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Original Article

Study on phenotypic characteristics of *Salmonella gallinarum* and *Sallmonella pullorum* isolates based on biochemical and antimicrobial susceptibility tests in Iran

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ABSTRACT

Salmonellosis is a very important disease of avian species because of its huge economic impact, worldwide distribution and difficulty posed in its control. Fowl typhoid and pullorum disease, is caused by *Salmonella enterica* subsp *enterica* serovar Gallinarum biovar Gallinarum and Pullorum. The purpose of this study was to investigate the biochemical characteristics and antimicrobial susceptibility of *Salmonella gallinarum* and *Salmonella pullorum*. A total of 13 *Salmonella* isolates, identified by biochemical tests and specific antisera including *Salmonella gallinarum* (n=10) and *Salmonella pullorum* (n=3). All were found to be susceptible to gentamicin. Also 7 (53.8 %), 6 (46.1%) and 5 (38.4%) isolates were resistant to streptomycin, cephalexin and nalidixic acid respectively. Multidrug resistance to three or more antibiotics was observed in 6 (46.1%) isolates and overall 9 antibiotic resistance patterns were recorded. The results showed that poultries as a source of antimicrobial resistance could pose a serious risk to public health via food chain transfer. Hence more epidemiological surveillance programs and antibiotic susceptibility investigations are advised.

Keywords: *Salmonella gallinarum*, *Sallmonella pullorum,* Antimicrobial Susceptibility Test, Iran

INTRODUCTION

Fowl typhoid and pullorum are two distinct septicemic diseases largely specific to avian species and caused by *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and Pullorum, respectively (OIE 2012). Clinical signs in chicks and poults include anorexia, diarrhea, dehydration, weakness and death. In mature birds pullorum disease is less severe but decreased egg production, poor hatchability and some increased mortality may occur. Fowl typhoid is a more acute septicemic condition which mainly affects mature birds and may be particularly severe in commercial laying flocks (OIE 2012, Shivaprasad 2003). These diseases are economically important and without an integrated control program under supervision of national regulatory authorities (NRA), having a sustainable production in poultry industry, would be impossible (Aragaw *et al* 2010, Williams 1978). *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and pullorum are non-motile, host adapted avian pathogens belonging to *Salmonella* serogroup D (Capita *et al* 2008). Considering antigenic structure

Salmonella Gallinarum and *Salmonella Pullorum* are very similar biovars; however they are responsible for distinct and different diseases in chickens (Proux *et al* 2002, Threlfall 2002). The differentiation between *Salmonella gallinarum and Salmonella pullorum* is very important in epidemiological and preventive perspectives. Epidemiological studies can be carried on the basis of phenotypic and genotypic methods; however the latter are more costly in terms of equipments, reagents, and analyzing software, and demand more expertise. Different phenotypic techniques that have been used for epidemiological analysis of *Salmonella* isolates include antimicrobial susceptibility, biotyping and serotyping (Ribeiro *et al* 2009). Serotyping is still the only reliable method for epidemiological studies of *Salmonella* isolates, however it cannot differentiate between closely related biotypes like *Salmonella gallinarum* and *Salmonella pullorum* (Capita *et al* 2008, Kwon *et al* 2002, Rajagopal & Mini 2013), but differentiation of these two biotypes can be carried out through biotyping. The main biochemical characteristics assessed are the capacity to use dulcitol by *Salmonella gallinarum* and ornithine decarboxylation by *Salmonella pullorum* (Shivaprasad 2003). In Iran, only a few reports reflecting epidemiological studies of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and pullorum have been carried out (Shapoury *et al* 2010). Also Sharifi-Mood et al. (2006) reported a case with empyema due to *Salmonella gallinarum*. In another report two cases of *Salmonella gallinarum* septicaemia in two immunocompetent patients have been reported (Yousuf *et al* 2001). Therefore, the aim of the present study was comparison of *Salmonella gallinarum* and *Salmonella pullorum* by biochemical and antimicrobial susceptibility test.

