International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 5(2): March-April 2015

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Life Sciences **Research Article……!!!**

Received: 15-04-2015; Revised: 18-04-2015; Accepted: 19-04-2015

PREVALENCE, BACTERIOLOGY AND PATHOGENESIS OF *PROTEUS* SPECIES IN SICK LAYER CHICKENS IN AJMER REGION OF RAJASTHAN

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Keywords:

Salpingitis, Omphalitis, Yolk sac infection, Mortality

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ABSTRACT

A study was conducted to determine the distribution of *Proteus* species in visceral organs of clinically sick chickens (*Gallus gallus*). A total of 48 tissue samples consisting of 24 liver, 12 small intestine, and 12 large intestine samples were aseptically collected from 12 fowls of 4 selected poultry farms of Ajmer suspected for salpingitis and omphalitis (yolk sac infection) and subjected to bacteriology and biochemical examination. 6 *Proteus vulgaris* (from liver) and 6 *Proteus mirabilis* isolates (3 from small intestine and 3 from large intestine) were isolated following standard procedures. Processed tissue from each sample was cultured primarily on Brain Heart Infusion (BHI) broth and then secondarily on 7% defibrinated sheep blood (Blood Agar) and MacConkey agar. Presumptive colonies of bacterial agents were subjected to conventional biochemical characterization. The result of biochemical test identified the *Proteus* species from the samples of clinically sick chickens; *Proteus vulgaris* (25%; in liver) and *Proteus mirabilis* (25%; in small and large intestine). *Proteus* infection was incriminated as the cause of severe depression, coma, and high mortality in successive broods of chicks. The pathological lesions comprised congestion of lungs, liver, and kidneys and mucus exudation in the trachea. A high incidence of pathogenic strains of *Proteus* bacteria from sick layer chickens was observed. This suggests that the isolates may have contributed to the mortality and reduced hatchability recorded in the farms investigated.

INTRODUCTION

Various species of *Proteus* which mainly exists as saprophytes are known to cause septic infections in man[1] and animals[2-4] under certain conditions. The organism has been incriminated in omphalitis and persistent yolk sac in chickens[5]. This paper describes the clinico-pathological features of a natural septicemic disease attributed to *Proteus* infection in chickens. *Proteus* species occasionally cause embryonic death, yolk sac infections, and mortality in young chickens, turkeys and ducks[6].

Yolk sac infection is the main infectious cause of chick mortality during the first week of the post-hatching period[7, 8] and is the main cause of chicks mortality accounting for large economic losses to the poultry industry[9]. It can cause mortality rate of about 5-10%; however the condition has also been associated with much higher mortality especially in chicks during first week of age[10]. Contamination of unhealed navels has been suggested as a cause of yolk sac infection in newly hatched chicks[11].

Different types of bacterial agents are attributed for causation of yolk sac infection/omphalitis in chicks[12]. Among these bacteria *Proteus* spp. is one of the bacterial agent that have been isolated from yolk sac infections in chicks in different locations all over the world. In Ajmer region, investigations on poultry diseases in general and yolk sac infections (omphalitis) in particular have received little attention. Till now no significant research has been reported in the Ajmer region of Rajasthan pertaining to yolk sac infections (omphalitis) and salpingitis indicating towards the presence of *Proteus* spp. infection and continued to be the most neglected and devastating diseases of chicken. Therefore, the objectives of this study were to assess the prevalence of yolk sac infection and salpingitis, to isolate and identify yolk sac infection-associated bacteria and salpingitis-associated bacteria i.e. *Proteus* spp.

Proteus mirabilis is a well known cause of human and animal urinary tract infections and several outbreaks of hospital acquired infections have been attributed to it. However, except for urinary tract infections, the organism is not considered an important pathogen in veterinary medicine and a recent report of septicaemic *Proteus* infection in Japanese quail[13] appears to be the only report of disease due to *P. mirabilis* in an avian species.

This study was therefore aimed at determining the distribution of *Proteus* spp. in visceral organs (liver and intestine) of clinically sick chickens.

