The Pharma Innovation Journal 2017; 6(2): 42-47

www.ThePharmaJournal.com

The Pharma Innovation

ISSN: 2277- 7695
TPI 2017; 6(2): 42-47

www.thepharmajournal.com
Received: 09-12-2016
Accepted: 10-01-2017

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In vitro evaluation of druggability prioritization targets of *Salmonella gallinarum*

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Abstract
An experimental study was conducted to evaluate the druggability prioritization targets of *Salmonella gallinarum* in commercial broilers in two experimental phases. In phase I of the experiment total proteome of pathogen Vs host comparison using comparative genomics was done followed by protein docking of the target proteins of the pathogen and drug target identification and then calculation of drug prioritization parameters for therapeutic target. Pharmacological evaluation of *in vitro* anti-biogram test of *Salmonella gallinarum* was done in phase II of experiment. 3965 proteins of *S. gallinarum* were compared with 24,068 proteins of *Gallus gallus* by BLASTP analysis. Out of 12 ligands, which were the lead molecules based on *in silico* virtual screening, used *in vitro* agar diffusion (for MIC), none of them was found effective as there was no inhibition. Further, minimum inhibitory concentration (MIC) was assessed with seven antibiotics (enrofloxacin, moxifloxacin, azithromycin, erythromycin, chloramphenicol, cefpodoxime and clindamycin), *Salmonella gallinarum* showed moderate efficacy in terms of zone of inhibition to one antibiotic (Cefpodoxime).

Keywords: *In-silico*, Druggability, *Salmonella gallinarum, Gallus gallus*

1. Introduction
Fowl typhoid is a disease of domestic birds caused by *Salmonella gallinarum*. It is distributed worldwide and the course of the disease is either acute or chronic. Fowl typhoid has been eradicated from those countries which have an intensive poultry rearing system for many years and is now of particular importance in those countries which are beginning the system e.g. countries in Latin America, South America, Middle East, the Indian subcontinent and parts of Africa [36, 40].

Fowl typhoid, an acute septicaemic disease of avian species caused by *Salmonella gallinarum* [37], affects all age groups of chickens. Morbidity is high among all age groups of the birds, whereas mortality may range widely from 10% to 90% [26]. Maintaining a disease free status is a challenging exercise due to the rapid expanding nature of the industry. This is indicated by the fact that a number of Salmonella outbreaks reported in the world are a result of injudicious introduction of infected birds [30]. Thus, poultry industry is facing great setbacks due to frequent outbreaks of salmonellosis [15]. Since its discovery, many efforts have been made to control and prevent the occurrence in commercial poultry farming. However, outbreaks of Salmonellosis still remain a serious economic problem in countries where control measures are not efficient or in those areas where the climatic conditions favour the environmental spread of these microbes [6].

The economic losses are chiefly due to morbidity, mortality, reduced growth rate, reduced feed conversion efficiency, drop in egg production, decreased fertility and hatchability [29]. The endemicity of the disease has been established in India including Haryana, where the infection was first detected in Gurgaon area in 1981 [3]. [28] Studied major diseases affecting broiler chickens during 1987-1990 in Haryana and observed that fowl typhoid was the one that caused maximum mortality (10.54%) and accounted for the second highest number of outbreaks (198) after *E. coli* (266).

Control of fowl typhoid is difficult [42] due to endemicity of the disease, facultative intracellular nature of causative organism, both vertical [34] and horizontal [13] modes of transmission, presence of carrier stage and multiple drug resistance. Fowl typhoid can be controlled by a combination of stringent management procedures and chemotherapy. The widespread and indiscriminate use of antibiotics in the treatment of poultry diseases has lead to antimicrobial resistance of resistant Salmonella strains [14] which is of a global public health concern [1].

However, [14] has reported that *Salmonella gallinarum* strains are becoming more resistant to antibiotics than other avian salmonellas, which meant that it is more difficult to treat infected flocks successfully. However, this view may change, as its true prevalence becomes known with improved diagnostic tests, and with the likely failure of antibacterial agents to control disease in the future and with the emphasis on curtailing the spread of disease in poultry.

2. Materials and Methods

2.1. Isolation and Identification of *S. gallinarum*

2.1.1 Collection of samples

Samples (intestine and liver) were collected at necropsy of chickens for bacteriological study. For bacteriological study the cotton swabs were kept into the test tubes containing tetrathionate broth (TTB) which inhibits the growth of all bacteria except salmonella.

