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Isolation, Screening and Charecterization of "Salcols" of *Salmonella* from Meat

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Salmonella **is a Gram negative facultative rod shaped bacteria which live in the intestine of warm and cold blooded animals. Some species are ubiquitous and others are specifically adaptive to a particular host. In human *Salmonella* are the cause of two diseases called Salmonellosis and enteric fever (typhoid). These bacterial pathogens are crucial zoonotic agents in the veterinary as well as medical field. The organism now named *Salmonella enterica* serotype *typhi* was discovered in 1880. Most *Salmonella* outbreaks are associated with the consumption of contaminated products of animal origin. The widespread distribution of *Salmonella* in meat and poultry makes meat products as a good source for the isolation of *Salmonella*.**

Key words: Bacteriocin, *Salmonella,* Sensitivity, antimicrobial activity.

Microbes produce an extra ordinary array of microbial defense systems. These include classical antibiotic metabolic by products, lytic agents, numerous proteins, exotoxins and bacteriocins. Gene coding antimicrobial peptide have been observed in virtually every living organism and these produced by bacteria are termed bacteriocins. These potential chemotherapeutic agents are defined by their bactericidal or bacteriostatic action on strains closely related to the producer bacteria.

Classes of Bacteriocins

Bacteriocins are subdivided into four classes in terms of their biochemical and genetic characteristics.

Class I

It comprises of lantibiotics small molecules characterized by the presence of residues of two modified aminoacid, lanthionine and methyl lanthionine.

Class II

It comprises of heat stable non lantibiotic bacteriocins whose molecular size is under 5Kda. There are three subdivisions.

Class II A

It is defined in terms of aminoacid sequences present in the N-terminus and by the strong antilisterial activity.

Class II B

Comprises the bacteriocins whose activity is dependent on the complementary activity of two different peptides.

Class II C

Is formed by non lantibiotic bacteriocins

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that failed to be ranked under classes of IIA and IIB.

Class III

These bacteriocins are larger in size (above 30KDa), and are heat labile proteins.

Class IV

It is formed by complex proteins that require association with lipids and carbohydrates in order to exhibit antimicrobial activity.

Use bacteriocin instead to kill pathogens

In recent scenario there is a very fast emergence of multidrug resistant strains and the problem to tackle the infections caused by these strains has become very difficult; as an alternate to this "The Designers Drug" – Bacteriocins can be used.

The bacteriocin produced by *Salmonella typhimurium* LT2 has antibacterial activity against *E. coli* K-12Lu which is is beyond the definition of bacteriocins that they are strain specific (Ben-Gurion *et a*l., 1979). So the bacteriocin produced by *Salmonella* is assayed in depth.

In the present study *Salmonella* were isolated from meat samples, identified and confirmed based upon the morphological and biochemical characteristics. The possibility of screening for new *Salmonella* bacteriocin producers is considered to be one of the major interests in bacteriocin research, Hence this study was undertaken to identify and partially characterize the salcols, bacteriocins produced by *Salmonella*.

Further the bacteriostatic activity of the bacteriocin was tested among the same species. Finally to detect the virulent nature of these isolates serum sensitivity assay and antiobiogram was done.

MATERIAL AND METHODS

Collection and Processing of Samples

A total of 100 samples were studied. These samples were obtained from different places in Coimbatore and were a random collection, during April 2007 to June 2007. Swabs from slaughter houses (working table, knife, meat surface) of poultry, goat and cow were collected aseptically in sterile test tubes containing selenite F-broth and immediately brought to the laboratory for processing. The broths were incubated at 37°C for 24 hrs. Primary isolation was done using XLD as a selective medium.

Isolation of *Salmonella*

Suspected colonies from XLD agar were inoculated into MacConkey agar. Charaterization and preliminary identification of suspected *Salmonella* cultures were made on the basis of morphology by microscopic examination (Gram negative rods) and cultural characterististics. Biochemical reactions were done to confirm the isolates.

Identification of *Salmonella*

Typical Salmonella colonies from XLD agar that were non lactose fermentors on Macconkey agar were subjected to the following biochemical tests.

Oxidase Test

A single colony of the suspected culture (24hrs) was removed from the agar surface and was placed on an oxidase disc and the disc was observed for colour change to purple with in ten seconds and the results were recorded.

