JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 175-179

Study of Drug Resistance in *Salmonella* spp. Isolated from Native Eggs of Iran's Southern Region

Ali Ghorbani Ranjbary[1], Hossein Kargar Jahromi[2]*, Nazanin Ghorbani Ranjbary[1], Sheyda Asmarian[3], Fatemeh Juibar[1] and Navid Kalani[2]

[1]Young Researchers and Elite Club, Kazerun Branch, Islamic Azad University, Kazerun, Iran.
[2]Zoonoses Research Center, Jahrom University of Medical Sciences, Jahrom, Iran.
[3]Departement of Phamacology, Faculty of Veterinary, Kazerun Branch, Islamic Azad University, Kazerun, I.R. Iran.

(Received: 25 April 2015; accepted: 21 July 2015)

Salmonellas are short bacteria, gram negative, some of them have caps, facultative aerobic and anaerobic have high resistance against physical and chemical factors. *Salmonella* causes bacillary white diarrhea in Avian. Its transference in Avian is vertical and horizontal. In human, it causes food poisoning too. The egg is the most important source of salmonella. Native eggs have been collected and transferred to the lab in order to study their contamination. These eggs were collected from areas around Shiraz (Iran). In this research the pattern of drug resistance of bacteria was studied. The egg shell is purified with Ethanol 70%, and then the contents of Fifty eggs are mixed in a dish and incubated with soab in Selenite-f. After 24 hour incubation at 37ºC the Selenite-f sample was transferred to salmonella spp and after incubation at 37ºC was analyzed in terms of suspected colonies to Salmonella. The suspected colonies were inoculated into lysine decarboxylase broth and TSI agar environment. The bacteria that had reactions were related to salmonella, and were analyzed by the PCR test, with special primer for salmonella spp, such as *S. enteritidis* and *S. typhimurium*. The results of this study indicated five samples of mixed eggs (at least 0/33% of eggs) were contaminated with salmonella of serotype *S. enteritidis*. Among the separated Salmonellas 85.9 % were resistant to Ampicillin, 14.5% to Tetracycline, and 42.9% to Kanamycin, but all of them were sensitive to Norfloxacin in this antibiotic resistance test. Excessive use of antibiotics and also having no information on sensitivity and bacterial resistance to antibiotics can make the antibiotic resistances more complex.

Key words: Salmonella spp., Salmonella enteritidis, Drug resistance.

Salmonella are a large group of negative Gram bacillus, 1 to 3 micron in length, and 0/5 to 0/8 micron in width, all of them have antigen H of the flagellum, and can move, except two serotypes of *Salmonella gallinarum* and *Salmonella pullorum* (Please check the entire document)[1, 2]. *Salmonella* have high resistance to chemical agents, bile, also physical factors such as temperature and disinfectant material. According to the Tajbakhsh and Nazar, 1974 study, Salmonella Abortus ovis can survive more than 5 months in the soil and maintain its ability to ability to create disease. So far, more than 2,500 serotypes of Salmonella have been identified[3,4]. In poultry, Salmonella can transmit horizontal and vertical and usually creates stable infection in contaminated flocks. Different serotypes of salmonella cause some diseases such as Pullorum diseasewhich is caused by s*Salmonella pullorum*. Its transmission is usually vertical. Poultry Typhoid is caused by salmonella Gallinarum (review) which almost always transmitted horizontally and by bird faeces.

* To whom all correspondence should be addressed.
E-mail: hossein.kargarjahromy@yahoo.com;
Tel: +989361554955

