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Pathobiological Studies on Multi Drug Resistant *Staphylococcus aureus* Associated with Gangrenous Dermatitis affected Chickens

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A B S T R A C T

Thorough postmortem examination of 195 broiler poultry carcasses brought to the Department of Veterinary Pathology; LUVAS was carried out to find out the cause of mortality. Gangrenous dermatitis condition was encountered in 3.58% of broilers of 6-12 weeks age group. In these cases mixed infection of *Staphylococcus aureus (all cases), E. coli (one case)* and *Salmonella* Gallinarum (one case) was found. *Staphylococcus* isolates were found to be most sensitive to ofloxacin (66.66%), followed by polymixin B (53.34%) and enrofloxacin (46.66%), whereas they were found resistant to cefixime (100%), ciprofloxacin, (100%), erythromycin (100%) followed by ceftriaxone tazobactam, amoxyclav, cefoparazone, tetracycline (86.6%). Overall 100% *Staphylococcus aureus* isolates showed multidrug resistant pattern (MDR). Grossly there was presence of exudative cellulitis in the subcutaneous tissues on lower abdominal, thigh and back regions and necrotization of feather follicles was observed. The liver appeared congested, hemorrhagic mottled along with paleness of bone marrow tissue. Main bacteria isolated from these chicks were *Staphylococcus aureus*. One chick was associated with *Salmonella* ser. Gallinarum infection showing bronze discoloration of liver along with enlargement, mottling and presence of multiple necrotic foci.

Introduction

Gangrenous dermatitis (GD) is a disease of chickens and turkeys that causes severe economic losses in the poultry industry worldwide (Li G *et al.*, 2010). The prevalence and severity of dermatitis has increased over the last several years due to ban on antibiotic use. The main causes of GD are considered to be *Clostridium septicum*, *Clostridium perfringens* type A, and occasionally *Clostridium sordellii*, however *Staphylococcus aureus* and other aerobic bacteria have also shown to be involved in some cases of the disease (Gornatti *et al.*, 2018). The prevalence of cellulitis is relatively low; however, the

disease can be devastating in the individual flocks affected (Umar *et al.,* 2015). The route of transmission of GD is poorly characterized. There are several infectious and environmental immunosuppressive factors that can predispose birds to GD. Among them skin lesions are considered to be the main route of entry for the microorganism for the development of disease (Gornatti *et al.,* 2018). GD is characterized by acute onset of mortality associated with gross skin and subcutaneous tissue lesions consisting of variable amounts of serosanguineous exudate together with emphysema and hemorrhages. Due to relatively less common condition among the broiler industry, there is a lack of extensive and conclusive published scientific findings of the conditions in the literature. Therefore present study was conducted to study the pathobiology of the disease and its associated causative organisms.

Materials and Methods

The present study was carried out on 195 poultry broiler carcasses brought to the Department of Veterinary Pathology, LUVAS, Hisar for postmortem examination from August, 2018 to January, 2019. Prior to post mortem examination the details regarding identification of all the carcasses viz. species, age, sex etc. were recorded as per requisition form. The detailed gross, histopathological examination, microbiological studies were carried of the carcasses which were suspected for gangrenous dermatitis.

Microbiological studies

Representative samples of affected subcutaneous tissue, liver and heart blood were collected aseptically for bacteriological isolation studies from birds suspected with gangrenous dermatitis disease condition. The samples were inoculated aerobically on nutrient agar, MacConkey's Lactose agar and Eosin-Methylene blue agar (EMB), for the isolation of bacteria. Anaerobic isolations were not carried out in the present study. Pure colonies of the bacterial growths so obtained were primarily identified on the basis of colony characteristics and Gram's staining reaction and further characterization and confirmation was done employing various biochemical tests by Vitek-2 system (BioMerieux, Inc. Hazelwood, MO, USA).

Antimicrobial sensitivity testing and Multiple Drug Resistance (MDR) patterns

All the isolates obtained were subjected to *in-vitro* drug sensitivity testing using antimicrobials by the disc diffusion method (Bauer *et al.,* 1966). Concentration of various drugs used in *in-vitro* chemotherapeutic sensitivity test is presented in Table 1. MDR pattern studies were conducted to know the multiple antibiotic resistances of bacterial isolates. The isolates showing resistance to more than three antibiotic groups/combinations were placed in MDR category (Oteo *et al.,* 2005).