Urease Test

Christenson's urea agar was prepared and a single colony of the suspected culture was streaked on to the slant and incubated at 37°C for 48hours and then the results were recorded.

Indole Production Test

A single colony of the suspected culture was inoculated into peptone broth. After 24hours of incubation a few drops of kovac's reagent was added to the tubes and the results were recorded.

Methyl Red Test

The suspected cultures were inoculated into MR broth and the tubes were incubated at 37°C for 24hours and after incubation 5 – 6 drops of MR indicator was added.

Citrate Utilisation Test

Simmons citrate medium was prepared, dispensed in tubes, sterilized at 121 C for 15minutes and was allowed to set as slopes. A loop full of suspected culture was streaked onto citrate agar slant and the slant was incubated at 37 C for 24hours after which the results were recorded.

Voges Proaskauer Test

The suspected cultures were inoculated onto MRVP broth and the tubes were incubated at 37 C for 24hours. After incubation a few drops of Barrits reagent was added and the results were recorded.

TSI

Triple Sugar Iron agar medium was prepared, dispensed in tubes, sterilized at 121°C for 15 minutes and was allowed to set as slope with butt a loopful of suspected culture was inoculated by stab and streaked method on to TSI agar and the slant was incubated at 37°C for 24hours after which the results were recorded.

Antibiogram

In vitro susceptibility of the Salmonella isolates to 9 different antimicrobial agents (concentration in mcg) used were Cefazolin (Cz; 30mcg), Ceftazidime (Ca; 30mcg),Ciprofloxacin (Cf; 5mcg), Nalidixic acid (Na; 30mcg), Chloramphenicol (C; 30mcg), Gentamycin (G; 10mcg), Norfloxacin (Nx; 10mcg), doxycycline (Do; 30mcg) and Cotrimoxazole (Co; 30mcg).

Screening for Bacteriocin Producers of cultures of each producer strain were incubated for 24 hours. The cells were separated by centrifugation at 10,000rpm for 10mts. The activity of the bacteriocin in the supernatant was determined by agar well diffusion method with the indicator strain at a concentration of 10^6 cells/ml. the sediment was treated with a few drops of chloroform, shaken well, washed, dissolved in PBS and tested for bacteriocin activity by the same method Patankar and Joshi .

Bacteriocin Assay

The qualitative analysis of the antimicrobial activity was measured in arbitary units per milliliter (Au/ml). The supernatant was diluted in phosphate buffered saline. Aliquots of 80µl from these dilutions were inoculated into the wells. The sediments were treated with a few drops of chloroform, shaken well and dilute in PBS and tested for the titre of bacteriocins. The agar plates were incubated at 35°C for 18 hours, after which inhibition halos were measured. All the experiments were carried out in triplicate with three different supernatants prepared as described before.

Bacteriocin Characterization

Effect of enzymes, pH, and temperature on the bacteriocin activity

The sensitivity of the bacteriocin for different enzymes were checked. Cell free supernatant and sediment were treated with trypsin and lysozyme at a final concentration of 0.5mg/ml; and their antimicrobial activity (Bacteriocinogenic) was assayed by agar well diffusion method.

To study the effect of $_pH$, culture supernatant and sediment was adjusted with $_pH$ values from 1-12 with 4M HCl and 4M NaOH. The activity was then determined by agar well diffusion method.

Effect of temperature on the activity of bacteriocin was assessed by heating the cell free supernatant and sediment at 40°, 50°, 60°, 70°, 80°, 90°, and 100° for 30minutes in a water bath. Samples were withdrawn after 30minutes. At all these temperatures and the activity was checked by the same method. (Farida Khalid *et a.l*, 1999).

Molecular Weight analysis

The molecular weight of the bacteriocin was determined by 15% SDS-PAGE which was performed using the bacteriocin from supernatants of overnight cultures (24hours) which where concentrated by ammonium sulfate precipitation (30% W/V) followed by centrifugation at 12,000rpm for 30minutes at 4°C (Dzung & Diep *et al*., 2006, Alessandra. *et al*., 2007).

The bacteriocin pellets were then suspended in distilled water and the aliquots, which are cell free supernatants were applied (used for loading the wells in the gel) to the gels. After electrophoresis gels were stained with coomassie blue and the molecular weight was observed.