MATERIALS AND METHODS

Source of sample: Samples used for this investigation were collected from 4 selected poultry farms of Ajmer region of Rajasthan state in India.

Postmortem (Necropsy) Examination: All the chicks were subjected to necropsy before sampling in order to record any gross lesion on their viscera with special reference to the yolk sac infections. Postmortem examination was done according to the procedure recommended for poultry by Chauhan and Roy[14]. All applicable corresponding institutional guidelines for the care and use of animals were followed.

Sample size: A total of 12 mostly dead and almost clinically sick chicks were necropsied for bacteriological examination.

Sampling of clinically sick chickens: Tissue samples collected from 12 clinically sick chickens submitted for diagnosis were liver, small intestine and large intestine. Tissue samples were aseptically collected comprising of 24 liver, 12 small intestine, and 12 large intestine samples, giving a total of 48 tissues samples.

Sample collection and Transportation: After necropsy, varying tissue samples were collected aseptically in normal saline solution. The collected samples were labeled, packed and transported to the Microbiology Laboratory in the Microbiology Department of Jawahar Lal Nehru Medical College, Ajmer, Rajasthan.

Culture and Bacterial Isolation: All chicks with gross lesions of yolk sac were sampled into liver, small intestine and large intestine. Each sampled organ was seared with spatula and incised with a small sterile scalpel blade. Pieces of sampled organs were inoculated directly and aseptically cultured on media such as blood agar and MacConkey agar and incubated aerobically for first isolation of the causative agent. All inoculated media were incubated at 37 ºC and inspected for growth after 24 hours of incubation. Based on macroscopic and microscopic appearance, the developed colonies were selected from each sample and subcultured on appropriate differential media for further identification. Tissue samples were cultured indirectly by inoculating brain heart infusion broth (BHI), incubated at 37ºC for 24 hours and then streaked into MacConkey agar and Blood agar plates. Presumptive colonies were subjected to Gram staining for cellular morphology. Cultural and morphological examinations were conducted as described by Barrow and Felthan[15]. Colonies representing each bacterial species were identified and characterized using standard biochemical methods according to the methods described by Barrow and Felthan[15]. The biochemical reagents and

tests used included: Triple sugar iron agar, PPA (Phenyl pyruvic acid), Urease, Simmons citrate, Indole, Methyl Red and Motility. Oxidase test and 3% KOH sting test were performed on presumed *Proteus vulgaris* and *Proteus mirabilis* isolates.

Bacterial Identification: Identification of the pure isolates was done on the basis of staining, colony morphology, cultural, physiological and biochemical character of pure isolates by using standard bacteriological and biochemical procedures as described by Cowan and Steel[16], Cruickshank *et al.*[17], Quinn *et al*.[18] and Swayne *et al.*[19].

RESULTS

Bacterial Isolation Rates: From the 48 tissue samples consisting of 24 liver, 12 small intestine, and 12 large intestine samples were examined and analyzed microbiologically, a total of 2 aerobic bacterial species of *Proteus* genus were isolated. The distribution of bacteria in tissues of clinically sick chickens were: liver 6 (25%), small intestine 3 and large intestine 3 (25%). *Proteus vulgaris* (25%) was isolated from liver and *Proteus mirabilis* (25%) was isolated from intestinal contents of clinically sick birds i.e. small and large intestine.

External (Symptoms and Traits) findings: A higher mortality rate was the first signal of *Proteus* infection in chicks. Normal birds rapidly became depressed, then recumbent and died with legs extended backwards. Depression, anorexia and frothy diarrhoea were the most consistently observed signs.

