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RESEARCH ARTICLE
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Isolation and prevalence of *Salmonella* from chicken meat and cattle milk collected from local markets of Patna, India

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Abstract

Aim: To evaluate the hygienic quality of raw chicken meat and raw milk sold in the local markets of Patna with reference to isolation of *Salmonella* and antibiotic resistance pattern of *Salmonella* against commonly used antibiotics.

Materials and Methods: A total of 370 samples comprising of 228 chicken meat and 142 market milk samples were processed for isolation and serotyping, supplemented with molecular detection of isolates targeting *inv*A gene of *Salmonella*. All the isolates were tested against commonly used antibiotics (13 nos).

Results: Out of 370 samples, 23.7% (54/228) chicken meat and 7.7% (11/142) milk samples were found positive for *Salmonella* based on biochemical reactions. The serotyping of *Salmonella* isolates showed an incidence of 6.1% of *Salmonella typhimurium*, 2.6% of *S. newport*, 1.7% of *S. gallinarum* and 0.4% each of *S. enteritidis*, *S. infantis* and *S. worthington* in chicken meat; and 2.1% of *S. typhimurium* and 1.4% of *S. newport* in market milk samples. Polymerase chain reaction targeting *inv*A gene showed positive presence of *Salmonella* in 18.42% chicken meat and 5.6% market milk samples.

Conclusion: The antibiotic susceptibility test revealed the presence of multiple drug resistant *Salmonella* in chicken meat and milk. The present study indicates high prevalence of *Salmonella* in raw chicken meat and milk due to poor hygienic practices and therefore emphasizes the need for adopting these hygienic practices.

Keywords: antibiotic resistance, *inv*A , *Salmonella*, serotypes.

Introduction

Salmonella enterica is a leading cause of enteric diseases in humans and animals with millions of illness reported worldwide. The nontyphoidal *Salmonella* serovars are predominantly associated with food of animal origin such as eggs, milk, poultry, beef and pork meat responsible for zoonotic transmission [1, 2]. The incidence of salmonellosis has been reported in many developing countries including India, Egypt, Brazil and Zimbabwe [3]. *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the most frequently encountered species from foods like poultry, pork and beef products [4]. Incidence of *Salmonella* in chicken meat and milk has been reported by several workers using different methods and the frequency of detection ranges from 6.79% to 97.6% [5] in chicken meat and 0.17% to *28.6% [6]* in raw milk. Although, isolation of *Salmonella* by growth in a culture medium followed by serotyping is considered as the gold standard for confirmation of *Salmonella*, it is also time consuming and labour intensive.

Therefore techniques like PCR are increasingly being used for rapid detection and confirmation of *Salmonella* [7]. Amplification of *inv*A gene of *Salmonella* has been reported as a suitable target for PCR amplification, with potential diagnostic applications [8]. Emergence of multidrug-resistant *S. enterica* serovars has been increasing and has become a major health concern [1]. One of the contributing factors for the widespread dissemination of multi drug resistant bacteria has been the indiscriminate prophylactic and therapeutic use of antimicrobial agents in food animals [9].

Therefore the present study was aimed to study the prevalence of *Salmonella* and also characterize the distribution of the drug resistant pathogen in chicken meat and market milk of Patna, India.

Materials and Methods

Sample collection: A simple random method was adopted to collect a total of 370 samples constituting fresh chicken meat (n=228) and milk (n=142) from different vendors of Patna, Bihar between September, 2010 to March, 2013. The samples were maintained on ice, transported to the laboratory and processed within 1 hr of collection.

Isolation and biochemical characterization: Ten grams of chicken meat and 0.5 ml of milk samples were used for pre-enrichment in buffered peptone water (BPW), at 37 °C for 18 hrs. One ml of pre-enriched broth was transferred into Selenite Cystine broth and

Table-1. Incidence and relative occurrence of *Salmonella* serovars in chicken meat and milk

