VETERINARSKI ARHIV 76 (5), 421-429, 2006

Recognition of virulence parameters in phospholipase A producing *Salmonella*

Shammi Kapoor[1]*, Davinder Kumar Sharma[2], Shukriti Sharma[3], and Mohinder Singh Kalra[1]

[1]*Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab, India*

[2]*Department of Livestock Products Technology, Punjab Agricultural University, Ludhiana, Punjab, India*

[3]*Department of Epidemiology and Preventive Veterinary. Medicine, Punjab Agricultural University, Ludhiana, Punjab, India*

KAPOOR, S., D. K. SHARMA, S. SHARMA, M. S. KALRA: Recognition of virulence parameters in phospholipase A producing *Salmonella*. Vet. arhiv 76, 421-429, 2006.

ABSTRACT

Thirty-two strains of *Salmonella* belonging to six serovars were subjected to antimicrobial drug sensitivity, surface hydrophobicity assay, haemolytic activity assay, hydrolytic activity assay and enterotoxigenicity of enzyme phospholipase A. The five strains that produced phospholipase A were resistant to most of the antibiotics. Using various concentrations of ammonium sulphate for salt aggregation test, a high degree of correlation was observed between phospholipase A production and hydrophobicity. The strain S_{30} (*Salmonella* Enteritidis) producing the maximum phospholipase A was selected for studying the haemolytic and hydrolytic activities, as well as enterotoxigenicity of phospholipase A fractions. The intracellular fraction of the enzyme phospholipase A showed more haemolytic activity and hydrophobic activity than extracellular enzyme. The intracellular enzyme was non-haemolytic to sheep RBC's but hemolyzed all other mammalian erythrocytes. Enterotoxigenic studies revealed maximal gut index with crude intracellular phospholipase A fraction (0.61) followed by crude extracellular fraction (0.57). The purified fractions of the enzyme showed mild to moderate gut reaction as compared to crude fractions, which gave moderate to marked reaction. The role of phospholipase A in exhibiting virulence has been discussed.

Key words: antibiogram, enterotoxigenicity, hydrophobicity, phospholipase A, virulence

Introduction

Salmonella has been recognized as an important cause of food poisoning in human beings, in addition to certain urinary tract infections (SAXENA et al., 1985). This organism is widely present in various food products including meat, milk and their products. The

* Contact address:
Dr. Shammi Kapoor, Associate Professor, Department of Microbiology, College of Basic Sciences, Punjab Agricultural University, Ludhiana- 141 004, India, Phone: +0161 2401960 Ext. 430; E-mail: skapoorpau@rediffmail.com

ISSN 0372-5480
Printed in Croatia

occurrence of phospholipase has been well established in some microorganisms, viz. *E. coli* (FUNG and PROULX, 1969). Although some workers have tried to correlate the pathogenicity of salmonellae and phospholipase A production (SHETTY et al., 1994) little information is available. In view of the importance of this organism in classic enteric infections, the present study was undertaken as an attempt to evaluate the multiple drug resistance, the surface hydrophobicity, hemolytic and hydrolytic activities and the enterotoxigenicity of the phospholipase A-producing strains of *Salmonella* isolated from foods of animal origin.

Materials and methods

Source of cultures. Thirty-two strains belonging to six serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Weltevreden, *S.* Infantis, *S.* Saintpaul and *S.* Bareily) isolated from various food products and handlers formed the basis of the study. Of the 32 strains, only 5 strains, i.e. 15.6% (S_4, S_8, S_{16}, S_{18}, S_{30}) showed the production of phospholipase A enzyme (KAPOOR et al., 2003). These strains were then screened for various virulence parameters.

Drug sensitivity of Salmonella strains. All 32 strains were subjected to antimicrobial drug sensitivity using filter paper discs obtained from Pasteur Biological Laboratories (Span Diagnostics, Gujarat) following the method of BAUER et al. (1966).

