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Effect of serogroup, surface material and disinfectant on biofilm formation by avian pathogenic *Escherichia coli*

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) are responsible for significant economic losses in the poultry industry and are difficult to eradicate. Biofilm formation by APEC has the potential to reduce the efficacy of cleaning and disinfection. In this study, biofilm formation on materials used in poultry facilities by APEC strains from laying hens was determined. APEC strains were analysed for an association between biofilm forming capacity and O serogroup. The abilities of two routinely used disinfectants, hydrogen peroxide (H_2O_2) and a quaternary ammonium compound (QAC), to kill adherent cells of two strong APEC biofilm producers (05/503 and 04/40) and a non-biofilm producer (05/293) on polystyrene (PS) and polyvinylchloride (PVC) surfaces were tested. Most APEC strains were moderate (PS) or strong biofilm producers (polypropylene, PP, and PVC). Strains in serogroup O2 more often belonged to the moderate (PS) or strong (PP and PVC) biofilm producers than to other groups, while most O78 strains were weak biofilm producers. O78 strains were stronger biofilm producers on stainless steel than on PP and PVC, while O2 strains were stronger biofilm producers on PP and PVC. A concentration of 1% H_2O_2 killed all adherent bacteria of strains 05/503 and 04/40 on PP and PVC, while 0.5% H_2O_2 killed all adherent bacteria of strain 05/293. QAC at a concentration of 0.01% killed all adherent cells of strains 05/503, 04/40 and 05/293 under equal conditions. In conclusion, biofilm formation by APEC was affected by serogroup and surface material, and inactivation of APEC was dependent on the disinfectant and surface material.

Introduction

Avian pathogenic *Escherichia coli* (APEC) cause significant economic losses in the poultry industry and are difficult to eradicate, possibly due to their ability to form biofilms and their associated resistance to disinfectants. They form an important hindrance during cleaning and disinfection. In all-in all-out systems, cleaning and disinfection are normally performed between different batches of birds to reduce the levels of pathogens (Moustafa Gehan et al., 2009). However, little is known about the capacity of APEC to form biofilms on different materials used in poultry facilities.

Biofilm formation by APEC is dependent on many factors, such as growth medium and phylogenetic group; for example, nutrient deficient media often induce biofilm formation (Skyberg et al., 2007). Several non-pathogenic and intestinal pathogenic *E. coli* strains have been analysed for biofilm formation on different surface structures (Pratt and Kolter, 1998; Vidal et al., 1998; Prigent-Combaret et al., 2000; Ryu et al., 2004; Ryu and Beuchat, 2005). The efficacy of routinely used disinfectants to eradicate APEC biofilms has not been studied in detail, although it is known that formation of a biofilm can reduce the efficacy of disinfectants against *E. coli* (Somers et al., 1994; Ntsama-Essomba et al., 1997; Ryu and Beuchat, 2005).

In the present study, we analysed the ability of APEC strains to form biofilms on materials used in poultry facilities. Two disinfectants routinely used in poultry facilities, hydrogen peroxide (H_2O_2) and a quaternary ammonium compound (QAC), were tested for their ability to kill bacteria in biofilms.

Materials and methods

Bacterial strains, media and growth conditions

APEC strains ($n = 93$) isolated from the caecum, liver, heart/pericardium, trachea, peritoneum, oviduct, down, faeces or bedding of caged layer hens with colibacillosis were collected from 49 farms throughout Belgium from 2000 to 2005. The APEC strains belonged to serogroups O1 ($n = 7$), O2 ($n = 30$) and O78 ($n = 56$) (Oosterik et al., 2014).

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Disinfectants

Two active ingredients often included in routinely used commercial disinfectants in the poultry industry were selected: (1) 50% benzalkonium chloride (alkyl dimethyl benzyl ammonium chloride; Sigma-Aldrich), a QAC; and (2) H_2O_2 at 42.1% W/V in H_2O (EcoClearProx, ABT).