MATERIALS AND METHODS

Samples. Thirteen isolates of *Salmonella* were obtained from Razi Type Culture Collection (RTCC), Karaj, Iran. Biochemical tests, antimicrobial susceptibility testing and serotyping of all 13 *Salmonella* were performed at the Microbiology Department of Razi Vaccine & Serum Research Institute.

Biochemical characteristics. To determin isolates' biotypes, the biochemical characteristics of all isolates were tested. ,including; Triple Sugar Iron (TSI) agar, Lysine Iron agar, Urea agar, Indole, Methyl red-Voges-Proskauer (MR-VP) , Simmon's citrate, Motility, and fermentation of sugars (such as glucose, maltose, rhamnose, dulcitol), ornithine and lysine decarboxylation. (Merck, Darmstadt, Germany) (Aragaw *et al* 2010, Lee *et al* 2003, Mirmomeni *et al* 2009, OIE 2012).

Serotyping. All isolates were serotyped using slide agglutination test with standard antiserum (Mast, Bootle, England) for somatic and flagellar antigens identification according to the Kauffman – White classification scheme (Grimont & Weill 2007).

Antimicrobial Susceptibility Testing. The isolates were examined for susceptibility against 15 antimicrobial drugs by the agar disc diffusion method. Antibiotic discs used in our study, were as follows: amoxicillin (25µg), cefazolin (30µg), cefotizoxim (30µg), cephalexin(30µg), cephalothin(30µg), chloramphenicol(30µg), ciprofloxacin (5µg), co-trimoxazole(25µg), entamicin(10µg), kanamycin (30µg), nalidixic acid (30µg), neomycin(30µg), nitrofurantoin (30µg), streptomycin (10µg) and tetracycline (30µg) (Padtan Teb Co. Tehran, Iran). *Escherichia Coli* ATCC 25922 was included for quality validation. The interpretive criteria were recommended by the Clinical and Laboratory Standards Institute 2011(Clinical and Laboratory Standards Institute 2011).

RESULTS

Biochemical characteristics of 10 *Salmonella gallinarum* and 3 *Salmonella pullorum* were shown in Table 1. Ten out of 13 isolates fermented dulcitol and decarboxylated lysine but not ornithine. On the other hand, 3 out of 13 did not ferment dulcitol and decarboxylate lysine but succeeded to carboxylate ornithine. On the basis of these 3 biochemical tests, 10 out of 13 isolates were recognized as *Salmonella*

gallinarum and 3 *Salmonella pullorum*. All isolates were serologically identified as *Salmonella enterica* subsp. *enterica* serovar Gallinarum. Serotyping could not differentiate between two biotypes *Salmonella gallinarum* and *Salmonella pullorum*. The results of the antimicrobial susceptibility testing by disc diffusion method using 15 antimicrobial agents are summarized in Table 2. All isolates were found to be susceptible against gentamicin. Also 7 (53.8 %), 6 (46.1%) and 5 (38.4%) isolates were resistant to streptomycin, cephalexin and nalidixic acid respectively. We observed 9 antibiotic resistance patterns by antimicrobial susceptibility testing (Table 3). Also multidrug resistance (resistance to three or more antibiotics) was observed in 6 (46.1%) isolates.

