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Research Paper

Characterization of Salmonella Enteritidis from two separate outbreaks of acute enteritis in Japanese quail (Coturnix coturnix japonica) and turkey (Melleagris gallopavo)

Biomedical

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ABSTRACT

Two independent outbreaks of acute gastroenteritis in Japanese quails and turkeys were investigated and 44 Salmonella Enteritidis isolates were recovered from clinical samples. The isolates were subjected to phenotypic and genotypic characterization. All of them produced amplicons of ~1103 bp, ~700bp and ~617 bp specific for sefC, pefA and stn genes, respectively, by a multiplex PCR. Molecular typing by ERIC-PCR, RAPD-PCR and REP-PCR techniques revealed genotypic similarity among all the 44 isolates. The investigation indicated the possible involvement of contaminated feed for both the outbreaks. The results also indicated that molecular techniques are rapid in comparison to conventional methods for epidemiological investigation of salmonellosis. Presence of highly virulent S. Enteritidis in food animals is a great public health concern, because poultry birds are one of the commonest sources of meat and meat products for human consumption.

INTRODUCTION

Salmonella Enteritidis is one of the major causes of food-borne illness and is considered to be the most important pandemic zoonosis produced under natural conditions. *Salmonella* Enteritidis infection may be transmitted to humans through the food production chain. Therefore, undercooked or raw eggs and poultry meat are of high risk to humans [Gillespie, et al., 2005]. Currently, incidence of watery diarrhoea caused by *Salmonella* Enteritidis and other bacterial pathogens are on the increase despite vaccination and proper medications as per sequel of various incriminating factors. Severe outbreaks of salmonellosis occur due to certain virulence factors like cytotoxin production, expression of fimbriae [Thorns, 1995] and high cell surface hydrophobicity, which are responsible for colonization and subsequent cell damage [Rahman et al., 2001].

Conventional methods of disease diagnosis are highly laborious, time consuming and have certain limitations in understanding the role of the pathogenic factors, origin and transmission of pathogens and in determining the incidence of disease and its economic importance. DNA based techniques, particularly the detection of virulence genes in the isolated organisms along with other commonly employed tests provide a rapid, specific and highly sensitive diagnostic procedure to ascertain an outbreak caused by any specific agent.

The present study was, therefore, undertaken to find out the role of some of the important virulence factors by Congo Red binding assay (CRDA), salt aggregation test (SAT) and multiplex PCR assay for detection of Salmonella enterotoxin (*stn*), *Salmonella* Enteritidis fimbriae (*sefC*) and plasmid encoded fimbriae (*pefA*) genes. The heterogenicity among isolates was studied by molecular biological techniques such as enterobacterial repetitive intergenic consensus (ERIC)-PCR), repetitive extragenic palindromic (REP)-PCR) and single primer PCR.

MATERIAL AND METHODS

Bacteria: Two independent outbreaks of gastroenteritis in adult flocks of Japanese quail and turkeys, maintained in Mizoram, India, were recorded within 6 months. The causative agent was isolated from intestinal content, heart blood, liver and rectal swabs and maintained on nutrient agar slant as well as in lyophilized ampoules for further studies.

All the isolates were conventionally identified based on their cultural, morphological and biochemical characteristics followed by *in vitro* antibiotic sensitivity test [Bauer et al., 1966] by disc diffusion method employing 20 commonly used antibacterial agents. Isolates were also serotyped at National Salmonella & Escherichia Center, Central Research Institute, Kasauli, Himachal Pradesh, India.

Congo Red dye adsorption assay (CRDA): All the isolates were subjected to CRDA [Ishiguro et al., 1985] using 0.05% Congo Red dye and incubated at 37^{0}C for 36 hours. Observations were recorded at 18 h, 24 h and 36 h of incubation. *Salmonella* Enteritidis (ATCC 13076) strain was used as negative control.

Salt aggregation test (SAT): SAT was performed as per the method of Quadri et al. [1988]. Fresh colonies of each isolate were washed in PBS, pH 6.8 and resuspended to a final concentration of 10^8 bacterial cells/ml and 5ml was mixed with equal volume of different dilutions of ammonium sulfate (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 2.0, 2.5 and 3.0 M) in 0.02 M sodium phosphate buffer (pH 6.8). To detect the rough variants, autoaggregation in physiological saline was done before performing the SAT.

Multiplex PCR for detection of virulence genes: A multiplex PCR was carried out using three sets of oligonucleotide primers [Rahaman et al., 2001] for *stn*, *pefA* and *sefC* genes. The PCR mixture (25 µl) contained 1X PCR buffer, 1.5 mM of $MgCl_2$, 20 nM each primer, 200 µM each dNTPs, 1.0U *Taq* polymerase and 2.0µl of template DNA lysate. The PCR reaction was performed in a thermal cycler using the following standard cycling procedure: an initial denaturation at 95^{0}C for 5 min, followed by 30 cycles of denaturation at 94^{0}C for 1 min, annealing at 59^{0}C for 1 min and extension at 72^{0}C for 1 min and a final extension at 72^{0}C for 6 min.

