Epidemiological Studies of Congo Red *Escherichia coli* in Broiler Chickens

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ABSTRACT

This prospective cohort study was designed to confirm the association between Congo red binding *Escherichia coli* (CREC) and *E. coli* airsacculitis in commercial broilers. It was also designed to evaluate CREC as an airsacculitis risk factor and to explore the CREC relationship to other airsacculitis risk factors (poultry house temperature, air-ammonia levels, and presence of other diseases). In addition, this study was used to assess a possible role of the broiler-breeder flocks and hatchers in the spread of CREC airsacculitis.

Congo red *E. coli*-associated airsacculitis risk was based on CREC exposure of the chicks in the hatchers. Breeder flocks with greater than 30 CREC colonies/plate from hatcher air sampling tests were placed in the high risk group; flocks with less than five CREC colonies/plate were placed in the low risk group.

Increased risks of death due to airsacculitis (RR = 2.26), and increased death rates due to CREC airsacculitis (RR = 9.45) in high-risk flocks, identified CREC as an important airsacculitis risk factor. The attributable risk percent of CREC airsacculitis from hatcher exposure of CREC was 89.4%, pointing to the hatcher as the source of CREC infection. The association of specific broiler-breeder flocks to high levels of CREC in the hatchers, and subsequent airsacculitis, suggests that the broiler-breeders are the ultimate source of CREC.

RÉSUMÉ

Cette étude prospective avait pour but 1° de vérifier les liens existant entre *Escherichia coli* liant le colorant rouge congo (ECRC) et l'aérosacculite du poulet à griller et 2° d'identifier les facteurs de risque et de les comparer aux autres facteurs associés à la maladie (température, niveaux d'ammoniac, présence de maladies concomitantes). La possibilité de dispersion du ECRC de l'aérosacculite par les troupeaux reproducteurs a aussi été étudiée. L'aérosacculite associée aux ECRC a été étudiée en relation avec l'exposition des poulets dans les incubateurs. Les troupeaux reproducteurs chez lesquels on a retrouvé plus de 30 colonies d'ECRC par milieu de culture à partir de l'air des incubateurs, furent classés dans les groupes à hauts risques, tandis que les troupeaux ayant moins de 5 colonies par milieu de culture furent classés dans les groupes à faibles risques.

L'augmentation de mortalité reliée à l'aérosacculite (RR = 2,26) et l'augmentation des taux de mortalité dans les troupeaux à hauts risques (RR = 9,45) démontrent que l'ECRC serait un facteur à risque important relié à cette maladie. Le pourcentage de risque attribuable à une exposition à l'ECRC dans les incubateurs fut de 89,4 %, indiquant que l'incubateur serait la principale source d'infection. La relation établie entre les troupeaux reproducteurs atteints d'aérosacculite et les hauts niveaux d'ECRC dans les incubateurs démontrent que les troupeaux reproducteurs seraient la principale source de contamination et de propagation de la maladie. (*Traduit par D* Pascal Dubreuil*)

INTRODUCTION

Airsacculitis is a leading source of mortality and a significant cause of carcass condemnation in broilers. Gempesaw and Gulczynski estimated that 26% of all poultry condemnations in the United States were due to airsacculitis (1). Airsacculitis is a respiratory disease of poultry, frequently caused by *Escherichia coli*. It is characterized by thickened, inflamed airsacs with fibrinous exudate, pericarditis, and perihepatitis as sequelae to a colisepticemia (2,3). *Escherichia coli* airsacculitis and septicemia can occur in broilers at any time during their growing period, but is most common between five and seven weeks of age (3). In general, *E. coli* airsacculitis infections are believed to be secondary infections (2,4). However, outbreaks of airsacculitis caused by *E. coli* acting as a primary pathogen have been documented (2,5,6). The epidemiology and pathogenesis of *E. coli* airsacculitis outbreaks have not been well understood, as it has been difficult to differentiate the virulent *E. coli* strains from the relatively nonvirulent secondary invaders (2,7,8).

