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Clinically important *E. coli* strains can persist, and retain their pathogenicity, on environmental plastic and fabric waste☆

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ABSTRACT

Plastic waste is ubiquitous in the environment and there are increasing reports of such waste being colonised by human pathogens. However, the ability of pathogens to persist on plastics for long periods, and the risk that they pose to human health, is unknown. Here, under simulated environmental conditions, we aimed to determine if pathogenic bacteria can retain their virulence following a prolonged period on plastic. Using antibiotic selection and luciferase expression for quantification, we show that clinically important strains of *E. coli* can survive on plastic for at least 28-days. Importantly, these pathogens also retained their virulence (determined by using a *Galleria mellonella* model as a surrogate for human infection) and in some cases, had enhanced virulence following their recovery from the plastisphere. This indicates that plastics in the environment can act as reservoirs for human pathogens and could facilitate their persistence for extended periods of time. Most importantly human pathogens in the plastisphere are capable of retaining their pathogenicity. Pathogens colonising environmental plastic waste therefore pose a heightened public health risk, particularly in areas where people are exposed to pollution.

1. Introduction

Hospital wastewater effluents are known to harbour pathogens, including those with antimicrobial resistance (AMR) (Rodriguez-Mozaz et al., 2015; Zhang et al., 2020). Wastewater treatment plants (WWTPs) process a wide range of domestic, clinical, and industrial effluents prior to their discharge to receiving waters (Al-Aukidy et al., 2018). A proportion of wastewater can bypass treatment if diverted through combined sewer overflows (CSOs); the direct discharge of untreated effluent to receiving waters via CSOs has attracted global attention (Sojobi and Zayed, 2022) due to the impacts on water quality, which is particularly amplified when discharged during low flow conditions (Whelan et al., 2022).

Discharge of untreated hospital effluent to receiving waters has the potential to deliver multiple drug resistant human pathogens of significant public health concern into the environment, and increase the probability of bacterial infections that are difficult, or sometimes impossible, to treat (WHO, 2020). Untreated discharge from WWTPs can also include sewage-associated plastics such as disposable wet wipes, cotton bud sticks, and sanitary products (Mourgkogiannis et al., 2018; Okoffo et al., 2019) colonised by faecal bacteria and potential human pathogens (Keswani et al., 2016; Metcalf et al., 2022a). Distinct microbial populations colonise environmental plastic debris in what is collectively known as the 'plastisphere' (Zettler et al., 2013); such communities are highly variable, diverse, and genetically distinct from the free-living communities in environmental matrices, implying that plastic provides a novel environmental niche for colonisation (Kirstein et al., 2019; Wu et al., 2020; Wang et al., 2021). Plastic waste colonised by bacterial pathogens is being increasingly found on public beaches and other environments where there is a high likelihood for human exposure (Keswani et al., 2016; Metcalf et al., 2022b).

A group of pathogens found with increasing frequency in WWTP and hospital effluents, are diarrheagenic *E. coli* (DEC) (Gomes et al., 2016). DEC are a primary source of diarrhoeal disease, which is the second leading cause of deaths in infants under five years old and responsible annually for over 1.53 million deaths (Abbafati et al., 2020; IHME, 2020). Outbreaks of diarrhoeal disease are a persistent global problem particularly in areas with limited sanitation and poor hygiene, with almost 90% of diarrhoeal deaths occurring in south Asia and sub-Saharan Africa (Naghavi et al., 2017; Troeger et al., 2017). In the

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UK, the number of deaths linked to diarrhoeal diseases increased from 120 to 1418 between 1990 and 2019; and from 682 to 11,726 in the USA during the same period (Dadonaite et al., 2018).

Conventionally, DEC are characterised into eight pathotypes, including enterohaemorrhagic *E. coli* (EHEC); enterotoxigenic *E. coli* (ETEC); enteropathogenic *E. coli* (EPEC); adherent-invasive *E. coli* (AIEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAEC); diffusely adherent *E. coli* (DAEC); and cell-detaching *E. coli* (CDEC) (Kaper et al., 2004; Pawłowska and Sobieszczańska, 2017). Such enteric *E. coli* can also cause infections at extraintestinal sites including uropathogenic *E. coli* (UPEC), which is a major cause of community-acquired urinary tract infection (Terlizzi et al., 2017). Enteric *E. coli* are predominantly transmitted via the faecal-oral route, often through consumption of contaminated food sources or ingestion of contaminated water, and through direct person-to-person contact (Berger et al., 2010). Importantly however, pathogenic *E. coli* can also persist in the environment, for example, ETEC are often found in soil, manure, and freshwater, and via environmental contamination can persist on the leaves and roots of plants (Gonzales-Siles and Sjöling, 2016). UPEC can survive sewage treatment and have been found in water downstream of WWTPs (Anastasi et al., 2010; Anastasi et al., 2012), whilst antibiotic resistant EAEC, EHEC, EIEC, EPEC and ETEC have all been recovered from surface water (Bolukaoto et al., 2021).

During the last decade research on the plastisphere has increased considerably and advanced our understanding of the role of plastisphere communities as a potential reservoir for human disease (Keswani et al., 2016; Rodrigues et al., 2019; Laverty et al., 2020; Galafassi et al., 2021). Recently however, it has been highlighted that out of 111 plastisphere studies, only 12 had identified potential pathogens, and only three had confirmed the presence of virulence-related genes (Beloe et al., 2022). Studies isolating faecal indicator organisms (FIOs) or quantifying antimicrobial resistance genes have not confirmed virulence and studies utilising metagenomic profiling and culture-based approaches only allow for identification of potential pathogens to the genus or species levels (Wright et al., 2020). Furthermore, gene presence does not always translate into transcribed and functional protein (e.g., DNA from viable but non culturable [VBNC] or extracellular DNA [eDNA] released from dead cells can also give positive identification of virulence genes that do not pose a direct threat to public health) (Masters et al., 1994; Wolffs et al., 2005; Li et al., 2013).