DISCUSSION

Salmonellosis is a very important disease of avian species because of its huge economic impact, worldwide distribution and difficulty posed in the control of the disease (Rajagopal & Mini 2013). Efficient laboratory methods for isolation, identification and typing of *Salmonella* are essential elements in *Salmonella* monitoring and control program (Shivaprasad 2003). Fowl typhoid and Pullorum disease, caused by *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum and pullorum, respectively (OIE 2012). The differentiation of *Salmonella pullorum* and *Salmonella gallinarum* cannot be made clearly from the disease symptoms and also lesions produced by certain strains of *Salmonella gallinarum* in chicks are indistinguishable from those produced by *Salmonella pullorum* (OIE 2012, Rajagopal & Mini 2013). These bacteria are also very similar in terms of antigenic and biochemical properties. The biochemical reactions are traditional distinctive method between *Salmonella gallinarum* and *Salmonella pullorum* (Rajagopal & Mini 2013). They are both characterized as *Salmonella enterica subsp. enterica* (group D, somatic antigen 1, 9 and 12), both lacks flagella and grows slowly in cultured media (Pomeroy & Nagaraja 1991). They have some differences in biochemical tests. *Salmonella Gallinarum* ferments dulcitol, maltose and glucose without producing gas but not rhamnose and do not decarboxylate ornithine while, *Salmonella Pullorum* ferments rhamnose and glucose with gas but not dulcitol and maltose and decarboxylate ornithine (Pomeroy & Nagaraja 1991, Rajagopal & Mini 2013, Snoeyenbos 1991). In our study, between 13 isolates of *salmonella*, 9 isolates fermented glucose, maltose and dulcitol without producing gas and 1 isolates did not ferment maltose and 1 isolates did not ferment dulcitol. Eight isolates did not ferment rahamnose and 1 of them decarboxylated ornithine. Between 13 isolates 2 of them indicated typical characteristics of *Salmonella Gallinarum*. On the other hand among the 13 isolates in our study, 3 of them fermented glucose along with producing gas and rahamnose, but did not maltose and dulcitol. Amongst them, one did not decarboxylate ornithine. Two of our *salmonella* isolates indicated typical characteristics of *Salmonella Pullorum*. The results obtained were almost in accordance with standard biochemical characteristic (Aragaw *et al* 2010). Christensen et al. (1992) reported that ornithine was weakly decarboxylated and also rhamnose fermented late (>2 days) by their isolates. Lee et al. (2003) also showed that there were some differences in dulcitol fermentation and ornithine decarboxylation among their isolates. Our data is similar to the results reported by Christensen and Lee (Table 2). In another study was done by Selvam et al. (2010) *Salmonella* isolated from 6 birds. All the isolates in their study fermented glucose and dulcitol and were confirmed as *Salmonella pullorum* that our results is similar to their study. Eight of our isolates had different results in rhamnose that is in contrary to results in Bergey's Manual of systematic Bacteriology. Also, one of our *Salmonella Gallinarum* had different results in Dulcitol and Maltose that is in contrary to the results reported by Lee et al. (2003). Use of antimicrobials in any environment creates selective pressures that favor the survival of antibiotic resistant pathogens. The emergence of multidrug resistance among *salmonella*

Table1. Biochemical characteristic of isolated *Salmonella gallinarum* and *Salmonella pullorum*

Sample	Citrate	Ornithine	Lysine	TSI	Glucose	Maltose	Dulcitol	Rhamnose	Indole	MR	VP	motility
S1	-	+	+	Alk/A, SH_2	+/g	-	-	+	-	+	-	-
S2	-	+	+	Alk/A, SH_2	+/g	-	-	+	-	+	-	-
S3	-	+(delay)	+	Alk/A, SH_2	+/g	-	-	+	-	+	-	-
S4	+	-	+	Alk/A, SH_2	+	+	+	+(2times)	_	+	_	_
S5	+	_	+	$_{+}SH_2$ Alk/A	+	+	+	+(2times)	-	+	_	_
S6	+	_	+	Alk/A, SH_2	+	+	+	+(2times)	-	+	-	-
S7	_	-	+	Alk/A, SH_2	+	+	-(3times)	+(2times)	-	+	-	-
S8	+(3times)	_	+	Alk/A, SH_2	+	+	+	+(2times)	_	+	-	-
S9	-	+(2times)	+	Alk/A, SH_2	+	-3times)	+	+(2times)	-	+	_	_
S10	+	-	+	Alk/A, SH_2	+	+	+	+(2times)	-	+	-	_
S11	+	-	+	Alk/A, SH_2	+	+	+	+(2times)	-	+	-	-
S12	+	_	+	Alk/A, SH_2	+	+	+	-	_	+	-	-
S13	+	_	+	Alk/A, SH_2	+	+	+	-	_	+	_	-