Serum Sensitivity Assay

Normal goat serum was collected, pooled and stored at 4°C. For the serum killing assay bacteria were grown to an O.D 600 of 0.3, then 0.2ml of the culture was removed and centrifuged at 10,000rpm for 2 minutes. The bacterial pellet was then suspended in 1ml PBs with 0.1% gelatin and the bacterial suspension (500µl) was added to the same volume of goat serum. Samples were obtained at 0, 5, 15 and 30 minutes. After incubation at 37^0C, the number of bacteria was calculated by serial dilution and plating onto XLD agar. Heat inactivated serum (56 C for 30minutes) used was a control (Miki Nishio. *et al*., 2005)

RESULTS AND DISCUSSIONS

Salmonella isolates appeared as transparent colonies with or without black colonial appearance on XLD agar and as colourless (NLF) colonies on

MacConkey agar. These colonies were futher differentiated into species based on the biochemical parameters shown in (Table 1)

It has been estimated that approximately 13 million cases of Salmonellosis occur worldwide annually, of which about 70% reports came from China, India and Pakistan (Bhat *et al.,* 1983). In this study, 75 (75%) *Salmonella* were recovered from 100 swab samples from slaughter houses of poultry (35 samples), goat (45 samples) and cow (20 samples). In this, 33 (94.2%) in poultry, 25 (55.5%)in goat and 17 (85%) in cow showed the presence of *Salmonella* (Table 2). Poultry is found to be the largest reservoir of *Salmonella*. The isolation rate was higher than the previous reports that were reported by other workers (Murugar *et al.,* 2005)

Of the 75 *Salmonella* isolates, 37 (49.3%) were *Salmonella paratyphi* A, 28 (37.3%) were *Salmonella paratyphi* B and 10 (13.3%) were *Salmonella typhimurium. Salmonella typhimerium* was found to be higher in poultry (21.21%). *S. paratyphi* A was widely distributed among the three sources, 52% in goat; 36.36% in poultry and 70.5%

Table 1. Biochemical differentiation of salmonella species

S.No.	Test	*Salmonella paratyphi* A	*Salmonella paratyphi* B	*Salmonella typhimurium*
1	Gram stain	-	-	-
2	Motility	+	+	+
3	Oxidase	-	-	-
4	Urease	-	-	-
5	Indole production	-	-	-
6	Methyl red	+	+	+
7	Voges – proskauer	-	-	-
8	Citrate	+	+	+
9	TSI (H_2S/ Gas)	-/+	+/+	+/-
10	Lysine decarboxylase	+	+	+
11	Gelatin hydrolysis	-	-	-
12	Nitrate Reduction	+	+	+
13	Acid Production			
	a) Lactose	+	+	+
	b) Manntol	+	+	+
	c) Sucrose	-	-	-
14	Catalase Production (24)	+	+	+

Table 2. Prevalence rate of salmonella in different sources

S.	Total samples n = 100		
No	Goat n = 45(%)	Poultry n = 35(%)	Cow n = 20(%)
1	25 (55.5)	33(94.2)	17(85)

Table 3. Distribution of salmonella species amoung various sources

S.	Sourse n = 75								
No.	Goat n = 25			Poultry n = 35			Cow n = 20		
	A(%)	B(%)	*typhimurium*(%)	A(%)	B(%)	*typhimurium*(%)	A(%)	B(%)	*typhimurium*(%)
1	13	10	2	12	14	7	12	4	1

in cow. The presence of *S.para*typhi B was also found to be higher in poultry(42.42%) and goat(40%). The distribution of different *Salmonella* species in various sources are shown in (Table:3)

Drug Resitogram

In recent years, antibiotic resistance in *Salmonella* has assumed alarming proportions worldwide(Agarwal *et al.,*1983, Dorn *et al.,*,1992). Monitoring drug resistance pattern among the isolate not only gives vital clues to the clinician regarding therapeutic regime to be adopted against individual cases, but is also an important tool to devise a comprehensive chemoprophylactic and chemotherapeutic drug schedule on herd basis within a geographical area.