PCR based molecular typing of the isolates: The ERIC-PCR and REP-PCR were conducted using primers targeting the palindromic sequences of ERIC and REP, respectively according to Versalovic et al. [1991]. RAPD-PCR was performed using a single microsatellite primer targeting variable repetitive regions as described by Dabo et al. [2000].

Amplified products from all the PCR reactions were separated by agarose gel (2% in 1X Tris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with ethidium bromide (0.5 µg/ml). Standard molecular size markers (100 bp and 500 bp DNA ladder) were included in each gel. DNA fragments were ob-

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served by ultra violet transilluminator and photographed in a gel documentation system (Alpha imager, Germany). All the PCR reactions were performed three times to ensure the repeatability of the techniques and to make sure that isolates were correctly assigned to respective patterns.

RESULTS

Epidemiological features of the outbreak: Out of 250 birds in flock of Japanese quails, 139 were affected and 48 were found dead within one week with an overall mortality and case fatality rate of 19.2% (48/250) and 34.53% (48/139), respectively. Similarly, out of 255 birds in a turkey flock, 64 were affected and 22 were found dead within one week. Overall mortality and case fatality rate were 8.63% (22/255) and 34.38% (22/64), respectively. In both the outbreaks, the affected birds showed anorexia, emaciated, dull and depressed appearance with ruffled feathers and progressive somnolence with closed eyes. Majority of the birds were shivering and huddled near the source of heat. All the affected birds exhibited profuse watery diarrhoea followed by severe dehydration. Histopathologically, severe lesions of enteritis accompanied with focal necrotic lesions in the mucosa of small intestine were prominent in majority of cases. Spleens and livers were swollen and congested with haemorrhagic or necrotic foci. Fibrinopurulent perihepatitis and pericarditis and omphalitis were also recorded in few cases.

Bacterial Isolation: A total of 44 bacterial isolates obtained from two outbreaks of severe gastro-enteritis were identified as *Salmonella* Enteritidis on the basis of standard biochemical tests and serotyping at reference laboratory. Twenty three isolates obtained from 70 samples from Japanese quails were designated as JQSE1 to JQSE23. Twenty one isolates obtained from 59 turkey birds were numbered as TBSE1 to TBSE21.

Antimicrobial sensitivity test: Majority of the isolates were highly resistance to Amikacin (86.36%), Penicillin (86.36%), Carbenicillin (81.82%), Oxytetracyclin (77.27%), Lyncomycin (77.27%) and Ampicillin (72.73%). The best antimicrobial agent in terms of sensitivity was Chloramphenical (77.27%) followed by Norfloxacin (68.18%), Erythromycin (63.64%) and Enrofloxacin (59.10%).

Congo Red Dye Absorption (CRDA) test: All the 44 isolates were found to be positive to Congo Red binding test.

Salt aggregation test: A total of 37(84.38%) isolates aggregated in 1.2 M, 4 isolates aggregated in 2.0 M and 2 isolates from feed were aggregated in 3.0 M ammonium sulfate.

Multiplex PCR for virulence genes: All the 44 isolates were found positive for all the three virulence genes (Fig. 1).

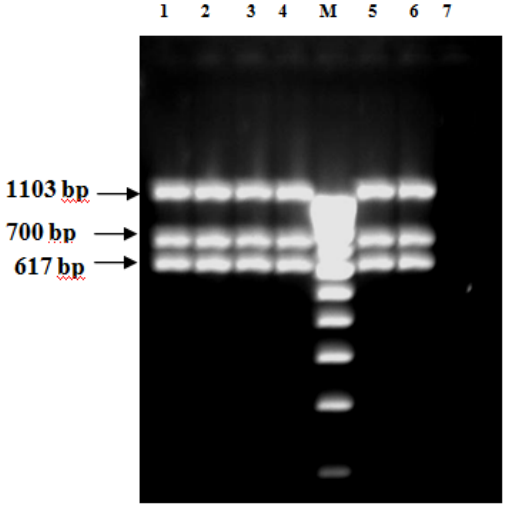

Figure 1. Multiplex PCR detection of Salmonella Enteritidis virulence genes. Lane M: 1000bp DNA ladder; Lane 1-6: Representative isolates of Salmonella Enteritidis from turkey and quail; Lane 7: Negative control.

PCR based typing of bacterial isolates: ERIC-PCR generated amplified products (n=7 bands) in the range of 800 to 150 bp, had identical profile in all the 44 isolates. RAPD-PCR amplified products in the range of 1500 bp to 400 bp (n= 6 bands) also provided a single banding profile in all the 44 isolates. REP-PCR generated the products (n=13 bands) in the range between 1500 bp and 50 bp and the banding patterns were identical.