Berkhoff and Vinal evaluated Congo red dye as a marker for pathogenicity of avian *E. coli* strains. They found that Congo red binding *E. coli* (CREC)

caused septicemia, whereas non-Congo red binding *E. coli* were apathogenic (6). Similar associations between virulence and Congo red binding have been shown for other bacteria (9,10,11).

Because CREC could be readily isolated from hatching chicks, as well as from air sampled from hatcheries, Berkhoff and Vinal hypothesized an association between CREC and the hatcher (6). The warm, humid hatcher provides an ideal environment for proliferation of *E. coli*, which the chicks then inhale. A pilot study by Corbett *et al* gave weight to this hypothesis (12).

The objectives of the present study were to: 1) examine the association of CREC to *E. coli* airsacculitis in commercial broilers, 2) determine the association of CREC with other risk factors, and 3) investigate the role of the breeder flocks and hatchers in the spread of CREC.

MATERIALS AND METHODS

A prospective cohort study was designed to examine the relationship between CREC and broiler airsacculitis mortality and condemnation. Two integrated poultry companies were involved in the study. To select the broiler-breeder flocks for this study, only hatcher units containing eggs from a single broiler-breeder flock were used.

Congo red agar plates were prepared as described previously (6). For hatcher air sampling, the Congo red agar plates were exposed in the hatchers for 3 min by placing an open plate on top of each rack in the hatchers just prior to removing the chicks from the hatchers. The ventilation units of the hatchers were kept running during the sampling period. After 72 h and a two-step incubation procedure (6), the total number of bacterial colonies and the number of CREC colonies were counted and recorded. The CREC colonies were identified by their dark red color and dry, wrinkled appearance. The Congo red-negative *E. coli* colonies were mucoid and pale pink or white (6,12).

In order to qualify for inclusion in this study, hatchers with eggs from one broiler-breeder flock were tested a minimum of three times. This was to determine an accurate CREC shedding rate, since many breeder flocks' shedding rates of CREC were found to vary over time. Extensive field testings have shown that most flocks have a low level of CREC exposure, ranging from zero to ten CREC/hatcher plate. Very few flocks show exposures as high as 30 CREC/plate (unpublished observations). Final broiler/breeder flock selection criteria for our study were: 1) that all Congo red plate readings had consistent numbers of CREC (i.e. < 5, 5 to 30, or > 30); 2) that the number of CREC colonies/plate was either < 5 CREC colonies/plate or > 30 CREC colonies/plate; and 3) that enough eggs were being produced by the broiler-breeder flock to supply sufficient chicks for at least one complete broiler production house. Of the initial 155 breeder flocks surveyed, 79 met the above criteria and were eligible for the study. The flocks were dichotomized to efficiently examine the difference between broiler flocks highly exposed and nonexposed to CREC. The broiler flocks selected for the study population were tested once more at hatching to insure the accuracy of their CREC exposure status.

The final study population was 157,000 commercial broilers from 11 broiler-breeder flocks, involving two poultry companies. Of these, two flocks had high exposure to CREC in the hatcher. The *E. coli* counts were over 30 CREC/plate by hatcher air sampling. Therefore, these two flocks were hypothesized to be at high risk for airsacculitis, condemnation, and mortality. The other nine flocks had counts of less than five CREC/plate in hatcher air-sampling and were hypothesized to be low-risk. Overall mortalities, total airsacculitis mortalities, CREC airsacculitis mortalities, and condemnations at the processing plant were recorded for each flock.

Other risk factors associated with airsacculitis (poultry house temperature, air-ammonia levels, and the presence of other diseases) were assessed during the study. House temperatures were continuously recorded with Tempscribe temperature clocks. Air-ammonia levels were measured twice weekly using a Matheson-Kitagawa ammonia meter. The presence of other diseases were monitored using serology, necropsies, and presence/absence of clinical signs of disease. Every week for five weeks, 25 representative live birds were collected from each flock. The birds were bled, sacrificed, and the airsacs swabbed and cultured for CREC. Their serum antibody titers for infectious bursal disease, infectious bronchitis, and Newcastle disease were determined.

