International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Screening of extracellular enzymes as virulence factors in *Salmonella* isolates from the rectal swabs of broiler and backyard chicken

M. Bhuvaneswari, *P. Sivagurunathan, C. Uma and S. Aruljothi

Division of Microbiology, Faculty of Science, Annamalai University, Annamalai Nagar, Chidambaram, Tamilnadu, India.
*Corresponding author: *sivaguru1981@yahoo.com*

Abstract

The aim of this study was to determine the presence of virulence factors in 46 *Salmonella* isolates obtained from free range chickens in urban and rural environments, detected by biochemical approach. Rectal swabs were collected from the backyard and broiler chicken in April 2014 and further confirmations were done bacteriologically. Out of the 80 samples collected, 46 *Salmonella* isolates were identified from both broiler and backyard chicken. More than 70% of the isolates demonstrated proteolytic and hemolytic activity. No significant difference was observed between the enzyme activities of *Salmonella* isolates in backyard and broiler chicken. *Salmonella* isolates, apart from causing serious problems, secrete enterotoxins and other virulent enzymes including protease, lipases and hemolysin, which find their way into the host cells with ease for colonization and propagation.

Keywords: CLSI; hemolysin; *Salmonella*; virulent factors

Introduction

Salmonella organisms are facultative anaerobic gram-negative rods within the family of Enterobacteriaceae (Yan *et al.,* 2003). *Salmonella* infections range from gastrointestinal infections that are accompanied by inflammation of intestinal epithelia, diarrhea and vomiting, to typhoid fever – a life threatening infection (Hensel, 2004). *Salmonellae* are widely distributed in nature and are responsible for a spectrum of diseases in man, animal and birds. Poultry eggs, meat and their products are the commonest carriers and transmit *Salmonella* to humans. Poultry can become infected by horizontal transmission through infected litter, faeces, feed, water, dust, fluff insects, equipment, fomites, diseased chicks and rodents contaminated with *Salmonella* (Poppe, 2000). Backyard chickens can also be infected through contact with wild animals, domestic mammals and commercial poultry that are carriers of *Salmonellae*, and consequently may play a role in the transmission of the organism to other animals and humans. Broiler chicken is the main type of chicken consumed as poultry in many countries. Large percentage of Broiler chicken are colonized by *Salmonellae* during their growing phase and the skin and meat of carcasses are frequently contaminated by the pathogen during slaughter and processing. In India, salmonellosis is hyper-endemic and there is an urgent need to strengthen the monitoring and surveillance of salmonellosis using suitable diagnostic tools so as to prevent and control its occurrence. There are more than 2,435 known serotypes of *Salmonellae* of which 209 serovars have been documented in India (Rahman, 2002) and many of these serotypes are well documented in human pathogens (Kwang *et al.,* 1996). The pathogenicity of *Salmonella* is a complex cascade of microbial and metabolic events. All *Salmonella* express virulence regardless of source and host status (Ohl and Miller, 2001). Pathogenicity is mediated by certain factors such as strain virulence, infectious dose, route of infection and host susceptibility

(Groisman *et al*., 1990). The idea that pathogenic microbes are endowed with certain components that confer upon them the capacity for virulence is the central theme of the virulence factor concept. The virulence factor concept has unquestionably led to the identification of important microbial attributes of virulence that have greatly furthered our understanding of microbial pathogenesis. Extracellular hydrolytic enzymes seem to play an important role in *Salmonella* overgrowth, as these enzymes facilitate adherence and tissue penetration and thus invasion of the host. Expression of virulence factors may be associated with specific characteristics of *Salmonella* isolates such as geographical origin or type of infection, knowledge of such correlation may help us understand the epidemiology of these infections (Price *et al*., 1982). Using the *Salmonella* isolates obtained from urban and rural chicken, this study aims to determine the presence of the virulence factors.

Materials and Methods

Samples and study site

Surveillance on the prevalence of *Salmonellae* in poultry was setup at Chidambaram, Cuddalore district, Tamil Nadu during April 2014. A total of 80 samples were analyzed in the study. Backyard chickens were sampled with 42 rectal swabs while the broiler chickens were sampled with 38 rectal swabs from different farms in Cuddalore district. The rectum of the chickens were swabbed using a transportable sterile swab (Hi-Media) applied with film rolling pressure. The swabs were placed immediately in a sterile container with selenite F broth, stored in ice box and transported to the Microbiology Laboratory, Annamalai University, Tamilnadu.

Bacteriological analysis

Specimens collected and transported to the laboratory following standard methods (Cheesbrough, 2006; Winn *et al*., 2006) were used for bacteriological analysis (Cappuccino and Sherman, 2004). The swab was aseptically streaked on *Salmonella-Shigella* agar (SS) plates for the isolation of *Salmonella*. The inoculated plates were incubated at 37º C for 18–48 hrs, after which they were examined for typical colonies of *Salmonella*. Suspected colonies were streaked onto nutrient agar slants and were stored at 4º C for further analysis.

