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ISOLATION AND IDENTIFICATION OF GRAM NEGATIVE OXIDASE POSITIVE BACILLI ISOLATED FROM BROILER CHICKENS

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ABSTRACT

Miscellaneous Gram negative bacteria are seriously affecting broiler chickens and poultry industry in Egypt. The present study aimed to isolate and completely identify oxidase positive Gram negative bacterial pathogens recovered from different pathological lesions in broiler chickens by conventional biochemical tests, API20NE and microbact24e system. Samples were taken from 200 Hubbard and Ross broiler chickens of different ages (3-5weeks), from different farms in Beni-Suef and El-Fayoum Governorates during the period from January 2016 to April 2016. Bacteriological examination showed that Gram negative bacteria were 165 (82.5%) of isolates of which 105 isolates (52.5%) were oxidase positive while 60 isolates (30%) were oxidase negative. The most prevalent bacterial isolates were *Pseudomonas aeruginosa* (43 isolates), *Aeromonas hydrophila* (35 isolates), *Pasteurella gallicida* (12 isolates), *Plesiomonas shigelloides* (10 isolates), and *Vibrio vulnificus* (5 isolates) with incidences of 21.5%, 17.5%, 6% 5%, and 2.5%, respectively.

Key words: Gram negative, oxidase positive, broiler chickens.

INTRODUCTION

Poultry are regarded without any doubt the most appropriate source of protein supply of high nutritive value for man. This is due to efficiency cost of production and its short life cycle. Starting from this point much interest oriented toward maximizing the factors increasing the conversion rate and weight gain as genetic improvement and selection, nutrition balance, and management control, besides minimizing factors inversely affecting conversion rate as diseases and stress factors. Among factors inversely affecting poultry production are diseases and infections (De Haan *et al.*, 2001 and McKissick, 2006).

Pseudomonas aeruginosa (*P. aeruginosa*) is the most predominant *Pseudomonas* species causing infection, mortality among birds and clinical signs including septicemia, diarrhea and respiratory signs (ElShafii, 1992 and Tanios and Kamel, 1999).

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Pasteurella multocida is the causative agent of numerous, economically important diseases, including avian fowl cholera and other disorders (De Alwis, 1992). Fowl cholera is a serious disease of poultry and can present in either acute or chronic forms. Obvious clinical signs of acute fowl cholera may not occur until very late in the infection and include depression, ruffled feathers, fever, anorexia, mucous discharge from the mouth, diarrhoea and an increased respiratory rate (Rhoades and Rimler, 1989).

Aeromonas infections in poultry have been reported in different parts of the world with devastating effects (Dashe *et al*., 2013). A higher occurrence of Aeromonas from chicken source suggests that chicken could be a potential host for the spread of Aeromonas infection and present a possible threat to public health due to the ubiquitous nature of Aeromonas in aquatic, clinical and environmental sources (Smita and Brahmabhatt, 2011).

Plesiomonas shigelloides (previously *Aeromonas shigelloides*) are ubiquitous, facultative anaerobic, flagellated, Gram-negative rods (San Joaquin, 1994). *P. shigelloides* have been isolated from a variety of environmental sources (Jeppesen, 1995).

The genus Vibrio includes several food-borne pathogens that cause a spectrum of clinical conditions including septicemia, cholera and milder forms of gastroenteritis. Several *Vibrio* species are commonly associated with food-borne transmission including *V. cholerae*, *V. parahemolyticus*, and *V. vulnificus* (Azwai *et al*., 2016).

The purpose of this study is to identify the most prevalent Gram negative oxidase positive bacilli affecting broilers.

MATERIALS AND METHODS

2.1. Samples

Samples were taken from 200 Hubbard and Ross broiler chickens of different ages (3-5weeks), from different farms in Beni-Suef and El-Fayoum Governorates during the period from January 2016 to April 2016.