Necropsy (Gross lesions) findings: The most consistent lesion in the majority of chicks was a dark and usually enlarged spleen accompanied in the majority of cases by frothy intestinal content. In most of the cases, the liver, spleen, heart, lungs, kidneys and other organs were congested with unabsorbed yolk sac. The major gross lesions observed in chicks died of yolk sac infection were unabsorbed yolk sac, congestion and discoloration of the yolk (greenish yellow; dark brown to bright yellow), retained caseous yolk sac and edematous yolk (especially sac infection included unabsorbed/ retained yolk sac). The yolk sac infection was usually associated with peritonitis, pericarditis, peticheal and ecchymotic hemorrhages on the serosal surface of visceral organs (the liver and intestine).

Bacteriological examination: Gram smears of primary and secondary Blood agar plates inoculated with liver and intestine tissues stained with crystal violet stain solution disclosed numerous gram-negative rods as shown in Fig. 1.

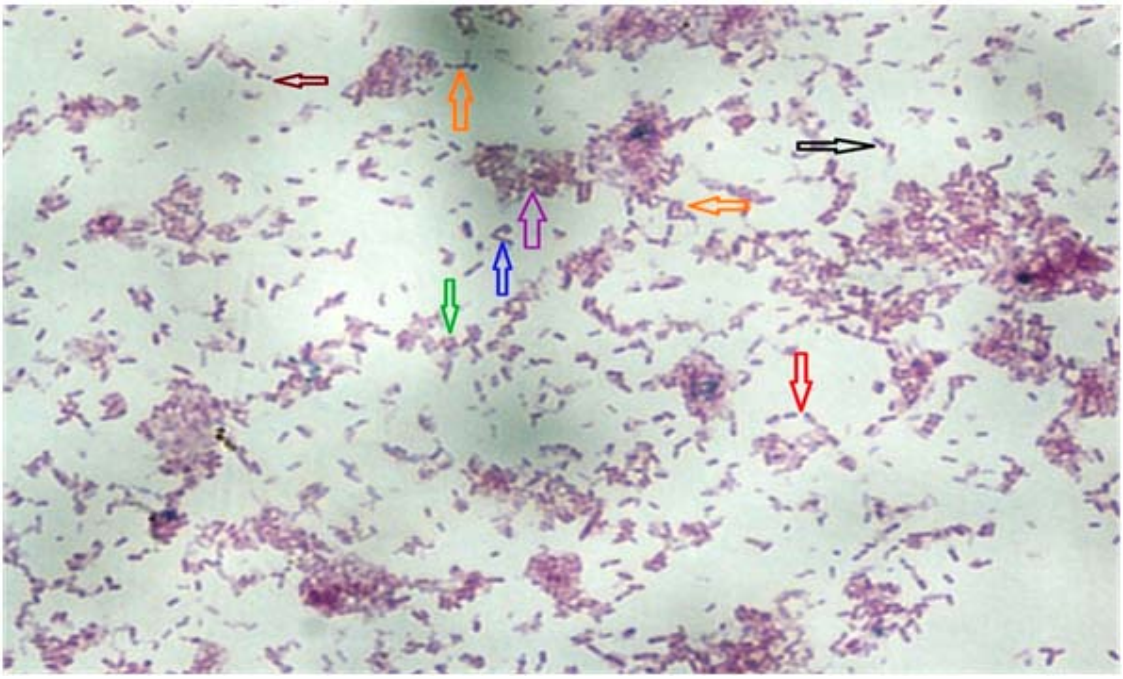

Fig-1-Photomicrography of gram stained slide prepared from BHI inoculated with infected small intestine tissue – black arrow head represents numerous gram negative bacilli

Pure cultures of these gram-negative rods were isolated from liver, small intestine and large intestine tissue samples. *Proteus* was isolated in pure culture from liver, small and large intestine on Blood agar plates showing characteristic swarming and non-hemolytic colonies. The cultures produced typical swarming growth on Blood agar plates and grew as NLF (Non-Lactose Fermenting) colonies on MacConkey agar at 37°C (Fig. 2 & 3).