In the present study highest number of isolates showed resistance against doxycycline followed by cefazpline and ceftazidime. All the isolates were resistance to cefazoline. The presence of antibiotic residues in foods of animal origin may result in increased drug resistance (Murugkar *et al.,*, 2005). High sensitivity was observed towards nalidixic acid, norfloxacin, ciprofloxacin and gentamycin. Moderate resistance was observed towards ceftazidime, ciprofloxacin, gentamycin and norfloxacin in *S. paratyphi A* (Table 4). *S. paratyphiB* was found to be highly sensitive to gentamycin and nalidixic acid (Table 4, 5).

Variation in drug sensitivity was shown by the isolates in the present study. *S. typhiurium*

Table 4. Antibiogram of *Salmonella paratyphia*

S.	Antibiotics	*S. paratyphia* n = 37					
		No of highly sensitive strain		No of moderately sensitive strains		No of resistance strain	
		No	%	No	%	No	%
1.	Cefazolin (cz) (30 mcg)	-	0	-	0	37	100
2.	Ceftazidime (ca) (30 mcg)	-	0	-	13.51	32	86.46
3.	Chloramphenicol (C) (30 mcg)	33	89.19	4	10.18	-	0
4.	Ciprofloxacin (cf) (5mcg)	36	97.29	-	0	1	2.70
5.	Cotrimoxadzole (Co) (25 mcg)	36	97.29	-	0	1	2.70
6.	Doxycycline (do) (30 mcg)	31	83.78	3	8.10	-	0
7.	Gentamycin (G) (10mcg)	30	81.08	6	16.21	1	2.70
8.	Nalidixic acid (na (30mcg)	34	91.89	3	8.10	-	0
9.	Norfloxacin (nx) (10mcg)	29	78.37	6	16.21	2	5.40

Table 5. Antibiogram of *Salmonella paratyphia* B

S.	Antibiotics	*S. paratyphia* n = 28					
		No of highly sensitive strain		No of moderately sensitive strains		No of resistance strain	
		No	%	No	%	No	%
1.	Cefazolin (cz) (30 mcg)	-	0	-	0	28	100
2.	Ceftazidime (ca) (30 mcg)	-	0	3	10.17	26	92.85
3.	Chloramphenicol (C) (30 mcg)	27	96.42	1	3.57	-	0
4.	Ciprofloxacin (cf) (5mcg)	27	96.42	-	0	1	3.57
5.	Cotrimoxadzole (Co) (25 mcg)	1	3.57	-	0	26	92.85
6.	Doxycycline (do) (30 mcg)	1	3.57	1	3.57	26	92.85
7.	Gentamycin (G) (10mcg)	27	96.42	-	0	1	3.57
8.	Nalidixic acid (na) (30mcg)	28	100	-	0	-	0
9.	Norfloxacin (nx) (10mcg)	26	92.85	1	3.57	1	3.57

was observed to be sensitive to gentamycin and chloramphenicol. It also showed higher resistance to cefazoline and ceftazidime. All the *S.typhimurium* isolates were resistance to atleast one of the 9 antibiotics tested (Table 6). This could be due to the wide and varied use of different antibiotics infeed. Antibiotic resistance has been reported to be more common in *S. typhim*

Detection of bacteiocin production was done according to the method described by Fredicq. A small loopfull (1.0mm) of overnight nutrient broth cultures of the test strains were stale inoculated into thick well dried nutrient agar plates. In a single plate, eight inoculation were done. The

Table 6. Antibiogram of *S. typhimurium*

S. No.	Antibiotics	*S. typhimurium* n = 10					
		No of highly sensitive strain		No of moderately sensitive strains		No of resistance strain	
		No	%	No	%	No	%
1.	Cefazolin (cz) (30 mcg)	-	0	-	0	10	100
2.	Ceftazidime (ca) (30 mcg)	-	0	1	10	9	90
3.	Chloramphenicol (C) (30 mcg)	9	90	-	0	1	10
4.	Ciprofloxacin (cf) (5mcg)	9	90	-	0	1	10
5.	Cotrimoxadzole (Co) (25 mcg)	10	100	-	0	-	0
6.	Doxycycline (do) (30 mcg)	-	0	1	10	9	90
7.	Gentamycin (G) (10mcg)	9	90		0	1	10
8.	Nalidixic acid (na (30mcg)	9	90	-	0	1	10
9.	Norfloxacin (nx) (10mcg)	9	90	-	0	1	10

plates were incubated at 37 C for 48hours. The surface growth was killed by exposing to chloroform for 30 minutes. Chloroform was evaporated and the plates were overlayered with 3-4ml of molten soft agar containing 0.1ml of overnight culture of the induator strain (*E. coli* KL-12). They were incubated overnight . Bacteriocins producing strains showed a zone of inhibition around their stab inoculated growths.

