Investigation of *in vitro* conditions required for biofilm formation in *Escherichia coli* isolated from mare reproductive tract

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Biofilm formation has been suggested to be important for chronic bacterial endometritis of mares. The crystal violet (CV) assay is used commonly in human medicine to evaluate the biofilm forming potential of bacterial isolates. The CV assay evaluates the early steps in biofilm formation, i.e. bacterial attachment and extracellular polymatrix (EPS) production. The CV dye binds to both cells and EPS, and it is a measure of total biofilm biomass formed. However the CV assay is not standardized across biomedical disciplines, as published methodologies vary considerably among laboratories. The aim of this project was to evaluate the effects of two incubation conditions (time and orbiting) on the biofilm forming potential of *Escherichia coli* (*E. coli*) isolates from the mare reproductive tract. We hypothesized that different methods of incubation affect biofilm development. The biofilm forming potential of 101 *E. coli* (49 clitoral fossa and 51 uterine isolates) was assessed under differing incubation conditions using the CV assay: orbiting for 24 h (O-24), non-orbiting for 24 h (NO-24) and orbiting for 48 h (O-48) at 37°C. Aside from the specific conditions being tested, all isolates were tested with the same CV assay protocol. Isolates with an optical density (OD) of greater than 0.4 were considered strongly adherent (a strong biofilm forming isolate). Additionally, 25 strong biofilm forming *E. coli* isolates were evaluated at 4 h intervals under orbiting incubation at 37°C for biofilm formation using the CV assay. The mean number of isolates and OD of the different incubation conditions and times were evaluated using t-test. Significance was set at P<0.05. The number of strong biofilm forming isolates for O-24, NO-24 and O-48 h, were 30, 28 and 27, respectively (*n*=101), with no significance between treatments. Similarly, there was no significant difference between the mean ± SEM OD of strong biofilm forming isolates at the end of each incubation period (1.54 ± 0.12, 1.51 ± 0.14, 1.28±0.11 for O-24, NO-24 and O-48 h, respectively). The mean ± SEM OD at 4, 8, 12, 16, 20 and 24 h were 1.09 ± 0.11, 1.57 ± 0.12, 1.77 ± 0.13, 1.83 ± 0.13, 1.89 ± 0.13 and 1.76 ± 0.13, respectively. Although the OD for the 4 h incubation time was significantly lower than any group, all incubation periods resulted in OD > 0.4 (strong biofilm formation). Our results show some *E. coli* isolates collected from the endometrium and clitoral fossa demonstrate strong biofilm forming potential when tested *in vitro*, and formation occurs rapidly and repeatedly under variable incubation conditions.

**Keywords:** Equine endometritis, bacterial biofilm, crystal violet assay