Veterinary Medical Journal-Giza (ISSN 1110-1423)
Faculty of Veterinary Medicine,Cairo University
Accredited from national authority for Quality Assurance and Accreditation
Giza, 12211, Egypt

Studies on the pathogenicity of Enterobacter sakazakii infection to 1-day old specific pathogen free chicks.

Amer, M.M.[1]; Manl A. Ali[1];Zeinab M.S. Amin Girh[2]; Kh. M. Elbayoumi[2]; Eman R. Hassan[2] and Elmarakby, E.S.I[3].

1- Dept. of Poult. Dis., Facul. Vet. Med . , Cairo University, Giza, Egypt.
2- Dept. of Poult. Dis., Vet. Res. Division, National Research Centre, Dokki, Giza, Egypt.
3- Vet. Service Division, Ministry of Defense

Abstract

Sixty specific pathogen free (SPF) chicks were divided into 3 equal groups; 20 each. Chicks of the 1st group were subcutanously (s.c) infected; Chicks of the 2nd group were intra-crop infected. Each chick was infected with 1 ml. of 24 hours *Enterobacter sakazakii* (*E. sakazakii*) broth culture containing 1.5× 10^8 viable microorganisms. Chicks of 3rd were kept as non infected control. The chicks were reared and kept under observation for 10 days. Mortality rate in E.sakazakii intra-crop infected SPF chicks was (4/20) 20% in the 1st 24 hours post infection and 12.5% in chickens during 10 days; with total deaths of 30% . Mortality rate in s.c injected SPF chicks with *E. sakazakii* was 50% at the 1st 24 hours post infection and 20% out of the remaining 10 chickens in 10 days, with total mortality of 60%. *E. sakazakii* could be reisolated from all dead chicks. Both s.c and intra-crop infections resulted in microscopic lesions in liver, spleen, kidneys and intestine. Shedding of *E. sakazakii* in dropping was found to be an intermittent shedding.

Clinical signs on chicks hatched from infected eggs were dullness, depression, sleepy appearance, ruffled feathers, brownish diarrhea and coughing. Post mortem lesions are congested lung, air sacculitis, hepatitis, and distended gall bladder, congested and inflamed kidney. The results of performance indicated that infection with *E. sakazakii* decrease body weight and feed conversion rate (FCR).

In conclusion, our findings indicate pathogenicity of *E. sakazakii* to 1-day old SPF chicks with bad impact on performance. The economical and public health importance of *E. sakazakii* are still need more investigation.

key words: pathogenicity, E. *sakazakii*, SPF chicks, intracrop and s.c infection
Corresponding Author: *Amer, M.M.* Profdramer@yahoo.com

Introduction

Enterobacter species are found in natural environment (water, sewage, soil and vegetables). Some species are found in human and animal species **(Nazarowec-White and Farber 1997)**. Enterobacter spp. particularly *E. cloacae, E. aerogenes* and *E. Sakazakii*, are important nosocomial pathogens responsible for various infections, including bacteremia, lower respiratory tract infection, skin and soft-tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteo-myelitis and ophthalmic infections. This bacterium's virulence similar to other members of the Enterobacteriaceae, family seems largely to be due to an endotoxin that it produces ***(Fraser et al., 2008).*** Recently, a taxonomic reclassification of this pathogen to consist of 5 species within a new genus "*Cronobacter*" was proposed **(Baumgartner et al., 2009)**. *E. sakazakii* has been recovered from milk powdered infant formula products in several countries **(Muytjens et al., 1988)** . Unheated milk, spoiled tofu, lettuce, fermented bread, and rinsed beer mugs have been source of *E.*

sakazakii. A recent study reported recovering this organism from eight of nine food processing plants and from 5 of 16 households (**Moats, 1979**).