Surface hydrophobicity assay. The salt aggregation test (SAT) was performed as per the method of LINDAHL et al. (1981). The test cultures were inoculated into 100 mL of nutrient broth and incubated at 37 °C for 20 hr. The contents were centrifuged at 7500 rpm for 20 min at 4 °C. The cell pellet was washed twice with physiological saline and cells were suspended in 0.05 M phosphate buffer (pH 7.6) to a concentration of 10^7-10^{10} cells/mL. Serial dilutions of ammonium sulphate solution (0.2 M to 3.2 M) were prepared. Equal amounts (20 mL) of each test suspension were added to the different dilutions. The mixture was shaken gently and allowed to settle for 5 min. The ammonium sulphate concentration giving visible bacterial aggregates was regarded as relative hydrophobicity.

Hemolytic activity assay. The haemolytic activities of both the extracellular and intracellular phospholipase A on erythrocyte membranes of various mammalian species (human, sheep, rabbit, rat and horse) were studied according to the method of LENKISH and VOGT (1972). Blood samples from human, sheep, rabbit, rat and horse were collected in Alsever's solution. The mixture was centrifuged at 3000 rpm for 20 min. The sediment erythrocytes were collected and washed three times with physiological saline. One per cent solution was then prepared in physiological saline to which 0.25 mM calcium chloride was added. One mL of crude phospholipase A enzyme was added to 5 mL of 1% erythrocyte suspension and incubated at 37 °C for one hr with intermittent shaking. The mixture was centrifuged at 3000 rpm for 20 min. The absorbance of clear supernatant was determined at 545 nm on Spectronic-20 spectrophotometer. Five mL of 1% erythrocyte suspension incubated with 1 mL of phosphate buffer instead of the enzyme served as control.

Hydrolytic activity assay. The blood samples were collected in Alsever's solution and centrifuged at 3,000 rpm for 20 min. The sedimented erythrocytes were used to study the hydrolytic activity of enzyme phospholipase A produced by *Salmonella* strains. Membrane phospholipids were extracted from erythrocytes in chloroform: methanol mixture (2:1 v/v) mixture and then filtered through Whatman No. 1 filter paper. The filtrate was concentrated in a stream of nitrogen and known amount was spotted on thin layer chromatographic (TLC) plates. The phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were identified using standards. The spots were scraped off and the phosphorus content in mg mL^{-1} was determined by the method of AMES (1966). Total phospholipids were then quantified by multiplying the phosphorus content by a factor of 25 to give phosphatidyl choline and phosphatidyl ethanolamine (mg mL^{-1}).

Phospholipase and enterotoxigenicity. The enterotoxigenicity of the phospholipase A was studied by the use of rabbit ileal loop assay (TAYLOR et al., 1958). Shigella dysenteriae was used as the positive control. Rabbits weighing 1.5-2.0 kg were fasted for 24 hr before the experiment and allowed access to water only. Rabbits were anaesthetized by sodium pentobarbitone intravenously 30 mg/kg body mass. Diethyl ether was used to maintain the anaesthesia during the experiment. The rabbit was secured in dorsal recumbency. The skin on the ventral side of the abdomen was shaved and a cut 2.5 cm in length was made parallel to the midline of the abdomen. The intestine was exposed, taken out and gently flushed with sterilized normal saline solution. Loops approximately 8 cm in length and 4 cm apart were alternately tied by using a surgical silk thread so that the 4 cm segments acted as locks between the segments to be used for experimental purpose. Each segment received 100 μg proteins (enzyme or culture) in 1 mL of phosphate buffer saline (pH 7.6). Phosphate buffer saline served as the control. Ligated intestine was pushed back and abdomen sutured. Animals were sacrificed after an 18-hour interval, the abdomen opened and the intestine taken out. The macroscopic appearance of the intestine was noted. The length of each segment (in cm) and volume of accumulated fluid was recorded. The enterotoxigenicity index was based on gut index (ileal loop ratio) and calculated as follows:

$$\text{Gut index} = \frac{\text{volume of fluid}}{\text{lenght of loop (cm)}}$$

Results

The results of the antimicrobial drug sensitivity of 32 strains revealed that gentamicin and chloramphenicol were effective against 93.8 and 90.6 per cent of the strains, respectively. Carbenicillin, penicillin and triple sulfa were the least effective (Table 1). Although little correlation could be established between phospholipase A producer strains and drug resistance, the strains which produced phospholipase A were more resistant to the antibiotics.