Serotype	Chicken meat		Market milk	
	Number isolated	Relative occurrence (%)	Number isolated	Relative occurrence (%)
S. typhimurium	14 (6.1%)	51.85	03 (2.1%)	60.0
S. newport	06 (2.6%)	22.22	02 (1.4%)	40.0
S. gallinarum	04 (1.7%)	14.81	-	-
S. enteritidis	01 (0.4%)	3.70	-	-
S. infantis	01 (0.4%)	3.70	-	-
S. worthington	01 (0.4%)	3.70	-	-
Total	27		05	

further incubated at 37 °C for 24 h for enrichment. Selective plating was performed using Hektoen enteric agar (HEA) (HiMedia, India) with overnight incubation at 37°C. Typical black color colonies surrounded by narrow green margin on HEA were biochemically tested by Indole (I), Methyl Red (M), Vogus Proskeur (Vi), Citrate (C), Triple Sugar Iron (TSI) and Urease Test. The colonies showing *Salmonella* specific IMViC pattern (-+-+) were inoculated on TSI slant. Furthermore, the colonies producing alkaline slant (pink) and acidic butt (yellow) with or without H_2S production (blackening) were tested for urease production on urea agar slant. All the urease negative isolates were considered as biochemically confirmed and were submitted to National Salmonella and Escherichia centre, Central Research Institute Kasauli, Himachal Pradesh, India for serotyping.

Optimization of PCR: A PCR protocol was standardized targeting *inv*A gene of *Salmonella* using vaccine strain E2375 of *Salmonella typhimurium*. A 26-bp forward primer (5'- GTG AAA TTA TCG CCG CGT TCG GGC AA3') and a 22-bp reverse primer (5' TCA TCG CAC CGT CAA AGG AAC C 3') [10] were used to obtain a 284 bp product. Amplification was carried out in a total volume of 25 µl containing 10 pmols of each primer, 50 µM of each dNTP, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 1X PCR buffer and 5 µl template DNA. Template DNA was prepared by boiling and snap chilling method [11]. A positive and negative control containing the template DNA from *Salmonella typhimurium* vaccine strain E2375 and nuclease free water, respectively, was included in every experiment. The reaction condition was optimized with initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 51 ^{0}C for 1 min and extension at 72 °C for 1 min. Finally, an additional extension was achieved for 5 min at 72 °C. The PCR product was electrophoresed on a 1·5 % agarose gel at 100 V. The agarose gel was stained with ethidium bromide (0·5 µg ml^{-1}) and visualized under gel documentation system (Biorad, USA).

Antimicrobial susceptibility test: The antibiotic susceptibility test of isolates was performed using agar disc diffusion method [12]. The antibiotic discs were impregnated with Ampicillin (10µg), Azithromycin (15µg), Amikacin (30µg), Clindamycin (2µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Erythromycin (15µg), Gentamicin (10µg), Ofloxacin (5 µg), Penicillin (10 units), Teicoplanin (30 µg), Tetracycline (30 µg) or Vancomycin (30 µg) (HiMedia, India). The isolates were grown on autoclaved Mueller Hinton broths (HiMedia, India) for 18 hrs at 37 °C. About 100 µl of the inoculums was spread on Mueller Hinton agar using sterile disposable L shaped spreader and antibiotic discs were placed onto the plate using sterile forcep. The plates were incubated at 37 °C for 24 hrs and observed for zone of inhibition. The results were categorized as sensitive, moderately sensitive and resistant based on diameter of zone of growth inhibition corresponding to different isolates.

Results and Discussion

A total of 370 samples comprising of 228 raw chicken meat samples and 142 raw milk samples were processed for isolation and identification of *Salmonella* spp. by both growth enrichment on culture media and molecular techniques. *Salmonella* isolates were obtained from enriched samples by selective plating on HEA. Typical black colored colonies surrounded by narrow green margin were selected for biochemical characterization which showed the presence of *Salmonella* in 23.7% (54/228) and 7.7% (11/142) chicken meat and market milk samples, respectively. The serotyping of isolates showed the prevalence of *Salmonella* in 11.8% (27/228) of chicken meat and 3.5% (5/142) of market milk samples. Among different serotypes of *Salmonella*, *S. typhimurium* was detected in 6.1% (14/228), *S. newport* in 2.6% (6/228), *S. gallinarum* in 1.7% (4/228), *S. enteritidis*, *S. infantis* and *S. worthington* in only 0.4% (1 each/228) of chicken meat samples, whereas, 2.1% (3/142) and 1.4% (2/142) of *S. typhimurium* and *S. newport*, respectively were detected in market milk samples (Table-1). Our results are in agreement with the earlier findings reported from different geographic regions [13-17]. However, other investigators have reported 6.7 to 97.6% prevalence of *Salmonella* in chicken carcasses and milk [5, 18] which further substantiate the present finding. Many of these serotypes have been reported earlier in cultures from various sources and places. The relative occurrence (Table-1) of *Salmonella* serovars in the present study revealed the maximum prevalence for *S. typhimurium* (51.85%) followed by *S. newport* (22.2%), *S. gallinarum* (14.8%), *S. entritidis* (3.7%), *S. infantis* (3.7%) and *S. worthington* (3.7%) which is in accordance with previous finding [4, 18, 19].