Detection of extracellular enzymes

A total of 46 isolates from the rectal swabs of backyard and broiler chicken were tested for the production of extra-cellular enzymes (amylase, gelatinase, hemolysin and lipase).

Test for amylase production

This test is used to detect the enzyme amylase, which breaks down starch. Isolated colonies were streaked in straight lines in starch agar plates with starch as the only carbon source. After incubation at 37º C for 24–48 hrs, individual plates were flooded with Gram's iodine. The colonies which were showing zone of clearance in starch agar plates were maintained on to nutrient agar slants. If starch has been hydrolyzed a reddish color or a clear zone appears around the bacterial growth; if it has not been hydrolyzed, a black/blue area indicating the presence of starch develops (Holdeman *et al.*, 1977).

Test for gelatinase and lipase production

For gelatinase production test, the organisms were inoculated on 0.4% gelatin agar. After satisfactory growth, the cultures were flooded with mercuric chloride solution, which rapidly denatured and rendered opaque any unhydrolyzed gelatin. Lipolytic organisms produced a restricted opacity and pearly layer on the medium (Nagmoti *et al.*, 2008).

Test for hemolysin production

Hemolysin production was tested by inoculating the test organism to the blood agar plate (5% blood). The results were read after incubation of 18 hrs (Coque *et al*., 1995).

Results and Discussion

Salmonellae usually infect their hosts via gastrointestinal tract. In the absence of other microflora, the organisms are apparently able to adhere, multiply and colonize at any point along the GI tract of chicken (Soerjadi *et al*., 1982), may be shed in the feces, and form a source of contamination for other animals, humans and the environment (Poppe, 2000). Cloacal swabs have been used to provide evidence of persistent intestinal colonization by *Salmonella*e in individual birds (Gast and Beard,

1990). When compared with fecal culture, rectal swabs were 64% sensitive and 90% specific. Rectal swabs are of moderate diagnostic utility for detection of *Salmonella* and may be useful when collection of fecal samples is impractical (Kotton, 2006). Thus, rectal swabbing offers an easy method of surveying the carrier rate of a specific flock. In this study, among the 80 samples collected, 38 were from broiler chicken brought to Chidambaram from different farms of the urban areas in Tamilnadu and 42 from backyard chicken reared at rural areas of Chidambaram. The region wise distribution of the samples were listed in Table 1.

Table 1 Details about the rectal swabs collected from broiler and backyard chicken

S.No	**Number of Rectal Swabs**			
	Broiler Chicken (N_1 = 38)		**Backyard Chicken (N_2 = 42)**	
	Location (Different farms from urban areas)	**N(%)**	**Location (Rural areas)**	**N(%)**
1.	Tiruchirapalli	3(7.8)	Puduchathiram	7(16.6)
2.	Pollachi	7(18.42)	Periyapattu	5(11.90)
3.	Udumalaipet (Coimbatore)	2(5.26)	Chinnandikuzhi	6(14.28)
4.	Namakkal	4(10.5)	Velangipattu	5(11.90)
5.	Kodaikanal	2(5.26)	Sakkangudi	4(9.5)
6.	Thirunelveli	9(23.68)	Killi	5(11.90)
7.	Rameshwaram	6(15.78)	Chethalapatti	4(9.5)
8.	Palani	5(13.15)	Sivapuri	6(14.28)
	Total = 38(47.5)		Total = 42(52.5)	

Salmonella was isolated from the rectal samples of broiler and backyard chickens with a prevalence of 41.25% and 16.25%. The distribution pattern was higher in the broiler chicken and moderate in the backyard chicken. The literature shows that there is little information about the *Salmonella* infection status in backyard chickens. Bouzoubaa *et al*. (1992) assessed 500 cloacal swabs, as 100 pooled samples, taken from village chickens in 50 different farms in Morocco, and stated that three cultures were isolated: two were *S. pullorum*, and one was *S. gallinarum*.

The overall isolation rate of 13(16.25%) (ten isolates from 80 samples) indicates that there is at least a moderate prevalence of infection in the backyard chicken, posing a risk to industrial poultry farms, and public health. Therefore, any prophylactic program aimed at controlling *Salmonella* infections must also take into account the backyard chicken.