Table 1. Antimicrobial sensitivity of various *Salmonella* strains

S.No.	Antimicrobial	Symbol	Number of strains		
			Sensitive	Intermediate	Resistant
1	Chlortetracycline	A	02 (6.2)	07 (21.9)	23 (71.9)
2	Doxycycline	VB	06 (18.8)	13 (40.6)	13 (40.6)
3	Oxytetracycline	O	02 (6.2)	18 (56.3)	12 (37.5)
4	Tetracycline	T	16 (50.0)	13 (40.6)	03 (9.4)
5	Gentamycin	J	30 (93.8)	00	02 (6.2)
6	Kanamycin	K	25 (78.1)	05 (15.6)	02 (6.2)
7	Neomycin	N	22 (68.8)	05 (15.6)	05 (15.6)
8	Streptomycin	S	20 (62.5)	08 (25.0)	04 (12.5)
9	Erythromycin	E	04 (12.4)	14 (43.8)	14 (43.8)
10	Chloramphenicol	C	29 (90.6)	00	03 (9.4)
11	Amoxycillin	AM	17 (53.1)	00	15 (46.9)
12	Ampicillin	I	21 (65.6)	02 (6.3)	09 (28.1)
13	Carbenicillin	CB	00	08 (25.0)	24 (75.0)
14	Cloxacillin	V	02 (6.2)	00	30 (93.8)
15	Penicillin G	P	01 (3.1)	02 (6.3)	29 (90.6)
16	Cephalexin	CP	11 (34.4)	07 (21.9)	14 (43.7)
17	Cephaloridine	H	23 (71.9)	05 (15.6)	04 (12.5)
18	Bacitracin	B	24 (75.0)	03 (9.4)	05 (15.6)
19	Polymyxin B	X	24 (75.0)	06 (18.8)	02 (6.2)
20	Co-trimoxazole	Q	22 (68.8)	02 (6.2)	08 (25.0)
21	Triple Sulpha	TS	01 (3.1)	02 (6.3)	29 (90.6)
22	Furazolidone	FZ	11 (34.4)	08 (25.0)	13 (40.6)
23	Nitrofurantoin	F	22 (68.8)	05 (15.6)	05 (15.6)
24	Nalidixic Acid	NA	17 (53.1)	10 (31.3)	05 (15.6)

Figures in parenthesis indicate percentage

Hydrophobicity by salt aggregation test (SAT) of five phospholipase A-producing strains is given in Table 2. Cells of strain S_{16} and S_{30} were aggregated by 0.4 M ammonium sulphate, while S_8 and S_4 were aggregated by 0.6 and 1.0 M, respectively. Cells of the strain S_{18} were not aggregated even at 3.2 M. A high degree of correlation was observed

between phospholipase A production and hydrophobicity. The strains (S_{30} and S_{16}) which produced the highest levels of enzyme were aggregated by even lower concentrations of ammonium sulphate (0.4 M), whereas the reverse was true for low phospholipase A producing strains.

Table 2. Determination of surface hydrophobicity of *Salmonella* strains by salt aggregation test

S. N^o	Ammonium sulphate conc. (M)	Strains				
		S_4	S_8	S_{16}	S_{18}	S_{30}
1	0.2	-	-	-	-	-
2	0.4	-	-	+	-	+
3	0.6	-	+	+	-	+
4	0.8	-	+	+	-	+
5	1.0	+	+	+	-	+
6	1.2	+	+	+	-	+
7	1.8	+	+	+	-	+
8	2.4	+	+	+	-	+
9	3.2	+	+	+	-	+

Table 3. Hemolytic and hydrolytic activities of phospholipase A from *Salmonella* strain S_{30} on mammalian erythrocytes