With emerging evidence that some pathogens can become 'enriched' on plastics compared to natural materials, such as fine sands and decaying seaweed (Pham et al., 2021; Metcalf et al., 2022a), there is an urgent need to determine whether clinically important human pathogens colonising abiotic debris in the environment pose a heightened public health risk by increasing the capacity for exposure to potentially dangerous pathogens. Therefore, the aims of this study were to: (1) quantify the potential for clinically important groups of *E. coli*, including EHEC, ETEC, AIEC and UPEC to colonise and persist on plastic waste under environmental conditions, and (2) determine the virulence of these strains following their recovery from the plastisphere. To address this, we have employed a *Galleria mellonella* model of infection to determine if plastisphere-associated pathogens can retain their virulence and thus represent a potential public health risk.

2. Materials and methods

2.1. Bacterial strains, generation of luciferase expression, and growth conditions

Pathogenic *E. coli* used in this study included the ETEC strains 239-1*lux* (which contains a heat stable toxin gene) and 239-2*lux* (which contains a heat labile toxin gene); AIEC strain LF82*lux*; UPEC strain CFT073*lux*; and EHEC strain TUV93-O*lux*. Wild type 239–1, 239–2, LF82, CFT073 and TUV93-O were transformed with p16Slux to allow constitutive luminescent tagging through site-specific integration into the 16S locus of the bacterial chromosome (Riedel et al., 2007); this integration also conferred erythromycin resistance. All isolates were grown in Luria-Bertani (LB) broth (Invitrogen, UK) containing 500 μg/ml erythromycin (Thermo Scientific, UK) at 37 °C with shaking at 120 rpm, unless otherwise stated.

2.2. Determination of minimum inhibitory concentration (MIC)

Individual colonies of each *E. coli* strain were picked off agar plates, grown overnight in LB broth, and subjected to MIC analysis to determine resistance and sensitivity to erythromycin (which ranged from 16 mg/ml to 0.25 mg/ml in two-fold serial dilutions). Cultures were grown in LB containing no antibiotic as a positive control for growth; a negative control of LB containing no bacteria was also included. Bacterial cultures were adjusted to an optical density at 600 nm (OD_{600nm}) of 0.1, and 100 μL added to each well of a 96-well plate. Samples were incubated statically overnight, at 37 °C. Growth was measured using OD_{600nm}, and the MIC determined as the lowest concentration of antibiotic that inhibited 50% of bacterial growth, relative to the no antibiotic control. Three independent replicates were used, and results presented as the mean ± standard error.

2.3. Formation of natural biofilm on cotton and plastic material

Bespoke high-grade stainless-steel frames (200 mm × 140 mm x 1.5 mm) with six 40 mm x 40 mm cut outs were designed (Fig. 1a) and purchased from Dunblane Light Engineering (DLE, Scotland, UK). Sheets of blue high-density polythene (HDPE) (20 μm; Thali outlet, Leeds, UK) or individual squares of blue cotton canvas fabric (240 GSM; Amazon Ltd, UK) were sandwiched between two frames revealing six individual windows of plastic or cotton to which independent samples could be added. To form a natural biofilm on the surface of the plastic or cotton, frames were placed in 12-litre glass tanks and submerged in surface water (subsequently called 'SW'; Fig. 1b) collected from Cocksburn Reservoir (Pendreich, Scotland, UK). The SW was tested to ensure the absence of erythromycin resistant *E. coli* prior to use. Three replicate tanks were used, each containing 7 L of SW, supplemented with a trace element solution to enhance biofilm formation ($CuCl_2.2H_2O$, 15 mg/L; $NiCl_2.H_2O$, 25 mg/L; $Na_2MoO_4.2H_2O$, 25 mg/L; $ZnCl_2$, 70 mg/L; $MnCl_2.4H_2O$, 100 mg/L; $CoCl_2.6H_2O$, 120 mg/L; $FeCl_3$, 4 g/L; EDTA, 2 g/L; HCl [25%], 6.5 ml/L) (Eguchi et al., 1996). The SW was aerated continuously using a Pond Air Pump (Swell, UK), and the frames left submerged for 96 h. Tanks were covered with opaque black plastic to prevent evaporation and inhibit algal overgrowth and left at ambient room temperature (ca. 18–21 °C).

Conductivity, pH, and turbidity of SW were measured using a portable probe (Combo pH and EC, Hannah Instruments Ltd., UK) and a HI-88703-02 Bench Top Turbidity Meter (Hanna Instruments Ltd., UK) (Supplementary Table S1).