Biofilm formation

Polystyrene, polypropylene and polyvinylchloride plates – A protocol was modified from previous studies (O'Toole et al., 1999; Stepanović et al., 2004; Merritt et al., 2005; Skyberg et al., 2007). APEC strains were grown in lysogeny broth (LB) medium for ~16 h in a shaking incubator (Innova 4200, New Brunswick Scientific; 230 rotations per minute); thereafter, cultures were diluted 1:100 (V/V) in nutrient deficient media, i.e. 1/20 diluted tryptone soya broth (TSB; CM0129, Oxoid) in distilled water. The diluted cultures (200 µL) were seeded into wells of sterile 96-well polystyrene (PS; BD Biosciences), polypropylene (PP; VWR International) and polyvinylchloride (PVC; Corning) microtitre plates. PP and PVC are used for flooring and water distribution systems/automatic drinkers in poultry facilities.

APEC strains were tested in triplicate on three different plates; to prevent contamination and evaporation, the wells on the outside of the plate were not used. APEC strains 05/503 (O78; PS), 05/39 (O2; PP) and 04/40 (O1; PVC) were strong biofilm producers, while APEC strain 05/293 (O78) was a non-biofilm producer. These strains were added in triplicate to every plate as positive and negative controls, respectively. The plates were incubated for 24 h at 25 °C, since growth at this temperature generated stronger biofilm formation than 20 °C, 30 °C and 37 °C (data not shown).

Stainless steel

Seventeen of the 93 APEC strains were tested for biofilm formation on stainless steel (type 316; Metaleuven), a material used in water distribution systems. The 17 APEC strains belonged to serogroups O1 ($n = 2$), O2 ($n = 6$) and O78 ($n = 9$). Stainless steel coupons (SSCs) with a diameter of 10 mm were obtained by notching a stainless steel sheet (thickness 3 mm). The SSCs were cleaned, disinfected and autoclaved. A SSC was placed in each well of a 24-well tissue culture plate (VWR International) and 500 µL of the 1:100 diluted cultures in nutrient deficient media were added in triplicate to the wells and incubated for 24 h at 25 °C. The strains were tested on three different plates. Positive control strain 05/503 and negative control strain 05/293 were added in triplicate to every plate.

Quantification of biofilm formation

Biofilm formation was measured after incubation of the diluted cultures for 24 h in diluted TSB. First, the plates/SSCs were washed three times with sterile phosphate buffered saline (PBS) and air dried, then the biofilms were heat fixed at 60 °C for 60 min. The biofilms were stained with crystal violet (0.1% in distilled water; 200 µL per well) at room temperature (RT) for 30 min. Next, the plates were washed three times under running distilled water (washing of SSCs was performed in three separate Falcon tubes containing distilled water). The plates/SSCs were air dried and a mixture of 80:20 ethanol:acetone (plates: 200 µL; SSC: 500 µL) was added to each microtitre plate well for destaining and incubated for 20 min at RT. Part of the solution (150 µL) was transferred to a new microtitre plate and the optical density (OD) was measured at 540 nm (OD_{540}) with a microtitre plate reader (Victor^2 1420-012, Perkin Elmer).

The strains were classified according to their capacity to produce a biofilm using a method adapted from Stepanović et al. (2004). The average OD_{540} of the negative control strain (05/293) plus three times the standard deviation (SD; two times the SD for SSC) from the mean was calculated for the three replicates per plate to derive the cut-off OD (ODc) per plate. The average OD_{540} of the strains for the three replicates per plate was calculated and the following scheme was used: (1) non-biofilm producer: ODc > OD; (2) weak biofilm producer: 2× ODc > OD > ODc; (3) moderate biofilm producer: 4× ODc > OD > 2× ODc; and (4) strong biofilm producer: OD > 4× ODc. Three biofilm capacities per strain were obtained and the most frequent capacity was selected.

Biofilm cell killing with H_2O_2 and quaternary ammonium compounds

APEC strains 05/503, 04/40 and 05/293 were grown for 24 h in 12 wells/strain for each plate type (PS 200 µL, PVC 150 µL per well). The plates were washed three times with sterile PBS (PS 220 µL, PVC 200 µL per well). Two-fold serial dilutions of H_2O_2 (final concentrations of 2%, 1% and 0.5%) and QAC (final concentrations of 0.02%, 0.01% and 0.005%) in saline, as well as plain saline, were added to each well in triplicate (PS 200 µL, PVC 150 µL per well) and incubated for 30 min at RT. Each plate was washed with sterile PBS (PS 220 µL, PVC 200 µL per well), then sterile PBS was added to each well (PS 200 µL, PVC 150 µL per well) and the plates were covered with a sterile film. The plates were sonicated at 47 kHz for 3 min in a water bath sonicator (Branson 2210, VWR International), the PBS was transferred to Eppendorf tubes and 10-fold dilutions were plated in duplicate on LB agar. The plates were incubated for 24 h, then bacterial numbers, expressed as colony forming units (cfu)/well, were determined.