2.1.2 Culture

All the samples were collected on tetrathionate broth. Then subculture was performed on different selective and enriched media of Salmonella such as Salmonella-Shigella (SSA) agar, triple sugar iron (TSI) agar, and brilliant green agar (BGA) to get pure culture and to study cultural characteristics [12, 31, 33].

2.1.3 Morphological characterization

The representative Salmonellae colonies on BGA were characterized microscopically using Gram's stain according to the method described by [31]. The motility test was performed according to the method described by [12] to differentiate motile Salmonellae from the non-motile.

2.1.4 Biochemical test

Five basic sugars such as dextrose, sucrose, lactose, maltose, and mannitol were used for sugar fermentation test. Several types of biochemical tests such as TSI agar slant reaction, methyl red (MR) test, Voges Proskauer (VP) test, indole test and dulcitol fermentation test were also performed in this study according to the methods described by [12, 31, 33].

Table 2.1: Biochemical Investigation of *S. pullorum* and *S. gallinarum*

	***Salmonella* Pullorum**	***Salmonella gallinarum***
TSI glucose (acid formation)	+	+
TSI glucose (gas formation)	V	–
TSI lactose	–	–
TSI saccharose	–	–
TSI hydrogen sulphide	V	v
Gas from glucose (medium with Durham tube)	+	–
Urea hydrolysis	–	–
Lysine decarboxylation	+	+
Ornithine decarboxylation	+	–
Maltose fermentation	– or late +	+
Dulcitol	–	+
Motility	–	–

□ = 90% or more positive reaction within 1 or 2 days; – = No reaction (90% or more); v = Variable reactions.

2.2. Anti-biogram test

The antibiogram test was carried out on isolates of *Salmonella gallinarum* inoculated in each test of sensitivity to the antibiotics. The antibiogram test was designed on the basis of micro dilution method in broth medium [47, 18].

2.3 Chemicals and Reagents

A. Drugs

Table 2.2: Antibiotic Concentration Strips (Varied Concentrations, 0.002-256mcg/ml) (Part A)

Sl. No	Name	Concentration	Company
1	Cefpodoxime	0.016-256mcg/disc	Himedia
2	Erythromycin	0.016-256mcg/disc	Himedia
3	Moxifloxacin	0.02-32mcg/disc	Himedia
4	Clindamycin	0.016-256mcg/disc	Himedia
5	Chloramphenicol	0.016-256mcg/disc	Himedia
6	Enrofloxacin	0.02-32mcg/disc	Himedia
7	Azithromycin	0.016-256mcg/disc	Himedia

Table 2.3. Lead Compounds: (Part D)

S. No	Name	Concentration	Company
1	Labetolol (100 mg tab)	2 tabs of 100mg/100ml=2mg/ml 5ml of this+5ml=1 mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.12 mg/ml 5ml of this+5ml=0.06 mg/ml 5ml of this+5ml=0.03 mg/ml	Sun Pharma
2	Fenofibrate (145 mg tab)	2 tabs of 145mg/100ml=29mg/ml 1ml of this+5ml=14.5mg/ml 1ml of this+5ml=7.25 mg/ml 1 ml of this+5ml=3.62 mg/ml 1 ml of this+5ml=1.81 mg/ml 1ml of this+5ml=0.90mg/ml 1ml of this+5ml=0.45 mg/ml	Knoll
3	Paracetamol (500 mg tab)	2 tabs of 500mg/100ml=10mg/ml 50ml of this+50ml=5mg/ml 25ml of this+25ml=2.5 mg/ml 10ml of 'b' +40ml=1 mg/ml 5ml of this+5ml=0.5mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.125 mg/ml	HSN International
4	Glucosamine (500 mg tab)	2 tabs of 500mg/100ml=10mg/ml 50ml of this+50ml=5mg/ml 25ml of this+25ml=2.5 mg/ml 10ml of 'b' +40ml=1 mg/ml	Elder Pharma

