Epidemiological Study of the Relationship between Congo Red Binding *Escherichia coli* and Avian Colisepticemia

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ABSTRACT

An epidemiological prospective (longitudinal) study design was used to evaluate the association of Congo red positive *Escherichia coli* and avian colisepticemia. High and low risk exposure groups of chickens were identified at hatching, and placed in separate identical houses on the same farm. Approximately 14,000 birds were placed in each house for the seven week grow-out period, during which all birds which died were necropsied and cultured, together with a representative sample of birds which were culled weekly. The findings implicated *Escherichia coli* as the etiological agent of avian colisepticemia. A relative risk of 6.5 and attributable risk of 73.5% supported the hypothesis that the Congo red medium identifies a virulent form of *Escherichia coli* which causes airsacculitis-colisepticemia in poultry.

Key words: Colisepticemia, *Escherichia coli*, Congo red, poultry, airsacculitis.

RÉSUMÉ

Cette expérience consistait en une étude épizootiologique prospective destinée à vérifier la relation entre les souches d'*Escherichia coli* positives au rouge Congo et la speticémie aviaire à colibacilles. Les auteurs identifièrent à cette fin des groupes de poussins fraîchement éclos, à haut et à faible risque, et ils les logèrent dans des poulaillers identiques, sur la même ferme. Environ 14,000 poussins se retrouvèrent dans chaque poulailler, pour la période de sept semaines qui s'écoule entre leur éclosion et leur abattage. Tous ceux qui moururent, ainsi qu'un nombre représentatif de ceux qu'on réforma hebdomadairement, subirent une nécropsie et un examen bactériologique. Les résultats permirent d'incriminer *E. coli* comme l'agent étiologique de la septicémie précitée. Un risque relatif de 6,5% et un risque réel de 73,5% appuyèrent l'hypothèse selon laquelle la gélose au rouge Congo permet d'identifier une variété virulente d'*E. coli* qui cause l'inflammation des sacs aériens et la septicémie aviaire à colibacilles.

Mots clés: septicémie à colibacilles, rouge Congo, volailles, inflammation des sacs aériens.

INTRODUCTION

Recently, avian colisepticemia (syn. colibacillosis, *Escherichia coli* airsac disease) has resurfaced to cause major losses in the poultry industry (1). The losses to the large producer can be significant, especially during the winter months (1). Direct losses to the North Carolina broiler industry from respiratory diseases are estimated to be $26 million annually, of which *E. coli*-associated respiratory/septicemic infection (colisepticemia) has been identified as the major part of this specific loss. In the U.S. annual losses resulting from colisepticemia have been estimated at $100 million (1). This situation can be expected to continue until prevention and control methods for this disease are developed and implemented. Congo red (CR), a simple acid dye, has been used to differentiate invasive pathogens such as *Shigella* and *Yersinia* spp. (2,3,4). The method was used in this laboratory to differentiate systemically invasive from noninvasive *E. coli* (5). Although the nature of Congo red binding is not understood, Berkhoff and Vinal speculated that Congo red becomes bound to bacterial surface components that may be required for, or that are linked to, an undescribed virulence factor of *E. coli* (5).

Pilot studies at our school indicated that the CR dye was identifying virulent *E. coli* in poultry. This study was designed to evaluate whether or not an association exists between *E. coli* strains that bind CR and an airsac-colisepticemia complex in broiler chickens. Insight for possible prevention and/or intervention measures is provided.

MATERIALS AND METHODS

An epidemiological prospective (longitudinal) study design was employed to evaluate the association of CR-positive *E. coli* and avian colisepticemia. High and low risk exposure groups of chickens were identified at hatching. This was achieved by placing culture plates containing selective CR agar medium in the hatcher towards the end of the hatching period. Relative numbers of CR-positive *E. coli* colonies that were isolated on the plates after overnight incubation were used as the determinant for the high and low risk groups (5). The culture medium consisted of 0.03% Congo red and 0.15% bile salts no. 3 (Sigma Chemical Co., St. Louis, Missouri) added to a trypticase soy agar base (BBL, Bioquest, Cockeysville, Maryland). Plates were exposed inside the hatcher for a period of ten minutes. The plates were incubated for 18-24 hours at 37°C and then placed at room temperature (approx. 22°C) for 48 hours to allow accurate identification of the *E. coli* CR phenotype colonies (6).