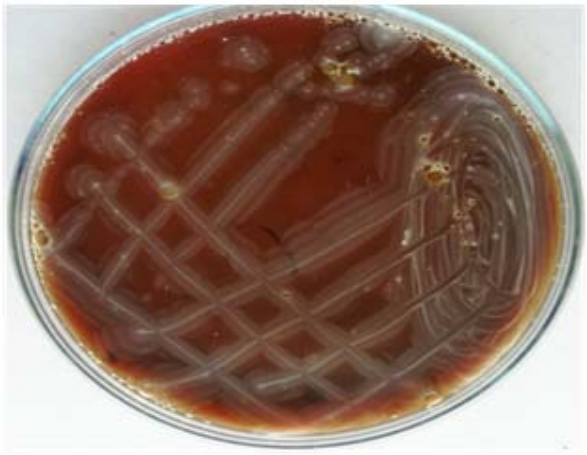

Fig-2-Petri plate containing pure isolates or colonies of *Proteus vulgaris* prepared from Sub BA of BHI of liver

Fig-3-Petri plate containing pure isolates or colonies of *Proteus mirabilis* prepared from Sub BA of BHI of large intestine

The isolates from MacConkey agar and Blood agar were identified by biochemical tests[20] as *P. vulgaris* and *P. mirabilis*, respectively. Biochemically, both the organism (*P. vulgaris* and *P. mirabilis*) were found to produce H_2S as indicated with the production of black colour and to ferment glucose, thereby producing acid and gas. Lactose, sucrose, dulcitol, mannitol, and maltose were not fermented. Both species produce K/A slant with no fermentation, along with butt showing fermentation of sugars and acid production (i.e. only fermentation of glucose took place) indicated by pink slant/black butt. The *P. vulgaris* isolate was positive for PPA (Phenylpyruvate) test, urease, methyl red, citrate and indole (Fig. 4). The *P. mirabilis* isolate was positive for PPA test, urease, methyl red, and negative for indole and citrate utilization (Fig. 5). The organisms were typed *Proteus vulgaris* and *Proteus mirabilis* as per Bergey's Manual of Determinative Bacteriology[21]. *Proteus* species were also confirmed by special confirmatory tests such as oxidase test (negative), 3% KOH string test (positive) and motility test (positive), respectively.

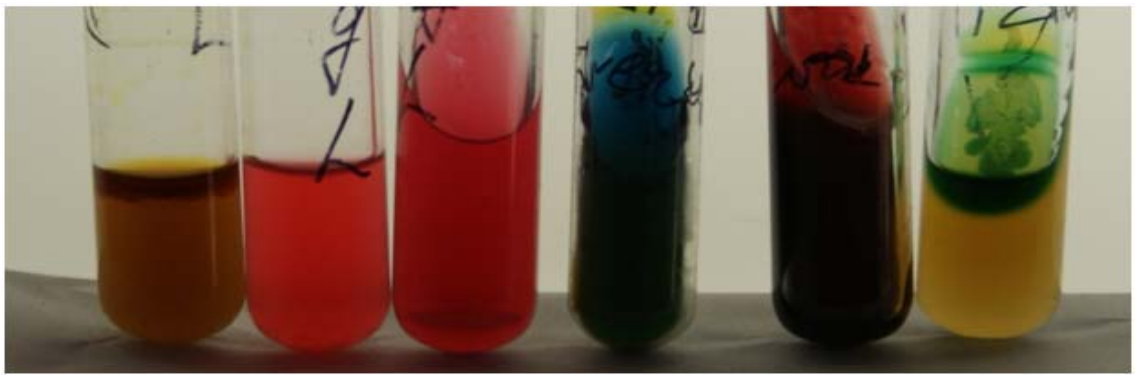

Fig-4-NLF set of biochemical test (I MR U C TSI PPA) from sub BA plates of BHI of liver showing positive I, MR, U, C, PPA test and pink slant/black butt along with H_2S production in TSI test, confirmed the presence of *Proteus vulgaris* in the infected liver tissues

Fig-5-NLF set of biochemical test (I MR U C PPA TSI) from sub MA plates of BHI of small intestine showing positive MR, U, PPA test and negative I & C test and pink slant/black butt along with H_2S production in TSI test, confirmed the presence of *Proteus mirabilis* in the infected small intestine tissues

DISCUSSION

Proteus organisms have been reported to cause septicemic disease in man and animals having serious underlying illness[6, 1]. Two factors might have been responsible in the culmination of the disease in these birds: (a) an overwhelming infection, possibly of incubator or brooder origin coupled with climatic stress, and (b) inadequate incompetence of the chicks to cope with infection during the early brooding stage.