Confirmation of the biofilm matrix

To confirm the results of crystal violet staining and quantification of cells in an untreated 24 h biofilm, cells adhering to PS were stained with wheat germ agglutinin-Alexa fluor 488 (WGA) according to the method described by Burton et al. (2007). APEC strains 05/503 and 04/40 (strong biofilm producers) and 05/293 (non-biofilm producer) were first grown for 24 h in a PS microtitre plate. All strains were tested 18-fold, at six-fold per plate repeated over three different plates. Plain 1/20 diluted TSB was used to derive the background value. Plates were washed twice with sterile PBS and adherent cells were stained with 200 µL WGA (5 µg/mL; VWR International), then washed and destained as described by Burton et al. (2007). Emission of WGA was measured at 535 nm after excitation at 485 nm using a multilabel microtitre plate reader (Victor^2 1420-012, PerkinElmer). Average fluorescent readings per plate were corrected for the average background value (1/20 diluted TSB) per plate.

Statistical analysis

The OD_{540} values of the positive and negative control strains on crystal violet staining were analysed for a normal distribution with Analyse-it Software in Microsoft Excel 2010. The WGA fluorescent measurements per strain, and cell numbers in biofilms before and after treatment with H_2O_2 and QAC, were analysed for significant differences between multiple groups with the Kruskal–Wallis test and between each pair of groups with the Wilcoxon rank sum test. The χ^2 test was used to analyse significant differences between the biofilm-forming capacities of strains within and between serogroups (O1, O2 or O78). $P \leq 0.05$ was considered to be significant.

Results

Effect of surface materials on biofilm formation

APEC strains ($n = 93$) were tested for their biofilm-forming capacity, determined by crystal violet staining, on PS, PP and PVC in nutrient deficient medium (Table 1). OD_{540} values of the control strains (05/503 and 05/293) had normal distributions for each material tested.

On PS, 6/93 (6.5%) strains were non-biofilm producers, 28/93 (30.1%) strains were weak biofilm producers, 42/93 (45.2%) strains were moderate biofilm producers and 17/93 (18.3%) strains were strong biofilm producers. Strains from serogroups O2 belonged significantly more often to the moderate biofilm producers than to the non-, weak or strong biofilm producers ($P < 0.05$), while O78 strains were significantly more often classified as weak or moderate biofilm producers (39/56, 69.6%) than as non- or strong biofilm producers (17/56, 30.4%).

On PP, 13/93 (14.0%) strains were non-biofilm producers, 23/93 (24.7%) strains were weak biofilm producers, 18/93 (19.3%) strains were moderate biofilm producers and 39/93 (41.9%) strains were strong biofilm producers. Six out of seven (85.7%) O1 strains were strong biofilm producers. Serogroup O2 strains were significantly

Table 1
Numbers and percentages of avian pathogenic *Escherichia coli* (APEC) isolates from different serogroups in relation to their capacity to form biofilms on various materials.

Serogroup (number of isolates)	Material[a]	Biofilm producer (%)			
		Non-producing	Weak	Moderate	Strong
O1 (7)	PS	14.3	14.3	42.9	28.6
	PP	0.0	14.3	0.0	85.7
	PVC	14.3	0.0	0.0	85.7
O2 (30)	PS	0.0^{A}	23.3^{A}	63.3^{B}	13.3^{A}
	PP	0.0^{A}	6.7^{A}	23.3^{A}	70.0^{B}
	PVC	0.0^{A}	10.0^{A}	23.3^{A}	66.7^{B}
O78 (56)	PS	10.7^{A}	35.7^{B}	33.9^{AB}	19.6^{AB}
	PP	25.0	35.7	17.9	21.4
	PVC	14.3	37.5	21.4	26.8
Total (93)	PS	6.5^{A}	30.1^{BC}	45.2^{C}	18.3^{AB}
	PP	14.0^{A}	24.7^{AB}	19.3^{A}	41.9^{B}
	PVC	8.6^{A}	25.8^{B}	21.5^{AB}	44.1^{C}

Different capital letters show significant differences within the row ($P < 0.05$).
[a] PS, polystyrene; PP, polypropylene; PVC, polyvinylchloride.