

Acid tolerance, bile salts and serum resistance of *Salmonella* Gallinarum from hens

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ABSTRACT

A total of ten *Salmonella* Gallinarum strains in log-phase of growth isolated from internal organs of dead hens with acute fowl typhoid were investigated for potential virulence factors. The strains differed in their acid tolerance, bile salts and serum resistance. *S. Gallinarum* cells were 70% resistant just 1 h post challenge at pH 2.0 and 0% at 4 and 24 h. When surviving at pH 2.5 and 3.0 it was found that the same cultures survived at these pH values much better than at pH 2.0. Half of the strains showed resistance to 20% and more than 20% bile salts, while the other half of strains showed resistance to 10% bile salts concentration. Seventy percent of the strains were resistant to blood serum from men and cattle, 60% to blood serum from women, 50% to blood serum from sheep, 40% to blood serum from swine and 90% to blood serum from chickens. This study established for the first time a range of apparently inherent tolerance in *S. Gallinarum* strains, and independently, that all of the strains *S. Gallinarum* isolated from dead hens with acute fowl typhoid showed different acid tolerance, bile salts and serum resistance.

Key words: chicken, *Salmonella* Gallinarum, acid tolerance, bile salts, resistance, serum resistance

Introduction

Salmonella Gallinarum produces fowl typhoid, which is a disease of major economic importance in many countries (SHIVAPRASAD, 1997). We are still far from understanding the pathogenesis of *S. Gallinarum* infection

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and the search for relevant pathogenic factors remains a necessary task. Upon entering a suitable host, *Salmonella* spp. encounter and must be able to resist the action of acidic pH of the stomach (ROWBURY et al., 1989; JONES and FALKOW, 1996), the action of bile salts (SUKUPOLVI and VAARA, 1989; Van VELKINBURGH and GUNN, 1999), bactericidal action of the blood serum (TAYLOR, 1983; D'AOUST, 1991). The success or failure of a bacterial pathogen during infection of a host relies upon its ability to sense and respond to its immediate environment. *Salmonella* spp. encounter numerous different environments. Many of these environments are potentially lethal to the bacterium; therefore, the requisite survival response includes mechanisms of resistance to these lethal factors. Responses to such stresses may influence subsequent ability to survive and cause disease (FOSTER and HALL, 1990; ROWBURY et al., 1989). Although the relationship between possession of different potential factors and virulence has been demonstrated in different bacterial pathogens (MEKALANOS, 1992; HUMPHREY et al., 1996; WILMES-RIESENBERG et al., 1996; CARRAMINANA et al., 1997) nothing is known about the presence of these potential factors in *S. Gallinarum* isolates from poultry with respect to acid tolerance, bile salt and serum resistance.

Materials and methods

Bacterial strains used in this study included 10 field *S. Gallinarum* strains isolated from internal organs of dead hens with acute fowl typhoid and which were stored in Dorset medium at 4 °C. Throughout the course of the experiments the strains were subcultured daily on sheep blood agar incubated at 37 °C to maintain their pathogenicity. Brain heart infusion broth cultures of the strains to be tested were grown for 3h at 37 °C to obtain log phase cells. The initial levels of *S. Gallinarum* were approximately 10^5 cells per ml. As the growth phase of an organism can influence its resistance to stressful conditions, we were careful to ensure that cultures were in the same phase in comparative studies. Samples from the above cultures were inoculated into brain heart infusion broth (1 ml in 9 ml broth), adjusted to pH values 2.0, 2.5 and 3.0, using 1 mol/l/-1 salty acid (pH was monitored using a pH meter OP-288 /Hungary radelkis, Budapest/). Viable counts were determined immediately prior to acid

challenge and at 1, 2, 4 and 24h post challenge (HUMPHREY et al., 1993). Standard minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays of bile resistance log phase were subjected to various concentrations of bile salts (No. 3 Difco Lab., Detroit, MI, USA) from 25%- 20%- ... to 5%. All assay mixtures were incubated overnight at 37 °C and analysed for MICs. MBCs were determined by plating tube cultures exhibiting no apparent growth in the MICs assay (VAN VELKINBURGH and GUNN, 1999). Resistance against blood serum from healthy men, women, sheep, cattle, swine and chickens was done by inoculation of 500 µl of broth, including 20% fresh serum with 50 ml of bacterial suspension in log-phase. The suspension of bacteria in serum was incubated without shaking and viable counts were made at 0 and 3h (SUSS et al., 1982). Complement-inactivated serum (heated for 20 min at 56 °C was always included as a control).

Results

The *Salmonella* Gallinarum strains differed in their tolerances of acid, bile salts and serum resistance. Studies characterizing these parameters of *S. Gallinarum* involved the use of clinical isolates. As shown in Fig. 1 *S. Gallinarum* cells at pH 2.0 were 70% resistant just 1h post challenge, 20% by 2h post challenge and 0% at 4 and 24h. When surviving at pH 2.5 and 3.0 it was found that the same cultures survived at these pH values much better than at pH 2.0. Although bile resistance in *Salmonella* spp. has been known for some time, the MIC and MBC of bile salts for *S. Gallinarum* strains were: half showed resistance to 20% and over 20% bile salts, while the other half were resistant to 10% bile salts concentration (Table 1). Surprisingly, the MICs of bile salts for *S. Gallinarum* were identical to the MBCs (data not shown).