S. N^o	Species	Hemolytic activity of phospholipase-A		% hydrolysis of phosphatidyl choline		% hydrolysis of phosphatidyl ethanolamine	
		Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
1	Human	++	+	35.70	16.30	24.70	12.30
2	Rabbit	+	+	14.20	09.60	17.80	12.10
3	Rat	+	-	21.60	18.40	16.30	07.10
4	Sheep	-	-	00.00	00.00	27.10	14.30
5	Horse	++	++	31.10	24.20	14.00	16.20
6	Control	-	-	-	-	-	-

Table 4. Enterotoxigenicity of phospholipase A fractions from *Salmonella* strain S_{30} tested by ligated ileal loop response

Sr.N°	Test Fraction	Loop positive/ tested	Gut index*	Gut reaction
1.	Inoculated broth	2/3	0.46	Mild to Marked
2.	Extracellular enzyme	3/3	0.57	Marked
3.	Intracellular enzyme	3/3	0.61	Marked
4.	Extracellular enzyme (pure)	1/3	0.16	Mild
5.	Intracellular enzyme (pure)	2/3	0.29	Mild
6.	Positive control	3/3	0.74	Marked
7.	Negative control	0/3	0.00	Negative

*Gut index = fluid accumulated (mL)/length of loop (cm)

The strain S_{30} (*Salmonella* Enteritidis) producing the maximum phospholipase A was selected for studying the haemolytic and hydrolytic activities of extracellular and intracellular phospholipase A fractions (Table 3). The results showed that intracellular enzyme was non- haemolytic to sheep erythrocytes but hemolyzed all other mammalian erythrocytes. The extracellular enzyme was less haemolytic than intracellular fraction. Similarly, both phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were hydrolyzed by both enzyme fractions. The intracellular enzyme was more hydrolytic than extracellular fraction as intracellular enzyme hydrolyzed up to 35.70 per cent of PC and 27.10 per cent PE, while its counterpart could hydrolyze only 16.30 and 16.20 per cent of PC and PE, respectively.

Results of enterotoxigenicity of phospholipase A-producing strains showed positive ligated ileal loop response. *Salmonella* Enteritidis (strain S_{30}), showing the maximum phospholipase A production was selected for assessing the effect of various fractions of this enzyme. The crude fractions of the enzyme showed the maximal gut index (Table 4). The purified fractions of the enzyme showed mild to moderate gut reaction as compared to crude fractions, which gave moderate to marked reaction. The maximal gut index was obtained with crude intracellular phospholipase A fraction (0.61), followed by crude extracellular fraction (0.57). The fluid accumulated in the intestine consisted of cell debris, mucous and blood in crude fractions, while it was less viscous and relatively clearer for purified fractions.

Discussion

Drug sensitivity testing revealed that gentamicin and chloramphenicol was most effective against the *Salmonella* strains and that carbenicillin, penicillin and triple sulfa were the least effective. The high degree of drug resistance could be due to the

indiscriminate use of antibiotics, and in too inadequate doses (ESCAMILLA et al., 1988). Although little correlation could be established between phospholipase A producer strains and drug resistance, the strains which produced phospholipase A were more resistant to the antibiotics.

A high degree of correlation was observed between phospholipase A production and hydrophobicity. An increase in hydrophobicity has been linked with an increase in association of *E. coli* and *Salmonella* sp. to human cells (JAAN et al., 1981). As the hydrophobicity interactions are more important for the adherence of microorganisms to host cells (LINDAHL et al., 1981), the correlation between phospholipase A production and hydrophobicity could provide an explanation for the basis of phospholipase A producing strains being more pathogenic/virulent.

The hyperphospholipase-A producing strain S_{30} was selected to study the haemolytic and hydrolytic activities of extracellular and intracellular phospholipase A fractions. The intracellular enzyme was non-haemolytic to sheep erythrocytes but hemolyzed all other mammalian erythrocytes. The extracellular enzyme was less haemolytic than intracellular fraction. TAKANO et al. (1990) have shown that -toxin from *Clostridium septicum* is required to enhance the haemolytic activity of phospholipase A to rabbit erythrocytes. The non-hemolytic property of phospholipase A to sheep erythrocytes in the present study could therefore be due to absence of certain co-factors required for activity of this enzyme. Both phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were hydrolyzed by both enzyme fractions. The intracellular enzyme was found to be more hydrolytic than extracellular fraction. Such a situation is not rare because the non-haemolytic but hydrolytic action of bee and cobra venom phospholipase A upon human erythrocytes has been reported by several workers (ZWAAL et al., 1973; WILBERS et al., 1979; DEUTRICKE et al., 1981). These workers have also reported that phospholipase A acted on PC and PE fractions of erythrocyte membrane, which is consistent with the findings of the present study.