DISCUSSION

Phenotypically, all the isolates had similar cultural and morphological characteristics. Moreover, even biochemical techniques were not useful in establishing the relationship between isolates of salmonellae and in detecting the source of infection. Antibiotic sensitivity studies revealed that except chloramphenicol, none of the commonly used antibacterial agents were effective in more than 75% level against the isolates and also could not provide any justifiable information to correlate with the isolates from the birds and feed samples.

Adherence and binding of the entero-pathogenic organisms to cells of the gastrointestinal tract are believed to be important processes in the pathogenesis of diarrhoeal diseases [Reed & Williams, 1978]. Therefore, the outer envelopes of these bacteria were studied intensively. In several reports, CRDA and SAT have been linked directly to virulence and pathogenicity [Quadri et al., 1988]. In the present study, all the 44 isolates from the clinical samples of the dead birds were positive for CRDA and at the same time they exhibited high level of cell surface hydrophobicity indicating the virulence property of the organisms except 2 isolates that showed salt aggregation at 3.0M ammonium sulfate.

Epidemiology and pathogenic properties of *Salmonella* Enteritidis are largely dependent on number of factors that act in tandem and ultimately manifest the typical symptoms of salmonellosis [Murugkar et al., 2003]. Some of the major important factors are *Salmonella* enterotoxin (*stn*), *S.* Enteritidis fimbriae (*sef*) and plasmid encoded fimbriae (*pef*). In the present study, all the 44 isolates exhibited the presence of all the 3 genes (Fig. 1), which indicated that the *stn*, *sef* and *pef* genes are widely distributed in all the isolates. The results are in agreement with earlier reports of Murugkar et al. [2003], where *sefC* gene was present only in *S.* Enteritidis and *S.* Gallinarum. Rahman [1999] found the *sefC* gene only in *S enteritidis* and *S.* Gallinarum and not in *S.* Typhimurium, S. Newport, S. Kentucky, S. Weltervreden and S. Indiana. In case of *pef* gene, no serotype specific pattern was recorded. Murugkar et al. [2003] recorded the *pef* gene in 31 of 36 isolates of *S.* Enteritidis. Besides the CRDA capabilities and SAT properties, presence of all the three virulence genes may be attributed to high level of mortality and case fatality in the adult birds. The virulence genes specific PCR assays employed during the study correctly identified all the genes in all the isolates, therefore, multiplex PCR assay for *stn*, *sef* and *pef* can be used to identify the virulence factors of *Salmonella* strains from different animals, including birds and human beings. PCR assay can provide rapid, sensitive and specific identification of *Salmonella* virulence factors with relatively uncomplicated methodology.

Phenotypically, all isolates had similar cultural and morphological characteristics. Moreover, biochemical and pathogenicity tests were not found useful in establishing the relationship between two outbreaks. Conventional methods provide limited characterization of isolates and are not capable of differentiating strains to the extent required for epidemiological studies [Wilson et al., 1993]. All the 44 isolates exhibited an identical fingerprinting profile by all the three methods (ERIC-PCR, REP-PCR and RAPD-PCR). PCR based typing techniques were found to be rapid, highly sensitive and discriminatory to identify and differentiate strains [Shivachandra et al., 2005]. In the present investigation, 23 and 21 isolates of *S.* Enteritidis from two outbreaks were identified as same by all the three techniques employed. No technique could distinguish these two clones from two outbreaks, indicating a close genomic relationship between these strains. The involvement of the same clones indicated its significant disease-inducing potential, and suggests that host associated and environmental factors may have been of lesser significance in these outbreaks.

Immediately after the first outbreak in Japanese quail flocks, all the birds were culled and the cages were disinfected and left unused for 4 months and the same cages were further used for rearing of turkey birds. Another common material used for both the flocks was the feed stock. Obtaining similar fingerprinting profiles in all the isolates indicated that the infection might have entered in the flock through a single common source. The initial entry of *S.* Enteritidis might have happened through the contaminated feed. It is very difficult for *S.* Enteritidis to survive in metal cages for 4 long months after thorough disinfection. Gast R. K [2003] also mentioned that contaminated feeds, particularly those containing animal proteins, have often been identified as likely sources of *Salmonella* Enteritidis.

Salmonella Enteritidis is one of the most common food-borne zoonotic agents of human being. Therefore, presence of the organism in food animals possesses a serious threat to public health. The present investigation also indicates that DNA based techniques, which are found to be superior to and more rapid than conventional techniques for detection and differentiation, should be performed in parallel with standard bacteriological methods in studies of outbreak. The present study also reveals the importance of molecular techniques that could be efficiently employed to understand the virulence properties of *Salmonella* isolates that appear difficult by phenotypic techniques.

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