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# Occurrence of weak mutators among avian pathogenic *Escherichia coli* (APEC) isolates causing salpingitis and peritonitis in broiler breeders

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ABSTRACT

A collection of 46 avian pathogenic *Escherichia coli* (APEC) isolates was examined for the presence of mutators by determining the rate of mutation to rifampicin resistance. The collection included 34 *E. coli* isolates obtained in pure culture from chronic lesions of salpingitis and peritonitis in 34 broiler breeders, of which 12 were associated with the development of secondary septicemia. Twelve additional isolates were obtained from a clonal outbreak (ST95) of *E. coli* peritonitis syndrome (EPS), the lesions of which changed gradually over time into a subacute/chronic form. The hypothesis of the present study was that mutation rates would be higher for chronic infection isolates than for isolates from acute infections/exacerbations. The distribution of mutation rates followed a pattern similar to that found for other clinical isolates of *E. coli*, with a modal/median value of $1.47 \times 10^{-8}$. Of the 46 isolates, 24% ($n = 11$) were weakly hypermutable ($2.00 \times 10^{-8} \leq \mu < 2.00 \times 10^{-7}$), however, no strong mutators were detected ($\mu \geq 2.00 \times 10^{-7}$). Chronic salpingitis isolates had the highest proportion (45%, $P = 0.001$) of weak mutators and also, significantly higher mutation rates ($P = 0.003$) compared to isolates that caused septicemia (4%). In addition, mutation rates were significantly lower among ST95 isolates ($P < 0.0005$), and among isolates from the same clonal group as ST95 ($P = 0.027$), when compared to isolates from other groups. Although a clear association with the time phase of infection (as lesions of EPS became more chronic) could not be observed ($\rho = 0.523$, $P = 0.081$), a higher frequency of weak mutators among chronic infection isolates suggests that increased mutation rates play a role in adaptation of APEC to long-term persistence in an infected host environment.



## 1. Introduction

Constitutive mutators have been reported in natural populations of *Escherichia coli* in all phylogenetic groups, including commensal and pathogenic strains (LeClerc et al., 1996; Matic et al., 1997; Denamur et al., 2002; Baquero et al., 2004). However, an increased mutation rate seems to be beneficial when there is a need for adaptation to certain environments. For example, uropathogenic *E. coli* (UPEC) were demonstrated to have the highest frequency of mutators and also, significantly higher mutation rates compared to commensal and bacteremia isolates (including urosepsis isolates) (Denamur et al., 2002). In addition, *in vitro* and *in vivo* studies using the UPEC strain CFT073 and its mutator mutant for the mismatch repair (MMR) gene *mutS* showed that this mutator phenotype was

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selected in urine and in the late (chronic) stages of infection in a mouse model of urinary tract infection (UTI) (Labat et al., 2005). These observations suggested that the frequency of *E. coli* mutator strains vary considerably among different ecotypes (Denamur et al., 2002), and that high mutation rates may confer an advantage in the pathogenesis of chronic *E. coli* infections (Labat et al., 2005). Indeed, chronic infections provide more time and, thus, more opportunities for relevant adaptive changes to occur. In this situation, the mutator phenotype is presumed to be advantageous because it will generate the 'pathoadaptive' mutations (Sokurenko et al., 1999) needed to cope with the host's immune system and the highly stressful environment encountered during prolonged infection (*e.g.* pus, blood, urine, oxidative burst, antimicrobial drugs) (Labat et al., 2005). As a result of pathoadaptive diversification and selection, chronic infection isolates derived from an initial acute infecting strain may present different colony morphotypes (*e.g.* mucoid, small colony variants), different phenotypes of susceptibility to antibiotics (Foweraker et al., 2005) and different mutation rates (Martínez-Solano et al., 2008; Turrientes et al., 2010).

Although the presence of mutators has been widely studied in *E. coli* isolates of human origin, their frequency in *E. coli* isolates that infect chickens has not been documented. Avian pathogenic *E. coli* (APEC) is the main cause of reproductive tract infections (salpingitis) in layers and broiler breeders (Jordan et al., 2005). These infections are caused by multiple and endemic APEC lineages (Pires-dos-Santos et al., 2013), presumably acquired independently by each chicken from the intestinal reservoir (Ewers et al., 2009). Chickens with salpingitis are likely to be infected early in their reproductive life, with most salpingitis infections evolving toward chronicity (Jordan et al., 2005); however, acute septicemia may complicate up to 28% (19/68) of the cases of chronic salpingitis (Pires-dos-Santos et al., 2013). We have recently reported that one particular clonal group defined by multilocus sequence typing (MLST), which we designated APEC 1 (ST95, ST420, ST117, ST135, ST428, ST429, ST131, ST352 and ST2024), was characterized by extensive virulence profiles and caused the majority of salpingitis infections in 68 broiler breeders from multiple farms in Denmark (Pires-dos-Santos et al., 2013). Members of this clonal group were significantly more likely to induce secondary septicemia, when compared to other isolates that caused chronic salpingitis (Pires-dos-Santos et al., 2013).