2.4. Inoculation of frames

Bacterial overnight cultures were grown as previously described. The following morning, cultures were diluted 1:100 into fresh, pre-warmed LB (to 37 °C) and grown to an OD_{600nm} of 0.3. During this growth period, frames were removed from the tanks and air dried for at least 3 h, or until visibly dry. Frames containing the cotton were disassembled to ensure areas between the individual cotton squares were dry and did not represent a risk for cross contamination between squares (due to the rapid rate of drying, this was not necessary for the frames containing plastic). Once cultures had reached an OD_{600nm} of 0.3, 15 ml were pelleted by centrifugation (4000 rpm, 10 min, 4 °C). Forty millilitres of SW were removed from each tank, and human faecal material (which had been stored frozen, and subsequently sterilised by autoclaving after thawing) was added at a concentration of 100 mg/ml and thoroughly homogenised by vortexing (this faecal suspension is subsequently called

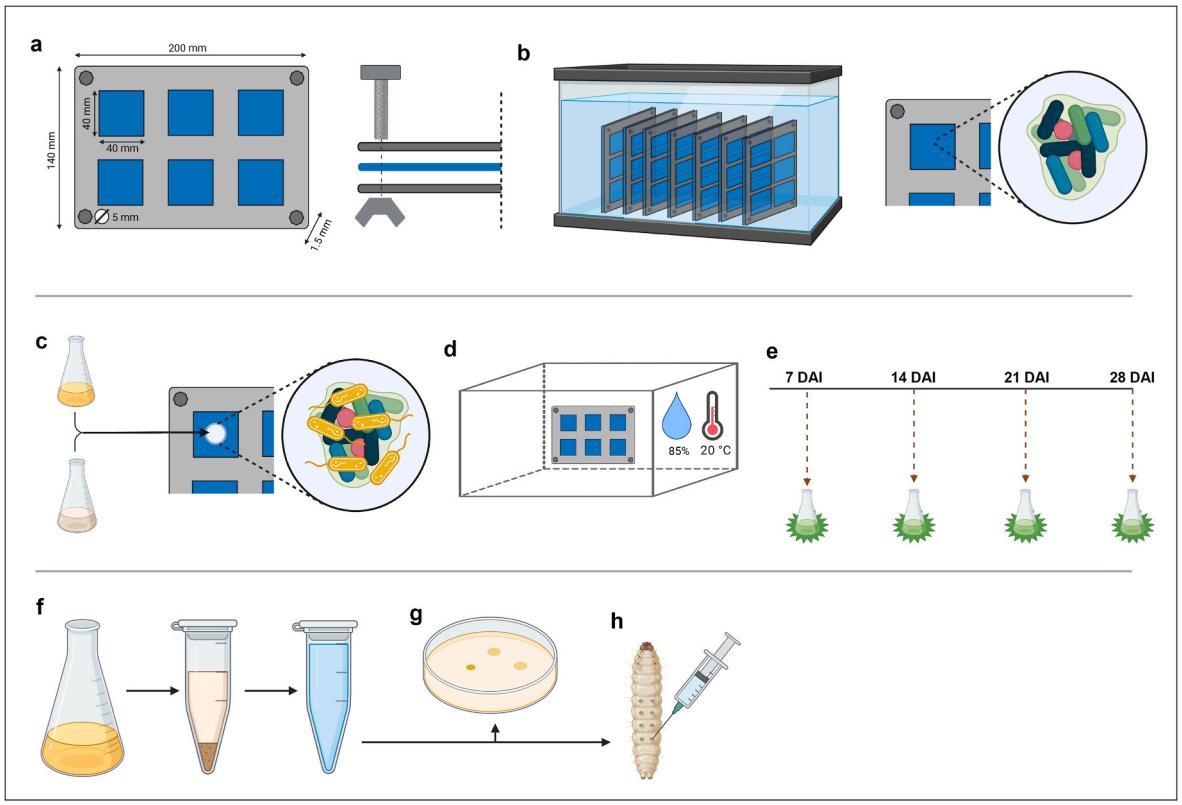

Fig. 1. Schematic overview of the sampling procedure for pathogen persistence and subsequent virulence. Two steel frames secured with wingnuts were designed to house six independent squares of plastic or cotton (**a**). Frames were submerged in surface water (SW) for 96 h to allow a natural biofilm to form on the material (**b**). Bacterial cultures of each individual pathogen were added to a faecal suspension (FS) and pipetted onto the air-dried plastic or cotton in the square of each frame (**c**). Frames were housed in plastic boxes at 20 °C (**d**). At 7, 14, 21, and 28 DAI, samples were removed and enriched overnight in LB + 500 μg/ml erythromycin; luciferase expression was subsequently quantified relative to control FS samples (**e**). At each timepoint, samples were grown in LB + 500 μg/ml erythromycin, washed, and resuspended in PBS (**f**) before CFU enumeration (**g**) and injection into *G. mellonella* (**h**). Figure generated using BioRender (https://biorender.com/).

'FS'). Bacterial pellets were then resuspended in FS ('bacterial FS'). Frames were reassembled, and 300 μl of bacterial FS was pipetted onto each square (Fig. 1c). This represented approximately 1×10^8 bacterial cells, with concentrations comparable between isolates and replicates (Supplementary Fig. S1a). As controls, squares of cotton and plastic were inoculated with 300 μl of FS (and are subsequently called 'FSC' and 'FSP,' respectively). Frames were then placed into a sealed plastic box (70 L; 810 × 620 × 225 mm) in which a humid environment (85% relative humidity) was generated through the addition of moistened paper towels (200 ml water per box). All boxes were placed into a controlled environment chamber (Microclima 1750E, Snijder Scientific, Tilburg, Netherlands) at 20 °C (Fig. 1d); when needed, an additional 100 ml of water was added to each box when the paper towels appeared dry, in order to ensure a humid environment was maintained. An i-Button temperature logger chip (iButtonLink, WI, USA) was added to each box to record temperature every 1 h for the duration of the experiment. Finally, glycerol stocks (40%) of each inoculum were made and stored at −80 °C. The exact concentration of bacteria in each inoculum was determined retrospectively by serial dilution and enumeration of colony forming units (CFU).