spp. is an increasing concern (White *et al* 2001). Surveillance for antimicrobial resistance is crucial for monitoring the emergence and spread of antibiotic resistance in *Salmonella* isolates. In the present study, the results of the antimicrobial susceptibility testing showed that all isolates were found to be susceptible to gentamicin. Seven (53.8 %), 6 (46.1%) and 5 (38.4%) isolates were resistant to streptomycin, cephalexin and nalidixic acid respectively. We observed 9 antibiotic resistance patterns. Also multidrug resistance was observed in 6 (46.1%) isolates. Threlfall (2002) stated that in developed countries, antimicrobial resistance of zoonotic Salmonellosis has been attributed to the injudicious use of antimicrobials in food producing animals including poultry. Under this contention the isolates from this study were subjected to antibiogram test with ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfonamides, tetracyclines, trimethoprim, ciprofloxacin, consisting the most commonly used antibiotics in poultry flocks. They showed resistance to up to six commonly used antimicrobials and about 15% of isolates also exhibited decreased susceptibility to ciprofloxacin. Capita et al. (2008) reported that *Salmonella pullorum* isolates were

multidrug resistant (MDR) to amoxycillin, fluoroquinolones, sulphadiazine and tetracycline. The observation in our study was almost similar to his study except for the amoxicillin.

Table2. Antimicrobial susceptibility of *Salmonella gallinarum* and *Salmonella pullorum* by disc diffusion method

ANTIBIOTIC DISC	No. (%)		
	R	I	S
Amoxycillin(penicillin)	1 (7.6%)	0	12 (92.3%)
Streptomycin(Aminoglycosid)	7 (53.8%)	1(7.6%)	5 (38.4%)
Kanamycin(Aminoglycosid)	2 (15.3%)	6 (46.1%)	5 (38.4%)
Neomycin(Aminoglycosid)	1 (7.6%)	5 (38.4%)	7 (53.8%)
Gentamicin (Aminoglycoside)	0	0	13 (100%)
Cephalexin(Cephalosporines)	6 (46.1%)	2 (15.3%)	5 (38.4%)
Cephalothin(Cephalosporines)	3 (23%)	4 (30.7%)	6 (46.1%)
Cefotizoxim(Cephalosporines)	0	1(7.6%)	12 (92.3%)
Cefazolin(Cephalosporines)	2 (15.3%)	2 (15.3%)	9 (69.2%)
Chloramphenicol(Phenicol)	1 (7.6%)	4 (30.7%)	8 (61.5%)
Co-trimoxazole(Sulphonamid)	1 (7.6%)	0	12 (92.3%)
Nalidixic acid(quinolone & fluoroquinolone)	5 (38.4%)	3 (23%)	5(38.4%)
Ciprofloxacin(quinolone & fluoroquinolone)	1 (7.6%)	0	12 (92.3%)
Nitrofurantoin(Nitrofuran)	3 (23%)	0	10 (76.9%)
Tetracycline(Tetracycline)	3 (23%)	1(7.6%)	9 (69.2%)

