µl of the lysis solution was added to each tube, and the tubes were repeatedly inverted to mix the contents, and left the tubes for 3 minutes for cell lysis. Each tube received 150µl of the neutralizing solution before being repeatedly inverted. Bacterial chromosomal DNA was now visible as a whitish precipitate.

In a microfuge, the tubes were centrifuged at 18000 g´ for 5 minutes. 250µl of isopropanol were poured to each of the labelled, sterile eppendorf tubes before they were put into a rack. Without disrupting the precipitate, the tubes were gently taken out from the microfuge. The white precipitate was avoided as much as possible when removing the supernatant. The new set of labeled eppendorf holding the isopropanol were filled with the liquid phase.

The tubes were spin in a microfuge for 30 seconds at 18000 g´ after being vortexed for 10 seconds. The precipitated plasmid DNA appeared as a white pellet. The pellet was washed with 70% ethanol, briefly vortexed, and after the supernatant was decanted in response to centrifugation at high speed for 30 seconds. Each tube received 50µL of TE buffer, and the pallet was reconstituted and kept at -20°C until further use.
For the detection of *S.entritidis* reported primers and thermo cycler condition were applied for the amplification of product as described by the Nisbet and Ziprin (2001).

Protein Profiling

Bacteria were harvested under controlled temperature centrifuge machine under a spin of 4000g´for 10 min at 4°C. Cells were re-suspended in 1mL of the sample buffer after being washed three times in normal saline, then the suspension was boiled for 5 minutes (Kustos *et al.,* 1998).

SDS-PAGE

The molecular weight of entire proteins of cell was calculated using SDS PAGE. For this, various stock solutions were prepared, including the following: Acrylamide Solution, 2M Tris-Cl buffer with a pH of 8.0, 0.5M Tris-Cl buffer (pH 6.8), Ammonium Per Sulphate (APS) solution 10%, SDS 10%, 5x Electrophoresis Buffer, Sample buffer, Staining Solution, Destaining Solution, 10% resolving gel and stacking gel 4%**.**

All of the resolving gel's components were combined, placed onto the glass plates used for gel formation, and allowed to polymerize. After the resolving gel had polymerized, the stacking gel's combined components were poured on top of it, and the comb was then put on it.

Electrophoresis

The gel assembly was fixed in the electrophoresis chamber after the comb was taken out. After loading each well with 25µl of samples and a protein marker, the running buffer was poured into the buffer chamber, and then the gel was powered up at 100 V.

Staining and De staining of Gel

After electrophoresis, the gel assembly was removed from the electrophoresis chamber, the spacers were taken out, and the plates were gently dispersed. The gel was scooped up and stained for the entire night. Gel was transferred to the de staining solution after staining. After distaining gel, distilled water was added until the backdrop was sufficiently transparent to reveal the bands. The gel was taken after de staining, and molecular weights were calculated using protein markers.

Results and Discussion

Ten *S. enteritidis* isolates were examined in the current investigation. Salmonellosis caused by serotypes that are not host-adapted is a serious food-borne enteric illness in people. According to studies, 13 million cases of salmonellosis are recorded worldwide each year, with India, China, and Pakistan accounting for 70% of those instances (Prakash *et al.,* 2005). Salmonella-Shigella agar (SSA), MacConkey's agar, buffered peptone water, and tetrathionate broth were employed to revive Salmonella isolates (OIE terstrial manual, 2010). On these medium, bacterium colonies with physically unique characteristics were seen, such as smooth, tiny, colourless colonies on MacConkey's agar with dark centers from H_2S generation, and light yellow colonies on SS agar. Related colonial characteristics were observed and reported by Gast *et al.,* 2004.

The susceptibility pattern of various antibiotics against the isolate is shown in table 1. According to CLSI guidelines, there were different set of antibiotics that were used against respective indigenous isolate, and the susceptibility patterns of each antibiotic were obtained and interpreted as per NCCLS, 2000. However, *S. enteritidis* was found 100% resistant to Ampicillin. Contrary, the isolate was seen 100% sensitive to

Levofloxacin and rest of the antibiotics i.e. amoxicillin, gentamicin, ciprofloxacin and chloramphenicol were observed 40%, 60%, 80% and 50% sensitive respectively. According to the findings of in vitro susceptibility testing using a standard disc, all isolates were completely insensitive or resistant to ampicillin (100%) and were then equally resistant to amoxicillin (60%). Chloramphenicol and Ciprofloxacin resistance rates overall were recorded as 30% and 20% respectively. Whilst, the increased susceptibility to levofloxacin and Ciprofloxacin was shown as 100% and 80% respectively. Another investigation found a pattern of susceptibility to levofloxacin and ciprofloxacin against *S. enteritidis* by Smith *et al.,* 2010.

However, Salmonella isolates are becoming more and more resistant to antimicrobials; the options for treating these infections have grown more and more limited. In the past, trimethoprim-sulfamethoxazole, chloramphenicol, and ampicillin were the drugs of choice for treating Salmonella infections. Due to the emergence of multidrug-resistant Salmonella, fluoroquinolones and the third-generation cephalosporins had also been recognized as the preferred treatments for Salmonella infections (Bakeri *et al.,* 2003).

