Diverse bacterial species contribute to antibiotic-associated diarrhoea and gastrointestinal damage

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HIGHLIGHTS

- A diverse range of bacteria may cause antibiotic-associated diarrhoea (AAD)
- Non-*C. difficile* AAD pathogens include antimicrobial resistant clinically important species
- Pathogens isolated show virulence potential based on encoded factors
- Isolates tested *in vivo* cause gut damage, targeting host processes essential for gut homeostasis

Diverse bacterial species contribute to antibiotic-associated diarrhoea and gastrointestinal damage

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Running title: Bacterial causes of non-*C. difficile* AAD

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ABSTRACT

Objectives. Antibiotic-associated diarrhoea (AAD) caused by *C. difficile* is one of the most common nosocomial infections, however, little is known about infections related to antimicrobial use for pathogens other than *C. difficile*. We therefore aimed to provide insight into other bacterial causes of AAD, and how infection with these pathogens causes damage in the dysbiotic gut.

Methods. Clinical isolates from *C. difficile*-negative AAD patients were whole genome sequenced for *in silico* analysis of potential virulence factors and antimicrobial resistance determinants. A mouse model of infection was developed to assess the capacity of these isolates to cause gastrointestinal damage, which was analysed by studying specific markers in the gastrointestinal mucosa of infected mice.

Results. Several bacterial pathogens were isolated from patients with *C. difficile*-negative AAD. Each isolate showed the potential for virulence based on encoded virulence factors, as well as most showing antimicrobial resistance *in vitro*. Isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were tested in the mouse model of infection, inducing damage primarily in the small intestine, affecting adherens junction integrity, cellular polarity, and cellular proliferation.

Conclusions. Several pathogens of clinical importance other than *C. difficile* are able to cause gastrointestinal infection following antimicrobial-mediated dysbiosis. The

virulence potential and multidrug resistance identified in these isolates illuminates the importance of further diagnostic screening in cases of *C. difficile*-negative AAD.

Keywords: Antibiotic-associated diarrhoea, antimicrobial resistance, bacterial pathogenesis, *Clostridium difficile*.

INTRODUCTION

Antibiotic-associated diarrhoea (AAD) can occur when antimicrobials disrupt the resident gut microbiota, pre-disposing patients to gastrointestinal infection with opportunistic pathogens, resulting in diarrhoeal disease. This is complicated by the overuse of broad-spectrum antimicrobials, and the emergence of antimicrobial-resistant pathogens. *Clostridium difficile* is the most common known cause of infectious AAD, and therefore its involvement is often suspected prior to laboratory diagnosis, and empiric antimicrobial therapy is often pursued based on this presumption (1, 2). However, only approximately 25% of AAD cases can be attributed to *C. difficile* infection (3), with other known infectious aetiologies including *Clostridium perfringens* and *Staphylococcus aureus* (4-8). Several non-infectious mechanisms of AAD pathogenesis have also been identified (9), however these will not be discussed here. It has been noted that empiric antimicrobial therapy aimed at *C. difficile* is unlikely to be successful at treating infection with some non-*C. difficile* AAD pathogens, while some infections may not require antimicrobial treatment, with a resolution of symptoms achieved by the cessation of the inciting antimicrobial and symptomatic care. However, even when *C. difficile* is not detected, screening for other potential causative organisms is often not performed and therefore the contribution of non-*C. difficile* pathogens to AAD is relatively unknown. For many candidate AAD pathogens, little work has been done to support their role in this disease. Although the human and financial costs of AAD infections overall are not known, in the USA there are up to 500,000 *C. difficile* cases per year at an estimated cost of $6.3 billion (10, 11). Considering that *C. difficile* infections only account for a quarter of AAD cases, this suggests that the cost of all AAD disease to human health worldwide is enormous.

In this study, we isolated and characterised several bacterial pathogens from cases of non-*C. difficile* AAD, and explored the capacity of these isolates to cause gastrointestinal disease using a mouse infection model of non-*C. difficile* AAD, utilising a multi-disciplinary approach for the analysis of gastrointestinal damage. In doing so, we found evidence of a role for several clinically important organisms in causing AAD in humans that are not being identified in current diagnostic screens. We have also provided evidence of the capacity of these isolates to cause gastrointestinal disease, and gained new insights into the mechanisms of pathogenesis and subversion of host processes by AAD-causing bacteria.