2.5. Persistence of bacterial isolates

Seven, 14, 21 and 28-days after inoculation (DAI), individual boxes were removed (Fig. 1e), and plastic and cotton squares removed from each frame using a sterilised scalpel blade and forceps. Each square was aseptically transferred to an individual vial containing 10 ml LB and 500 μg/ml erythromycin. Samples were grown overnight at 37 °C, with shaking at 120 rpm. The following day, the presence of each *E. coli* strain was determined by measuring OD_{600nm} and luciferase expression, relative to the respective FS control samples, and sterile LB. Persistence was confirmed when both OD_{600nm} (>0.5) and luciferase expression thresholds (>1000 RFU) were reached. Glycerol stocks (40%) of each sample were made and stored at −80 °C for subsequent analysis.

2.6. Challenge of Galleria mellonella

G. mellonella larvae (obtained from Livefood, Axbridge, UK), were kept in the dark at 15 °C and used a maximum of one week after the lab receiving delivery. Healthy larvae, measuring from 2 to 2.5 cm in length and showing no signs of melanisation were used for all experiments.

Glycerol stocks from each timepoint were grown in LB containing 500 μg/ml erythromycin at 37 °C, with shaking (120 rpm) to an OD_{600} of 0.3 (Fig. 1f). Cells were pelleted by centrifugation and the inoculum resuspended in PBS. Inocula were serially diluted and plated on LB agar plates for retrospective enumeration (Fig. 1g). To determine virulence of each *E. coli* strain following its persistence on either the plastic or cotton, groups of 10 larvae were each injected with 10 μl of bacterial suspension (approx. 5×10^4 or 5×10^5 CFU; Supplementary Fig. S1b) into the hemocoel via the last right pro-limb. *Galleria* were challenged with ~5×10^4 CFU for isolates 239-1*lux*, 239-2*lux*, CFT073*lux* and TUV93-O*lux*; whereas a higher inoculum of ~5×10^5 CFU was required for LF82*lux*. Control samples at 5×10^4 (for comparison with 239–1*lux*; 239-2*lux*; CFT073*lux*; and TUV93-O*lux*) and 5×10^5 (for comparison with LF82*lux*) CFU were included to determine the effect of FS (Supplementary Fig. S1b). For comparison, the initial inoculum that was added to the frames at day 0 was grown and prepared in the same way for *Galleria* challenge. Following challenge, larvae were placed in an incubator at

37 °C. Survival was assessed for 72 h, with larvae considered dead when non-responsive to touch. Experiments were conducted in biological triplicate. Virulence was assessed relative to the initial frame inoculum for each isolate. For all challenge experiments, an inoculation of PBS was used as a control ($n = 10$ larvae per experiment) to account for mortality caused by physical injury or infection by a contaminant.

2.7. Statistical analysis

Statistical analyses were conducted using GraphPad Prism Software. Analysis of variance (ANOVA) was used to compare the survival of *G. mellonella* at 72 h post injection (hpi). Data are reported as mean ± standard error (SE). P values < 0.05 are considered significant.

3. Results

3.1. Luciferase expression and antibiotic selection for E. coli isolation

Modified variants containing genes for luciferase expression and erythromycin resistance were generated for each clinical *E. coli* isolate, to allow subsequent isolation from the surface of plastic and cotton. Wild-type variants of each strain were unable to express luciferase, while the transformed variants, designated '*lux*,' expressed luciferase at greater than 1×10^5-fold higher levels (Fig. 2a and b). No luciferase expression was detected in the FS control.

The MIC of erythromycin for each strain was determined and indicated that following incorporation of p16Slux, LF82*lux*, CFT073*lux* and TUV93-O*lux* were more tolerant to higher concentrations of erythromycin than isolates 239-1*lux* and 239-2*lux* (Supplementary Table S2). For this reason, 500 μg/ml was selected as adequate to suppress the growth of background flora and allow comparable growth of the *lux* strain variants. Growth rates of Wild-type (WT) strains compared to *lux*-transformed variants demonstrated that incorporation of the p16Slux plasmid did not greatly alter fitness as determined through growth (Fig. 2c; doubling times WT vs *lux*: 239-1 – 126 min vs 144 min; 239-2 – 138 min vs 150 min; LF82 – 156 min vs 204 min; CFT073 – 132 min vs 180 min; and TUV93-O – 210 min vs 246 min), as previously demonstrated (Riedel et al., 2007). The growth of microbial communities in the FS was impeded by the addition of erythromycin.

3.2. Clinical E. coli can survive on plastic and cotton for up to 28 days

After several days, samples had become dried on both plastic and cotton and were completely desiccated by day 28 (Fig. 3a). ETEC strains 239-1*lux* (Figs. 3b) and 239-2*lux* (Fig. 3c), AIEC strain LF82*lux* (Fig. 3d), and UPEC strain CFT073*lux* (Fig. 3e) all persisted on both plastic and cotton for 28 days under simulated environmental conditions. EHEC isolate TUV93-O*lux* was unable to survive on plastic under these conditions (Fig. 3f), however, it did persist on cotton for 21 days. No luciferase expressing bacteria were recovered from the control samples at any timepoint (Fig. 3g).