In poultry industry the organism was reported to contaminate Fertilized eggs and may result in weak chicks, poor chick growth and low FCR (**Ramnoff, 1960**), increased mortality of embryos, lower hatchability and increased early chick mortality (**Ljudmila Milakovic-Novak and Estella Prukner, 1990**). The organisms was also reported on the eggshell surface, cloacal swabs, commercial eggs and fertilized eggs (**Al-Bahry et al., 2010**) and **Asma- Abd-Ellatif , 2013**). **Fang Hai et al. (2012)** isolated 11 bacteria from the affected chicks and identified as *Enterobacter*, and stated that these isolates were highly pathogenic to chickens by experimental infection and this explain the high mortalities occurred when *E.sakazakii* inoculated into chicks. **Asma-Abd-Ellatif (2013)** reported pathogenicity of *E. sakazakii* to broiler chickens with clinical signs, mortality, pathological lesions and decreased feed conversion rate (FCR). Poultry remains a vehicle of important pathogens such as *Enterobacteriaceae* (**Threlfall et al., 1993 and Weinstein, 1993**).

This study was carried out to investigate the pathogenicity of *E. sakazakii* after intracrop or s.c infection of SPF chicks and its effect on performance.

Material and methods

SPF chicks:

A total of 60, a day old SPF chicks were used in this experiment were obtained by hatching of 80 fertile eggs from Koum Oshem, Fayoum.

Culture techniques:

Isolation of *E.sakazakii* was adopted as recommended by FDA (2002) ; the enrichment of samples using enrichment broth, incubated at 37 c for 24 hr. from each enrichment culture, a loopful was inoculated into violet red bile agar plates , incubated overnight at 36 °C. then colonies were streaked onto trypticase soya agar and incubated at 25 °C for 48-72 hr. Only yellow pigmented colonies were selected and confirmed as *E. sakazakii* by oxidase test and API 20E.

Inoculums preparation:

Inoculum was grown in 100 ml of trypticase soy broth for 24 h at 35 C°. *Mc Ferland* standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. A sample of the medium was removed aseptically and a plate count was performed to confirm inoculums concentration according to ***Jones et al. (2002)***.

Experimentally infection:

The experimental groups were inoculated subcutaneously or intra-crop with 1 ml of 24 hr. *E.sakazakii* broth culture containing 1.5×10^8 cfu/ml. Then the birds or eggs were kept under daily observation for mortality, clinical signs and post mortem picture according to (**Asma- Abd-Ellatif , 2013**).

Histopathological examination:

Specimens from kidneys, spleen, intestine and liver were immediately taken after scarification, then fixed in 10% buffered neutral formalin solution for 24 hr and then transferred to 70% alcohol in which they were preserved till processed. Parts from the taken tissue specimens were then washed, dehydrated, embedded in paraffin, sectioned at 4-5 micron thickness and stained with H&E as a routine work for histopathological studies according to (**Bancrof and Stevens, 1996**).

Experimental design:

Sixty, 1 day-old SPF chicks were divided into 3 equal groups; 20 each. Chicks of the 1st group were s.c inoculated with 1 ml. of 24 hrs *E.sakazakii* broth culture containing 1.5×10^8 viable microorganisms. Chicks of 2nd group were infected intra-crop with 1 ml. of 24 hrs *E. sakazakii* broth culture containing 1.5×10^8 viable microorganisms. Chicks of the 3rd group were kept as control. The chicks were reared for 10 days, kept under observation and samples from dead chicks within this period were collected. Postmortem examination was done and samples from liver, spleen, kidney and intestine were collected for histopathology. The results are shown in tables (1and2) and Figs. (1and 2).

Results

The performance parameters of *E. sakazakii* infected SPF chicks by intra-crop or s.c infected were seen in table (1). In s.c infection and intra-croup groups weekly body weight, weekly feed consumption, weekly body weight gain and FCR in 1st week after infection were 125 gm, 140 gm, 90 gm and 1.55; respectively and these results were the same as control group. In the 2nd week these parameters were different from each other, in s.c injection parameters were 245 gm, 245 gm, 120 gm and 2.1 respectively, while in intra-croup infection parameters were 255 gm, 257 gm, 130 gm and 1.9; respectively. Those parameters in control group were 300 gm, 298 gm, 175 gm and 1.7; respectively.