Table 1. Antibiotics sensitivity zones of an individual isolates and susceptibility pattern

Antibiotics	Diameter of Zone of Inhibition in mm against number of Isolates										Susceptibility pattern (%)		
	1	2	3	4	5	6	7	8	9	10	S	I	R
AM [a]	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	-	-	100%
AX [b]	20(S)	35 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	28 (S)	0 (R)	22 (S)	40%	-	60%
CN [c]	19(S)	17(S)	14(I)	17(S)	14 (I)	15 (S)	18 (S)	17 (S)	14 (I)	13 (I)	60%	40%	-
CIP [d]	31(S)	35(S)	31(S)	14(R)	34 (S)	13(R)	24 (S)	25 (S)	24 (S)	25 (S)	80%	-	20%
LEV [e]	23(S)	20(S)	30(S)	21(S)	32 (S)	18 (S)	30 (S)	32 (S)	30 (S)	27 (S)	100%	-	-
C [f]	20(S)	22(S)	19(S)	0(R)	0 (R)	20 (S)	14 (I)	19 (S)	13 (R)	16 (I)	50%	20%	30%

Where: S: Sensitive; I: Intermediate; R: Resistant
a: Ampicillin
b: Amoxicillin
c: Gentamycin
d: Ciprofloxacin
e: Levofloxacin
f: Chloramphenicol
(NCCLS, 2000)

Table 2. Approximate Molecular weight of proteins

Distance of bands (cm)	R_f Value	Log of Mol. Wt. (From Graph)	Mol. Wt. of Proteins (kD)
0.8	0.17	5.09	123
1	0.24	5	100
1.3	0.3	4.91	79
1.4	0.31	4.88	77
1.5	0.33	4.87	75
1.8	0.42	4.78	61
2.2	0.52	4.58	37
2.8	0.65	4.35	21
2.9	0.66	4.32	20
3	0.68	4.26	18
3.1	0.73	4.12	13
3.2	0.75	4.1	12
3.3	0.78	4.0	10

Distance was covered by solvent front= 4.3

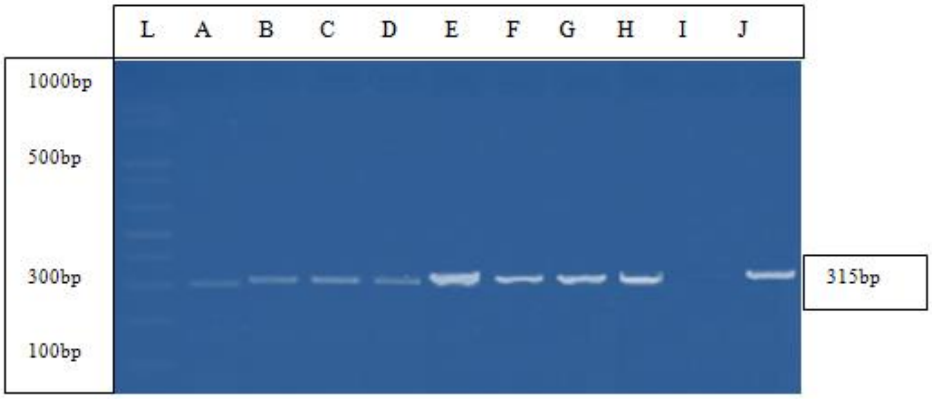

Fig.1. Band Showing Presence of plasmid of resistant isolates on 0.7% agarose gel
Where: **L**=Ladder; **A** to **J**= Showing the resistant strain with a product size of 315 **bp**=Base paid

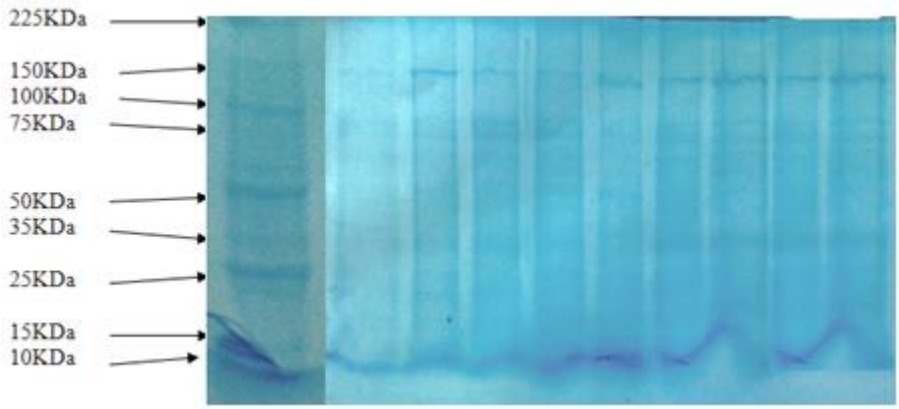

Fig. 2. Protein Profiling of Isolates of *S. enteritidis*

Profiling of Plasmid

Alkaline lysis was used to extract the plasmids from all of the resistant and susceptible isolates as demonstrated by Chu *et al*., (2009), and then both isolates were run on 0.7% agarose gel and record the results. Following the plasmid profiling of ten resistant isolates, a single plasmid type containing a unique heavy weight plasmid was identified in all isolates. All isolates are members of the same serotype, according to a single plasmid profile. Results revealed that all resistant isolates shared a solitary, sizable plasmid (Fig 1), while sensitive isolates contained no plasmid.