3.3. Clinical E. coli can retain virulence in the plastisphere

Following the recovery of isolates 239-1*lux*, 239-2*lux*, LF82*lux* and CFT073*lux* from both plastic and cotton at 28 DAI, and isolate TUV93-

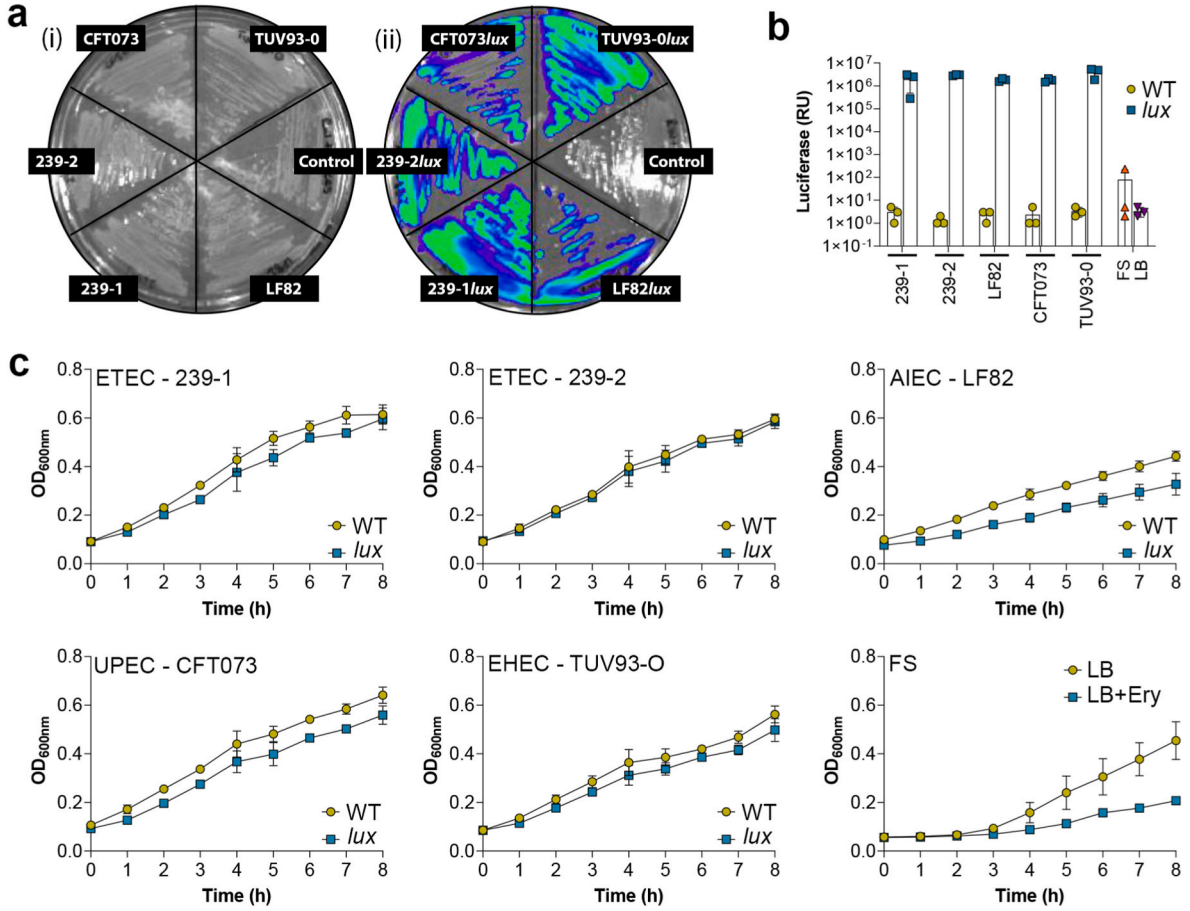

Fig. 2. Incorporation of p16Slux into isolates allowed for selection but did not greatly alter growth. Wild-type (WT; [i]) and luciferase expressing (*lux*; [ii]) isolates of 239–1, 239–2, LF82, CFT073, TUV93-O, compared with the control (FS) (**a**). Levels of luciferase expression in transformed *E. coli* strains (**b**). Growth rates of *E. coli* strains over an 8 h period; and the rate of growth of FS in LB and LB supplemented with erythromycin (LB + Ery) (**c**).