Multiple hatchers were checked over time with this sampling procedure

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in order to identify hatchers that consistently yielded high or low CR colonies. A low hatcher colony count was five or less and a high was ≥20-30 colonies per plate. Two hatchers were so identified. Thus, eggs entering a hatcher consistently demonstrating ≤5 CR+ colonies per plate became the low risk group on hatching. The hatchlings from the other hatcher (>20-30 CR+ colonies/plate) became the high risk group. Continued sampling of the two hatchers was conducted weekly for an additional eight weeks to verify that counts obtained of CR-positive and CR-negative *E. coli* were consistent and that the results were reproducible with incubating eggs from specific breeder farms.

To avoid erroneous results due to the effect of confounding factors such as management practices and personnel, paired houses on one farm were utilized. In this way, variability between groups could be reduced to a minimum. Low risk and high risk birds were housed in separate identical houses on the same farm. No birds were added to the flocks during the study. Approximately 14,000 birds were placed in each house for the seven week grow-out period. This design was deemed important to minimize differences in vaccination, stress, crowding, dust, ammonia, hygiene, litter, pressure and humidity. Such variable factors have been implicated as having a potential role in the development of colisepticemia (7,8,9,10). In addition, cross contamination between houses was minimized by the wearing of separate protective clothing.

Two sampling techniques were used. First, all birds dying during the seven week grow-out period were necropsied and appropriate samples were taken for bacteriology and serology. The cause of death was determined from gross pathology studies and by the above diagnostic procedures. A diagnosis of airsacculitis-colisepticemia was based on pathognomonic necropsy findings (10). Second, once weekly for seven weeks, a representative sample of clinically healthy birds from each flock was necropsied and CR culture media inoculated. Selection by random sample methods would have been ideal, but this was impossible to accomplish in this type of setting. In this study, the poultry house was divided into seven equal sections and approximately the 700th bird in each section was selected for evaluation (approximately 1% of the birds). Finally, the paired flocks were followed to slaughter, at which time the condemnation rates and body weights were obtained.

A 2 × 2 contingency analysis was used to evaluate whether or not an association existed between disease and CR-positive *E. coli* (11). A double blind study procedure was used in sampling, testing, and culturing phases of this study to prevent potential bias. Data was initially evaluated for simple frequencies and distributions, with mortality rates being generated for "cases" (CR+) and "controls" (CR−). Risk factors such as ammonia, dust, temperature, humidity, and pressure were evaluated at three day intervals and analyzed by appropriate univariate analysis to determine if any difference existed between houses over the course of the study.

RESULTS

During this study involving 14,000 chickens in each group, a total of 222 high risk and 141 low risk birds died. No statistical difference could be found between the houses with respect to the risk factors identified as potential confounding variables (temperature, humidity, ammonia, etc.). Figure 1 shows the number of birds in each group that died in each of the seven weeks. Colisepticemia was diagnosed in 101 birds from the high risk group and 16 birds from the low risk group. Mortality rates for colisepticemia were 7.2 per 1000 for the high risk group and 1.1 per 1000 for the low risk group. For total deaths, the rates were 15.6 per 1000 and 10 per 1000, respectively. Differences in mortality rates were found to be statistically different, with a chi-square analysis [$X^2(1)=60.5$, $P<0.001$-colisepticemia deaths; $X^2(1)=17.86$, $P=0.023$-all deaths].