These chickens were suffered from respiratory manifestations (coughing, sneezing, ralling, nasal discharge and some times swelling of infra orbital sinuses eitherbilateral or unilateral). The chickens were subjected to clinical, postmortem and bacteriological examination of the affected tissues including liver (*n=95*), kidney (*n=50*), pericardium (*n=29*) and air sacs (*n= 26*).

2.2 Bacteriological examination

The collected samples were cultivated under aseptic conditionin to Tryptone Soya broth. All inoculated media were incubated aerobically at 37ºC for 24 hrs. Then loopful from the inoculated broth were streaked onto MacConkey's agar, tryptone soya agar (TSA) and dextrose starch agar. The colonies were examined for their cultural characters and morphological appearance according to Mahon *et al*. (2015).

Medium sized colonies from MacConkey's agar and dextrose starch agar were picked up for purification on TSA and incubated aerobically at 37C for 24-72hours. Smears from separate colonies and from livers of suspected fowl cholera cases were stained with Gram's and Leishman's stains and examined microscopically. Colonies revealed pure Gram negative bacilli, showing bipolarity from blood film were inoculated onto nutrient slopes.
Foreach plate, one single colony representing typical colonial appearance and morphological character was picked up and inoculated into 12%glycerol broth then kept at -20ºC for further investigation.

All the recovered Gram negative, medium size and non sporulated isolates (with Gram's stain smears) were further examined biochemically.

2.3. Biochemical identification of the obtained bacterial isolates.

2.3.1 By using conventional biochemical tests.

The following tests were adapted for identification of bacterial isolates: oxidase, TSI, indole production, methyl red (MR), Voges Prauskeur (VP), citrate utilization, hydrogen sulphide (H_2S) production on TSI, urease activity, nitrate reduction, gelatin liquefaction and sugar fermentation for glucose, lactose, sucrose, mannose, arabinose, maltose and mannitolaccording to Collee *et al*. (1996). Other tests such ashaemolysis onto blood agar (β), motility, growth at 4ºC, Growth at 42ºC and pigment production were included.

2.3.2. Identification by using API kit

The appropriate API kit (API20NE, Oxoid) was selected.API strips should only be used to identify pure cultures of an unknown organism. Gram stain (plus catalase and oxidase if appropriate) were done before inoculating a test strip. It was used according to the manufacturer's instruction.

2.3.3. Microbact-24E bacterial identification system

Is a commercial microsystem simplify the identification of Enterobacteriaceae and common miscellaneous Gram-negative bacilli, including oxidase positive GNBs, consists of dehydrated substrates distributed in the wells of microtitre trays with the Thermo Scientific™ Oxoid™ Microbact™ GNB that Kit is a complete, self-contained biochemical based identification system. It Uses 24 different biochemical tests in microplate format to produce easy-to-read, distinct color reactions, generally following overnight incubation. Interpret using the Thermo Scientific™ Microbact™ Identification Package) and this was done according to the manufacturer's instruction.

RESULTS

3.1. Prevalence of bacterial isolation from the diseased chickens.

The results recorded in table (1) showed that out of 200 chickens (aged 3-5 weeks) the total number of recovered isolates were 193 (96.5%). Gram negative bacterial isolates were 165 (82.5%) of which 105 isolates (52.5%) were oxidase positive and 60 isolates (30%) were oxidase negative. Moreover, 28 isolates (14%) were Gram positive. On the other hand, 7 samples (3.5%) had no bacterial isolation (negative isolation).

Table 1: Prevalence of bacterial pathogens obtained from diseased chickens:

Total No. of samples	Positive isolation										Negative isolation	
	Gram negative						Gram positive		Total isolation			
	Oxidase positive		Oxidase negative		Total							
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
200	105	52.5	60	30	165	82.5	28	14	193	96.5	7	3.5

%: calculated according to the total number of samples.

3.2. Biochemical identification of oxidase positive Gram negative bacterial isolates.

Identification of oxidase positive Gram negative bacterial isolates using traditional biochemical tests as well as API 20NE and Microbact systems revealed thatthe isolates were identified as *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Pasteurella gallicida*, *Plesiomonas shigelloides* and *Vibrio vulnificus*.