The gross lesions observed in chicks died of yolk sac infection included unabsorbed/ retained yolk sac and edematous yolk which was also reported by different workers[24-27]. This is the first report in Ajmer, Rajasthan pertaining to yolk sac infection in clinically sick chickens. Involvement of *Proteus* species has also been reported previously[22-24].

In conclusion, the results of the present study in poultry farms of Ajmer region entailed that the importance of yolk sac infection (YSI) in causing the high mortality of chicks and thus posing a great threat to the poultry industry in Ajmer, Rajasthan. However, the disease has received little attention. Moreover, a further study on epidemiological investigation of Yolk sac infection in this region, economical impact of YSI, experimental studies on breed susceptibility to Yolk sac infection and on the solutions to prevent and control the disease is therefore, encouraged. This study has shown that *Proteus* species were widely distributed in visceral organs of clinically sick chickens in this region. Further study to elucidate the virulence factors and associated economic impact of these organisms is recommended. In this study, prevalence rate of 25% was recorded in dead sampled chickens (*Gallus* gallus).

A study was undertaken to assess the virulence of *P. vulgaris* and *P. mirabilis* in infected chicks. It is clear from the report of Sah *et al.*[13] and from the outbreaks just described that *P. mirabilis* has the potential to cause fatal septicaemic infections in large numbers of chicks. *Proteus mirabilis* is often reported to be responsible for urinary tract infections in human beings[28]. *Proteus vulgaris* causes arthritis in fowls especially males characterized by painful and swollen joints and bones of leg (hind-leg) due to which the males becomes unable to mount-on females resulting in reduced production of fertilized egg or ova in females. This causes huge economic loss to poultry as if where at least 85 fertilized eggs should be produced out of 100 eggs laid by hens (female bird), only 20-25 fertilized eggs would come out from the body of females. Thus, arthritis in spur of males leads to infertility.

Proteus mirabilis causes salpingitis in female bird characterized by swollen oviduct of females. The ova seems to be a bunch of grapes from which few ova are detached one by one from bunch and fall into the oviduct, where they transfer throughout the entire length. A thin membrane of albumin secretes over the yolk (ova) in the proximal part of oviduct and the process of deposition of material and membrane continues till it reaches the distal part of oviduct i.e. cloaca, where $CaCO_3$ (Calcium Carbonate) gets deposited on the egg forming the egg shell. When the oviduct of females get swollen, the ova fall very rapidly in it one by one and reaches the cloaca without undergoing any deposition of the material as the oviduct does not obstruct their path. As a result of which the ova become mis-shapened as only a thin membrane is found outside it. They become long, narrow, oval in shape and fragile instead of a definite round shape. At the end, the ova get disrupted as the membrane gets ruptured and yolk comes out in the cloaca. Sometimes, a thin membrane of $CaCO_3$ get deposited on these mis-shapened immature ova and these travel back to the gut of bird due to peristaltic movement of gut or stomach as the end point of oviduct also opens into the part of gut i.e. cloaca. So in birds having salpingitis, few shelled immature small ova or egg can be found in the gut of bird while performing necropsy of bird. Thus, *Proteus mirabilis* also causes huge economic loss to poultry as they affect the females mosty and thereby, reduce fertility of female fowls.

ACKNOWLEGEMENT

We greatfully acknowledge the technical assistance of Dr. V. K. Mishra, Retired Govt. Veterinary Doctor, Ajmer in performing necropsy of birds and his invaluable advice and encouragement in preparing the manuscript.

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