Broiler farms for study flocks were selected based on availability, capacity, and willingness of the farmers to raise test flocks. Only the research team and the upper management of each company were aware of the flocks' CREC exposure status. Each flock was vaccinated and handled in the hatcheries by company standards. Antibiotics other than an anticoccidial agent were not used on any of the flocks under study.

All dead and culled birds were collected each day from each flock. Necropsies were done to assess the presence or absence of airsacculitis. The number dead and the total number of airsacculitis cases were recorded. Airsacculitis was defined as fibrinous pericarditis, fibrinous perihepatitis, or fibrinous airsacculitis. All airsacculitis cases were cultured for CREC (12,13). Primary *E. coli* airsacculitis cases were defined as any airsacculitis case from which CREC was cultured. The total airsacculitis rate included the CREC airsacculitis cases, plus airsacculitis cases of undetermined etiology.

The flocks were marketed through the respective processing plant of each company. The meat inspectors were unaware of each flock's CREC status. Condemnation data of numbers and type of condemnation were collected for each flock.

Broiler mortality rates were the number of dead and culled birds divided by the number of birds placed on each farm. Percent livability was the percentage of birds reaching the processing plant. Total airsacculitis mortality rates were calculated by multiplying the number of airsacculitis cases/birds necropsied by the broiler mortality rates. The CREC airsacculitis rates were calculated by multiplying the number of CREC airsacculitis cases/total airsacculitis cases by the airsacculitis mortality rate. The total condemnation rates and airsacculitis-specific condemnation rates were calculated by dividing the number of birds condemned by the number of

TABLE I. Crude outcomes for each flock

Flock	Risk	Initial pop.	Total[c] death rate	Airsac[c] death rate	CREC airsac[c] death rate	Liveability (%)	Total condemn.[d]	Airsac condemn.[d]
1	low	10,924	23.25	2.30	0.14	97.67	0.97	0.29
2	low	10,040	66.04	6.60	0.88	93.40	1.71	0.49
3	low	19,093	41.90	1.83	0.18	95.81	0.84	0.35
4	low	9,815	62.15	9.27	0.34	93.79	1.08	0.48
5	low	10,511	43.38	2.79	0.40	95.70	2.02	0.68
6[b]	low	13,080	42.20	5.23	2.08	95.78	0.21	0
7[a]	low	13,832	43.67	13.74	0.16	95.63	1.14	0.57
8[a]	low	14,280	92.16	9.19	0.28	90.78	1.43	0.70
9[a]	low	26,119	86.72	19.42	0.92	91.33	1.78	0.61
10[b]	high	17,093	55.75	26.00	6.61	94.42	1.94	0.53
11[b]	high	12,571	53.06	12.34	4.44	94.69	0.34	0.08

[a] Flocks with disease outbreaks other than airsacculitis
[b] Flocks from one company
[c] Death rate per 1000 birds
[d] Condemnation in %

birds reaching the plant. Both rates were expressed as percentages. The variables were compared between the CREC exposure groups using relative risks with their confidence intervals and attributable risk percent (14). Flocks which experienced any other increased risk factor for airsacculitis (including any illness other than airsacculitis) were removed from the analysis and the remaining flocks reanalyzed. The low-risk flocks remaining in the analysis were called the healthy low-risk flocks.

Whole flock analysis was done using nonparametric statistics, the Mann-Whitney two sample test. Analysis by flock assumed that each chick was not independent of the other flock members, i.e. no intraflock exposure variation occurred.

Geometric mean serum antibody titers of both the broiler-breeders and the commercial broilers were compared to the standards of the poultry companies for each serological test. Any serum titers outside the normal ranges were recorded.