Pathological studies

The detailed post mortem examination was conducted on all the poultry carcasses. All poultry carcasses suspected for gangrenous dermatitis were examined critically for gross pathological alterations in various internal organs. Representative tissue pieces from organs showing lesions were collected in 10 percent buffered formalin for routine histopathological examination. The fixed tissues were washed in running tap water, dehydrated in graded ethyl alcohol, cleared in benzene and embedded in paraffin wax (melting point 60-62^{o}C). Paraffin sections were cut at the thickness of 4-5µ and stained with routine haematoxylin and eosin stain using Lilly Mayer's haematoxylin and 2 percent water soluble eosin (Luna, 1968).

Results and Discussion

Out of 195 broiler carcasses examined, gangrenous dermatitis condition was encountered in 3.58% of broilers of 7 wks age group. In these cases mixed infection of *Staphylococcus aureus* (all cases), *E. coli* (one case) and *Salmonella* ser. Gallinarum (one case) was found.

Microbiological studies

On nutrient agar plates, *Staphylococcus aureus* colonies appeared as small, round, yellowish pigmented, raised from surface on nutrient agar, while no growth was obtained on MLA agar, *Escherichia coli* colonies appeared as pale, round, slightly raised while in Mac Conkey's lactose agar plates colonies appeared as pink coloured round and raised indicating presence of lactose fermentation. On EMB agar, colonies were small, circular, slightly raised with metallic sheen indicating the presence of *E. coli*. *Salmonella* ser. Gallinarum colonies appeared as dew drop whitish coloured with pale coloured (non lactose fermenting type) colonies on MLA.

After preliminary identification of bacterial isolates, the pure culture of bacterial colonies was taken from nutrient agar for further identification of bacteria by Vitek-2 system. Average analysis time for *E. coli* was 4.75 hrs, average probability was 99.00% and confidence was excellent. Average analysis time for *Staphylococcus aureus* was 4.5 hrs, average probability was 99.00% and confidence was excellent. Average analysis time for *Salmonella* ser. Gallinarum was 5.5 hrs, average probability was 95.00% and confidence was very good.

The results of *in-vitro* chemotherapeutic drug sensitivity to *Staphylococcus* isolates have been represented in Table 2. *Staphylococcus* isolates were found to be most sensitive to ofloxacin (66.66%), followed by polymixin B (53.34%) and enrofloxacin (46.66%), whereas they were found resistant to cefixime (100%), ciprofloxacin, (100%), erythromycin (100%) followed by ceftriaxone tazobactam, amoxyclav, cefoparazone, tetracycline (86.6%).

Multidrug resistance (MDR) pattern studies revealed that about 100% *Staphylococcus aureus* isolates showed MDR pattern. About 40% *Staphylococcus aureus* isolates were resistant to all antibiotic group /combinations studied, while 26.7% isolates were resistant to 9 antibiotic groups /combinations, 20% isolates were resistant to 8 antibiotic group /combinations and 13.3 % of isolate were resistant to 7 antibiotic group /combinations.

Pathological studies

In gangrenous dermatitis affected broiler chickens, grossly there was presence of exudative cellulitis in the subcutaneous tissues on lower abdominal, thigh and back regions and necrotization of feather follicles. The liver appeared congested, hemorrhagic and mottled. There was paleness of bone marrow tissue. But in one case from which *Salmonella* ser. Gallinarum was isolated there was bronze discoloration of liver along with enlargement (Fig. 1), mottling and presence of multiple necrotic foci. Lungs were congested. Serosal congestion and haemorrhages throughout the intestinal tract were seen.

Histopathological lesions revealed severe inflammatory reaction in sub cutaneous tissue with infiltration of leucocytes including heterophils, lymphocytes and macrophages (Fig. 2). The lungs and liver sections revealed vascular changes with congested blood vessels and haemorrhages in parenchyma. Liver in GD affected chick associated with *Salmonella* ser. Gallinarum revealed mild to severe congestion of sinusoids and central veins in

liver (Fig. 3), haemorrhages, vacuolar degeneration and multifocal areas of coagulative necrosis of hepatocytes. Multifocal areas of suppurative hepatitis characterized by presence of islands of eosinophilic necrotic hepatocytes floating in pool of heterophils were the typical lesion found. Heterophilic infiltration in portal triads along with pyknotic and karyorrhectic nuclei of necrotic hepatocytes was also observed. Thickening of mucosal folds of proventriculus due to edema was observed. Intestinal sections revealed necrotic villous epithelium along with severe congestion in lamina propria of villi and infiltration of heterophils (Fig. 4). The results indicated that mixed infection cases revealed more severe pathological lesions.