There were a total of 350 samples analyzed during winter (November–February) and 50 samples in early spring (March–April). The imported broiler carcasses were 10.62 times (Odds ratio 10.62) more likely to yield *Salmonella* in the hot season as compared to the winter season. (Naraptidahal, 2007**)**. These differences in isolation might be due to the fact that *Salmonella* is more prevalent in the hotter season (Fossler *et al.*, 2005; Liljebjelke *et al.*, 2005). Similarly in a study in Nepal, the prevalence of *Salmonella* was found highest during the months of April and May (Maharjan *et al.*, 2006) which envisaged our results of higher *Salmonella* prevalence during the month of April. Table 2 depicts the week-wise incidence of the *Salmonella* isolates from both backyard and broiler chicken. The highest incidence of *Salmonella* occurred in the last week of April showing an isolation rate of 13(40.62%) from broiler chicken and 8(25%) from backyard chicken when compared to earlier weeks (2nd and 3rd week) of sample collection.

Table 2 Variations in the isolation of Salmonella during the study period classified by the week of sample collection

Variations in *Salmonella* isolates during the month of April 2014					
2nd week(N=19)		3rd week(N=38)		4th week(N=32)	
Backyard	Backyard	Backyard	Broiler	Backyard	Broiler
3(15.78%)	7(36.84%)	2(5.26%)	13(34.21%)	8(25%)	13(40.62%)

The microbiological examination of the rectal swabs showed 78 samples to be positive for bacteria out of 80 rectal samples collected. Based on the culture characteristics, 46 samples were noted and subcultured as *Salmonella* and 45 samples gave positive culture for bacteria other than *Salmonella*. Fifteen samples were found to be culture positive for both *Salmonella* and other bacteria based on colony morphology. Highest number of *Salmonella* was isolated from broiler chicken (42.3%) in comparison with backyard chicken (16.6%) (Table 3).

Table 3 Bacteriological analysis of the rectal swab samples collected from broiler and backyard chicken

Isolation Details	**Backyard Chicken N(%)**	**Broiler Chicken N(%)**
No. of isolates	42(52.5)	49(61.25)
No. of *Salmonella*	13(16.25)	33(41.25)
No. of other Bacteria	29(36.25)	16(20)
Gram negative rods	26(89.7)	13(81.25)
Gram negative cocci	3(10.3)	3(18.75)
Gram positive rods	–	–
Gram positive cocci	–	–

Enzyme secretions such as phospholipase, proteinase, amylase and hemolytic activity were considered as virulence determinants, and their activities were assayed. The results of the variable determinants are tabulated in Table 4. More than 70% of the isolates demonstrated proteolytic and hemolytic activity. No significant differences were observed between the enzyme activities of Salmonella isolates in backyard and broiler chicken. Thus, the *Salmonella* isolates, apart from being so and causing serious problems, secrete enterotoxins and other virulent enzymes, including protease, lipase and hemolysin, which find their way into the host cells with ease for colonization and propogation

.

Table 4 Number (%) of enzymatic virulent determinants of *Salmonella* isolates

Extracellular enzymes	Backyard N = 13		Broiler N = 33	
	+ve No(%)	-ve No(%)	+ve No(%)	-ve No(%)
Amylase	12(92.30)	1(7.69)	26(78.78)	7(21.21)
Gelatin	7(53.84)	6(46.15)	25(75.75)	8(24.24)
Lipase	6(46.15)	7(53.84)	19(57.57)	14(42.42)
Haemolysin	10(76.9)	3(23.0)	30(90.9)	3(9.09)

Conclusion

Salmonella has developed several virulence traits enabling invasion of host tissues and avoidance of host defencive mechanisms. Virulence factors that contribute to this process are the hydrolytic enzymes. Most of them are extracellularly secreted by the bacteria. The most discussed hydrolytic enzymes produced by *Salmonella* are haemolysin, gelatinase,

lipases and amylases. They may play an important role in the pathogenicity of salmonellosis and their hydrolytic activity probably has a number of possible functions in addition to the simple role of digesting molecules for nutrition. Hydrolytic enzymes contribute to host tissue invasion by destroying cell membranes and by degrading host surface molecules. Moderate hydrolytic activity with broad substrate specificity has been found in most of the *Salmonella* isolates. More than 70% of the isolates demonstrated proteolytic and hemolytic activity. No significant differences were observed between the enzyme activities of Salmonella isolates in backyard and broiler chicken. Comprehensive knowledge on the enzymes secreted during infection can help us in developing an antibacterial agent to inhibit these virulence factors.

References

Bouzoubaa K, Lemainguer K, and Bell JG. Village Chickens a Reservoir of *Salmonella pullorum* and *Salmonella gallinarum* in Morocco. *Preventive Veterinary Medicine*, **12** (1992): 95–100.

Cappuccino JG, and Sherman N. *Microbiology: A Laboratory Manual*. Sixth edition. San Francisco: Benjamin Cummings, (2004): 491.

