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Study of antimicrobial resistance and physiological biomarkers with special reference to Salmonellosis in diarrheic foals in Punjab, Pakistan

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ABSTRACT

Antimicrobial resistance results in selective colonization in animals. In the present study, 447 diarrheic foals (235 horse foals, 165 donkey foals and 47 mule foal) were selected from Lahore and Sahiwal districts of Punjab, Pakistan. Fresh fecal and blood samples from diarrheic foals were collected for isolation and confirmation of Salmonella Polymerase chain reaction. Results revealed that 50 (11.25%) foals (horse n = 29, donkey n = 12 and mule n = 9) were positive. Fifty *Salmonella enterica* isolates belonging to 7 serovars *S. Paratyphi B* (15), *S. Saintpaul* (7), *S. Newport* (6), *S. Typhimu-rium* (11), *S. Kottbus* (4), *S. Lagos* (2), and *S. enterica ssp salamae* (5). Salmonella was common in foals that visited veterinary hospital, as compared to those in stud farms and individual foals reared in low income household. Out of the total 50 samples, 92% of isolates were resistant to three or more than three antimicrobials. The highest resistance (86%) was against Sulphamethoxazole (23.75 mg) and lowest (4%) against trimethoprime (5 mg). The isolates also showed resistance against Doxycycline (30 mg), Oxytetracycline (30 mg), Streptomycin (10 mg), Neomycin (30 mg), Amikacin (30 mg), chloramphenicol (30 mg), Ampicillin (10 mg), Amoxicillin (10 mg), kanamycin (30 mg), Norfloxacin (10 mg), Gentamicin 10 mg, Cefotaxime (30 mg), Ciprofloxacin (5 mg) and Ceftriaxone (30 mg). Blood analysis of salmonella infected foals showed That Hemoglobin, PCV and TEC were significantly higher and (while) TLC, PCV, Monocytes, Lymphocytes, Basophils, Eosinophil and Neutrophils were significantly lower than normal. Albumin were lower and BNU, Biluribin, ALT and creatinine were higher than normal values.

1. Introduction

Infectious diseases are serious threat for animal health and productivity in developing countries (Qayyum et al., 2016; Wen et al., 2016). Salmonellosis is a common problem in horses. It occurs in different clinical forms and is caused by *Salmonella enterica*. The disease results in severe diarrhea and fatal septicemia in foals while in colitis/typhilitis in equids of all ages (Kohn, 1982). The outbreak of the disease in hospitalized horses may results in economic losses to owners and affects the welfare of affected animal (Murray, 1996). The infection transmits normally through contaminated feed and water. It invades intestinal epithelium and start production of toxins (Gruenheid and Finlay, 2003). Stress is supposed to be an important risk factor of the disease both in foals and adult horses (Powell et al., 1988). Different serovars of salmonella are considered important pathogens of neonates that results in septicemia with diarrhea (Dunkel and Wilkins, 2004; Hollis et al., 2008). Peracute form of salmonellosis occurs only in foals. The symptoms of the diseases are; fever, leukopenia, increase heart rate, anorexia and septic shock. The acute form is reported in horses manifested by fever, neutropenia, anorexia and diarrhea. Diarrhea normally occurs in chronic form and develops from acute form of the disease (Smith, 1981).

Salmonella can transfer from effected horses to healthy horses. It was reported that (6–13) % of healthy horses presented to veterinary hospitals were positive for salmonella (Ernst et al., 2004; Ward et al., 2005). Salmonella was isolated from fecal samples in 6.77% of equines in India and in the beginning of 1980's the common serovar isolated was *S. abortu* (Singh et al., 2007; Gupta et al., 1987). Later *S. Typhimurium* was found common in horses (Rajasekhar and Babu, 1992).

Development of antibiotics resistance in pathogens due to the indiscriminate use of antibiotics for treatment of animals is an important problem worldwide (Murray et al., 1986). It may be due to selective colonization of pathogens in animals treated with antimicrobial drugs. Multi-drug resistant salmonella is common in horses and it is supposed

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to be due to the colonization of resistant salmonella in equines (Dargatz and Traub-Dargatz, 2004). Worldwide emergence of multiple drug resistant strains of salmonella of animal and human origin (Hald et al., 2003) and interaction of population with equines. The pathogen can transfer resistant genes among species and has a wide range of reservoir and vehicles (Raghunath and Banker, 1992; Singh et al., 1992). Hence, the detection of organism has become important for both animal and human health (Tillotson et al., 1997). In Pakistan, little or no data is available about the prevalence of salmonella ssp. in equines. An active surveillance is required to determine its prevalence and servers. Present study was designed to find the prevalence of multidrug resistant salmonella from foals of two districts of Punjab, Pakistan.

2. Materials and methods

2.1. Study population

The study was carried out in the rural and urban areas in Lahore and Sahiwal districts of Punjab, Pakistan. In the study area equines were kept as animals for carrying loads and other works and are mostly kept in households or in small farms and there is no organized commercial equine farming. Normally, no record is maintained regarding diseases and treatment history. The pattern of feeding in the selected foals were that the farmers normally feed mother milk up to six months which was supplemented with green grass. After weaning, they were normally kept with their mothers and were fed the same feed offered to their mothers. Due to the lack of record, no accurate data regarding the diseases and treatment history was available. Foals were selected by purposive convenient sampling technique from foals of those owners willing to get their foals sampled.