Paratyphoid is another disease with symptoms similar to Pullorum and is very important because it is a creating agent, which can be caused by Salmonella typhimurium, enteritidis and Salmonella Havana. However, these bacteria, especially Salmonella typhimurium and Salmonella enteritidis, do not inhabit a specific host, and they are the most important species involved in human salmonella contamination[1, 5]. Most research shows that salmonella enteritidis in most salmonellosis epidemics are involved with origin eggs[6,7]. Infection of reproductive system by salmonella enteritidis can be transmitted to egg and contaminate the egg's content. It seems that a major part of infection is upper site of Audact[8]. Other serotype Paratyphoids include antibiotic-resistant serotypes, Salmonella typhimurium can also be replaced in the egg[9]. Consumption of contaminated eggs is the most important source of Salmonella infections[10, 11]. A *Salmonella* bacterium does not have hard growth and after enrichment it can be separated from environment, clinical sample and nutrition. PCR technique provides precise and quick results[12,13]. Regarding the importance of detection, because of salmonella contamination in the poultry breeding industry, public health and also increasing antibiotic resistance of salmonella bacteria[6, 14, 15, 16,17], it is vital to detect , salmonella contamination of local eggs. So the aim of this research is the study of drug resistance in *Salmonella spp*. isolated from native eggs of the south of southern Iran.

MATERIALS AND METHODS

For isolation of salmonella bacteria, 1500 eggs were collected from the south of Iran and transferred to the lab. . In the laboratory, the egg shells were cleaned and disinfected using ethanol 70%, calcareous shell was broken with sterilized scissors, and the contents of each 50 eggs was mixed in a sterilized glass dish. After 24 h incubation at 37ºC the mixture was incubated and then or mixed with to selenite - F for enrichment The mixture was incubated for 24 to 48 hours at 37 ºC. After removal of incubation on solid medium of salmonella-shigella it was incubated for 24 h at a temperature of 37 ºC, Salmonella environment - Shigella agar for 24 hours incubated at 37ºC. The suspected Salmonella colonies were then examined and in case of negative result, the incubation was continued for 24 hours. Salmonella that had no color after this time or suspected pale or yellow colonies with gray and black center were negative and deleted, then suspicious colonies were removed from salmonella-Shigella agar medium and were cultured Surface and deeply in TSI and LIA medium. Also, we provide a spread from the suspicious colonies which were stained with Gram method. If the rods Gram-negative bacterium was observed, a pure culture was provided and it was checked for existence of Salmonella[18].

Recognition of *Salmonella* serotype *enteritidis* and *typhimurium* by PCR test

For DNA extraction of the identified salmonella above, colony suspensions in sterile distilled water were prepared. The suspension was centrifuged for 5 min at 18°C at a speed of 14,000 rpm and bacterial genome was extracted from the precipitate by phenol-chloroform bacterial genome method (reference). Genome, by using specific primers of invasion gene, was used for PCR to detect Salmonella. To determine the serotype, the primers of Fimbria gene (sefA) for Salmonella enteritidis and primers of Virulence Fimbriae (pefA) for Salmonella typhimurium were used in PCR (Table 1). PCR products on agarose gel 1.5 percent electrophoresis and 100 bases per pair were used to determine the molecular weight of DNA fragments. Gel was stained with ethidium bromide solution, which has a Fleur Scans color. UV radiation device was used for observing bright bands under the ultraviolet light[19].

Antimicrobial Resistance

To determine the sensitivity of isolated salmonella to the antibacterial compounds, a disk agar diffusion method was used. First, colonies in Moler Hilton (Mueller Hinton) liquid environment reached to half Mcfoarlan, then from that, a culture was provided on the agar bis culture environment and simultaneously a disk containing antibiotic was placed (from the Padtan Teb Company on the culture environment). After 24 h incubation at 37ºC, Salmonella growth inhibitions ring were determined. For antibiogram tests, four antibiotics disks containing: Ampicillin (10 ìg), Tetracycline (30 ìg), Kanamycin (30 ìg) and Norfloxacin (10 ìg) were used (Table 2). The result of salmonella isolation from eggs was estimated by the eggs contamination percentage. The results of

determining salmonella sensitivity to anti-bacterial effect on the base of the percentage were also estimated in 3 sensitive, semi-sensitive and-resistant levels (based on the Padtan Teb is written differently company's table from the number of salmonella samples) (using standard Kirby & Bauer technique)[20,6].