S=Sensitive, I=Intermediate, R=Resistant

Antibiogram studies by Jahan et al. (2013) indicated that the *Salmonella pullorum* and *Salmonella gallinarum* isolated by them were more or less susceptible to chloramphenicol, azithromycin, ciprofloxaxin, gentamycin and norfloxacin. Kang et al. (2010) reported that antimicrobials including aminoglycosides and fluoroquinolones are commonly used in commercial chicken farms to prevent or treat fowl typhoid in South Korea. They demonstrated that resistance to fluoroquinolone occurred in parallel with multiple aminoglycoside resistance. Recently, some authors have reported an increase in quinolone resistance in *Salmonella* (Kabir 2010, Lee *et al* 2003, Molbak *et al* 2002, Tuhin *et al* 2013) which also partially supports the findings of this study. Further studies that should be brought to attention in future research might be molecular characterization and genomic studies to get an idea over genes responsible for drug resistance of the *Salmonellae Gallinarum* and *Salmonella Pullorum.*

Table3. Antibiotic resistance patterns of *Salmonella gallinarum* and *Salmonella pullorum*

Resistotype	Antibiotic resistance pattern	N0. (%) of isolates
S1	*S*	1(12.5)
S9	*S, CN*	1(12.5)
S10	*S, TE*	1(12.5)
S8	S,NA,TE	1(12.5)
S5	S,CN,NA	1(12.5)
S11	S,CN,N	1(12.5)
S12	*CZ, FM, CF*	1(12.5)
S6	S,CN,NA,TE,CTX	1(12.5)
S13	CN, NA, CZ, FM, CF	1(12.5)
	Total	9

The high resistance of the isolates to quinolones and aminoglycosides, and the reduced susceptibility to fluoroquinolones, can probably be directly attributed to frequent use of the antimicrobials in chicken farms. The high frequency of MDR isolates would be plausible effect of the continuous use of antimicrobials in chickens. According to Sharifi-Mood *et al* (2006) case report about an isolation of *Salmonella gallinarum* from a patient and other researches on this field, it would be suggested that antimicrobial susceptibility testing should be performed as a routine test for ongoing monitoring of antimicrobial resistance in *Salmonella* isolates. This is the first study performed on *Salmonella Pullorum* and *Salmonella Gallinarum* isolates by biochemical tests and antimicrobial susceptibility test in Iran.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Aragaw, K., Terefe, L., and Abera, M. (2010). Prevalence of Salmonella Infection in Intensive Poultry Farms in Hawassa and Isolation of Salmonella species from sick and dead chickens. *Ethiopian Veterinary Journal* 14:115-124.

Capita, R., Alonso, C.C., and Prieto, M. (2008). Prevalence of Salmonella enteric serovars and genovars from chicken carcasses in slaughterhouses in Spain. *Journal Animal Science* 86:173-187.

Christensen, J.P., Olsen, J.E., Hansen, H.C., and Bisgard, M. (1992). Characterization of salmonella enteric serovar gallinarum biovars gallinarum and pullorum by plasmid profiling and biochemical analysis. *Avian & Exotic Animal Clinical Pathology Labs* 21:461-470.

Clinical and Laboratory Standards Institute (2011). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, Wayne, PA, USA.

Grimont, P.A.D., and Weill, F. (2007). Antigenic formulae of the Salmonella serovars Pasteur Institute, Paris, France.

Jahan, F., Kabir, S.M.L., and Amin, M. (2013). Identification and antimicrobial resistance profiles of Salmonellae isolated from the broiler dressing plants associated with their environments. *Advanced Research Journal of Microbiology* 1:001-009.

Kabir, S.M.L. (2010). Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *International Journal of Environmental Research and Public Health* 7:89-114.

Kang, M.S., Kim, A., Jung, B.Y., Her, M., Jeong, W., Cho, Y.M., Oh, J.Y., Lee, Y.J., Kwon, J.H., and Kwon, Y.K. (2010). Characterization of antimicrobial resistance of recent Salmonella enterica serovar Gallinarum isolates from chickens in South Korea. *Avian &Exotic Animal Clin Path Labs* 39:201-205.

Kwon, H.J., Kim, T.E., Cho, S.H., Seol, J.G., Kim, B.J., Hyun, J.W., Park, K.Y., Kim, S.J., and Yoo, H.S. (2002). Distribution and characterization of class 1 integrons in Salmonella enterica serotype Gallinarum biotype Gallinarum. *Veterinary microbiology* 89:303-9.