MATERIALS AND METHODS

Isolate collection and identification

Diarrhoeal faecal samples (taking the shape of the container into which they were put) submitted to the Enteric Laboratory at PathWest Laboratory Medicine (WA) in Perth, Australia, were chosen for culture of potential pathogens based on a documented history of "antibiotic-associated diarrhoea" and a negative *C. difficile tcdB* PCR performed using a BD Max platform (Becton Dickinson). Samples fulfilling these two criteria were cultured on MacConkey agar (Merck), cysteine lactose electrolyte deficient agar (Oxoid) and Sabouraud agar (Difco) aerobically, and blood agar supplemented with neomycin anaerobically in an A35 anaerobic chamber (Don Whitley Scientific). Agar plates showing a heavy growth of an apparently pure culture of any organism were chosen for further study. The predominant colonies were then identified using MALDI-TOF mass spectrometry and stored at -80°C in brain heart infusion broth plus 15% glycerol.

Isolation of genomic DNA and sequencing

Genomic DNA from all strains was isolated using the DNeasy Blood and Tissue kit (QIAGEN), as per manufacturer's instructions. Sequencing libraries were prepared with Nugen, Ovation Ultraflow System V2 (1-96) using protocol M01380v1, 2015. Sequencing was performed using Illumina MiSeq v2 to achieve paired end 150 bp reads. The *de novo* genome assemblies were prepared using SPAdes genome assembler and annotated using Prokka. Detailed information on the genomic analysis performed is available in Supplementary Material. All genome sequences can be found under BioProject no. **PRJNA430462**.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of all isolates was determined using the VITEK 2 system (bioMérieux) with the AST-N246 antimicrobial susceptibility card for Gram negative organisms (bioMérieux). Minimal inhibitory concentrations (MICs) were interpreted in accordance with guidelines set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (12).

Murine model of non-*C. difficile* AAD

Animal handling and experimentation was performed in accordance with Victorian State Government regulations and approved by the Monash University Animal Ethics Committee (Monash University AEC no. MARP/2014/145). Male 6-7 week old C57BL/6J mice (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) were pre-treated with an antimicrobial cocktail in the drinking water consisting of kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215

mg/ml), vancomycin (0.045 mg/ml) and cefaclor (0.3 mg/ml) for 7 days, ceasing three days prior to infection with approximately 10^{10} CFU per mouse by oral gavage. All mice were screened prior to infection to ensure they were not already carrying the infecting organism. Throughout the course of infection, mice were monitored for weight loss, changes in behaviour, and faecal consistency. Faeces was collected at 24 hour intervals up to 48 hours post-infection for enumeration of bacterial shedding. Animals were humanely euthanised by CO_2 asphyxiation at 48 hours post-infection, followed by collection of the gastrointestinal tract for histological analysis. Further details are available in Supplementary Material.

Histological staining and scoring

Small intestine sections were stained with haematoxylin and eosin (H&E), and large intestine sections were stained with periodic acid Schiff/Alcian blue (PAS/AB) using standard techniques. Detailed information on imaging and analysis is available in Supplementary Material.

Immunostaining

Immunostaining for E-cadherin, β-catenin, ezrin, and proliferating cell nuclear antigen (PCNA) were performed using standard techniques. Detailed information is available in Supplementary Material. Detailed information on imaging and analysis is available in Supplementary Material.

Statistical analysis

Statistical significance of data was determined by the Mann Whitney *U* test, performed using GraphPad Prism 7 software.

RESULTS

Isolation and characterisation of non-*C. difficile* AAD isolates from human patients

Screening of patients with *C. difficile*-negative AAD revealed several potentially novel bacterial AAD pathogens; one isolate each of *Enterobacter cloacae* (DLL7524), *Rahnella aquatilis* (DLL7529), and *Pseudomonas aeruginosa* (DLL7525), two isolates of *Klebsiella pneumoniae* (DLL7526 and DLL7528), and three isolates of *Escherichia coli* (DLL7527, DLL7530, and DLL7531). *In vitro* antimicrobial susceptibility testing detected resistance in 6 out of 8 isolates for the antimicrobials tested (Figure 1 and Supplementary Table 1). Each isolate that demonstrated resistance *in vitro* was resistant to at least two or more classes of antimicrobials, with *E. coli* DLL7531 being an extended-spectrum beta-lactamase (ESBL)-producer. Whole genome sequencing and bioinformatic analysis revealed many important virulence factors encoded by these isolates including adhesion factors, flagella, capsules, siderophores, secretion systems, and toxins (Supplementary Table 2–9), as well as antimicrobial resistance genes (Supplementary Table 10–17).

