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Bacterial isolation rate from fertile eggs, hatching eggs, and neonatal broilers with yolk sac infection

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ABSTRACT. Yolk sac infection (YSI) is a major cause of mortality of broilers during the first week post-hatching. The aim of the present study was to analyze the possible sources of fertile egg contamination and to establish the etiology of YSI. Sixty fertile eggs, sixty sawdust samples from the nest, sixty nonfertile 19 to 21 day old incubation eggs and liver and yolk sac samples from 216 dead, 1 to 7 day old chicks, were cultured. Five hundred and eighty eight colonies were isolated and further characterized using biochemical tests. *Escherichia coli* was the most common bacterium recovered from all samples except the sawdust and fertile eggs collected from the nest. Fertile egg contamination at breeder farm level was found to be minimal. In broilers, both mortality and the rate of *E. coli* isolation were increased with the time. These results suggest that egg contamination does not occur at the breeders farm, as previously has been reported. Bacterial contamination causing YSI in vertically integrated operations can occur at a latter stage. It can be considered that the main etiologic agent of YSI is *E. coli*, since YSI mortality was highly correlated with *E. coli* isolation.

Key words: *Escherichia coli*, yolk sac infection, poultry.

RESUMEN. La infección del saco vitelino (ISV) es la principal causa de mortalidad en pollo durante la primera semana. El objetivo del presente estudio fue identificar la posible fuente de contaminación del huevo fértil, para determinar la etiología de la ISV. Se seleccionaron sesenta muestras de cama de nido, huevo de nido, huevo a los 19 y 21 días de incubación, y muestras de saco vitelino e hígado de 216 pollitos muertos durante la primera semana. Se obtuvieron 588 aislados; la identificación bioquímica mostró que *Escherichia coli* era el microorganismo más común en todas las muestras, excepto en las de nido y el huevo de nido. La contaminación del huevo fértil en la granja de reproductoras fue mínima. En pollo de engorda, el aislamiento de *E. coli* se incrementó en relación con la mortalidad observada. Los resultados obtenidos sugieren que la contaminación del huevo no ocurre en la granja de reproductoras, como ha sido propuesto, ya que ésta puede ocurrir en etapas posteriores. Con respecto a la etiología de la ISV, *E. coli* fue el principal agente etiológico identificado.

Palabras clave: *Escherichia coli*, infección del saco vitelino, aves.

INTRODUCTION

In Mexico, poultry meat is the most important source of animal protein ingested by the general population. According to the Unión Nacional de Avicultores, the *per capita* consumption of poultry meat will increase from 21.68 kg in 2002 to 22.36 kg in 2003 (http://www.una.com.mx).

Yolk sac infection (YSI) is a major cause of mortality in broilers during their first week of life.[1,5,10] YSI occurs in all flocks resulting in decreased hatchability, increased mortality and increased cull rate due to retarded growth.[5,11,17] YSI occurs mainly due to bacterial contamination of the eggshell at the broiler breeder farm, shortly after the egg is laid, while the cuticle is still moistened. Contamination-promoting factors include lack of hygiene in the nests, presence of eggs on the floor, incubation of dirty eggs or eggs with eggshell defects, and collection of dirty and clean eggs at the same time.[5,10,17]

Alternatively, poor fertile egg storage conditions, poor egg disinfection, and high humidity levels during incubation may also promote YSI.[1] Another important route of contamination of the yolk sac is the bacterial penetration through a poorly healed navel.[5,17]

Proteus spp., *Enterobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Clostridium* spp., *Bacillus cereus*, and *Enterococcus*, are some bacteria that have been isolated from YSI-positive birds. Nevertheless the most common isolated bacterium is *E. coli*.[1,5,10,17]

Escherichia coli is an ubiquitous pathogen distributed throughout the world that causes significant economic losses to commercially produced poultry.[4,26,31]

In the present study, potential sources of fertile egg contamination were investigated to establish the etiology of YSI in a vertically integrated operation located at Central Mexico.

MATERIAL AND METHODS

All samples were obtained from a vertically integrated poultry operation located at Guanajuato State, Mexico.

Nest management and fertile egg collection. At the onset of lay, sawdust was placed in the nests and formaldehyde

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(10 g) was added. Dirty material was removed and replaced with clean sawdust. Egg collection was performed 4 times per day. Prior to egg collection, workers washed and disinfected their hands using a quaternary ammonium-based product. Once collected in plastic flats, eggs were disinfected by fumigation with formaldehyde gas and then stored at 18°C for 1-2 days until transferred to the hatchery.