RESULTS

Eight low-risk flocks belonged to the first of two integrated companies. One low-risk flock and the two high-risk flocks belonged to the second integrated company. Overall death rate, airsacculitis death rate, airsacculitis death rate due to CREC, total condemnation percent and airsacculitis-specific condemnation percent are shown in Table I. Figure 1 gives the
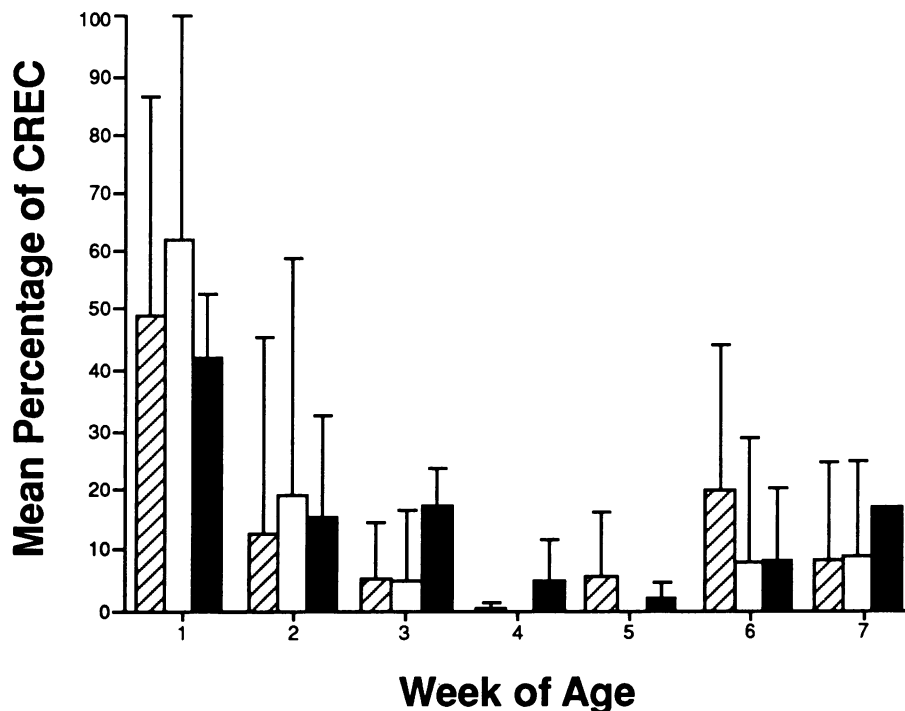

Fig. 1. Average Congo red *Escherichia coli* airsacculitis cases by week. Percentage of total counts by flock (mean and standard deviation). Hatched bars, all low risk flocks; plain bars, all low risk healthy flocks; solid bars, high risk flocks.

▨ All low risk flocks

☐ All low risk, healthy flocks

■ High risk flocks

percent CREC airsacculitis cases by week of life for the high-risk, low-risk, and healthy, low-risk groups.

Relative risks with 95% confidence intervals and attributable risk percents were calculated and are summarized in Table II. For this purpose each chick was assigned the CREC exposure

status of its broiler flock and considered as an independent observation.

Total airsacculitis rates and CREC airsacculitis rates were significantly increased in the high-risk flocks (p > 0.001 for both). Thirty-seven percent of the deaths in the high-risk flocks were due to airsacculitis and 10.4%

222

TABLE II. Relative risks (with confidence intervals) and attributable risk % between high and low CREC exposure flocks

	Relative risks (with 95% confidence intervals)	
	High-exposure flocks vs all low-exposure flocks	High-exposure flocks vs healthy low-exposure flocks
Overall death rate	0.93 (0.88–0.98)	1.20 (1.14–1.27)
Airsacculitis deaths	2.26 (2.05–2.49)	4.41 (3.85–5.06)
CREC airsac deaths	9.45 (7.22–12.38)	8.90 (6.39–12.39)
Total condemnations	1.01 (0.90–1.13)	1.19 (1.05–1.35)
Airsac condemnations	0.71 (0.57–0.88)	0.93 (0.74–1.18)

	Attributable risk % for high CREC exposure flocks	
	High-exposure vs all low-exposure flocks	High-exposure vs healthy low-exposure flocks
Overall death rate	0	16.9
Airsacculitis deaths	55.7	77.3
CREC airsac deaths	89.4	88.7
Total condemnations	0.7	15.7
Airsac condemnations	0	0

were due to CREC airsacculitis. By comparison, the low-risk flocks had 9.4% and 1.5%, respectively.