3.3. Prevalence of oxidase positive Gram negative bacterial isolates recovered from diseased chickens.

From the total collected samples (*n=200*) the most prevalent bacterial isolates were *Pseudomonas aeruginosa* (43 isolates), *Aeromonas hydrophila* (35 isolates), *Pasteurella gallicida* (12 isolates), *Plesiomonas shigelloides* (10 isolates), and *Vibrio vulnificus* (5 isolates) with incidences of 21.5%, 17.5%, 6% 5%, and 2.5%, respectively (Table2).

Table 2: Prevalence of oxidase positive Gram negative bacterial isolates recovered from diseased chickens.

Bacterial isolates	No.	%
Pseudomonas aeruginosa	43	21.5
Aeromonas hydrophila	35	17.5
Pasteurella gallicida	12	6
Plesiomonas shigelloides	10	5
Vibrio vulnificus	5	2.5
Total	**105**	**52.5**

No.: Number of isolates.

%: was calculated according to the total number of the examined cases.

3.4. Prevalence rates of bacterial pathogens in relation to site of isolation:

The result recorded in **table (3)** showed that the bacterial pathogens were isolated from samples from liver (*n=56*), kidney (*n=30*), pericardium (*n=14*) and air sac (*n=5*).*P. aeruginosa* was isolated from 21 livers, 8 kidneys, 9 pericardia and 5 air sacs with incidence rates of 37.5, 26.7, 64.3 and 100%, respectively. *Aeromonas hydrophila* was isolated from 17 livers, 13 kidneys and 5 pericardia with incidence rates of 30.4, 43.3 and 35.7 %, respectively. *Pasteurella gallicida* was mainly isolated from 8 livers and 4 kidneys with incidence rates of 14 .3 and 13.3%, respectively.

Plesiomonas shigelloides was mainly isolated from 6 livers, 4 kidneys with incidence rates of 10.7 and 13.3%. *Vibrio vulnificus* was mainly isolated from 4 livers and one kidney with incidence rates of 7.1, 3.3% respectively.

Table 3: Prevalence rates of bacterial pathogens in relation to site of isolation:

Site of samples	No. of isolates	Bacterial isolates									
		Pseudomonas aeruginosa		*Aeromonas hydrophila*		*Pasteurella gallicida*		*Plesiomonass higelloides*		*Vibrio vulnificus*	
		No.	%	No.	%	No.	%	No.	%	No.	%
Liver	**56**	21	37.5	17	30.4	8	14.3	6	10.7	4	7.1
kidney	**30**	8	26.7	13	43.3	4	13.3	4	13.3	1	3.3
Pericardium	**14**	9	64.3	5	35.7	-	-	-	-	-	-
Air sac	**5**	5	100	-	-	-	-	-	-	-	-
Total	**105**	**43**	**41**	**35**	**33.3**	**12**	**11.4**	**10**	**9.5**	**5**	**4.8**

%: was calculated according to the number (No.) of isolates.

DISCUSSION

No doubt that many microorganisms are considered to be the most important causative agents infect poultry causing significant economic losses are incurred in commercially produced poultry worldwide due to diseases caused by bacterial agents (Barnes *et al*., 2003).

Out of 200 examined broiler chickens; with respiratory disorders and kidney lesions by taking samples from liver, kidney, heart blood and air sac. The total number of Gram negative bacterial isolates were 165 while 28 isolates were Gram positive and the remaining 7 cases were bacteriologically negative (3.5%). Such obtained results are nearly coinciding with that reported by Harbi *et al.* (1982); Poornima and Upadhyde (1995) and Sedhom (2000). Samples with negative bacterial isolation could be attributed to viral, parasitic or fungal causatives (De Herdt *et al*., 2008 and Karki *et al.*, 2009).