The enterotoxigenicity of phospholipase A producing strains showed positive ligated ileal loop response. The crude fractions of the enzyme showed the maximal gut index. The purified fractions of the enzyme showed mild to moderate gut reaction as compared to crude fractions, which gave moderate to marked reaction. The maximal gut index was obtained with crude intracellular phospholipase A fraction followed by crude extracellular fraction. It has been concluded that the gastroenteritis caused by this organism is mediated by enterotoxin (SOBEH et al., 1984) and cytotoxic principles (BALODA et al., 1983). The role of *Salmonella* in causing gastroenteritis is well documented (AGGARWAL and CHOWDERI, 1983; WANI and GUPTA, 1990). The culture filtrates of several salmonellae have been shown to possess enterotoxic activity (THAPLIYAL and SINGH, 1978; WANI and GUPTA, 1990).

From the above observations, it is concluded that phospholipase A enzyme of *Salmonella* can be correlated with antimicrobial drug resistance, hydrophobicity, hemolytic and hydrolytic activity, as well as enterotoxigenicity.

References

AGGARWAL, P., A. N. CHOWDERI (1983): *Salmonella* aetiology of acute diarrhea. Salmonella Surv. Bull. 1, 11-18.

AMES, B. N. (1966): Assay of inorganic phosphate, total phosphate and phosphatases. In: Methods in Enzymology. VIII (Newfled, E. E., V. Giinasburg, Eds.) Academic Press. New York. pp. 115-118.

BALODA, S. B., A. FARIS, K. KROVACEK, T. WADSTROM (1983): Cytotoxic enterotoxins cytotoxic factors produced by *Salmonella enteritidis* and *Salmonella typhimurium.* Toxicon. 21, 785-796.

BAUER, A. W., W. M. M. KIRBY, J. C. SHEMIS, M. TRUCK (1966): Antibiotic susceptibility testing by standard simple disc method. Am. J. Clin. Path. 45, 493-496.

DEUTRICKE, B., M. GRIZE, B. FROST, P. LUETKEMUIR (1981): Influence of enzymatic phospholipids cleavage on permeability of erythrocyte membrane. Discrimination between the causal role of split products and of lecithin removal. J. Membrane Biol. 59, 45-51.

ESCAMILLA, J., M. E. KILAPATRICK, H. F. UGRETA (1988): Spurious sulphamethoxazole trimethoprim resistance of *Salmonella typhi*. J. Clin. Microbiol. 23, 205-211.

FUNG, C. K., P. PROULX (1969): Metabolism of phosphoglycerides in *E. coli*. 3. The presence of phospholipase. Can. J. Biochem. 47, 371-373.

JAAN, K., G. SCHMIDT, E. BLUMENSTOCK, K.VOSBECK (1981): *Escherichia coli* adhesion to *Saccharomyces cerevisiae* and mammalian cells: role of pilation and surface hydrophobicity. Inf. Immunity 32, 484-488.

KAPOOR, S., M. S. KALRA, D. K. SHARMA (2003): Production of phosphatase A by isolates of *Salmonella* spp. J. Res. Punjab Agricultural University 40, 221-225.

LENKISH, P. C., W. VOGT (1972): Direct hemolytic activity of phospholipase A. Biochem. Biophys. Acta. 277, 241-247.

LINDAHL, M., A. FARIS, T. WADSTROM, S. HERTEN (1981): A new task based on 'Salting Out' to measure salt hydrophobicity of bacterial cells. Biochem. Biophys. Acta. 367, 295-301.