Clinical AAD isolates cause gastrointestinal disease in a mouse model of non-*C. difficile* AAD

Due to their lack of antimicrobial resistance, *E. coli* DLL7527 and DLL7530 could not be confidently isolated from mouse faeces for enumeration, and therefore these strains were excluded from *in vivo* analysis. Preliminary trials also determined that colonisation could not be detected in mice infected with *R. aquatilis* DLL7529 or *E. cloacae* DLL7524, and therefore these isolates were also excluded from further analysis.

All remaining isolates were able to colonise the mice, shedding at high numbers in the faeces at 24 and 48 hours post-infection (Figure 2). Significant weight loss was observed at 24 hours post-infection for all infected groups, with a slight recovery in weight at 48 hours post-infection, however, weights remained significantly different from those of uninfected mice for all infected groups at this time point (Figure 2).

Histological analysis of gut tissue collected at 48 hours post-infection showed that each isolate tested was capable of causing damage, with the majority of severe damage observed in the small intestine (Figure 3A). This damage primarily affected the villi, presenting as superficial damage and sloughing of epithelial cells, development of Gruenhagen's space, loss of nuclei staining, and blunting and destruction of the overall villus structure. This is reflected by histological scoring, which showed that most of the damage caused by these isolates generally occurred in the proximal small intestine (Figure 3B). The amount of damage in the large intestine was generally lower in mice infected with each isolate, and this damage was overall less severe than that seen in the small intestine, largely presenting as superficial damage to the epithelium (Figure 3A). In most tissue where infection-mediated damage was present, it appeared in sporadic patches, with some healthy tissue remaining.

Disruption to epithelial integrity by clinical AAD isolates

Immunostaining of E-cadherin and β-catenin showed disruption to adherens junctions primarily in the villi of the small intestines for all isolates (Figure 4). This disruption presented as a loss of staining for both E-cadherin and β-catenin, or β-catenin alone. In mice infected with *K. pneumoniae* DLL7528, adherens junctions in the distal small intestine appeared to be intact. Analysis of adherens junction integrity also revealed

damage to the large intestine in mice infected with *E. coli* DLL7531, and *K. pneumoniae* strains DLL7526 and DLL7528, which appeared to target β-catenin only in these tissues. Immunostaining of ezrin revealed disruption to cellular polarity primarily in the small intestine for all isolates (Figure 5). In line with what was observed for adherens junctions, ezrin in the distal small intestine in mice infected with *K. pneumoniae* DLL7528 appeared intact. Similarly, in the large intestine, only minor disruption to ezrin was observed in the colon of mice infected with *K. pneumoniae* DLL7526.

Alterations to cellular proliferation in small intestinal crypts by AAD clinical isolates

Analysis of changes in cellular proliferation within the proximal small intestine was examined by PCNA immunostaining (Figure 6). Compared to proximal small intestinal tissue from uninfected mice, the percentage of proliferating cells within crypts increased significantly in mice infected with *P. aeruginosa* DLL7525, with a slight increase observed in mice infected with *K. pneumoniae* DLL7526 and *K. pneumoniae* DLL7528 (Figure 6). A slight decrease in proliferation was observed in mice infected with *E. coli* DLL7531 (Figure 6). In sections of the proximal small intestine where crypt damage was apparent, the density of crypts in the mucosa was significantly reduced in infected mice in comparison to uninfected mice (Figure 6).

DISCUSSION

The emergence of hypervirulent strains of *C. difficile* and the current worldwide focus on antimicrobial use and gut health has brought attention to conditions that arise as a consequence of antimicrobial use. However, focus remains on AAD caused by *C. difficile*, and therefore little is known about other infectious causes of dysbiosis-related

diarrhoea. In this study, we identified several bacterial pathogens which previously have not been associated with this disease from patients with clinically-diagnosed AAD, and showed the capacity of four of these isolates to cause gastrointestinal damage in a mouse model of infection. Of note, many of the organisms identified in this study were of clinical importance, including several ESKAPE (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii, P. aeruginosa*, and *Enterobacter* spp.) pathogens, which are known to be multidrug resistant and a leading cause of healthcare-related infections throughout the world (13).