Mortality rate in *E.sakazakii* intra-crop infected SPF chicks is (4/20) 20% in the 1st 24 hrs post infection and 2/16) 12.5% in the reared chickens for 10 days (Table 2). The total death was 6/20 with total mortality rate 30%. Rate of isolation of *E.sakazakii* from dead embryos was 20% while in chickens reared for 10 days after hatching is 12.5%. Mortality in s.c injected chicks with *E.sakazakii* broth culture (Table 2) was 50% at the 1st 24 hours post infection and 20% in the remaining 10 chickens for 10 days, while total mortality rate was 12/20 chicks with 60% total mortality rate. Rate of isolation of *E.sakazakii* from dead embryos is 50% in the 1st 24 hrs, while in chickens reared for 10 days after hatching was 20%. Clinical signs appear on chicks hatched from infected eggs are dullness, depression, sleepy, ruffled feathers brownish diarrhea and coughing. Post mortem lesions were congested lung, air sacculitis, and distended gall bladder, congested and inflamed kidney.

The shedding rate of *E.sakazakii* in dropping was also determined. There was an intermittent shedding from the 1st up to 5th day, no shedding was detected from 6th and 7th day and then the shedding occurred again from 8th - 10th day after infection in s.c infected group. Shedding of *C.sakazakii* in dropping of crop infected birds occured from the 1st day up to 4th day, while in from 5th day up to 6th day, no shedding was detected, and re-shedding was occurred again from 7th -10th day.

Histopathological lesion (Fig1) at 1 day post infection (dpi) of s.c infected SPF chick : liver showing dilated hepatoportal blood vessel and focal leucocytic cell infiltration, kidneys with necrosed renal tubules and congested interstitial blood vessel, spleen with atrophied follicles and Intestine with severely necrosed and disintegrated glands, while, in intra-crop infected SPF chick (fig 2) at 1 dpi showed liver with focal scattered necrotic area infiltrated with mononuclear cells infiltration, intestine with degenerated glands, kidneys with degenerated and atrophied glomerular tuft and spleen with focal area of necrosis.

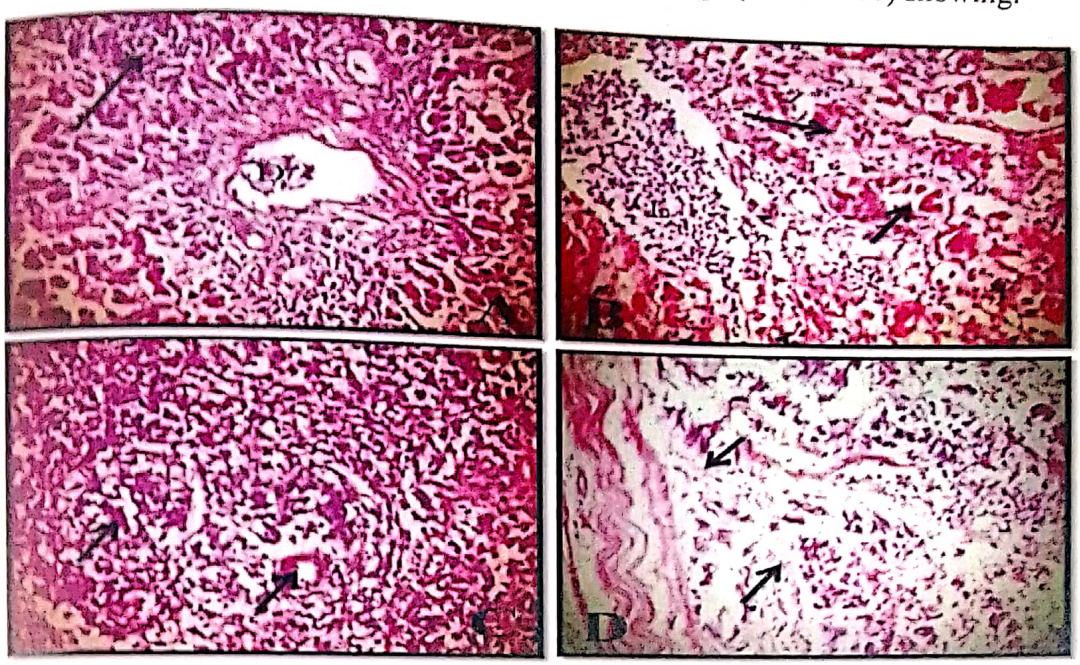
Table (1): Weekly performance parameters of *E.sakazakii* infected SPF chicks.