RESULTS

To increase the isolation sensitivity of Salmonella, the enrichment step prior to inoculation of the selected medium was done. From the 30 mixed samples of eggs, 5 mixed samples in biochemical tests and PCR contaminated with Salmonella were Salmonella enteritidis (Figure.1). In the pattern of studying drug resistance of isolated salmonella, resistance rate was Ampicillin 85.9%, Kanamycin 42.9% and Tetracycline 14.5% respectively, while there was no observed resistant sample to Norfloxacin. Regarding the antibacterial drugs used, the most sensitive was observed for Norfloxacin (Table 2).

Row M: Indicator of molecular weight DNA; Row 1: positive control (standard strain of Salmonella enteritidis ATTC 13076) rows 5,6,7 are eggs contaminated with Salmonella enteritidis and rows of egg-free from salmonella enteritidis are 2,3,4,, row 8 negative control.

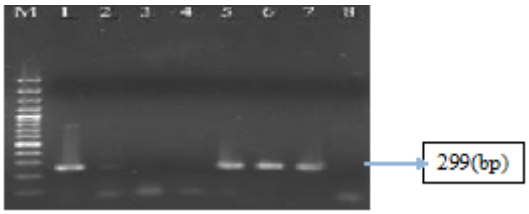

Fig. 1. Detection of Salmonella enteritidis, egg specimens native around Shiraz.

DISCUSSION

According to the method of preparing samples for bacterial culture, it was shown that at least 0.33 % of the eggs (one eggs in each mixed sample) or up to 16.66 % of eggs (all eggs in the mixture) were contaminated with salmonella. Salmonella contamination rate in Iran (Ahwaz) in native eggs was determined by using biochemical and serological tests and it was also determined that contamination rate was 1.66%[22]. In a study in the USA (Arkansas) salmonella contamination of content and shells of 1200 eggs were examined, just in 12 eggs, external contamination with Heidelberg salmonella was found, while all egg contents had no salmonella contamination[23]. In another study, the mass in the shell and content of egg were tested from more than 5700 eggs of 15 flocks that had been naturally contaminated with enteritidis salmonella.. The contents of 32 eggs (6%) were infected[9]. Organisms inside the egg through direct transfer or egg shell contamination or through oviduct penetrate to the egg[24] .Native chickens are usually kept with domestic animals and their egg laying also takes place on the bed, so the contamination probability to bacteria with intestinal origin, such as salmonellosis, will increase; also, control programs and salmonella eradications are not performed. Therefore, to reduce the risk of salmonella, it is proposed that:

1 - Fully cooking eggs enough (70 ° C during 10 minutes) can remove Salmonella (25). 2- Teaching the dangers of salmonellosis to people and preventing methods of salmonella transmission to the food in the kitchen which is the most practical method to prevent salmonellosis caused by eggs[5,26].

In patterns of studying drug resistance of isolated salmonella, resistance rate to the

Table 1. Oligonucleotide sequences used as primers in polymerase chain reaction, (PCR)

Primer	Sequence 5¹ to 3¹	Amplicon fragment(bp)	References
invA-1	TTGTTACGGCTATTTTGACCA	521	21
invA-2	CTGACTGCTACCTTGCTGATG		
sefA-1	GCAGCGGTTACTATTGCAGC	299	21
sefA-2	TGTGACACGGACATT TAGCG		
pefA-1	TTCCATTATTGCACTGGGTG	479	21
pefA-2	GGCATCTTTCGCTGTGGCTT		

Table 2. Pattern of drug resistance of salmonella isolated from the native eggs

Result	Ampicillin	Tetracycline	Kanamycin	Norfloxacin
Sensitivity	0	42.7	28.4	100
Semi-critical	14.1	42.8	28.7	0
Resistance	85.9	14.5	42.9	0
Total	100	100	100	100