Fertile egg/nest samples. Sixty non-disinfected eggs and sixty sawdust samples (10 g) collected from the same nest were analyzed.

Hatchery. At 19 days of incubation, (at the time of incubator-to-hatcher transfer) sixty infertile eggs were obtained from the same breeder flock. On hatch day, at sorting time, 60 piped, dead-in-shell eggs were taken.

Broilers. The offspring from the same breeder flock was followed up. Samples of liver and yolk sac were obtained from 216 birds that died during the first week of life. These samples were individually placed in sterile plastic bags for transportation. Samples were kept in refrigeration until they were analyzed within the two hours following collection.

Bacterial isolation. McConkey Agar (McC) and Trypticaseine Soy Agar (TSA) (DIFCO Laboratories, Detroit MI, USA) were used to culture all the samples. Eggs from the broiler breeder farm and eggs from the hatchery were rinsed with PBS (10 ml). One ml of this solution was inoculated onto the above-mentioned culture media. The egg content was sampled with a loop and plated onto McC and TSA.

Yolk sac samples from 21 day old eggs were grown in both McC and TSA. Liver and yolk sac samples were also obtained from birds that had died during the first week post-hatch and these were plated onto McC and TSA. All inoculated media were incubated at 37.5°C for 24 hours. Based on morphology, all different bacterial colonies were selected from each sample. These colonies were isolated in pure culture for further identification.

Bacterial identification. The identification of the isolated colonies was performed using standard bacteriological and biochemical procedures as described by Ewing[9] and Barrow & Feltham.[3]

RESULTS AND DISCUSSION

YSI is an economically important disease since it increases 8-day mortality and causes poor weight gain. In addition, birds that survive to a YSI outbreak show poor carcass quality.[27] Isolation frequency of the microorganisms found in the different samples from breeder farm, hatchery and broiler farm is summarized in Table 1. A total of five hundred and eighty eight strains were isolated. Even though isolates representing a great diversity of genera were recovered, *E. coli* (45.5%) and *Enterobacter aerogenes* (17.9%) were the most common. These results are similar to those obtained by Harry[12] who pointed out that *E. coli* was one of the microorganisms most frequently associated with YSI.

In the present study, fertile egg contamination at the broiler breeder farm level was found to be minimal. Only 22 isolates (22/588; 3.7%) were obtained from nest sawdust samples. Several authors had reported earlier that contamination of fertile eggs in the nest at the broiler breeder farm is the main cause of YSI.[10,17,24] However, in the present study *Staphylococcus aureus* was the most common bacterium isolated at the breeder farm (17 strains) and these had no significant impact in the mortality of broilers, since only one strain of this microorganism was isolated from dead chicks (Table 1). It is known that micrococci (*Staphylococcus* spp. and *Streptococcus* spp.) can be the predominant contaminants on the shell of clean eggs in the nest, however these organisms do not appear to be pathogenic for newly hatched chickens.[25]

Table 1. More common microorganisms isolated from different samples collected from the breeder farm, hatchery and broiler farm.

Strain	Breeders Farm		Hatchery		Broilers	
	Nest material	Fertile eggs	19th day	21st day	Mortality	Total
Escherichia coli		4 (0.7)	28 (4.8)	47 (8)	188 (32)	267 (45.5)
Enterobacter aerogenes			2 (0.3)	6 (1)	97 (16.5)	105 (17.9)
Klebsiella pneumoniae					56 (9.5)	56 (9.5)
Staphylococcus aureus	17 (2.9)*	2 (0.3)	12 (2)	20 (3.4)	1 (0.2)	52 (8.8)
Yeast				14 (2.4)	13 (2.2)	27 (4.6)
Streptococcus spp.	3 (0.5)		2 (0.3)	16 (2.7)	5 (0.8)	26 (4.4)
Others	2 (0.3)	2 (0.3)	14 (2.4)	2 (0.3)	35 (6)	55 (9.3)
Total	22 (3.7)	8 (1.3)	58 (9.9)	105 (17.9)	395 (67.2)	588

* Number inside the parenthesis represent the percentage with respect to the total of isolates (588)

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From non-disinfected eggs collected at breeder farm, only 8 strains were isolated (8/588; 1.3%), and 4 of them were identified as *E. coli*. These results suggest that egg contamination does not always occur at the broiler breeder farm, as previously reported.[10] Other bacteria such as *Pseudomonas aeruginosa* have been reported as a potential pathogens able to contaminate fertile eggs at the breeder farm,[30] but in our study the rate of contamination of eggs at the breeder farm was extremely low compared with later stages and most of the contamination was by gram positive bacteria (*S. aureus* and *Streptococcus* spp.), whereas *Pseudomonas* spp. was only isolated at broilers farm.