Group No.	Infection	Age/weeks	Mean body weight (g)	Feed intake (g)	Body weight gain (g)	FCR
1	s.c	1	125	140	90	1.55
		2	245	240	120	2.0
		Total	370	380	210	1.8
2	Intra-crop	1	125	140	90	1.55
		2	255	257	130	1.9
		Total	380	397	220	1.8
3	Control	1	125	140	90	1.55
		2	300	298	175	1.7
		Total	435	438	265	1.6

Table (2): The effect of *E. sakazakii* in intra-crop or s.c infected 1-day old SPF chicks (n=20).

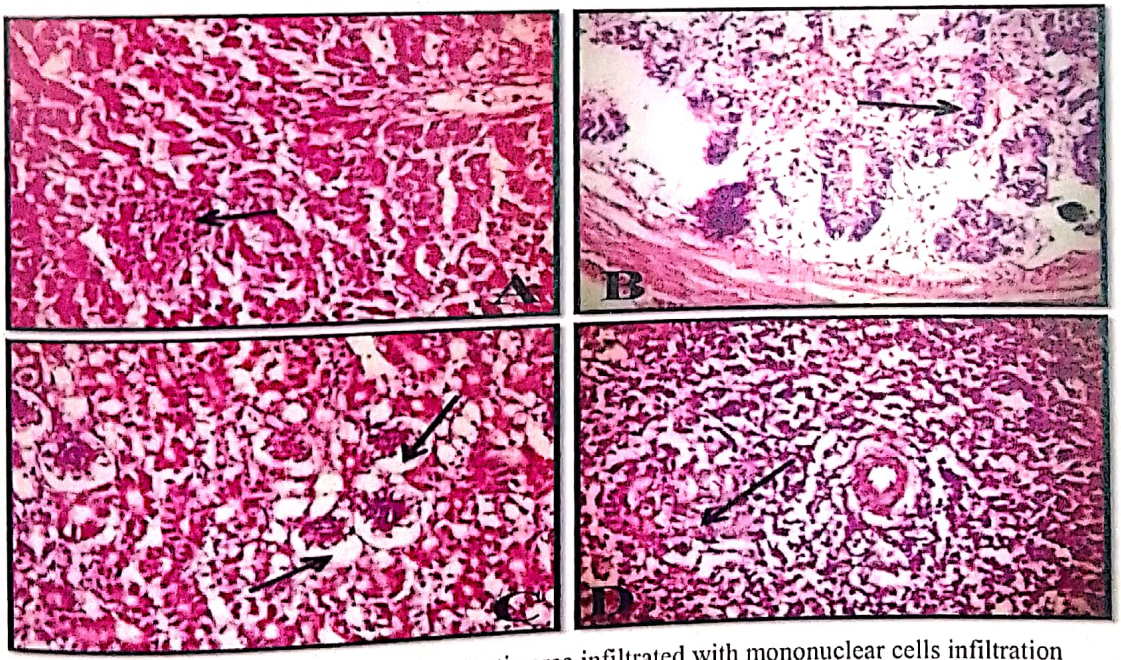
Group No	Route of infection	No. of chicks	24 hr deaths			10 days post-inoculation				No of total mortality	Total mortality rate (%)
			No of chicks	+ve reisolation	Mortality rate	No of chicks	No. of deaths	+ve reisolation	Mortality rate(%)		
1	S.c	20	10	10	50	10	2	2	20	12	60
2	intra-crop	20	4	4	20	16	2	2	12.5	6	30
3	Control	20	1	0	5	19	-	-	-	0	0

Fig (1): Sections of s.c infected SPF chick at 1 dpi (H&E ×400) showing:



A- Liver showing dilated hepatoportal blood vessel (D) and focal leucocytic cell infiltration (white arrow).
B- Kidneys showing necrosed renal tubules (arrows) and congested interstitial blood vessel (L)
C- Spleen showing atrophied follicles (arrows)
D- Intestine showing severely necrosed and disintegrated glands (arrows)

Fig (2): Sections of intra-crop infected SPF chick at 1 dpi(H&E ×400) showing:



A- Liver showing focal scattered necrotic area infiltrated with mononuclear cells infiltration (arrow).
B- Intestine showing degenerated glands (arrow)
C- Kidneys showing degenerated and atrophied glomerular tuft (arrows)
D- Spleen showing focal area of necrosis (arrow)

Discussion

Enterobacter spp. are the sixth most common cause of nosocomial infection and antibiotic resistant strains are observed with increasing frequency (**Peters et al., 2000**) . Enterobacter spp. are not primary human pathogens, however E. cloacae have been implicated in a broad range of clinical syndromes (**Kaminska et al., 2002 and Liu,et al., 2004**). *E. sakazakii* is an opportunistic pathogen causing meningitis, septicemia and enterocolitis in neonates (**Bar-Oz et al., 2001**), Multiple cerebral infarcts with resulting multicystic encephalomalacia in a premature infant with *E.sakazakii* meningitis *(Dubos et al., 2006).*

This work was designed to study pathogenicity of *E. sakazakii* in SPF chicks. In this experiment SPF chicks were experimentally infected with *E. sakazakii* by s.c injection or intra-croup. In s.c chicks infected 10 chicks were died (50 %) and *E. sakazakii* was isolated from internal organs (liver, kidney, spleen) of the 10 chicks. From the 10 survival chicks, 2 chicks were dying (20%) within 10 days after infection from which rate of reisolation was 20% (table 2).In chicks intra-croup infected, 4 chicks were died (20 %) and *E. sakazakii* was isolated from internal organs (intestine, kidney, spleen) of the 4 chicks. From the 16 survival chicks, 2 chicks were dying (12.5%) within 10 days after infection from the organism was risolation from 12.5% dead chicks (table 1). This results indicate pathogenicity of used isolate (**Ramnoff, 1960**), **Silva et al., 2011**), **Fang Hai, et al., 2012**, **Asma- Abd-Ellatif , 2013** and **Kothary** et al., **2007**) stated that *E. sakazakii* virulence factors are a proteolytic enzyme.

The performance parameters of *E. sakazakii* infected S.P.F. chicks by intra-crop or s.c infection were seen in table (2) .

These results indicate that infection with *E.sakazakii* decrease body weight and decrease FCR (**Ramnoff, 1960 and Asma- Abd-Ellatif , 2013**). Shedding of *E.sakazakii* in dropping was found to be intermittent shedding. this result can be supported by **Savov (1966)** who reported that excretion of E.coli from experimentally infected fowls continued for 20 days. Also, **Ardrey et al., 1968)** who recorded experimentally that infected *E. coli* carriers were continuous or intermittent rectal shedders. *Praxedes et al., 2012* identified *E.sakazakii* from Fecal samples of broiler submitted from the 15th to the 23th day of life

Histopathological picture of intra-crop and S.c infected SPF chick in Liver ,Kidneys , Spleen and Intestine were reported by (**Asma- Abd-Ellatif , 2013**).

Comparing our results with the available literature about public health importance and scant data about *E.sakazakii* in poultry, we can concluded that *E.sakazakii* needs more investigation especially under our field conditions.

References

Al-Bahry, S. N., Mahmoud, I. Y., Al-Zadjali, M., Elshafie, A., Al- Harthy, A. and Al-Alawi, W. (2010): Antibiotic resistant bacteria as bio-indicator of polluted effluent in the green turtles, Chelonia mydas in Oman. Mar. Environ. Res. 71, 139-144.

Ardrey, W.B., Peterson, C.F. and Haggart, M. (1968): Experimental collibacillosis and the Avian Diseases, 21, 505.

Asma- Abd-Ellatif M. (2013): Studies on enterobacteriaceae in chicken egg. MVSc, In Poultry Diseases Beni-Suef University.

Bancroft, J.D. and Stevens, A. (1996): Theory and Practice of Histological Technique pp 766, Churchl Livingstone, Edinburgh.

ISSN 1110-1423

Baumgartner, A.; Grand, M.; Liniger, M. and Iversen, C. (2009): Detection and frequency of Cronobacter spp. (Enterobacter sakazakii) in different categories of ready-to-eat foods other than infant formula. Int. J.Food Microbial. 136 (2): 189-192.

Bar-Oz, B.; Preminger, A.; Peleg, O.; Block, C. and Arad , I. (2001) : Enterobacter sakazakii infection in the newborn. Acta Paediatr. 90 (3) 356 –358.