Three of the low-risk flocks belonging to the first company experienced concurrent outbreaks of other diseases. One flock had clinical signs compatible with infectious bronchitis or infectious coryza, though an etiological diagnosis was not made. Another flock had an outbreak of ascites of unknown etiology. The third flock's illness was characterized by flock inappetence and depression and increased mortality, involving over 10% of the flock for approximately three weeks. These three flocks were kept in the general analysis, but when they were removed, and the healthy, low-risk flocks were compared to the high-risk flocks, the relative risk for death due to airsacculitis increased from 2.26 to 4.41. The attributable risk percent for CREC airsacculitis in the high-risk flocks remained unchanged.

Death rates between the risk groups were not statistically different (p > 0.1). High-risk flocks had significantly higher airsacculitis mortality rates (p 0.017). Neither the total condemnation nor the airsacculitis condemnation rates were significantly different. The same results were seen when the three sick flocks were removed from the analyses.

The broiler-breeder serum antibody titers for all 11 flocks were negative for *Mycoplasma gallisepticum*. The antibody titers for infectious bronchitis, infectious bursal disease, or Newcastle disease virus were all within the goals for healthy breeders of the respective companies. All of the 11 broiler flocks had postvaccination titers within the standards of each company. There was no serological evidence of infectious bronchitis, infectious bursal disease, and Newcastle disease up to the fifth week of age. To prevent excess stress on the flocks, no live birds were collected after five weeks of age.

In clinically normal birds collected from serological testing, CREC was cultured in low numbers from 0 to 8% of the birds. The high-risk flocks remained culture positive for the five weeks. By comparison, the low-risk flocks became culture negative at three weeks.

No other risk factor for airsacculitis (poultry house temperature and air-ammonia levels) was increased. No ammonia readings greater than 25 ppm were recorded. When the house temperature ranges were compared to industry standards, no extremes were recorded (data not shown).

When the eight low-risk flocks from the first company were compared to the one low-risk flock of the second company, large differences were seen in the CREC airsacculitis mortality rates. The integrated poultry company appeared to be a confounder. A confounder is an independent risk factor that is associated with CREC exposure and airsacculitis rates, and it masks the true effect of CREC exposure on airsacculitis rates. The low-risk flock from the company with the two high-

risk flocks also had the higher CREC airsacculitis mortality rates (Table I). The opposite was seen for condemnation rates and airsacculitis condemnation rates. Since only one company had high-risk flocks, potential confounding could not be fully addressed.

DISCUSSION

The results were analyzed by flocks and by chicks. For the by-flock analyses, the Mann-Whitney two sample test was used, since there was potential nonnormal distribution of the data and low numbers (n = 11). Earlier experiments determined that the hatcher air sampling was a good estimator of the level of CREC throughout the hatcher (unpublished data), and we concluded that the flock test was a good estimator of the individual chick's exposure to CREC. Therefore, relative risks with confidence intervals were calculated. The length of exposure for each chick was unknown, and the total CREC exposure for each chick was not known, but, the relative level of CREC was known for the hatcher, and all chicks tended to hatch within 24 h of each other.

A significant difference in the overall mortality rate was seen only between the high-risk flocks and the healthy, low-risk flocks. A relative risk of 1.2 with a 95% confidence interval of 1.14 to 1.27 implies that the impact of CREC exposure, and subsequent airsacculitis is small for overall mortality. A relative risk of 1.2 means the high-risk flocks had a 1.2 times greater risk of dying than the healthy, low-risk flocks. CREC airsacculitis accounted for only 10.4% of the overall mortality in the high-risk flocks. These results are consistent with results of a prior study where a relative risk of 1.6 was seen (12).

The total airsacculitis death rates of high- and low-risk flocks were statistically different in all analyses. When the sick flocks were removed from the analysis, the attributable risk percent for total airsacculitis cases increased from 55.7% to 77.3%. This last figure of attributable risk percent means that 77.3% of the airsacculitis cases can be attributed to CREC exposure in the hatcher. This demonstrates that other illnesses may disguise the effect of CREC in airsacculitis deaths.