Cheesbrough M. Microbiological tests. Chapter 7. In: Cheesbrough M. (editor). *District Laboratory Practice in Tropical Countries, Part II*. Second edition. Cambridge: Cambridge University Press, (2006): 9–267.

Coque TM, Patterson JE, Steckelberg JM, and Murray BE. Incidence of Hemolysin, Gelatinase and Aggregation Substance among Enterococci Isolated from Patients with Endocarditis and Other Infections and from Feces of Hospitalized Patients and Community Based Persons. *J Infect Dis*, **17** (1995): 1223–9.

Fossler CP, Wells SJ, Kaneene JB, Ruegg PL, Warnick LD, Bender JB, Eberly LE, Godden SM, and Halbert LW. Herd-Level Factors Associated with Isolation of *Salmonella* in a Multi-State Study of Conventional and Organic Dairy Farms. I. *Salmonella* Shedding in Cows. *Preventive Veterinary Medicine*, **70** (2005): 257–77.

Gast RK, and Beard CW. Production of *Salmonella enteritidis*-contaminated Eggs by Experimentally Infected Hens. *Avian Diseases*, **34** (1990): 438–46.

Groisman EA, Fields PI, and Heffron F. Molecular Biology of *Salmonella* Pathogenesis. In: *The Bacteria. Vol. **XI**: Molecular Basis of Bacterial Pathogenesis*. San Diego, California: Academic Press, Inc. (1990).

Holdeman LV, Cato E, and Moore WE. (editors) *Anaerobe Laboratory Manual*. Fourth edition. Blaksburg: Verginia Polytechnic Institute and State University (1977).

Hensel M. Review: Evolution of Pathogenicity Islands of *Salmonella enterica*. *International Journal of Medical Microbiology*, **294** (2004): 95–102.

Kotton C.N., Lankowski A.J. & Hohmann E.L. (2006). Comparison of rectal swabs with fecal cultures for detection of Salmonella Typhimurium in adult volunteers. Diagn. Microbiol. infect. Dis., 56, 123-126.

Kwang J, Littledike ET and Keen JE. Polymerase Chain Reaction for *Salmonella*. *Lett Appl Microbiol*, **22** (1996): 46–51.

Liljebjelke KA, Hofacre CL, Liu T, White DG, Ayers S, Young S, and Maurer JJ. Vertical and Horizontal Transmission of *Salmonella* within Integrated Broiler Production System. *Foodborne Pathogens and Disease*, **2(1)** (2005): 90–102.

Maharjan M, Joshi V, Joshi DD, and Manandhar P. Prevalence of *Salmonella* Species in Various Raw Meat Samples of a Local Market in Kathmandu, Part II. *Trends in the Study of Disease Agents*, **1081** (2006): 249–56.

Naraptidahal. Prevalence and Antimicrobial Resistance of *Salmonella* in Imported Chicken Cercases. *Avian Diseases*, **43** (2007): 611–15.

Nagmoti JM, Patil CS, Nagmoti MB, and Mutnal MB. Detection of Extra-Cellular Enzymes of Anaerobic Gram-Negative Bacteria from Clinically Diseased and Healthy Sites. *Indian Journal of Medical Microbiology*, **26**(1) (January–March 2008): 65–7.

Ohl ME, and Miller SI. Salmonella a Model for Bacterial Pathogenesis. *Annual Review of Medicine*, **52** (2001): 259.

Price MF,Wilkinson ID, and Gentry LO. Plate Method for Detection of Phospholipase Activity in *Candida albicans*. *Sabouraudia*, **20** (1982): 7–14.

Poppe C. *Salmonella* Infections in the Domestic Fowl. In: Wray C, and Wray A. (editors) *Salmonella* in Domestic Animals. *CAB International* (2000).

Rahman H. Some Aspects of Molecular Epidemiology and Characteristics of *Salmonella typhimurium* Isolated from Man and Animals. *Indian J Med Res*, **115** (2002): 108–12.

Soerjadi AS, Rufner R, Snoeyenbos GH, and Weinack OM. Adherence of *Salmonella*e and Native Gut Microflora to the Gastrointestinal Mucosa of Chicks. *Avian Diseases*, **26** (1982): 576–84.

Winn WC, Allen S, Janda W, Koneman E, Procop G, and Schreckenberger P. The *Enterobacteriaceae*. In: *Koneman's Colour Atlas and Textbook of Diagnostic Microbiology*. Sixth edition. Philadelphia: Lippincott Williams and Wilkins, (2006): 211–303.

Yan SS, Pandrak ML, Abela-Rider B, Punderson JW, Fedorko DP, and Foley SL. An Overview of *Salmonella* Typing Public Health Perspectives. *Clinical and Applied Immunology Reviews*, **4** (2003): 189–204.