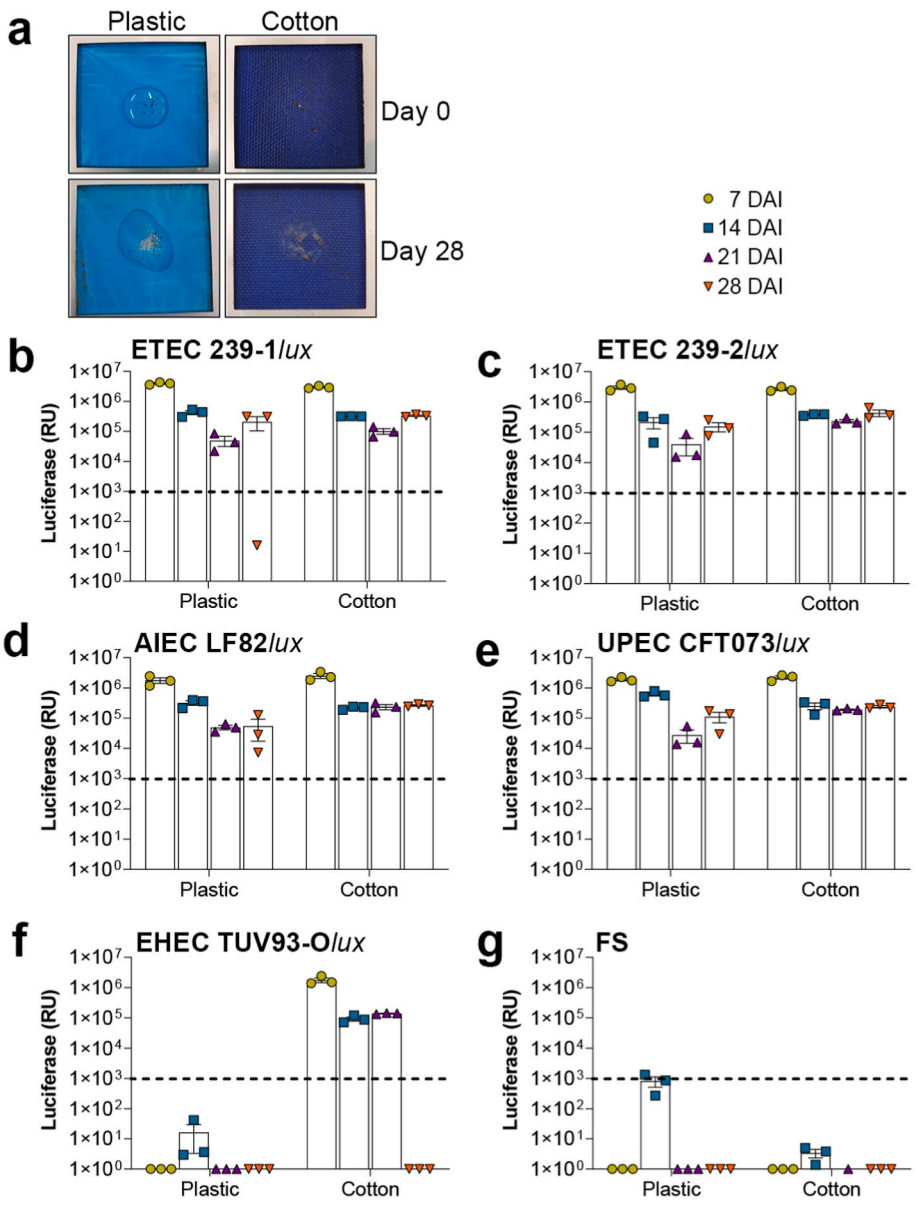

Fig. 3. Persistence of *E. coli* on plastic and cotton. Samples had begun to dry after several days and had become completely desiccated after 28 days (**a**). Persistence on plastic and cotton of isolates 239-1*lux* (**b**); 239-2*lux* (**c**); LF82*lux* (**d**); CFT073*lux* (**e**); TUV93-O*lux* (**f**); and control faecal suspension (FS) (**g**), was measured 7, 14, 21 and 28 DAI. Samples were considered positive for persistance when luciferase expression was above 1000 relative units (dashed line). Only two out of three replicates of the ETEC isolate 239–1 were recovered from the plastic at 28 DAI. Experiments were conducted in biological triplicate and are presented as mean ± SE.

O*lux* on cotton at 21 DAI, their pathogenicity was determined using a *G. mellonella* infection model (Fig. 4). In each case, the virulence of bacterial isolates recovered at 21 or 28 DAI were compared to the initial inoculum of each strain from day 0, and to the control material (FS) recovered from either plastic (FSP) or cotton (FSC). At 24 and 48 h post injection (hpi) of the larvae, there were no discernible differences in virulence between isolates recovered from cotton or plastic compared to their initial inoculum, except for isolate CFT073*lux* (Fig. 4d), which was significantly more pathogenic towards the *Galleria* larvae 24 hpi following its recovery from plastic ($p = 0.0001$). By 72 hpi, there were no significant differences in pathogenicity between isolates recovered from plastic and cotton relative to the initial inoculum added to the frames at day 0 (Supplementary Fig. S2), indicating that persistence on either plastic or cotton does not reduce virulence. When compared to the FS recovered from the plastic (FSP) at 72 hpi; 239-1*lux* ($p = 0.0103$), 239-2*lux* ($p = 0.021$), LF82lux ($p = 0.0037$) and CFT073lux ($p < 0.0001$) were all significantly more virulent towards the *Galleria* larvae. Similarly, when compared to the FS recovered from the cotton (FSC) at 72 hpi, 239-1*lux* ($p = 0.0002$), 239-2*lux* ($p < 0.0001$), LF82lux ($p = 0.0130$), CFT073*lux* ($p = 0.0001$) and TUV93-O*lux* ($p < 0.0001$) were all significantly more virulent towards the *Galleria* larvae. At 72 hpi, the initial inoculum of isolates caused significantly greater mortality than material recovered from FSP (239-1*lux* [$p = 0.0001$]; 239-2*lux* [$p = 0.0003$]; LF82*lux* [$p = 0.277$* not significant]; CFT073*lux* [$p = 0.0001$]; and TUV93-O*lux* [$p < 0.0001$]) or FSC (239-1*lux* [$p < 0.0001$]; 239-2*lux* [$p = 0.0003$]; LF82*lux* [$p = 0.0465$]; CFT073*lux* [$p < 0.0001$]; and TUV93-O*lux* [$p < 0.0001$]), indicating that the virulence was due to the presence of the pathogenic *E. coli* within the sample, and not due to residual microflora or faecal homogenate within FS.