It seems to be more likely that contamination occurs after the fertile eggs have been transported to the hatchery. In a study where eggs from large poultry breeding plants and other from family breeders were analyzed results showed that eggs from the family breeders were almost free of pathogens.[21] Moreover, the incidence of YSI increased after 1930 with the advent of commercial hatcheries[14] showing that a higher rate of fertile egg contamination occurs outside the breeder farm. This observation is supported by the increase of isolates at the hatchery compared with those obtained in the same batch at the breeder farm in our study.

Fifty-eight (58/588; 9.9%) bacteria were isolated from eggs collected at 19 days of incubation (Table 1). Several researchers[5,8,17,30] have emphasized the importance of YSI as a cause of mortality even in the hatchery, which could increase the number of so-called "dead-in-shell" eggs, where our samples were collected. Forty-two percent of dead-in-shell ostrich embryos contained bacteria, where the most common isolate was *E. coli*.[8] Other isolates from dead-in-shell ostrich eggs and chicks included *Pseudomonas mesophilia, P. pneumoniae, Serratia liquifaciens, Alcaligenes xylosopsid, Aeromonas hydrophilia* and *Enterobacter cloacae*. We also isolated *Alcaligenes* spp and *Enterobacter aerogenes* from the broiler embryos but the isolation frequency was low.

A total of 105 isolates (105/588; 17.9%) were obtained from 21 day old eggs (hatch day). *E. coli* (47 strains) was the most common bacteria isolated, followed by *S. aureus* (20 strains). At this stage, the presence of *Streptococcus* spp. (16 strains) and yeast (14 strains) was also observed. The high prevalence of gram negative bacteria, mainly *E. coli,* is likely due to the relatively simple nutritional requirements of *E. coli* and their ability to grow in the presence of the iron chelating agents like ovotransferrin.[25] More than one bacteria were isolated from 21 of the samples. In most cases *E. coli* or *S. aureus* were isolated with yeast and/or *Streptococcus* spp. (data not show), however, most of the later microorganisms were recovered from the eggshell and thus are probably not important because they had not crossed the eggshell barrier. Yeast have not been reported as pathogens in chickens, but they have been isolated from the air and soil of poultry brooding and rearing houses, old litter, wet feed and birds droppings. Most of the reports of yeast in poultry are related with spoilage of fresh and processed carcasses.[15,29] However it is known that yeast colonies possess proteolytic and lipolytic activity.[15] This ability may play a role in the YSI by breaking down yolk sac constituents, and making nutrients more readily available for bacterial growth. Bacteria like *Staphylococcus* spp and *Streptococcus* spp have been reported as major contaminants at the hatchery and in a few cases they were more prevalent than *E. coli*.[25]

In the present study, a positive bacterial isolation was obtained from one hundred and eighty eight (87%) of the dead chicks at the broiler farm. A total of 395 different strains belong to different genera were isolated: E. *coli* (188/588; 32%), *E. aerogenes* (97/588; 16.5%) and *K. pneumoniae* (56/588: 9.5%) were the most common. Of these *E. coli* has been previously reported as one of the most prevalent organisms involved in the development of YSI.[5-8,23,25]

Other bacteria like *Aeromonas salmonicida, Alcaligenes faecalis, Bacillus* spp., *Chromobacterium* spp. *Citrobacter freundii, Enterobacter cloacae, Klebsiella oxytoca, Klebsiella* spp. *Proteus mirabilis, Providencia retgerii, Pseudomonas fluorescens, Serratia* spp., *Streptococcus* spp., and non-identified yeasts were isolated out of several samples but in a lower proportion than with those described above. In a limited number of reports it has been mentioned that microorganisms other than *E. coli* may cause mortality during the first week.[30] Additionally, it has been reported that *S. aureus* is an important cause of disease in poultry[13,16] and as a common isolate from the yolk sac and liver of broilers and turkeys.[13,32] Moreover, *S. aureus* is principally associated with bone and joints infections, abscesses and dermatitis.[16,32] On the other hand, a study using pulsed-field gel electrophoresis (PFGE) to determine the origin of *S. aureus* strains from several hatcheries and broiler farms, revealed that the patterns of PFGE were related in both places, indicating an association between the presence of the strains in the hatchery and subsequent broiler flock infection.[16] However in our study this relationship could not be established because, despite the large amount of *S. aureus* strains recovered in breeders farm and hatchery, only one strain was found among the 216 examined chicks. *Pseudomonas aeruginosa* has been isolated from the hatchery and from chicks with omphalitis. *Pseudomonas aeruginosa* is mentioned as an opportunistic pathogen that is able to invade fertile eggs, causing death of embryos and creating a localized or systemic disease in newly hatched chicks.[30] However we did not find this