A PCR assay targeting *inv*A gene of *Salmonella*

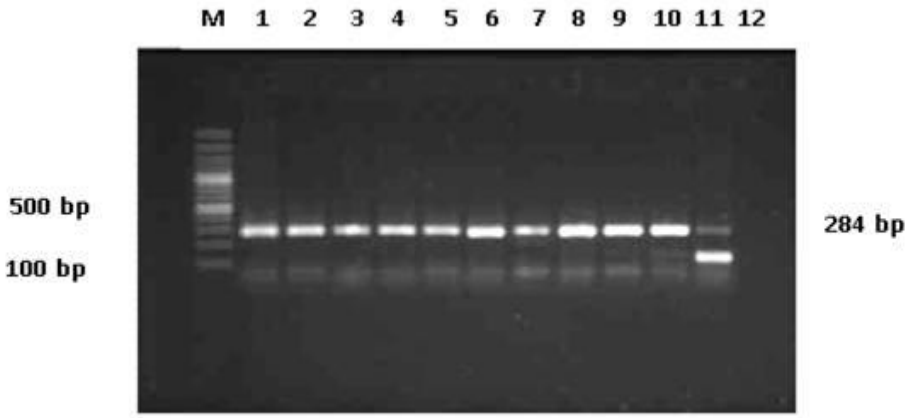

Figure-1. Electrophoresis of invA gene PCR products on 1.5% agarose.
Lane M : 100bp DNA ladder
Lane 1: Positive control
Lane 2-11: Samples isolates with positive amplicon
Lane 12: Negative control

was standardized for rapid detection and confirmation of *Salmonella* isolates. The assay relied on specific amplification of a 284 bp product (Fig.1). All the biochemically confirmed isolates of *Salmonella* (N=65) were tested for amplification of *invA* gene by PCR. Among these, 76.92% (50/65) showed specific amplification of *inv*A gene of *Salmonella*, which comprises of 18.4% (42/228) and 5.6% (8/142) isolates from chicken meat and milk samples respectively which is a higher distribution than that showed by serotyping of isolates. The PCR assay also detected some of the isolates which had originally been detected as negative in serotyping study. This is in concordance with the finding of Hamza [20]. This may be attributed to the presence of rough mutant strains which lack the specific side chains responsible for 'O' specificity or some additional abnormalities of the core structure [21] resulting in negative result in serotyping. Hence, the study recommends that PCR may be used for rapid and sensitive detection of Salmonella supported by the findings of Wang et al [7].

Since food of poultry origin and milk are some of the most common sources of human salmonellosis, the findings from this report may be correlated with the hygienic practices to reduce public health problem in the area of study.

The antibiotic susceptibility test of isolates revealed that all serotype were resistant to Gentamicin, Ampicillin, Penicillin, Erythromycin, Vancomycin, Amikacin and Clindamycin which are commonly used antibiotics. Most of the isolates were found to be highly sensitive to Azithromycin and Ceftriaxone and moderately sensitive to Ofloxacin, Ciprofloxacin and Tetracycline. The bacterium develops resistance to most of these commonly used antibiotics because of their inadvertent use for long duration or in suboptimal doses. Therefore, it is recommended to use antibiotics based on their antibiogram pattern only. Use of antibiotics based on earlier report of their effectiveness may not be effective at all times [13] because of the presence of resistant bacterium. The current study reveals that *Salmonella* isolates from chicken meat and milk are showing resistance to more than one antibiotic indicating the prevalence of multidrug resistant *Salmonella*, which substantiate the findings of Kumar *et al*., Siemon *et al*. and Kessel *et al*. [13, 18, 22].

Conclusion

The study concluded that non typhoidal *Salmonella* serotypes are prevailing in the poultry carcasses and milk and therefore act as a source of human infection. The level of prevalence can be reduced by adopting hygienic practices during poultry slaughter. The presence of multiple resistance in *Salmonella* isolates suggested that there is appreciable risk of infection to humans with multidrug resistant *Salmonella* from consumption of unpasteurized milk and undercooked chicken meat.

Authors' contributions

PK is a project leader and supervised the project. P K and Anjay carried out bacterial isolation and molecular characterization. SK, SKB and SD collected samples and carried out ABST. All authors contributed in drafting and revision of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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