4. Discussion

Although previous plastisphere studies have shown that plastics in the environment can act as vectors for the survival and dissemination of pathogenic organisms, the significance of this in terms of public health risk has been questioned (Beloe et al., 2022). In this study, we have addressed this knowledge gap in plastisphere research by demonstrating that isolates of DEC including ETEC, AIEC and UPEC were readily able to colonise and persist on plastic and cotton fabric for at least 28-days, and most importantly that these pathogenic organisms still retain their

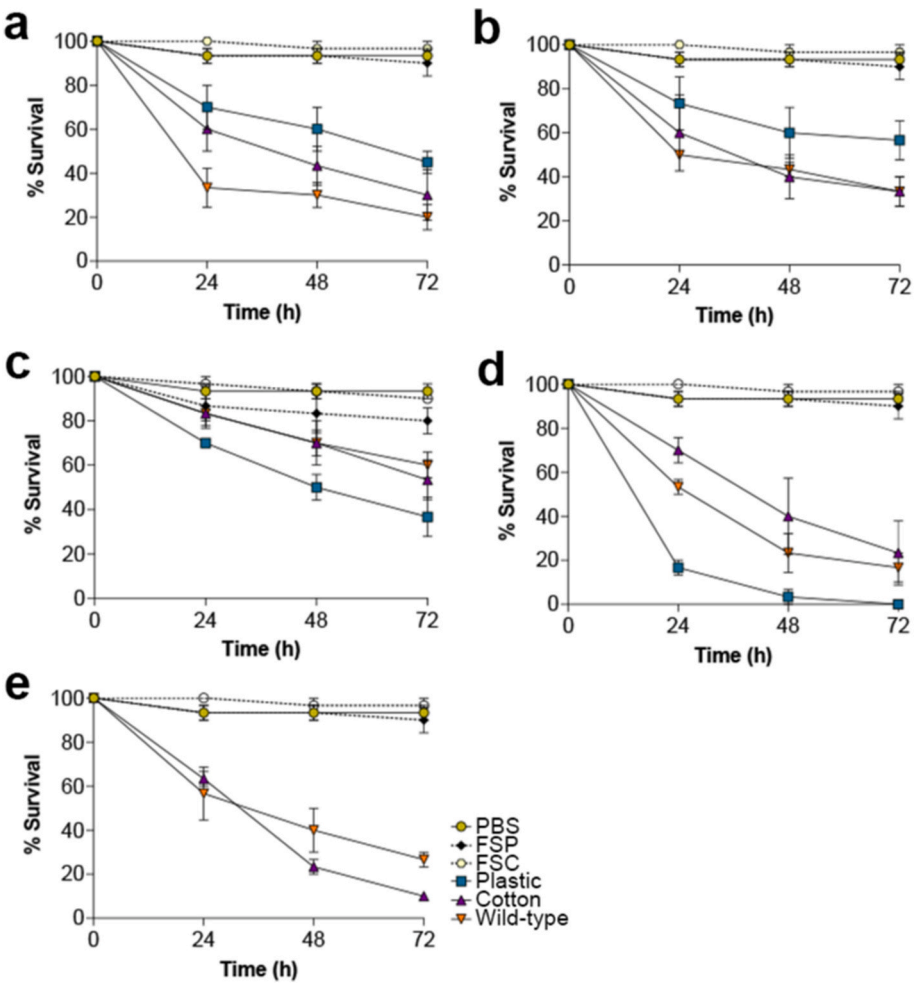

Fig. 4. Clinical *E. coli* recovered from plastic and cotton retained virulence in a *Galleria mellonella* model of infection. *Galleria* survival following challenge with *E. coli* strains 239-1*lux* (**a**); 239-2*lux* (**b**); LF82*lux* (**c**); CFT073*lux* (**d**); and TUV93-O*lux* (**e**). FSP and FSC represent the control faecal suspension (FS) recovered from the plastic and cotton control samples at 28 DAI, respectively. Data points (n = 10 *G. mellonella* larvae) represent the mean of three independent biological replicates ± SE.

virulence. Our findings therefore confirm that plastic and cotton fabric in the environment can act as reservoirs for dangerous clinical pathogens and represent a significant environmental and public health risk.

4.1. Persistence of pathogens on plastic and cotton

Diarrheagenic *E. coli* are being detected with increasing frequency in WWTP discharge and hospital effluents (Gomes et al., 2016) and represent a major source of diarrhoeal disease worldwide. In this study, ETEC strains 239–1 and 239–2; AIEC strain LF82; and UPEC strain CFT073 were able to persist on plastic under simulated environmental conditions for a prolonged period. Whilst the ability of pathogenic microorganisms to colonise environmental plastic debris has been reported previously (Metcalf et al., 2022b), this study provides the first indication that pathogenic microorganisms colonising plastic debris can remain viable for extended periods, even following desiccation. This has significant environmental and human health implications because the lightweight and buoyant properties of plastic allow for enhanced distribution through the landscape and can result in increased risk of plastic debris contaminating areas where direct human exposure can be high, for example river and bathing water beaches used for recreation (Keswani et al., 2016; Rodrigues et al., 2019; Xue et al., 2020). Our data indicate that such pathogens can survive on plastic debris for at least 28-days, signalling greater potential for interactions between humans and clinical pathogens than has previously been reported.

All isolates examined in this study were also readily able to persist on cotton fabric. However, EHEC isolate TUV93-O persisted for longer on cotton than on plastic. This could be due to the surface roughness and topographical features of the cotton fabric facilitating greater EHEC attachment compared to plastic, with surface roughness known to be an important factor for EHEC attachment to surfaces (Wang et al., 2009). Additionally, it is possible that the humidity used within our experiments hindered EHEC persistence on plastic, e.g., EHEC survive for longer on the surface of spinach when the humidity is higher (Choi et al., 2011). Further investigation under a wider range of conditions, including increased humidity, may reveal new insight into the longer-term persistence of EHEC in the environment.