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species but rather we isolated *P. fluorescens*. The ability of *Pseudomonas* spp. to cause infection of yolk sac of a chick is enhanced by its ability to degrade the proteins found in the yolk, creating the opportunity for other bacteria to multiply.[30]

The frequency of isolating *E. coli* from the different samples is summarized in Table 2. *Escherichia coli* was not found in sawdust samples which supports the hypothesis that nest is not the main source of contamination for fertile eggs. Four strains of *E. coli* were isolated from fertile eggs at the broiler breeder farm (4/267; 1.5%), 2 of them were isolated from the internal egg sample, whereas 2 were isolated from eggshell surface. Strains isolated from the eggshell could play an important role in the contamination of the embryo because it is know that pathogenic bacteria present in the faeces of breeders may contaminate the eggshell and penetrate the egg through shell pores.[2] Alternatively, if disinfection is performed inadequately, bacteria could remain in the surface of the egg and infect the chicks when they piped the eggshell at hatching. Sixteen of the 28 strains isolated at 19 days of incubation were obtained from inside the egg and 12 from the eggshell. Many of the strains isolated in the present study were found in the eggshell. This means that they had not crossed the eggshell barrier. A more extensive characterization of our strains is required; in order to establish the relationship between these strains and to look for possible pathogenic markers previously described in *E. coli* strains.[18-20,22,26,31] Information obtained from such work could determine possible mechanisms involved in the pathogenesis of the YSI.

In broilers, one hundred and eighty eight *E. coli* strains were obtained, 102 of these were obtained from the liver and 86 (86/267; 32%) were from the yolk sac. Dzoma reported similar results when they analyzed yolk sac from ostriches samples with 22% of 80 yolk sacs being infected with different bacteria.[8] Generally, many of these same strains were isolated from liver as well as the yolk sac.

A correlation between the chick mortality and the frequency of isolation of *E. coli* was observed in the first week of life (Table 3). The mortality peak* (25%) and *E. coli* isolation (37%) were observed at day 5. Mortality rate declined thereafter until 7 days post hatch which was the last day of our evaluation (Table 3). Our results show a similar pattern with those reported by Mosqueda & Lucio who indicated that the YSI mortality curve lasts 7-10 days, it peaks at 4-5 days and decreases during the following 3 to 5 days.[17] However, as mentioned above, mortality may not be the most important impact in this disease, as economic losses due to subsequent complications and condemnations at the slaughterhouse could seriously impact poultry production.

The results from this study indicate that bacterial contamination resulting in YSI in vertically integrated operations does not necessarily occur in the broiler breeder farm, but can occur at a latter stage. Despite the great diversity of bacteria isolated from yolk sac, *E. coli* appears to have been main cause of YSI. However, the mechanism that this microorganism use to cause the signs and lesions observed in this disease remains unknown. A more detailed study is needed in order to determine the relationship between different strains isolated here and to establish the pathogenic characteristics possessed by those strains. Ultimately, identification of potential virulence traits may allow for their use as specific markers for the diagnosis of pathogenic strains.

Table 2. Frequency of isolation of *E. coli* from different sources.

Sample	*Escherichia coli*	Total (%)
Nest		
Sawdust (Nest)	0	0
Fertile egg		
Eggshell	2	
Inner sampling	2	4 (1.5)
19th day		
Eggshell	12	
Inner sampling	16	28 (10.5)
21st day		
Eggshell	29	
Yolk sac (Embryo)	18	47 (17.6)
Mortality		
Yolk sac	86	
Liver	102	188 (70.4)
Total	267	267

Table 3. Association between mortality and *E. coli* isolation in broilers during the first week of life.

	Number (%)	
Day	Dead chicks	*E. coli*
1	9 (4.17)	5 (2.66)
2	22 (10.19)	1 (0.53)
3	46 (21.30)	21 (11.17)
4	24 (11.11)	20 (10.64)
5	54 (25)	70 (37.23)
6	37 (17.13)	39 (20.74)
7	24 (11.11)	32 (17.02)
Total	216	188

* This percentage was obtained taking the amount of birds dead during week one as 100%.

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