The abundance of these components in the isolates under study restricts the use of plasmid profiling as an epidemiological method for studying *S. entritidis*. It is well recognized that plasmids contain the genes that cause antibiotic resistance. Plasmid analysis of *S. entritidis* isolates in one research showed limited promise due to the detected plasmid's uncommon occurrence (Morshed and Peighambari, 2010). The isolates differ in terms of the quantity of plasmids and associated characteristics (Fernandes *et al.*, 2003; Bakeri *et al.*, 2003). In one investigation, 105 *S. entritidis* isolates from both human and non-human sources had 7 plasmid patterns (Fernandes *et al.*, 2003). In early studies, Bakeri *et al*. (2003) observed 9 and 17 plasmid patterns among 65 and 250 *S. entritidis* isolates from human and animal sources respectively. In our research, seven isolates showed single plasmid patterns.

Several Salmonella serotypes, including enteritidis, have been found to have virulence plasmids that are distinct to each serotype (Rychlik *et al.*, 2006). Although these virulence plasmids have a lot in common, each one seems to be unique to its host. 22 (100%) and 96% of the SE isolates examined included a plasmid that was unique to the 36 MDa serotype for pathogenicity (Fernandes *et al.*, 2003). Most of the resistant isolates were found to have the 38 MDa (55 kb) serovar-specific plasmid, according to plasmid profile analysis (Bakeri *et al.*, 2003). Our results were comparable to earlier findings in that 100% of our resistant isolates were found to carry a sizable plasmid (kb), which appears to be a virulence plasmid unique to a particular serotype.

Protein profiling SDS-PAGE

Figure 2 shows the SDS-PAGE of whole cell proteins isolated from all isolates. There were around 30 bands in the patterns, with molecular weights ranging from 10 to 123 kDa. No obvious changes in molecular weights were visible in the protein profiles of any isolates. These isolates' main differences were found in the

15-35 kDa and 75 kDa areas. The protein separation of entire bacteria exhibited no appreciable changes in the SDS-PAGE profile. In all strains, significant protein bands of 123, 79, and 37 kDa were seen.

There were no variations between the isolates pertaining to this serovar as protein profiles of *S. enteritidis* in poultry were investigated. The use of conventional identification and typing methods is still widely utilized in routine microbiological diagnostics (Durrani *et al.,* 2008). For categorizing, recognizing, and comparing bacteria, measuring the total quantity of protein is essential since bacterial protein profiles are a reflection of the strain's DNA (Aksakal, 2010). SDS-PAGE is another crucial molecular method used for species-level identification (Durrani *et al.,* 2008). In order to conduct an extensive recognition and description of the microorganisms used in the epidemiological assessment of outbreaks, advanced methods, like plasmid evaluation and whole cell protein analysis, are frequently required (Nakamura *et al.,* 2002; Acik *et al.,* 2005; Begum *et al.,* 2008).

In various studies on Salmonella serovars, SDS-PAGE has been utilized to evaluate the whole cell lysate (Ngwai *et al.,* 2006; Begum *et al.,* 2008). According to Nakamura *et al*. (2002), SDS-PAGE analysis of *S. enteritidis* total cell proteins revealed similarities, major bands at 71.4, 67.7, 44.0, and 30.3 kDa were produced by all strains. According to Ngwai *et al*. (2006), SDS-PAGE analysis of all of the cell proteins of S. enteritidis strains revealed the presence of proteins with molecular weights of 36.5 and 65 kDa though the total protein profiles of all strains were similar. Another study discovered that bands of 77, 55, 33, and 16 kDa were commonly present in the protein profiles of several Salmonella strains. Additionally, more than fifty Salmonella serovars, including *S. enteritidis*, *S. agona*, *S. anatum*, *S. virchow*, and *S. corvallis*, had their whole cell protein profiles examined using SDS-PAGE (Begum *et al.,* 2008). A protein band of 37.8 kDa was present in all serovars, and each serovar's protein profiles were identical. Another viewpoint is presented by Acik *et al*. (2005) who claim that electrophoretic patterns of bands obtained using the SDS-PAGE method are insufficient for definitively differentiating Salmonella species.

Conclusion

The following protein bands were identified in this study: 123, 100, 79, 77, 75, 61, 37, 21, 12, and 10 kDa. The 123, 79, and 37 kDa bands were shown to be significant bands in all strains. Therefore it is concluded that the results indicated a closed relationship between Salmonella strains.

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