Present study showed about 3.58% gangrenous dermatitis incidence among broilers affecting 6-12 wks age group. The cases were showing involvement of mixed bacterial infections of aerobic nature but *Staphylococcus aureus* was found associated in all the cases.

Microbiological studies

Antibiotic sensitivity studies revealed that the *Staphylococcus* isolates were found to be most sensitive to ofloxacin (66.66%), followed by polymixin B (53.34%) and enrofloxacin (46.66%), whereas they were found resistant to cefixime (100%), ciprofloxacin (100%), followed by ceftriaxone tazobactam, amoxyclave, cefoparazone, tetracycline (86.6%). It is also reported that more than 50% of the *Staphylococcus aureus* isolates shows resistance to ampicillin, tetracycline, erythromycin, penicillin and clindamycin, where as none of the isolates are resistance to cephalothin (Geidam *et al.,* 2012). It is also revealed that most of the strains of *Staphylococcus aureus* are resistant to bacitracin (87.5%), methicillin (67.5%) and penicillin G (53.8%) (Gundogan *et al.,* 2005). In the present study, the resistance to these antibiotics might be attributed mainly to the frequent and indiscriminate use of these antibiotics in treatment and prophylaxis and as feed supplements.

Upon studying the multidrug resistance (MDR) pattern; all the isolates of *Staphylococcus aureus* showed MDR pattern to more than 4 groups of drugs/combinations. The findings of the present study indicate towards the extensive use of antibiotics by poultry farmers.

In agreement to our findings higher percentage (77.2%) of multiple drug resistance to *Staphylococcus aureus* is also reported by Geidam *et al.,* (2012). Transfer of antibiotic resistant bacteria from animals to humans through food products has been well documented and pose a serious threat to public health (O' Brien, 2002; Sornplang *et al.,* 2011). Therefore continuous monitoring and surveillance is necessary to efficiently evaluate the resistance problem and will detect trends and changes in the pattern of resistance. This can help to develop efficient guidelines for the use of antimicrobials and limit the spread of resistant organisms.

Pathological studies

Pathological lesions observed in most of the cases were of exudative cellulitis and vascular changes in visceral organs and paleness of bone marrow. Earlier workers also observe that *S. aureus* is the most common non-clostridial bacterial species which is associated with GD (Andreasen 2013). This microorganism has found to be able to cause GD alone or in combination with one or more clostridial species. Other species of this genus which have been found associated in the outbreaks of GD in broiler chickens include *S. xylosus* and *S. Epidermidis* (Thayer and Miller, 2008).

Table.1 Concentration of various drugs used in *in-vitro* chemotherapeutic sensitivity test

S. No.	Group of antibiotics/ combinations	Drug	Concentration (mcg)/disc
1	**Penicillin+ Beta lactamase inhibitors**	Amoxyclav (AMC)	30
2	**Cephalosporins**	Cefixime (CFM)	5
		Cefoperazone (CPZ)	75
3	**Cephalosporin/Beta lactamase inhibitors**	Ceftriaxone/tazobactam (CIT)	30
4	**Chloramphenicol**	Chloramphenicol (C)	30
5	**Tetracycline**	Tetracycline (TE)	30
6	**Fluoroquinolone**	Ciprofloxacin (CIP)	5
		Enrofloxacin (EX)	10
		Moxifloxacin (MO)	5
		Ofloxacin (OF)	5
7	**Sulphonamide + DHFR inhibitor**	Co-Trimoxazole (COT)	25
8	**Macrolides**	Erythromycin (E)	15
9	**Aminoglycoside**	Gentamicin (GEN)	30
		Streptomycin (S)	25
10	**Miscellaneous**	Polymyxin B (PB)	25

Table.2 *In-vitro* chemotherapeutic drug sensitivity (% sensitivity) and resistance (% resistance) of *Staphylococcus aureus* isolates