Lee, Y.J., Kim, K.S., Kwon, Y.K., and Tak, R.B. (2003). Biochemical characteristics and antimicrobials susceptibility of Salmonella gallinarum isolated in Korea. *Journal of veterinary science* 4:161-6.

Mirmomeni, M.H., Naderi, S., Hosseinzadeh, A., and Sisakhtenezhad, S. (2009). Isolation of Salmonella enteritidis using biochemical test and diagnostice potential of sdfI amplified gene. *Research Journal of Biological Sciences* 4:656-661.

Molbak, K., Garner, S.P., and Wegener, S.P. (2002). Increasing quinolone resistance in Salmonella enterica serotype Enteritidis. Emerging infectious diseases 8:514-5.

OIE (2012) Fowl typhoid and pullorum disease. Chapter 2.3.11.

Pomeroy, B.S., and Nagaraja, K.V. (1991) Fowl typhoid. 9 ed. Iowa State University Press Ames., USA.

Proux, K., Humbert, F., Jouy, E., Houdayer, C., Lalande, F., Oger A., and Salvat G. (2002). Improvements required for the detection of Salmonella Pullorum and Gallinarum. *Canadian journal of veterinary research* 66:151-7.

Rajagopal, R., and Mini, M. (2013). Outbreaks of salmonellosis in three different poultry farms of Kerala, India. *Asian Pacific journal of tropical biomedicine* 3:496-500.

Ribeiro, S.A., de Paiva, J.B., Zotesso, F., Lemos, M.V., and Berchieri Janior, A. (2009). Molecular differentiation between Salmonella enterica subsp enterica serovar Pullorum and Salmonella enterica subsp enterica serovar Gallinarum. *Brazilian journal of microbiology: [publication of the Brazilian Society for Microbiology]* 40:184-8.

Selvam, A., Gunaseelan, I., Senthil, Kumar, K., and Seka, M. (2010). Assessment of carrier status of Salmonella Pullorum and Gallinarum infection in healthy flocks. *Tamil Nadu Journal of Veterinary and Animal Sciences* 6:99-101.

Shapoury, R., Rahnama, M., and Eghbalzadeh, S. (2010). Pervalence of salmonella serotypes in poultry meat and egge and antimicrobial resistance in zanjan. *The quarterly journal of biological Sciences* 3:62-71.

Sharifi-Mood, B., Metanat, M., and Salehi, M. (2006). Salmonella gallinarum empyema- The first case from Iran. *Journal of medical sciences* 6:180-182.

Shivaprasad, H.L. (2003). Pullorum Disease and Fowl Typhoid Iowa State University Press, USA.

Snoeyenbos, G.H. (1991). Pullorum disease. 9 ed. Iowa State University Press Ames, Iowa, USA.

Threlfall, E.J. (2002). Antimicrobial drug resistance in Salmonella: problems and perspectives in food- and water-borne infections. *FEMS microbiology reviews* 26:141-8.

Tuhin, A.F., Kabir, S.M.L., Amin, M.M., and Hossain, K.M.M. (2013). Identification and antimicrobial usceptibility of Salmonella species isolated from washing and rinsed water of broilers in pluck shops. *International Journal of Animal and Veterinary Advances* 5:1-8.

White, D.G., Zhao, S., Sudler, R., S. A., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D., and Meng, J. (2001). The isolation of antibiotic-resistant SalmonellaSalmonella from retail ground meats. *New England Journal of Medicine* 345:1147-1154.

Williams, J.E. (1978). Avian salmonellosis. 7 ed. Iowa State University Press Ames, USA.

Yousuf, M., Nadeem, A., and Irfan, A. (2001). Salmonella gallinarum septicaemia in humans. *Pakistan journal of medical science* 17:50-52.