		5ml of this+5ml=0.5mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.125 mg/ml	
5	Chlorpromazine (100 mg tab)	2 tabs of 100mg/100ml=2mg/ml 5ml of this+5ml=1 mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.12 mg/ml 5ml of this+5ml=0.06 mg/ml 5ml of this+5ml=0.03 mg/ml	Sun Pharma
6	Phenylephrine (50mg tab)	4 tabs of 50mg/100ml=2mg/ml 5ml of this+5ml=1 mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.12 mg/ml 5ml of this+5ml=0.06 mg/ml 5ml of this+5ml=0.03 mg/ml	Auro lab
7	L-glutamic acid (10 gr)	(1 gr) 1000mg/100ml=10mg/ml 50ml of this+50ml=5mg/ml 25ml of this+25ml=2.5 mg/ml 10ml of 'b' +40ml=1 mg/ml 5ml of this+5ml=0.5mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.125 mg/ml	Nouveau Medicament
8	Ethambutol (800 mg tab)	1 tabs of 800mg/100ml=8mg/ml 5ml of this+5ml=4 mg/ml 5ml of this+5ml=2 mg/ml 5ml of this+5ml=1mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25mg/ml 5ml of this+5ml=0.12mg/ml	Lupin laboratories Ltd
9	Thiamine (100 mg tab)	2 tabs of 100mg/100ml=2mg/ml 5ml of this+5ml=1 mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.12 mg/ml 5ml of this+5ml=0.06 mg/ml 5ml of this+5ml=0.03 mg/ml	Franko Indian
10	Topiramate (25 mg tab)	8 tabs of 25mg/100ml=2mg/ml 5ml of this+5ml=1 mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.12 mg/ml 5ml of this+5ml=0.06 mg/ml 5ml of this+5ml=0.03 mg/ml	Wyeth
11	Chlorpheniramine (10 mg tab)	10 tabs of 10mg/100ml=1mg/ml 5ml of this+5ml=0.5 mg/ml 5ml of this+5ml=0.25 mg/ml 5ml of this+5ml=0.12 mg/ml 5ml of this+5ml=0.06 mg/ml 5ml of this+5ml=0.03 mg/ml 5ml of this+5ml=0.015 mg/ml	Bayer
12	Galantamine (4 mg tab)	10tabs of 4mg/100ml=4mg/ml 1ml of this+5ml=2mg/ml 1ml of this+5ml=1 mg/ml 1 ml of this+5ml=0.5 mg/ml 1 ml of this+5ml=0.25 mg/ml 1ml of this+5ml=0.125mg/ml 1ml of this+5ml=0.06 mg/ml	Sun Pharma

Note: 10 µl of each concentration was used in MIC.

2.4 Preparation of Mueller-Hinton agar

The Mueller-Hinton agar was prepared as per the instructions provided by the manufacturer. After autoclaving the media at 121 ºC for 15 minutes, it was cooled to 50 ºC and approximately 30 ml to 50 ml was poured into the 15 x 150 mm Petri dishes. The depth of the agar in the petri dishes was maintained approximately at 4 mm. The freshly prepared plates were used on the same day. The pH of the medium was regularly tested for its consistency.

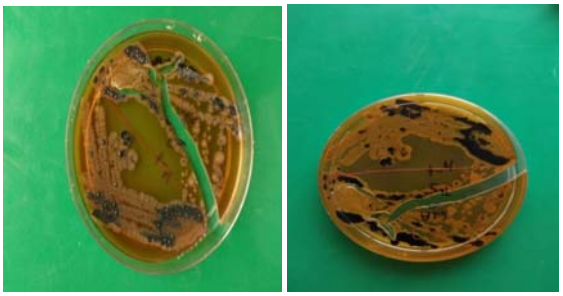
2.5 Composition and preparation of culture media and reagents

Muller Hinton II agar (e.g from BBL)
Beef extract 2.0 g
Acid hydrolysate of casein 17.5 g
Starch 1.5 g
Agar 17.0 g
Distilled water 1000 ml

Preparation
Dissolved the dehydrated medium in water by heating if necessary. Adjusted PH to 7.2-7.4 and transfered into bottles and autoclaved at 110 ^{0}C for 20 min. The agar plates were prepared then seven commercially prepared antibiotic strips were placed to each plates. Two non-antibiotic compounds were made into seven different dilutions as 5µg, 2.5 µg, 1.25 µg, 0.6 µg, 0.3 µg, 0.15 µg/10 µL for Labetalol and 14.5 µg, 7.2 µg, 3.6 µg, 1.8 µg, 0.9 µg, 0.4 µg, 0.2 µg/µL for Fenofibrate. (These two molecules were selected based on ABST) Afterwards seven wells were punched of different dilutions using block making holder and the drugs of different dilutions added to the wells. Plates are incubated for 16–24 h at 35 °C prior to determination of results.

2.6. MIC (minimum inhibitory concentration)
2.6.1 Disk diffusion test
The disk diffusion susceptibility method [22, 7, 10] is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately 1–2×10^{8}CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35 °C prior to determination of results. The zones of growth inhibition around each of the antibiotic strips are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) [10].