S. No.	Group of antibiotics + Combinations	Drug	*Staphylococcus aureus*	
			% sensitivity	% resistance
1	Macrolides	Erythromycin (E)	0	**100**
2	Amphenicol	Chloramphenicol (C)	40	60
3	Aminoglycoside	Streptomycin (S)	20	80
		Gentamicin (GEN)	33.33	66.66
4	Cephalosporins	Cefoperazone (CPZ)	13.34	86.66
		Cefixime (CFM)	0	**100**
5	Cephalosporins/ Beta lactamase inhibitors	Ceftriaxone Tazobactam (CIT)	13.34	86.66
6	Sulphonamide + DHFR inhibitor	Co-Trimoxazole (COT)	26.67	73.33
7	Fluoroquinolones	Enrofloxacin (EX)	46.67	53.33
		Moxifloxacin (MO)	26.67	73.33
		Ofloxacin (OF)	66.66	33.33
		Ciprofloxacin (CIP)	0	**100**
8	Tetracycline	Tetracycline (TE)	13.34	86.66
9	Penicillin+ Beta lactamase inhibitors	Amoxyclav (AMC)	13.34	86.66
10	Miscellaneous	Polymyxin B (PB)	53.34	46.66

Fig.1 Dark congested liver with bronze discoloration in gangrenous dermatitis case associated with *Salmonella ser. Gallinarum*

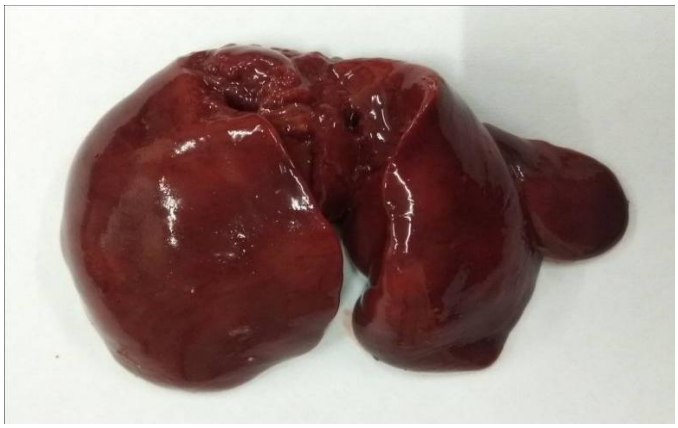


Fig.2 Inflammatory reaction in subcutaneous tissue with infiltration of leucocytes including heterophils, lymphocytes and macropahges (H&E stain 400X)

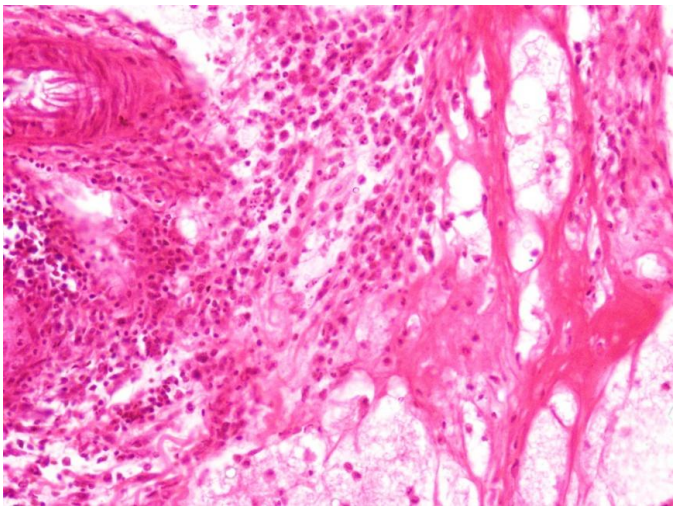


Fig.3 Congestion of sinusoids and central veins in liver of GD affected chicken associated with *Salmonella ser. Gallinarum* (H&Estain 200X)