2.2. Collection of samples

Diarrheic foals were selected by purposive convenient sampling technique of those owners willing to get their foals sampled. A total of 447 foals (235 horse foals, 165 donkey foals and 47 mule foal) with manifestation of diarrhea were sampled. Blood and freshly passed fecal samples were collected with care to avoid contamination. The collected samples were immediately transferred to labeled container and were brought to laboratory in icebox. Data was collected about foal's specie, sex, and visits to veterinary hospital, entry of new animals/foals without Quarantine, type of farm and severity of diarrhea (based on frequency and consistency of feces) on predesigned questioner.

2.3. Isolation of bacterial from fecal samples

All 447 fecal samples were extended in 10 ml buffered peptone water and incubated at 37 °C for 18 h. After pre-enrichment, the samples were selectively enriched in tetrathionate broth (TTB) at 42 °C for 24 h. After 24 h, a loop of inoculum was taken from the enriched sample and was streaked on brilliant green agar plates and the cultured plates were incubated at 37 °C for 24 h. Suspected colonies of salmonella (transparent colonies with reddish periphery) were re-cultured on brilliant green agar plates to get pure culture (Singh et al., 2007).

2.4. Confirmation of bacteria by polymerase chain reaction

After purification, DNA was extracted from the pure culture. For DNA extraction, 4–5 similar and isolated colonies were taken from pure culture and suspended in ultra-sterilized water to homogenize it. After homogenization, DNA was extracted using commercially available DNA extraction Kit (QIAGEN, Germany). The DNA fragment of 496-bp sequence of the J gene was amplified by polymerase chain reaction using primer 1 (5′ ACT GGC GTT ATC CCT TTC TCT GGT G) and 2 (5′ ATC TTG TCC TGC CCC TGG TAA GAG A) (Ernst et al., 2004). To amplify the DNA fragment a 25 µl PCR reaction mixture consisting of 12.5 µl of 2X green master mix (Promega, USA), 1 µl forward Primer, 1 µl reverse Primer, 2 µl DNA and 8.5 µl of nuclease free water was prepared. The conditions in thermocycler were set at an initial denaturation temperature of 94 °C for 5 min, 25 cycle of denaturation (1 min at 94 °C), annealing (1 min at 55 °C) and extension (1 min at 72 °C). The PCR product were then run on 1.2% agarose dissolved in Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer having ethidium bromide at 120 V in TBE buffer for one hour. The confirmed samples were send to school of biological sciences, Lahore for serotyping. The results were visualized under gel documentation system (Syngene, UK). The prevalence of salmonella in foals was determined by using chi-squared test.

2.5. Antibiogram study of salmonella isolates

Sensitivity of salmonella isolates against different antimicrobial was studied using disc diffusion method on Muller Hinton agar. The antimicrobials used were Amoxicillin 10 mg (AML), Ampicillin 10 mg (AMP), Norfloxacin 10 mg (NOR), Oxytetracycline 30 mg (OT), Gentamicin 10 mg (CN), Ciprofloxacin 5 mg (CIP), sulphamethoxazole 30 mg (SM), chloramphenicol 30 mg (C), Streptomycin 10 mg (S), trimethoprim 5 mg (TM), kanamycin 30 mg (K), Neomycin 30 mg (N), Amikacin 30 mg (AK), Cefotaxime 30 mg (CF), Ceftriaxone 30 mg (CI) and Doxycycline 30 mg (DO). Isolates were classified based on zone of inhibition as sensitive or resistant according to CLSI document M100-S23 (M02-A11) (Performance Standards, 2012). The strains resistant to three or more drugs were designated MDR strains.

3. Hematologic and serum biochemical analyses

Complete Blood Count (CBC), including total Red Blood Count (RBC) and White Blood Count (WBC), were obtained from anticoagulated blood by using hematology analyzer. Packed cell volumes (PCV) were obtained manually by using of 50-µl microhematocrit tubes after centrifugation for 5 min at 1520*g*. Differential white blood cell counts were performed manually. Absolute values of the various WBC were obtained by multiplying the percentage of the specific cell type by the total WBC count. Chemistry analyzer was used for serum biochemical values. All the obtained values were compared with established reference range values (Santos et al., 2002a).

3.1. Statistical analysis

Prevalence between foal's specie, sex, visit to veterinary hospital, entry of new foal/foals without quarantine and type of farm was compared using the chi-squared test wherever applicable. And Analyses of the data of hematology and serum chemistry were performed in a paired *t*-test.

4. Result

4.1. Detection and confirmation by PCR of salmonella in fecal samples

Salmonella colonies appeared as pink white surrounded by red zones on brilliant green agar. The suspected colonies were re cultured for purification and expressed same colony characteristics. The positive cultures were confirmed by using the genus specific primer, a product of 496 bp (Fig. 1) was generated for Salmonella species. A total of 447 samples were studied. Out of 447 samples, 235 were horse, 165 were donkey and 47 were mule foals. The result showed that out of the total 447 samples, 50 foals (horse n = 29, donkey n = 12 and mule n = 9) were positive for salmonella (Table. 1). The overall prevalence of salmonella in diarrheic foals was 11.25%. prevalence of Salmonella enterica in different species of equine was, 29 (12.34%) horse foals, 12 (7.3%) donkey foals and 9 (19.2%) in mule foals at *p*-value 0.053 (Table 1). The percentage prevalence of salmonella was higher in females (14.2%) as compared to males (8.6%). The salmonella was more