Since a prospective study design was used, a true relative risk [incidence rate of those with the risk factor (CR+) over the incidence rate of those without the risk factor (CR−)] could be determined (12). A relative risk of 6.5 was found for colisepticemia deaths. This suggests that birds from the high risk group were at 6.5 times greater risk of dying from colisepticemia than those from the low risk group. The relative risk for all deaths was 1.6, which indicates that there was no dramatic clinical difference between the two groups, even though there was a statistical difference.

Weekly random sampling revealed that birds from the high risk group had a 23 times greater chance (relative risk = 23) of having CR-positive *E. coli* cultured than the low risk group

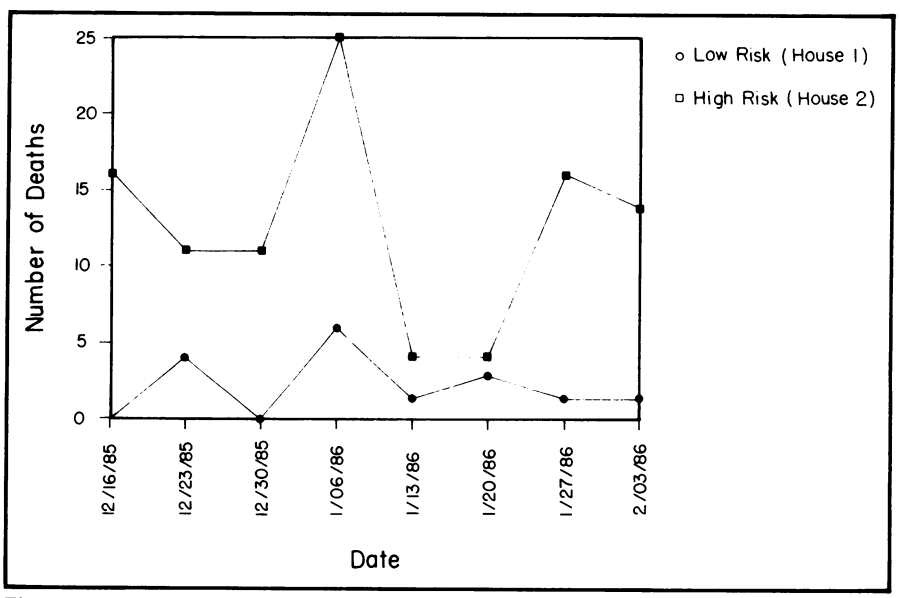

Fig. 1. Weekly report of the total number of deaths as a result of colisepticemia in high and low risk groups of chickens.

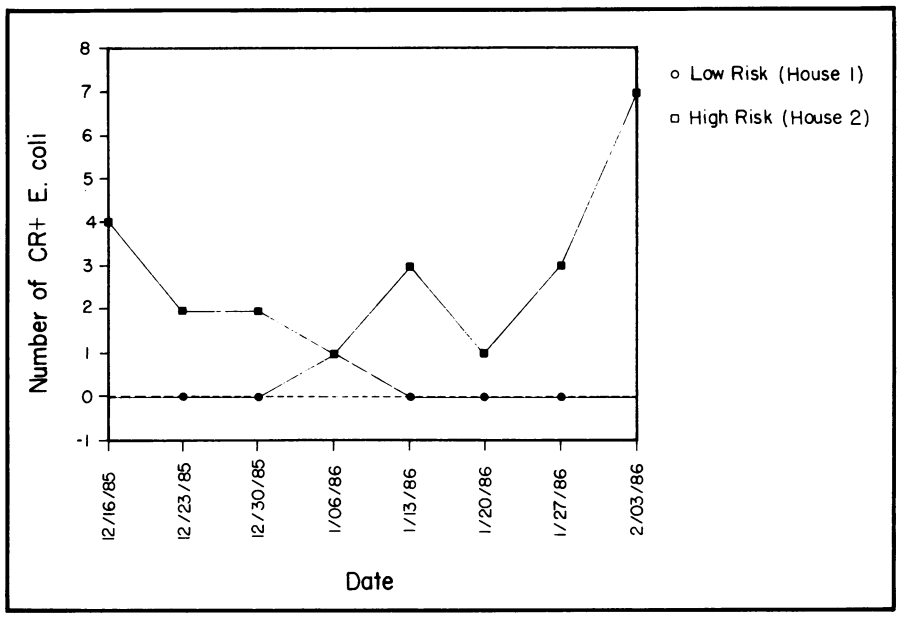

Fig. 2. Weekly random samples of high and low risk groups of chickens.