However, the reduced persistence of pathogens such as EHEC on plastic may not be concurrent with a reduced risk to public health. In the environment, cotton decomposes within five to six months, whereas plastics can persist for decades or even centuries (Chamas et al., 2020). The increased durability of plastic over cotton therefore provides increased opportunities for colonisation (and subsequent re-colonisation) by pathogens from environmental contamination. This may lead to the development of wider risks linked to horizontal gene transfer (HGT) and thus new and emerging pathogens with enhanced antimicrobial resistance and more extreme virulence mechanisms. Additionally, some strains used in this study, e.g., TUV93-O, that appeared unable to persist on plastic, may have entered a viable-but-non-culturable (VBNC) state (Gião and Keevil, 2014). Subsequent resuscitation (and retained virulence) following dissemination to an appropriate host is therefore theoretically possible.

4.2. Retention of virulence following recovery from plastic and cotton

In addition to surviving on plastic and cotton for at least 28 days, our

study has shown that pathogenic *E. coli* can retain their virulence. Addressing the previously highlighted limitations of plastisphere studies (Beloe et al., 2022), our results have confirmed that pathogenic organisms residing in the plastisphere can be virulent and thus pose a potential threat to human health. While all isolates examined here showed comparable virulence following their recovery from plastic and cotton 28 DAI relative to the inoculum added at day zero, UPEC isolate CFT073 showed enhanced pathogenesis in the *Galleria* infection model, suggesting that the plastisphere may even promote virulence. Biofilm formation is considered a major virulence trait in UPEC strains, allowing for increased persistence on abiotic surfaces and chronic recurrent infections *in vivo* (Naziri et al., 2021). Within the biofilm environment, bacteria often diverge into population subsets, for example, some cells have roles in structural formation, some in antibiotic tolerance, and some in shared resource production (Bisht and Wakeman, 2019). Consequently, cells dispersed from biofilms are physiologically distinct and often more virulent compared to both their planktonic and biofilm counterparts (Chua et al., 2014). Our data indicate that the plastisphere may promote diversification and that the fittest, most virulent variants persist for longer in an effort to enhance their survival and dissemination to new hosts. The increased nutrient levels found within the plastisphere may increase pathogen virulence by favouring transmission of fast-growing virulent strains, or by allowing pre-adaptation to efficient use of resources following dissemination to a nutrient rich host. It is possible that extending the timepoints further may result in more virulent variants of ETEC and AIEC being identified following their recovery from the plastisphere, also.

Few plastisphere studies have ascertained the virulent threat that plastic-associated pathogens pose to humans and to date, no studies have examined virulence following extended periods of environmental exposure (Beloe et al., 2022). The ability of the pathogens examined in this study to persist under environmental conditions, potentially allowing for greater dissemination through the environment, together with the ability of DEC to retain virulence following their recovery from plastic has significant implications for human health. With contaminated plastic waste being found in locations where human exposure can be high, there is a heightened potential for the spread of disease to the human population. Additionally, with human pathogens surviving for long periods in the environment, there is an increased chance of zoonotic transmission and potential evolution of novel pathogens, as animals often interact with abiotic debris in the environment. Anthropogenic changes to the environment can facilitate the spread of pathogens from animals into human populations (Shanks et al., 2022), and research is urgently needed to understand if environmental pollution and plastic waste could accelerate this (Krystosik et al., 2020).

Many pathogens, including DEC, can survive primary, secondary, and in some instances, tertiary wastewater treatment (Mbanga et al., 2020). While ensuring the proper inactivation of pathogens through treatment processes would drastically reduce their potential to enter the environment, pathogens are often released in CSOs and in illegal spills. Therefore, it is essential that we recognise the ability of wastewater pathogens to persist and retain virulence, regardless of how they have entered the environment, and to understand how long different pathogenic organisms can persist on different materials, and at what concentration. The diverse environmental conditions that pathogens encounter in the environment will likely impact their survival on different materials and in different environmental matrices. In this study, we have attempted to replicate environmental conditions through re-suspension of pathogenic isolates in human faecal suspensions (simulating wastewater), and using plastics already colonised by natural biofilms. However, the effect of other parameters on persistence and pathogenicity, such as UV, pH, temperature, salinity, chemical composition, and movement through different environmental matrices should be considered in the future. Understanding the mechanisms by which these pathogens colonise and persist on different substrates will be crucial for informing health legislation concerning plastics in the environment, and future policy related to material production and disposal.

5. Conclusions

Untreated sewage is often released into aquatic environments through illegal dumping and via CSO spills resulting in increased reports of clinical pathogens being found in sewage effluent and in environmental waters downstream of WWTPs. The release of clinical pathogens into the wider environment provides opportunities for their interaction with environmental debris. This work has demonstrated that human pathogens are able to colonise and persist on plastics and fabrics under simulated environmental conditions; and crucially retain virulence.

Author statement

MJO, **RSQ**: Conceptualisation; **MJO**, **HLW**, **RM**: Data curation; **MJO**: Formal Analysis; **MJO**: Writing-Original draft preparation; **RSQ**: Supervision; **MJO**, **HLW**, **RM**, **DMO**, **RSQ**: Writing, reviewing, and editing; **RSQ**: Funding acquisition.

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Statement of environmental impact

Plastics colonised by clinical pathogens have significant environmental and human health implications. The lightweight and buoyant properties of plastic allow for enhanced distribution through the landscape, which can result in increased risk of contaminating areas where direct human exposure can be high, for example, rivers and bathing water beaches used for recreation. Our data indicate that human pathogens can survive on plastic debris for at least 28-days, but most importantly they retain their virulence. This demonstrates the heightened co-pollutant risk of plastic pollution, and the increased risk of humans being exposed to dangerous clinical pathogens in the environment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121466.

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