The CREC-specific airsacculitis death rates also differed statistically between the high-risk and the low-risk flocks. The attributable risk for high-risk flocks ranged from 88.7% to 89.4%. This implies that almost 90% of the CREC airsacculitis cases in the high-risk flocks could have been prevented if the CREC levels were reduced in the hatchers to those of the low-risk flocks. That would mean about 10% of the CREC cases were from other sources or that the hatcher air test missed a percentage of the CREC in hatchers. The latter hypothesis could be valid since many CREC isolates lose their Congo red dye binding ability on subculture (6). Therefore, some CREC may be detected as Congo red-negative *E. coli* in the hatcher air test. This misclassification would underestimate the real effect of CREC on the broiler flocks.

The CREC status did not strongly affect total condemnation rates. The only significant difference seen was between the high-risk flocks and the healthy low-risk flocks (RR = 1.19). One reason for this was that two different broiler processing plants were used. This could create strong confounding, which could not be assessed in this study.

The only risk factors for airsacculitis, other than CREC, that could be assessed were the illnesses experienced by the low-risk flocks. When the sick flocks were removed from analysis, the relative risk became nonsignificant. Otherwise, the relative risk of airsacculitis condemnations between the low-risk groups and the high-risk groups suggest that CREC may have had a protective effect (RR = 0.71), i.e. that all broilers with airsacculitis died before processing.

As stated earlier, approximately 89% of the CREC-specific airsacculitis cases in the high-risk flocks could be attributed to CREC exposure in the hatchers. Two other results pointed to hatcher exposure of CREC as the source of CREC airsacculitis. These were the airsac cultures of the weekly live-bird samples and the timing of the CREC airsacculitis cases. The CREC were found in low numbers in the airsacs of both low-risk flocks and high-risk flocks. Clinical airsacculitis was not noted among these birds. The high-risk flocks continued to yield low levels

of CREC in their airsac cultures out to the fifth week when flock sampling was halted. In the low-risk flocks, airsac cultures became negative by the third week. Almost 50% of the CREC airsacculitis cases in both the high-risk and the low-risk groups occurred in the first week of life (Fig. 1). The general consensus is that any health problems seen in the first week of life of a chicken is usually traceable to the hatchery and/or the breeder flocks. Since the hatchers are sanitized prior to egg placement and are negative for CREC, the source of the CREC must be the hatching eggs. Egg shells would be contaminated with the *E. coli* bacteria when they pass through the hen's cloaca. By deduction, the source of CREC is probably the broiler-breeder hen and her eggs, and dissemination of the infection occurs in the hatchers (2,5,12).

Other researchers have implicated the role of hatchers in the development of primary *E. coli* airsacculitis (2,5). Experimental studies have shown that CREC airsacculitis can be contracted by one-day-old chicks via aerosol exposure, similar to what the broilers experience in the hatcher (13). Unpublished research has demonstrated the presence of culturable CREC on the outer egg membrane from 20-day-old eggs. The CREC has been cultured from the allantoic fluid of chicks which have "pipped" (broken the shell, but not hatched). These observations help support the hypothesis that the breeder hens are the source of CREC. The broilers exposed to CREC in the hatcher are then at high-risk of succumbing to CREC airsacculitis during their first week of life.

CONCLUSION

The results of this study showed that the level of CREC in the hatcher is a strong predictor of the level of CREC airsacculitis deaths. It strongly suggests that CREC is shed onto the hatching eggs by the breeder hens, with CREC dissemination occurring in the hatcher. In general, CREC exposure does not appear to have a strong effect on the overall death rates and condemnation rates, as CREC is found at a low level even in the high-risk flocks (5.7 cases per 1,000 birds). The presence of other

diseases affects the total airsacculitis mortality rate, but not the CREC airsacculitis mortality rate. Other risk factors for airsacculitis were not found in this study.

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