Dubos F.; Moullin F.; Gajdos V.; Desuremain N.; Biscardi S.; Lebon P.; Raymond J.; Breart G.; Gendrel D.; Chalumeau M.(2006): Serum procalcitonin and other biologic markers to distinguish between bacterial and aseptic meningitis .J. Pediatr. Jul.; 149 (1) :72- 76.

Fang Hai; Chen CuiZhen; Shi QiuMei; Zhang Yan Ying; Ma ZengJun and Zhang DongLin (2012): Isolation and identification of chicken pathogenic Enterobacter. Zhongguo Yufang Shouyi Xuebao/Chinese J. of Preventive Vet. Med. 34(5): 384-387.

Food and Drug Administration (2002): Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. Center for food safety and applied nutrition.

Fraser, S.L.; Arnett, M. and Sinave, C.P. (2008): Enterobacter infections. E-Medicine specialties-infectious diseases

Jones D. R., Anderson K. E., Curtis P. A and Jones F. T. (2002): Microbial Contamination in Inoculated Shell Eggs. Poultry Sci. 81:715–720.

Kaminska, W.; Patzer, J. and Dzierzanowska, D. (2002): Urinary tract infections caused by endemic multiresistant *Entero-bacter cloacae* in a dialysis and transplantation unit. J. Hospital Infection, 51: 215-220.

Kothary, M. H.; McCardell, B. A. ; Frazar, C. D.; Deer , D. and Tall, B.D. (2007): Characterization of the Zinc-Containing Metalloprotease Encoded by zpx and Development of a Species-Specific Detection Method for Enterobacter sakazakii. Appl. Environ. Microbiol. 73 (13) 4142 - 4151.

Liu, C.P.; Wang, N.Y.; Lee, C.M.; Weng, L.C.; Tseng, H.K.; Liu, C.W.; Chiang, C.S. and Huang, F.Y. (2004): Nosocomial and community-acquired *Enterobacter cloacae* bloodstream infection: risk factors for and prevalence of SHV-12 in multiresistant isolates in a medical centre. J. Hospital Infection, 58:63-77.

Ljudmila Milakovic-Novak and Estella Prukner (1990): Hygiene levels of eggs Faculty for Vet. Medicine, University of Zagreb (Yugoslavia).

Moats. W. A. (1979) : The effects of washing eggs under commercial conditions on bacterial loads on egg shells. Poult. Sci. (58)1228 - 1233.

Muytjens, HL, Roelofs-Willemse, H, Jaspar, GH (1988): Quality of powdered substitutes for breast milk with regard to members of the family Enterobacteriaceae . J. Clin. Microbiol. 26 (4) 743–746.

Nazarowec-White, M. and Farber, J.M. (1997): *Enterobacter sakazakii*: a review. International j. Food Microbiol.,34, (2): 103-113.

Peters, S.M.; Brtan, J. and Cole, M.F. (2000): Enterobacterial repetitive intergenic consensus polymerase chain reaction typing of isolates of *Enterobacter Cloacae* from an outbreak of infection in a neonatal intensive care unit. Amer. J. infection control, 28:123-129.

Praxedes, C. I. S.; Zuniga, N. O. C.; Bastos, P. A. M. B.; Franco, R. M. and Mano, S. B.(2012): Enterobacteriaceae identification of the broiler intestinal microbiota submitted to nitrofurans diet. Revista Brasileria de Ciencia Veterinaria. 19(1):46-49.

Ramnoff, A.L.(1960) : The avian embryo, Macmillan Company, New York: 1077.

Savov, D. (1966): Pathogenic E.coli carriage in fowl. Vet. Med. Nauki, Sof., 3,519.

Silva, M. C. A.; Melo, D. B.; Costa, W.L.R.; Fernandes, L. M. B.; Pinna, M. H. and Vieira Neto, J.(2011): Macroscopic and micro-scopic aspects of airsacculitis in slaughtered broilers in Brazil. Animal hygiene and sustainable livestock production. Proceedings of the XVth international congress of the international society for animal hygiene, Vienna, Austria, 3-7. 603-605.