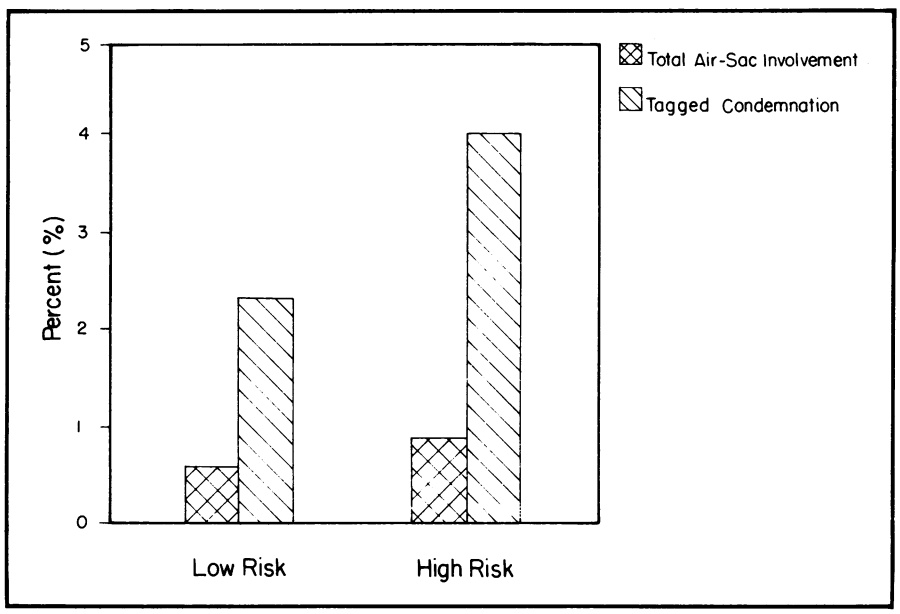

Fig. 3. Percent of the total number of air-sac involvement and tagged condemnation of high and low risk groups of chickens.

(Table I). The absolute numbers of positive cultures by week and group are given in Fig. 2.

In addition, an epidemiological analysis (attributable risk) indicated what proportion of the colisepticemia deaths and total deaths might be due to the presence of CR-positive *E. coli* (12). The attributable risk of CR-positive *E. coli* in colisepticemia was 73.5% and in all deaths it was 22.8%. This implies that there were 86 excess colisepticemia deaths and these can be attributed to the risk factor, CR-positive *E. coli*. Likewise, the attributable risk for all deaths implies that 22.8% of all deaths can be attributed to the presence of CR-positive *E. coli*.

Finally, the difference between tagged condemnation (total condemnation) and those condemned with airsac involvement in the two groups at slaughter is illustrated in Fig. 3. The total condemnation rates (2.33% for low risk versus 4.01% for high risk) were statistically significantly different [$X^2(1)=61.44$, $P<0.001$]. In addition, the birds in the high risk group weighed approximately 1.6% less per bird.

TABLE I. Random Sample of Clinically Healthy Birds for Congo Red-positive *Escherichia coli* Infection

		CR-positive *E. coli*	
Group	Number Tested	Present	Absent
High risk	168	23	145
Low risk	168	1	167

$X^2(1)=19.78$, $P<0.001$ Relative risk = 23

DISCUSSION

During the past decade, much research has been focused on the etiology of colisepticemia. The results have been conflicting or unclear (10). The disease has not been successfully reproduced in the laboratory setting with low numbers of *E. coli* (10). Therefore, it has been suggested that *E. coli* is probably an opportunistic organism and requires predisposing factors in order to produce the classical disease observed in the field. The classical disease shows lesions of fibrinous airsacculitis and fibrinous perihepatitis, with yellow caseous material usually found in and around the airsac and covering the lungs. In chronic cases, synovitis of the hock joints may be seen.