The objective of this study was to analyze the distribution of mutation rates in a collection of APEC isolates, the hypothesis being that the mutation rates of APEC isolates obtained from chronic infections are higher than those of isolates associated with acute infections/exacerbations. To test this hypothesis, the mutation rates of *E. coli* isolated from chronic salpingitis (with no septicemia involved) were compared to those of *E. coli* from chronic salpingitis accompanied by secondary septicemia. In addition, we analyzed the mutation rates of isolates sampled from a clonal outbreak of *E. coli* peritonitis syndrome (EPS), which evolved toward chronicity throughout a period of 16 weeks.

## 2. Materials and methods

### 2.1. E. coli isolate collection

The collection investigated included 34 *E. coli* isolates obtained from chronic salpingitis and peritonitis in 34 broiler breeders, 12 of which were accompanied by secondary septicemia. The remaining 22 only demonstrated chronic salpingitis and peritonitis. The 34 broiler breeders originated from eight different farms. The selected isolates belonged to multilocus sequence type (ST) 95 ($n = 9$), STs from the same MLST-based clonal group as ST95 (APEC 1) (ST117, ST131, ST420, ST428, ST2024, ST352, and ST429) ($n = 15$), and to STs of other clonal groups (non-APEC 1) (ST23, ST57, ST770, ST648, ST88, and ST93) ($n = 10$) (Pires-dos-Santos et al., 2013). Twelve additional isolates obtained from the longitudinal sampling of a clonal outbreak of EPS (ST95) in 2003 were included for comparison, as they represented isolates from both acute and chronic lesions. These isolates represented broiler breeders of the age of 25 ($n = 4$), 26 ($n = 2$), 30 ($n = 2$), 35 ($n = 2$), 40 ($n = 1$) and 41 ($n = 1$) weeks. The lesions were initially acute (age of 25 and 26 weeks) but changed gradually to subacute or chronic (and more productive) forms during the remaining weeks. Isolates of ST95 from the longitudinal study and epidemiologically unrelated single cases of salpingitis, shared similar (>85%) pulsed-field gel electrophoresis (PFGE) profiles during a period of seven years (unpublished data). A detailed list of the isolates analyzed is given as supplementary information in Table S1.

### 2.2. Determination of mutation rates

Mutation rates were determined using a standard fluctuation assay following an extended Jones protocol (Crane et al., 1996) that uses a maximum likelihood method (Gerrish, 2008). This method was chosen because it is well suited for all ranges of $m$ values ($m$ = mutations per culture) (Rosche and Foster, 2000). Briefly, *E. coli* isolates were cultured in 10 ml of Luria Bertani (LB) broth (pH = 7.4) (Oxoid, Basingstoke, UK) to an $OD_{600\ nm}$ equivalent to 1.5 and subsequently diluted to an $OD_{600\ nm} \sim 0.1$ in fresh LB broth. A 1:10 diluted fraction was passed through a fine needle syringe (25G) to ensure that the organisms did not aggregate. For each isolate, 2.5 μl of this suspension was inoculated into 250 ml of fresh LB, thoroughly mixed, and distributed into 24 parallel cultures of 10 ml (~1000 cfu per culture). The 24 cultures were incubated overnight and grown to saturation under aerobic conditions during 20 h, at 37 °C with agitation (125 rpm). After incubation, 100 μl from each culture were spread on 24 LB agar plates containing rifampicin (100 μg/ml) (Sigma–Aldrich), which were subsequently incubated for 24 h at 37 °C under aerobic conditions. A Miles and Misra plate count (Miles and Misra, 1938) was performed for five of the 24 cultures from each isolate, and each dilution was spotted in triplicate 10-μl drops onto blood agar plates to obtain the final number of cells in the cultures ($N_t$). The most likely number of mutations per culture ($m$) that gave rise to the distribution of mutants observed, was estimated using a recursive formula for obtaining markedly improved