antibiotics were Ampicillin 85.9%, Kanamycin 42.9% and Tetracycline 14.5% respectively, while there was no observed resistant sample to Norfloxacin. Regarding the antibacterial drug used, the highest sensitivity was observed for Norfloxacin, which is a result of these antibiotics not being used in the area, so no resistance to it was found. Having no complete sensitivity to Tetracycline and Kanamycin can result in excessive use of these antibiotics in treatment, so resistance to these antibiotic is not unexpected. Ampicillin resistance may be due to the greater effect of these antibiotics on Gram- positive bacteria than Gram-negative bacteria[27]. Graziani et al. examined antibiotic sensitivity of isolated Salmonella typhimurium from humans and animals, and also determined the resistance of salmonella (which was isolated from poultry) to Ampicillin (3. 54%), Gentamicin (2. 3%), Kanamycin (85 %), chloramphenicol (5 . 24%), Tetracycline (52.1%) and ciprofloxacin (0%)[15]. Pan et al., 2009 studied Antibiotic resistance of Salmonella Antryka which showed approximately 39/95% percent isolated bacteria indicating high resistance, particularly against ampicillin (40.2%), Streptomycin (58%) and Tetracycline (58.9%). During the years 1999-1962, all isolated salmonella were sensitive to Norfloxacin, but during the years 2007-2000 low resistance (17/5) to Floxacin was seen[16].

CONCLUSIONS

Nowadays, use of antibiotics in poultry is common. With regard to increasing antibiotic resistance, culture and antibiogram should be performed and prescription should be done with regard to antibiotic sensitivity of Microorganisms. Excessive use of antibiotics and also having no information on the sensitivity and bacterial resistance to antibiotics can make the antibiotic resistances more complex. According to this study, the most sensitive antibiotic for salmonella enteritis was Norfloxacin, and resistance to Ampicillin, Kanamaycin, and Tetracyclin is decreasing respectively.

REFERENCES

1. Hosseini Tabatabaei, A. Herbivorous bacterial disease. Tehran University Publication, Tehran., 2001; No 2492, 255-257.
2. Rajesh, C., Rao, V.D.P., Gomez-Villamandos, J.C., Shukla, S.K., Banerjee PS. Diseases of poultry and their control .1st ed., International Book Distributing Company, Indian., 2001; pp: 74-82.
3. Chu, C., Chiu, C.H. Evolution of the Virulenece plasmids of non-typhoid Salaonella and its association with antimicrobial resistance. *Microbes and infection*., 2006; **8**: 1931-1936.
4. Tajbakhsh, H., Nazar, A.A. Persistence of S.abortusovis in soll. *Rev. Med. Vet .pays Tvo.*, 1974; p.27:57-59.
5. Razavilor, V. Pathogenetic microbes in foodstuffs and epidemiology of food poisoning. Tehran University Press, Tehran, 2nd ed No,2431., 2003; 76-60.
6. Adesiym, A., Offah, N., Seepersadsingh, N., Rodrigo, S., Lashley, V., Musai, L. Antimicrobial resistance of Salmaonela spp.and Escherichia coli lsolated from table eggs. *food Control.*, 2007; **18**: 306-311.
7. St Louis, S.T., Morse, M.E., Potter, D.L., Demelifi, M.E., Guzewich, T.M., Taux J.J., and et al. The emergence of Grade A eggs as a major source of Salmonella enteritidis infections: New implications for the control of salmonellosis. *Journal of the American Medical Association.*, 1988; **259**: 2103-2107.
8. Humphrey, T.J., et al. Numbers of Salmonella entertidis in the contents of naturally contaminated hens eggs. *Epidemiology and Infection.*, 1991; **106**: 489-496.

9. Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. Diseases of poultary .11th ed ., Iowa State Press., 2003; pp: 583-599.
10. De Boer, E., Hahne, M. Cross Contamination with Campylobacter jejuni and Salmonella spp. From raw chicken products during food preparation. *Journal of Food Protection.,* 1990; **53**: 1067-1068.
11. Nunes, I.A., Helmuth, R., Schroeter, A., Mead, G.C., Santos, M.A., Solari, C.A. Phage typing salmonella Enteritidis from different sources in Brazil. *Journal of Food Protection.,* 2003; **66**: 324-327.
12. Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G., et al. Evaluation of a multiplex PCR assay for simultaneous identification of Salmonella sp ., Salmonella enteritidis and salmonella typhimurium from environmental swabs of poultry house. *Letters in Applied Microbiology.* 1999; **28**: 113-117.
13. Woodward, M.J., Kirwan, S.E.S. Detection of Salmonella enteritidis in eggs by the polymenrase chain reaction. *Veterinary Record.,* 1996; **138**: 411-413.
14. Cailhol, J., Lailler, R., Bouvet, P., LaVieille, S., Gauchard, F., and et al. Trends in antimcrobial resistance phenotypes in non-typhoid Salmonellae from human and poultry origins in France. *Epidemiology and Infection.,* 2006; **134**: 171-178.
15. Graziani, C., Busani, L., Dionisi, A.M., Lucarell, S., Owczarek Ricci, S, and et al. Antimicrobial resistance in salmonella enterica serovar Typhimurium from human and animal sources in Itali. *Veterinary Microbiology*., 2008; **128**: 414-418.
16. Pan, Z., Wang, X., Zhang, X., Geng, S., Chel, X., Pan, W., and et al. Changes in antimicrobial resistance among salmonella enterica subspecies enterica serovar? pullorun isolates in china from 1962 to 2007. *Veterinary Microbiology.,* 2009; **136**: 387-392.
17. Van Duijkeren, E., Wannet, W.J., Houwers, D.J, Van Pelt, W. Antimicrobial susceptibilities of Salmonela strains isolated fromhumans, cattle, pigs, and chickens in the Netherlands from 1984 to 2001. *Journal of Clinical Microbiology.,* 2003; **41**: 3574-3578.
18. Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.G., Leonard, F.C. Veterinary Microbiology and Microbial disease. First published. Blackwell Science, Oxford., 2002; pp: 113-118.
19. Cortez, A.L.L., Carvalho, A.C.F.B., Ikuno, A.L.L., Carvalho, A.C.F.B., Ikuno, A.A., Burger, K.P. Vidal-Martins A.M.C. Identification with Campyiobacter jejuni and Salmonela spp. Isolates from chicken abattoirs by multiplx-PCR. *Research in Veterinary Science.,* 2006; **81**: 340-344.
20. Quinn, P.J., Carter, M.E., Markey, B., Carter, G.R. Clinical Veterinary Microbiology. Wolf Press, London.,1994; pp: 95-102.
21. GhorbaniRanjbary, A., GhorbaniRanjbary, N., GhorbaniRanjbary, Z., Asmarian, S.H. Study on drug resistance in salmonella spp. isolated form native eggs in Fasa. *Journal of Modern Veterinary Researches,* 2012; **4**(1)., Pp: 1-7.
22. Jaafari, R.A., Fazlara, A., Delirania, A. The review of Salmonella in Consumption eggs in Ahwaz. The articles summary set of the fourth Assembly of Clinical Sciences Veterinarians of Iran., 2005; pp:325.
23. Schutze, G.E., Fawcett, H.A., Lewno, M.J., Flice, E.L., Kirby, R.S. Prevalence of Salmonella Enteritidis in poultry shell eggs in Arkansas. *South Med. J.,* 1996; **89**: 889-891.
24. Nazer, A.H.K., Safari, G.H. Bacterial flora from dead-in-shell-chicken embryos and their drug resistanci in Fars province of Iran. *Indian Journal of Animal Sciences.,* 1994; **64**: 1006-1009.
25. Rokni, N. The principles of foodstuffs health. Tehran University Publication, Tehran, 6th ed No. 2208., 2007; 10-4.
26. Sande-Bruinsma, N.; Grundmann, H.; Verloo, D.; Tiemersma, E.; Monen, J.; Goossens, H.; Ferech, M. Antimicrobial drug use and resistance in Europe. *Emerg. Infect. Dis*. 2008, **11**, 1722–1730.
27. Adams, R.H. Veterinary Pharmacology and Therapeutics. 7th ed. Iowa State University Press, Ames., 1995; pp: 1112-1114.