The lack of the ability to specifically identify the virulent *E. coli* strain involved in producing disease has been a major stumbling block to progress in the understanding of this disease. Recently, work by Berkhoff and Vinal has shown that certain avian *E. coli* can bind CR, a simple acid dye, which has provided a tool to better delineate the role of *E. coli* in the development of colisepticemia (5). *Escherichia coli* that were stained with the Congo red dye were hypothesized to be virulent, and we attempted to demonstrate this relationship between colisepticemia and CR-positive *E. coli* with this study. Although all epidemiological studies provide circumstantial evidence of the association of the risk factors involved with disease, the magnitude of the evidence in this study strongly suggests that this association is real. The high relative risk and attributable risk observed do not appear to be spurious. Also, since the effect on overall deaths is not marked, this would infer a focused specificity of the CR-positive *E. coli* with airsacculitis.

The pattern of deaths seen in Fig. 1 mimics what is seen in the poultry industry during a classic airsac disease epizootic; that is, an increased number of deaths at three to four weeks, with a second peak at six to seven weeks (1,10). During this study, weather conditions were such that ideal conditions for proper ventilation of poultry houses and higher than average temperatures provided better health conditions, as compared to the same season in previous years. These were field conditions conducive for the growth of viable poultry, but not for demonstrating dramatic differences between the high and low risk groups in this study. Yet, in these less than ideal experimental field conditions, marked differences were still noted. If one considers the birds carrying CR-positive *E. coli* uncovered in the random sample of clinically normal birds to be potentially latent or incubating infection, the second peak would have been much more dramatic, especially if cold and wet weather conditions had occurred. Speculating, one could envision that birds harboring detectable CR-positive *E. coli* in the random sampling might have died, if poor weather conditions had been encountered (our prediction of mortality of 13.6% or 23/168 for the high risk group). If this assumption was true, the potential was present for over 2000 chickens to have died from colisepticemia. Local poultry industries have records showing deaths due to colisepticemia to have comparable high levels in severe outbreaks (1).

The body weight differences obtained from slaughterhouse data must be viewed with care. Even though there was a 1.6% difference per bird between the groups, the difference cannot be attributed only to CR-positive *E. coli*. This is supported by the finding that the overall causes of death did not differ greatly. Thus, subclinical disease from the other etiologies must be considered, and needs further study.

Among the questions raised by the study, the first one to be addressed is, "Where is the source of the CR-positive *E. coli*?" In past epidemiological studies, it has been suggested that colisepticemia may be related in some way to the hatchery (13,14). Our preliminary studies showed that CR-positive *E. coli* could not be cultured from the hatchers before eggs were placed in them or during early stages of incubation. In fact, positive cultures were not obtainable until about one-half to two-thirds of the eggs had hatched. This seems to indicate that the organism may have survived on or in the shells and that, after hatching, the chicks may have become "living incubators" for the CR-positive *E. coli*. If true, the source or "reservoir of infection" would logically be the breeder flocks and the actual infection occurred in the hatchery. Further studies are need to clarify this issue.

Although these results provide only circumstantial evidence implicating CR-positive *E. coli* as the etiological agent for colisepticemia, it is strong epidemiological evidence. A relative risk of 6.5 and an attributable risk of 73.5% support our hypothesis that the CR medium may in fact be identifying a virulent form of *E. coli* causing airsacculitis/colisepticemia. This should be verified with additional studies. In addition, inoculating CR-positive *E. coli* and CR-negative *E. coli* strains into pathogen-free (SPF) chickens by aerosol is needed, in order to more clearly and precisely delineate this association.

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