Results of traditional biochemical tests for bacterial identificationare the same to the results obtained by Macfaddin (1976); Krieg *et al.* (1984); Sedhom (2000) and Emam (2006).

Concerning using MB24E and API20NE besides conventional biochemical methods for diagnosis; the MB24E identifications were found to be "correct". Thus in their hands, the MB24E gave 100% correct identification, while the API20E was "correct" to species level in 97.4% of cases (Mugg and Hill, 1981).

The MB24E gave very similar results to the API20E for the identification of common clinical isolates of both-. Enterobacteriaceae and non-fermenting Gram negative bacilli, and is suitable for use in a routine diagnostic laboratory. The API20E database is more extensive than that of the MB24E and contains more examples of rare organisms. Both systems are more reliable for the identification of clinical rather than environmental isolates (Ling *et al*., 1988). The most prevalent isolate was *P. aeruginosa* (43 isolates) with a prevalence rate of 21.5% followed by *Aeromonas hydrophila* (35 isolates; 17.5%) then *Pasteurella gallicida* (12 isolates, 6%), *Plesiomonas shigelloides* (10 isolates; 5%), and *Vibrio vulnificus* (5 isolates; 2.5%). Such results are in agreement with that of (El Sayed *et al*.,b2016) who recovered *P. aeruginosa* from liver of freshly dead chickens and yolk sac of dead -in-shell embryos 12/100 (12%) and 26 (52%) respectively and, the total isolation rate was 38/166 (22.9%). Enany *et al*. (1986) recovered 30 isolates of *P. aeruginosa* from 250 diseased broilers in Sharkia. This prevalence was higher when compared with the separate findings of different authors in Egypt, where, Younes *et al.* (1990); Riad (1994); Abd El-Gawad *et al.* (1998) Osman *et al.* (1998); Mahmoud and Moussa (2000) and Mohammed (2005) who reported prevalence of 4.9%, 7.4%, 8%, 9.43%, 6.6% and 9.3%, respectively. This result may be considered low if compared with that reported by Kurkure *et al.* (2001) who recorded a prevalence of 57%.

Tandomly; Kumar *et al*. (2000) reported a close prevalence of Aeromonas (16.7%) in poultry meat while Shinde *et al*. (2005) observed a moderate prevalence (24%) in poultry meat. Alhough, a very high prevalence (55%) was found in the studies of Smita *et al*. (2011). Presence of Aeromonas species in foods of animal origin, water environment and retail foods as evident from the available literatures may indicate that these products can act as possible source of vehicles for dissemination of food-borne Aeromonas gastroenteritis (Neyts *et al*., 2000; Bhowmik *et al*., 2009). The Present findings emphasized the need of more intensive study on distribution of Aeromonads in soil, water environment and foods of animal origin considering its public health implications. Hygiene at retail chicken shop where samples were collected was heavily compromised and water use for carcass washing may be important critical point in carcass contamination from wide number of gastrointestinal

food-borne zoonotic pathogens. It was observed that water kept in bucket for washing either fish or chicken carcass was not subject to frequent change and used for dipping of number subjects.

The isolation rate of *Pasteurella gallicida* was 6% (12 isolates,) which was in agreement with that of Mbuthia *et al.* (2008) who isolated *P. multocida* from 25.9% of healthy-looking ducks and 6.2% of chickens from free-range family poultry farms. Aslightly lower than that recorded by Mohamad *et al.* (2012) who studied the prevalence of *P. multocida* strains among 275 backyard chickens from different regions of Upper Egypt. A total of 21 isolates *P.multocida* were recovered in 21 out of 275 chickens tested (7.6%) and were confirmed using phenotypic characterization. While Varga *et al.* (2007) determined *Pasteurella* sp. by using biochemical characterization and PCR-based techniques and also determined lower incidence as 87% of the isolates belonged to *P. multocida*. 10% of the strains were *P. multocida* subsp. septica. Only 3% of the isolates were *P. multocida* subsp. *gallicida*.