Table 1. Minimal inhibitory concentration of bile salts for *Salmonella* Gallinarum strains

Strains	N°	Minimal inhibitory concentrations (% bile salts)					
		25	25	20	15	10	5
<i>S. Gallinarum</i>	10	2 (20)	1 (10.0)	2 (20.0)	0	5 (50.0)	0

A high percentage of the *S. Gallinarum* strains investigated showed serum resistance. Seventy percent of the strains were resistant to serum from men, 60% were resistant to serum from women, 70% to serum from

Table 2. Number (%) of blood serum resistant *Salmonella* Gallinarum strains

Strains	N°	Resistance to blood serum from					
		men	women	sheep	cattle	swine	chicken
<i>S. Gallinarum</i>	10	7 (70)	6 (60.0)	5 (50.0)	7 (70)	4 (40.0)	9 (90)

cattle, 50% to serum from sheep, 40% to serum from swine, and 90% to serum from chickens (Table 2). No significant reduction in bacterial growth was obtained when heat-inactivated sera from normal individuals were used.

Discussion

This study was the first to examine a range of apparently inherent tolerance in *S. Gallinarum* strains. Host-pathogen interactions are a dynamic process in which an invading microbe encounters diverse and often harsh environments within the host. As a result, survival and growth in the various locations require that this bacterium constantly adapts to its changing environment. In fact, there are numerous examples in the literature of virulent determinants that are regulated in response to different environmental factors (GRIFFITS, 1991; MEKALANOS, 1992; VAN VELKINBURG and GUNN, 1999). When enterobacteria are exposed to inhibitory chemicals, the inducing molecules generally cross the outer membrane and compartments of the cell membrane, which have sensory feelers into the periplasm, and respond to increased periplasmic concentration of the chemical by conformational changes which triggers internal production of effector molecules (STOCK et al., 1989)

It has not been possible to fully identify the cellular mechanisms responsible for enhanced acid and bile tolerance and serum resistance. Protein synthesis clearly has a role in the development of acid resistance

in *S. Enteritidis* (HUMPHREY et al., 1993) and in *S. Typhimurium* (FOSTER and HALL, 1991). VAN VELKINBURG and GUNN (1999) demonstrated that *Salmonella* spp. dramatically alters protein expression in response to bile and that PhoP-PhoQ two compounds the regulatory system in a major part of the bile resistance mechanism of *Salmonella*. Changes in LPS and membrane protein have been shown to affect bile salts tolerance (SUKUPOLVI and VAARA, 1989).

The results showed a high percentage of *S. Gallinarum* strains resistant to metabolic inhibition of different sera. It has been established that sera from chickens inhibited *S. Gallinarum* strains less efficiently than either bovine, human, sheep or swine sera. The blood serum resistant strains are frequently isolated as causative agents of infections involving tissue damage (TAYLOR, 1983). It has therefore been suggested that serum resistance is an important determinant of virulence.

There exists a remarkable difference in the invading capacity of different hosts. A few serovars are host-adapted and under normal circumstances will cause disease only in the host to which they are adapted (EKPERIGIN and NAGARAJA, 1998). Examples include *S. Gallinarum*-Pullorum, which will infect and cause fowl typhoid and pullorum disease in chicken and turkey, but will not naturally infect man, cattle, swine, or sheep. Occasionally, however, *S. Gallinarum* or *S. Pullorum* strains have been isolated from man and domestic animals (SHIVAPRASAD, 1997).

Previous studies (MORGAN et al., 1986; WILMES-RIESENBERG et al., 1996) showed that bacteria with enhanced tolerance to acid, bile salts and are blood serum resistant may be better able to survive in the organisms of animals, and facilitate infection. Our experimental strains of *S. Gallinarum*, however, showed independently that those isolated from dead hens with acute fowl typhoid have different survival profiles. It is possible that the pathogenicity of *S. Gallinarum* strains has multifactorial nature.

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SAŽETAK

Istraženi su mogući čimbenici virulencije u logaritamskoj fazi razmnožavanja u 10 sojeva bakterije *Salmonella Gallinarum* izdvojenih iz unutarnjih organa lešina kokoši uginulih od kokošjeg tifusa. Sojevi su se razlikovali po svojoj podnošljivosti prema pH te po otpornosti prema žučnim solima i serumu. Pri pH 2,0 čak 70% stanica *Salmonella Gallinarum* bilo je otporno u tijeku jednog sata. Sve bakterijske stanice su uginule nakon 4 sata držanja pri pH 2,0. Bakterije su preživjele pri pH 2,5 i 3. Polovica sojeva bila je otporna prema 20%-tnoj ili većoj koncentraciji žučnih soli, dok je druga polovica pokazivala otpornost prema 10%-tnoj koncentraciji žučnih soli. 70% sojeva bilo je otporno na krvni serum muškaraca i goveda, 60% na krvni serum žena, 50% na krvni serum ovce, 40% na krvni serum svinje i 90% na krvni serum pilića. U radu je po prvi put dokazana otpornost sojeva bakterije *Salmonella Gallinarum* prema pretraženim čimbenicima. Sojevi te bakterije izdvojeni iz lešina kokoši s akutnim kokošjim tifusom pokazivali su različitu podnošljivost prema pH i otpornost prema žučnim solima i serumu.

Ključne riječi: kokoš, *Salmonella Gallinarum*, kiselinska podnošljivost, žučne soli, serum, otpornost