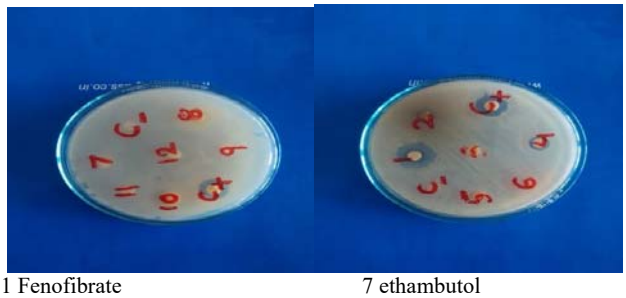
3. Results and Discussion
In this study, Salmonella *gallinarum* isolates were found resistant to enrofloxacin, moxifloxacin, azithromycin, erythromycin, chloramphenicol and clindamycin whereas cefpodoxime showed greater sensitivity to *Salmonella gallinarum*. Cefpodoxime had greater activity among antibiotics used with a range 0.016-256 µg/ml.
These findings are in agreement with research results of [21] who also used agar diffusion method for MIC detection in their research and showed *Salmonella gallinarum* isolates were resistant to tetracycline, streptomycin, kanamycin. But they reported their isolates were sensitive to cephalothin, tylosin. The results were also in agreement with reports of [44, 23]. [35] Who used agar diffusion method reported *Salmonella gallinarum* isolates were resistant to tetracycline and susceptible to ciprofloxacin similar to our findings. But they reported their isolates were resistant to gentamycin, kanamycin, amoxicillin. On the other hand [27] reported susceptibility to ampicillin resistant to chloramphenicol, erythromycin, streptomycin, tetracycline [38] described that the sensitivity of *Salmonella* species was 100% for ciprofloxacin followed by amoxicillin [20] and [39] stated that in *Salmonella* the highest antibiotic resistance observed was to erythromycin followed by streptomycin, tetracycline and chloramphenicol which was in accordance with the results of the present study [4] and [25] who also used agar diffusion method for MIC detection in their research reported *Salmonella gallinarum* isolates were sensitive to streptomycin and chloramphenicol which was contrary to the results of the present study
Ciprofloxacin had greater activity among antibiotics used with a range 0.016-0.125 µg/mL. [32] Investigated development of antibiotic resistance in *Salmonella gallinarum* and reported resistance to amoxicillin. However, in our research the *Salmonella gallinarum* isolates were more resistant to enrofloxacin, moxifloxacin, azithromycin, erythromycin, chloramphenicol and clindamycin. On the other hand [2] reported that their isolates were sensitive to ceftriaxone, chloramphenicol and resistance to streptomycin, oxytetracycline, ampicillin whereas chloramphenicol showing resistance to the *Salmonella gallinarum i*n accordance with the present study. None of the lead molecules were found effective *in vitro*.



Salmonella gallinarum colonies on salmonella *shigella* agar

Triple Sugar Iron Test Indole Test

Fig 3.1: Isolation and identification of *Salmonella gallinarum*

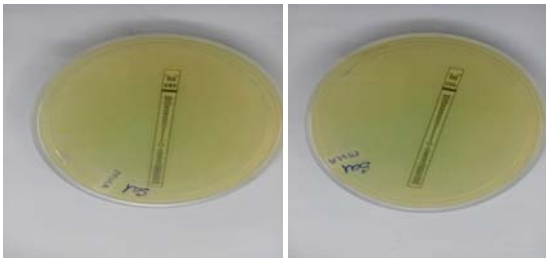


1 Fenofibrate
2 paracetamol
3 thiamine
4 labetalol
5 topiramate
6 chlorpheniramine
C $^+$ control positive
7 ethambutol
8 glucosamine
9 galantamine
10 chlorpromazine
11 L-glutamic acid
12 phenylephrine
C $^-$ control negative
(A) Resistant
(B) Resistant
(C) Resistant
(D) Resistant
(E) Resistant
(F) Resistant
(G) Resistant
(H) Resistant
(I) Susceptibility range (0.016-256 µg/mL)

Fig 3.2: Anti-biogram of *Salmonella gallinarum* with different molecules

(A) Enrofloxacin (B) Clindamycin

(C) Moxifloxacin (D) Azithromycin



(E) Chloramphenicol (F) Erythromycin

(G) Fenofibrate (0.2-14.5 µg/mL) (H) Labetalol (0.15-5 µg/mL)

(I) Cefpodoxime

Fig 3.3: Minimum Inhibitory Concentration of *Salmonella gallinarum* with different antibiotics and lead molecules

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