The result recorded in table (3) showed the prevalence rates of bacterial pathogens in relation to site of isolation *P. aeruginosa* was isolated with incidence rates of 37.5, 26.7, 64.3 and 100%, respectively. Meanwhile, *A. hydrophila* was isolated with incidence rates of 30.4, 43.3, 35.7 and 0%, respectively. *P. gallicida*, *P.shigelloides* and *V. vulnificus* were isolated only from liver and kidney samples. The incidence rates in liver were 14.3, 10.7 and 7.1 %, respectively, while incidence rates in kidney were 13.3, 13.3 and 3.3%, respectively.

P. aeruginosa was isolated from pericardium, liver, spleen, trachea, nasal discharge, intestinal contents and yolk sac of dead broiler chicks by (Kurkure *et al*. 2001). They added that it was responsible for early mortality in broiler chicks. Also the isolation of 11 isolates of *A. hydrophila* species from 3 different organs (bone marrow, heart and liver) of clinically sick chickens was an indication that avian species were susceptible to this organism. Despite that *Aeromonas species* have not been reported as an important poultry pathogen; there are documented reports that the organism caused a fulminating disease which was characterized with high mortality in wildlife birds such as canary birds (Franca *et al*., 2009). The low frequency of isolation of *A. hydrophila* from tissue samples of clinically sick birds could be due to indiscriminate administration antibiotics to birds by poultry farmers once any sign of disease is observed.

P. multocida was readily isolated, often in pure culture from visceral organs such as lung, liver and spleen, bone marrow, gonads or heart blood of birds that succumb to the acute bacteraemic form of the disease, or from the caseous exudate characteristic of chronic fowl cholera lesions (Curtis *et al.*, 1980 and Snipes *et al.*, 1988).

CONCLUSION

Fowls are susceptible to numerous infections attributable to many organisms. The present study focusing on oxidase positive Gram negative bacteria: the most prevalent isolates were *Pseudomonas aeruginosa* (43 isolates), *Aeromonas hydrophila* (35 isolates), *Pasteurella gallicida* (12 isolates), *Plesiomonas shigelloides* (10 isolates), and *Vibrio vulnificus* (5 isolates) with incidences of 21.5%, 17.5%, 6% 5%, and 2.5%, respectively.

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عزل وتصنيف البكتريا سالبه الجرام موجبه الاوكسيديز في بداري التسمين

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البكتريا سالبه الجرام موجبه الاوكسيديز لها بالغ التاثير على بدارى التسمين وصناعه الدواجن فى مصر. تم اجراء الفحوص البكتريولوجيه على عدد 200 من اصابات باثولوجيه مختلفه من بدارى التسمين عمر (٣-٥) اسابيع من مزارع مختلفه فى محافظتى بنى سويف والفيوم فى الفتره من يناير ٢٠١٦ الى ابريل ٢٠١٦ وقد تم التصنيف البيوكيميائي بواسطه الطرق البيوكيميائيه التقليديه وكيتسات ال api20ne بالاضافه الى نظام الميكروباكت ١٢٤ . الفحوص البكتريولوجيه كشفت عن وجود ١٦٥(٨٢.٥%) بكتريا سالبه الجرام منهم ١٠٥(٥٢.٥%) موجبه الاوكسيدبز و ٦٠(٣٠%) سالبه الاوكسيديز. وكانت الميكروبات التى تم عزلها هى: سودوموناس ايروجينوسا ٤٣ معزوله، ايروموناس هيدروفيلا ٣٥ معزوله، باستيريلا مالتوسيدا ١٢ معزوله، بلسيوموناس شيجيلويد ١٠ معزوله وفيبريو فالنيفيكس ٥ معزوله بنسبة ٢١.٥%، ١٧.٥% . ٦% ،٥% و ٢.٥%على التوالى.

الكلمات المفتاحية : البكتريا سالبه الجرام - موجبه الاوكسيديز– بداري التسمين.