Threlfall, E.; Rowe, B. and Ward, L. (1993) : A comparison of multiple drug resistance in salmonella from humans and food animals in England and Wales, 1981-1990. Epidemiol. and Infection, 111 : 189 -197.

Weinstein, P. (1993): Multiple resistant *staphylococcus aureus* (MRSA) infection in South Australia, 1983-1990. Comm. Disease Intel. 17, 25 : 590 -593 .

دراسات على الإصابة الإمراضية لبكتيريا إنتيروبكتر ساكازاكى للكتاكيت عمر يوم الخالية من المسببات المرضية

الملخص

تم إجراء هذا البحث على عدد ٦٠ كتكوت خالى من المسببات المرضية عمر يوم تم تقسيمهم إلى ثلاث مجاميع متساوية العدد (٢٠ كتكوت لكل مجموعة). المجموعة الأولى تم إصابتها عن طريق الحقن تحت الجلد ببكتيريا إنتيروبكتر ساكازاكى أما المجموعة الثانية فتم إحداث العدوى بها عن طريق الحويصلة وفى كلتا المجموعتين تم إحداث العدوى بإعطاء الكتاكيت ١ مللى من المستعمرة البكتيرية المرقية عمر ٢٤ ساعة والتى تحتوى على ١.٥×١٠^٨ من بكتيريا 'نتيروبكتر ساكازاكى الضارية. أما المجموعة الثالثة فقد تم الحفاظ عليها سالبة الإصابة المرضية . بعد العدوى المرضية تم وضع الكتاكيت فى الثلاث مجاميع تحت المراقبة لمدة عشرة أيام. وقد أظهرت النتائج أن معدل النفوق فى المجموعة التى تم إصابتها عن طريق الحويصلة كان ٢٠% (٢٠/٤) بعد أول ٢٤ ساعة من العدوى بينما وصلت إلى ١٢.٥% مع نهاية العشر أيام المراقبة بعد العدوى بمعدل نفوق كلى وصل إلى ٣٠% ، بينما وجد أن معدل النفوق فى الكتاكيت الخالية من المسببات المرضية والتى تمت العدوى بها عن طريق الحقن تحت الجلد ببكتيريا إنتيروبكتر ساكازاكى الضارية وصل إلى ٥٠% بعد ٢٤ ساعة من إحداث الإصابة بينما كان ٢٠% من اليوم الثانى وحتى العاشر بعد إحداث الإصابة المرضية بمعدل نفوق كلى وصل إلى ٦٠% مع نهاية التجربة كذلك وقد تم عزل بكتيريا إنتيروبكتر ساكازاكى الضارية من جميع الطيور النافقة بعد العدوى. فى الكتاكيت المصابة حدثت أفات مرضية ميكروسكوبية فى الكبد، الطحال، ، الكلى ، وكذلك الأمعاء فى كلتا المجموعتين المصابتين عن طريق الحويصلة أو بالحقن تحت الجلد . وقد لوحظ نزول متقطع لبكتيريا إنتيروبكترساكازاكى الضارية فى زرق الكتاكيت المصابة بالعدوى.

بالنظر للإعراض الإكلينيكية التى ظهرت على الكتاكيت الخالية من المسببات المرضية الفاقسة من بيض ملوث ببكتيريا إنتيروبكتر ساكازاكى الضارية فكانت بلادة، خمول، ترييش غير منتظم ، مع وجود إسهال بنى وسعال . الأفات التشريحية كانت إحتقان بالرئة ، إلتهاب بالأكياس الهوائية ، إلتهاب بالكبد مع تمدد بالحويصلة المرارية وإحتقان وإلتهاب بالكلى. نتائج معدلات الإنتاجية أظهرت إنخفاض فى الأوزان وكذلك معدل التحويل فى الكتاكيت المصابة .

خلصت النتائج أن بكتيريا إنتيروبكتر ساكازاكى قادرة على إحداث إصابة مرضية بالكتاكيت الخالية من المسببات المرضية وكذلك لها تأثير سلبى على الأداء الإنتاجى لها . إما بالنسبة لتأثير البكتيريا على الصحة العمة لمستهلكى الدواجن وكذلك الأهمية الإقتصادية لها فإنها تحتاج إلى دراسات أخرى.