The characteristics of the zones of inhibition produced by Salmonella strains were studied. Action spectrum on a set of *E.coli, Klebsiella, Shigella, Proteus, Pseudomonas* were also studied. Broth activity and cell bound activity were tested. For broth activity 10ml aliquots to cefazoline and ceftazidime. All the *S.typhimurium* isolates were resistance to atleast one of the 9 antibiotics tested (Table:6). This could be due to the wide and varied use of different antibiotics infeed. Antibiotic resistance has been reported to be more common in *S. typhimurium* than the other serovars (Threlfall. *et al.,* 1986). Least resistance by the isolates in the present study wa observed in case of norfloxain an ciprofloxaien .The findings are concurrent to the observations of other workers(Threlfail *et al.,*1986, Shivhare *et al.,* 2000).

Fluoroqeunolone group of antibiotics

Table 7. Activity spectrum of *Salmonella bacteriocin*

S.No.	Microorganism	Supernatant	Sediment
1	*Salmonella*	+	+
2	*E.coli*	+	+
3	*Klebsiella*	-	-
4	*Proteus*	+	+
5	*Shigella*	+	+
6	*Pseudomonas*	-	-

Table 8. Effect of enzymes on bacteriocin activity

S. No.	Enzymes	Sensitivity to proteolytic enzymes	
		Supernatant	Sediment
1	Trypsin	S	S
2	Lysozyme	S	S

R – Resistance : No change in Bacteriocin activity
S – Sensitive : Inhibition of Bacteriocin activity

have rapid and prompt bactericidal action at a very low minimum inhibitory concentrations against Salmonella (Hooper *et al.,*1995).However caution is warranted against their indiscriminate use as is evident from the resistance problems being faced by many developed countries.Excess use or inappropriate use of antibiotics in the rearing of farm animals represent a major factoring the emergence,persistence and spread of resistance *Salmoella* even in the humans who are the the cul-de-sac of the food chain. Hence it is imperative that judicious use of antibiotics use of antibiotics in the treatement and prophylaxis, after *in vitro* testing, be practiced to sustain the usefulness of the antibiotics in controlling Salmonellosis on long term basis.

Salcols of *salmonella*

Of the 75 strains only three (4%) produced bacteriocin. This is to compared to the varying incidence of 1-10% as reported by other authors (Agarwal *et al,*1964; Atkinson *et al.*, 1970; Levine et. al, 1954; Wray *et al.,* 1974).These three strains were among *paratyphi B* isolates.

Bacteriocin production in *S. paratyphi* A and *S. typhimurium* was completely absent. Agarwal (1964) reported that none out of the b3 strains tested of *S. paratyphi* b was bacteriocinogenic. It apparently seems that group B Salmonella have maximum bacteriocin producing strains (Patnakar *et .al.,* 1985). Action spectrum of these bacteriocins on different gram negative organisms were showm in (Table 7). These bacteriocins, salvols were active against the other strains of *Salmonella.* Activity was also observed against *E.Coli*, *Proteus* and *Shigella. Klebsiella* and *Pseudomonas* were not found to be inhibited (Table 8).

Bacteriocin production of both culture supernatant and sediment were assayed by agar well diffusion method. Bacteriocin activity was observerd till 1:40 dilution. Hence the arbitraty unit was found to be 40Au/ml. An arbitraty unit (Au/ml) was defined as the reciprocal of the highest dilution halo larger than 2mm in diameter (Kauramoto *et al.,*2002). Similar bacteriocin activity was reported in all the three strains. Atkinson (1973) has reported that *Salmonella* produces little bacteriocin in broth even after incubation of 3 days. Our findings too support Atkinson's view. The low broth activity may be because of two reasons firstly, the strains may not be capable of producing bacteriocins at high levels, Secondly, though the bacteriocin is produced in good amounts it may be that it is not released in the medium but remains adhering to the producing cell. In *Salmonella* strains the cell bound activity is significant.