SAXENA, S. N., M. L. MAGO, N. KUMARI (1985): Increasing prevalence of of high degree resistance strains of *Salmonella* to chloramphenicol and furazolidone in India. Indian J. Med. Res. 39, 97-105.

SHETTY, M., K. L. BAIRY, S. KRISHNANANDA, P. G. SHIVANANDA (1994): Correlation of phospholipase-A and entertotoxin production by *Salmonella typhimurium* with reference to virulence parameters. Indian J. Med. Res. 99, 162-166.

SOBEH, F., K. B. SHARMA, K. PRAKASH (1984): Enterotoxin production by various *Salmonella* serovars isolated from various parts of India. Indian J. Med. Microbiol. 2, 67-71.

TAKANO, S., M. NODA, I. KATO (1990): Activation of phospholipase A_2 in rabbit erythrocyte by a novel hemolytic toxin (H - toxin) of *Clostridium septicum*. Microbiol. Letters. 68, 319-320.

TAYLOR, J., P. MALTHY, J. N. PAYNE (1958): Factors influencing the response of ligated rabbit gut segment to *Escherichia coli*. J. Path. Bact. 76, 491-495.

THAPLIYAL, D. C., I. P. SINGH (1978): Enterotoxin activity of *Salmonella weltevreden* culture filtrates. Indian J. Expl. Biol. 16, 396-398.

WANI, S. A., R. S. GUPTA (1990): Mucosal invasion and morphological reaction in rabbit ileum experimentally infected with enterotoxigenic *Salmonella* bareily. Proceedings of the National Academy of Sciences. B56, pp. 163-167.

WILBERS, K. H., C. W. M. HAEST, M. VON BENTHERIM, B. DEUTRICKE (1979): Influence of enzymatic phospholipids cleavage on permeability of erythrocyte membrane. Protein mediated transfer of monosaccharides and amines. Biochem. Biophys. Acta. 554, 400-404.

ZWAAL, R. A. F., B. ROELOFSON, C. M. COLLEY (1973): Localization of red cell membrane constituents. Biochem. Biophys. Acta. 300, 159-163.

Received: 25 April 2005
Accepted: 8 September 2006

KAPOOR, S., D. K. SHARMA, S. SHARMA, M. S. KALRA: Prepoznavanje pokazatelja virulencije u salmonela koje proizvode fosfolipazu A. Vet. arhiv 76, 421-429, 2006.

SAŽETAK

Trideset i dva soja salmonela svrstana u šest serovarova pretražena su na osjetljivost prema antimikrobnim tvarima, površinskoj hidrofobnosti, hemolitičnoj aktivnosti, hidrolitičnoj aktivnosti i enterotoksigenosti enzima fosfolipaze A. Pet sojeva koji su proizvodili fosfolipazu A bilo je otporno na većinu antibiotika. Upotrebom različitih koncentracija amonijeva sulfata za test agregacije soli ustanovljen je visok stupanj korelacije između proizvodnje fosfolipaze A i hidrofobnosti. Soj S_{30} (*Salmonella* Enteritidis) s najvećom proizvodnjom fosfolipaze A odabran je za istraživanje hemolitičke i hidrolitičke aktivnosti kao i za enterotoksigenost frakcija fosfolipaze A. Intracelularna frakcija toga enzima pokazala je veću hidrolitičnu i hidrofobičnu aktivnost nego ekstracelularna frakcija. Intracelularni enzim nije bio hemolitičan za ovčje eritrocite, ali je bio hemolitičan za eritrocite ostalih vrsta sisavaca. Istraživanje enterotoksigenosti pokazalo je veći crijevni indeks s nepročišćenom intracelularnom frakcijom fosfolipaze A (0,61) nego s ekstracelularnom (0,57). Pročišćene frakcije enzima pokazale su blagu do umjerenu crijevnu reakciju u odnosu na nepročišćene frakcije, koje su davale umjerenu do jaku reakciju. Raspravlja se o ulozi fosfolipaze A u očitovanju virulencije.

Ključne riječi: antibiogram, enterotoksigenost, hidrofobnost, fosfolipaza A, salmonele, virulencija

---