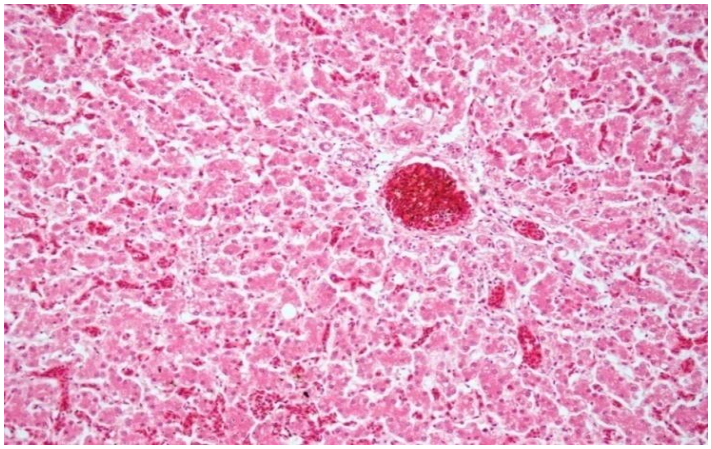
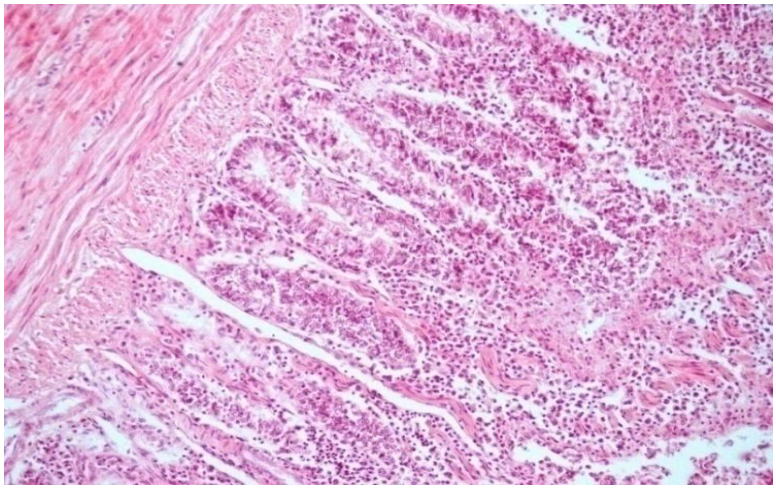


Fig.4 Intestinal sections revealed necrotic villous epithelium along with severe congestion in lamina propria and infiltration of heterophils and lymphocytes in GD affected chicken with *Salmonella ser. Gallinarum* (H&E stain 200X)



However, in contrary various reports from literature states that these microorganisms can also be found in skin and nares of healthy chickens, and isolation from chickens with GD does not necessarily confirm a causative role in this disease (Andreasen, 2013). It is reported in literature that *S. aureus* can produce several toxins, including hyaluronidase, deoxyribonuclease, fibrinolysin, lipase, protease, leucocidin, hemolysins, epidermolytic toxin, and dermonecrotic toxin (Hermans *et al.,* 2010) and experimental studies following intradermal inoculation in poultry and rabbits (Cervantes *et al.,* 1988), indicates that dermonecrotic toxin of *Staphylococcus aureus* induced severe dermal inflammation and skin necrosis at the injection site, which supports a possible role of this toxin in the pathogenesis of GD. Various studies indicate that *E. coli* isolates which have been obtained from cases of cellulitis, swollen head syndrome and colisepticemia in chickens are found to produce a vacuolating cytotoxin. This toxin is specific for avian cells and appears to be similar to the one produced by *Helicobacter pylori* (Salvadori *et al.,* 2001).

In broiler chickens, GD is mainly predisposed to by traumatic damage of the skin, usually associated with cannibalism and overcrowding. Such skin lesions provide a portal of entry for bacteria (Opengart, 2013). However, GD was also reported, especially in heavy broiler chickens with grossly intact skin. In those cases, the predisposing factor was considered to be anaerobiosis generated by subcutaneous tissue necrosis associated with trauma of the pectoral region, as a result of prolonged ventral recumbency. Also indiscriminate multiplication of the intestinal flora followed by absorption into the bloodstream promoted bacteremia, which is thought to be the origin of some of the GD lesions (Hoerr, 2010).

In corroboration to the present study findings other researchers also indicates that GD cases associated with *S. aureus* are usually characterized by ulceration of the epidermis and necrosis in dermis and subcutis (Shivaprasad, 2016). They also reported that in cases of GD complicated with *S. aureus* coinfection, gram-positive cocci mixed with variable numbers of heterophils can be observed (Shivaprasad, 2016). Similar to our

findings, lesions other researchers also observed randomly scattered, small foci of coagulative necrosis with bacterial colonies in the liver and spleen of affected birds indicating secondary hematogenous spread of bacteria from the skin, subcutis, and muscle (Opengart, 2013; Shivaprasad, 2016).

Controlling GD requires a multifactorial approach, with an emphasis on reducing causative organisms in both the bird and the environment, as well as by promoting immunity and reducing stress. From the findings of the present study it may be concluded that special attention must be attributed to the presence of multi drug resistant bacteria in affected birds and their environment to prevent the further transfer of resistance genes to other animals and humans.

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