The sensitivity of the bacteriocins to the proteolytic enzymes waas analysed by treating it with enzymes like trypsin and lysosyme. Both the supernatant and the sediment were sensitive to the enzymes. Inhibition of bacteriocin activity by these enzymes was proved by the absence of zone on the agar well diffusion assay (Table 9). This shows that the bacteriocin is a protein.

The S*almonella* bacteriocins was sensitive to heating for 30 minutes between 60-100°C.The bacteriocin was found to be stable at 50°C in the supernatant and in the sediment for 30 minutes(Table:9).Fredericing *et al.,*1965 opened that activity spectrum of a bacteriocin is important in classifying it into a certain group. This different colicins give different activity spectra.Our findings clearly indicate that the substances produced by Salmonella strains are related to colicins but are not identical with them.

The bacteriocin of salmonella were sensitive to both more acidic(1,2,3) and basic pH(8,9,10,11,12). Moderate activity was found at a pH ranges of 4,5,6. High activity of the Bacteriocin was found neutral pH. (Table 10). Many of them are considerably more tolerant to acid than alkaline $_{p}$H values (Tahara *et al.,* 1991).

The bactericidal activity of *Salmonella* bacteriocin is associated with a protein with an apparent molecular mass of 30 KDa. All the

Table 9. Effect of temparature on bacteriocin activity

S. No.	Temparature	Sensitivity to Temparature	
		Supernatant	Sediment
1	40 °C	+/R	+/R
2	50 °C	+/R	+/R
3	60 °C	-/S	-
4	70 °C	-/S	-
5	80 °C	-/S	-
6	90 °C	-/S	-
7	100 °C	-/S	-

Table 10. Effect on pH on bacteriocin activity

S.No.	pH	Activity on	
		Supernatant	Sediment
1	1	S	S
2	2	S	S
3	3	S	S
4	4	R	R
5	5	R	R
6	6	R	R
7	7	R	R
8	8	S	S
9	9	S	S
10	10	S	S
11	11	S	S
12	12	S	S

isolates of *Salmonella* were found to be resistantto goat serum antibodies on incubation at 37 C for 0, 5, 15 and 30 minutes. On serial dilution and plating onto XLD agar, the same n umber of colonies were obtained with that of the control. This may be an important virulent factor in *Salmonella,* suggesting the potential pathogen city of the isolates.

Serum resistance is an important virulence determinant in gram negative bacteria. In a study by Mider *et al.,* 1989, it was found that the pag c gene of S. enterica serovar choleraescis chromosome confers a high level resistance to the bactericidal activity of pooled normal swine serum when cloned into *E.coli* or *Salmonella* strains. In addition insertion and deletion mutations of the cloned pag c gene abolished serum resistance in the host strains. Pag c is a pho PQ – regulated gene required for intramacrophage survival and virulence of *Salmonella* in a mouse infection model.

CONCLUSION

Undoubtedly, the bacteriocins constitute of the most promising groups of antimicrobial peptides. Despite the efforts made to understand their mode of action, biosynthesis, and heterologous production, several points remain to be elucidated. As summarized, the overall mechanisms behind bacteriocin secretaion and regulation of bacteriocin production are fairly well described, but the molecular details involved in these mechanisms are only partly understood, and several fundamental questions remain unsolved. Extensive structural and genetic analyses of both the transporter proteins and the regulatory proteins, including the inducer peptide, are necessary to unravel their molecular mechanisms and how they interact in the cell.

In the future, detailed structural information obtained by NMR spectroscopy, X-ray crystallography, and site-directed mutagenesis of more class IIa immunity proteins will be required to get in-depth understanding, how they physically inhibit their antimicrobial activity.

Food application bacteriocins is appearing as a good alternative to protect foods against pathogenic microorganisms. Nevertheless, we also expect more applications of bacteriocins in the medical area as antibiotic complements, since encouraging examples have been reported for nisin in the treatment of infections caused by *Pseudomonas aeruginosa* and methicillin – resistant *Staphylococcus aureus* and Vancomycin-resistant *enterococci.*

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