E. cloacae is associated with a variety of opportunistic infections (14), and has been isolated from patients with AAD (15, 16), with an increase in *E. cloacae* intestinal colonisation being associated with antimicrobial treatment (14). Although the isolate identified in this study (DLL7524) was not analysed *in vivo*, it was antimicrobial resistant (Figure 1) and encoded several potential virulence factors including curli fimbriae, involved in *E. cloacae* biofilm formation (17); lectins; and putative virulence effectors with homology to SrfB and SrfC from *Salmonella* Typhimurium (Supplementary Table 2 and 10).

R. aquatilis is a rare member of the *Enterobacteriaceae* which has been associated with a variety of opportunistic infections, including gastroenteritis (18). Although the isolate identified in this study (DLL7529) was not able to be analysed *in vivo*, it was also antimicrobial resistant (Figure 1) and encoded several virulence factors, including a heat-labile enterotoxin (Supplementary Table 3 and 11), suggesting that it has the potential to cause gastrointestinal disease.

E. coli is a well known cause of gastrointestinal disease, however it has not previously been associated with AAD. The three *E. coli* strains isolated in this study each encoded factors that are known to be involved in the virulence of diarrhoeagenic *E. coli* (Supplementary Table 4–6), including several autotransporter proteins that have been associated with promoting gut colonisation, and cytotoxic effects (19-21). Despite encoding several putative antimicrobial resistance determinants (Supplementary Table 12 and 13), both *E. coli* DLL7527 (ST491, O57:H45) and DLL7530 (ST10, O107:H27) were susceptible to all antimicrobials tested (Figure 1), and were not able to be tested *in vivo*, however, the virulence determinants encoded by these strains suggest the capacity to cause disease. In contrast, *E. coli* DLL7531 (ST226, O11:H4) was an ESBL-producer (Figure 1), encoding the class A beta-lactamase TEM-1B (Supplementary Table 14).

P. aeruginosa is a notorious opportunistic pathogen, and a potential cause of AAD in susceptible patients (22, 23). Antimicrobials are a known risk factor for *P. aeruginosa* gastrointestinal colonisation (24, 25), which can also lead to extra-intestinal infection due to translocation out of the gut (25, 26). The isolate identified in this study, DLL7525 (ST309, O11), encoded several antimicrobial resistance determinants (Supplementary Table 15) and was antimicrobial resistant *in vitro* (Figure 1). It also encoded the cytotoxins exotoxin A, pyocyanin, and the T3SS effectors ExoU and ExoT, as well as many other virulence factors that may contribute to gastrointestinal disease (Supplementary Table 7).

K. pneumoniae commonly causes a range of opportunistic infections, and is of particular clinical concern due to increasing multidrug resistance (27). In this study, two

antimicrobial resistant *K. pneumoniae* strains were isolated from patients with AAD, DLL7526 (ST221, K39) and DLL7528 (ST1380, K31) (Figure 1), each encoding a range of virulence factors. This includes several types of fimbriae, which are known to facilitate biofilm formation and host cell adhesion (28, 29); filamentous haemagglutinin; and putative autotransporter proteins (Supplementary Table 8 and 9). *K. pneumoniae* has previously been isolated from AAD patients (30), as well as HIV-positive patients with a history of prolonged antimicrobial use and presenting with diarrhoea (31), however, its role in AAD has not previously been investigated.

In the non-*C. difficile* AAD mouse model of infection, mice challenged with *P. aeruginosa* DLL7525, *E. coli* DLL7531, *K. pneumoniae* DLL7526, and *K. pneumoniae* DLL7528 were successfully colonised (Figure 2) and presented with substantial damage in the small intestine (Figure 3). Although *C. difficile* is generally not associated with enteritis, damage to the small intestine is commonly seen in cases of non-*C. difficile* AAD involving pathogens such as *S. aureus* (32), and is a well-known effect of the *C. perfringens* enterotoxin *in vitro* (33). In reported cases of non-*C. difficile* AAD, colitis has been associated with the isolation of *S. aureus* (6), and *K. oxytoca* (1), however, the isolates tested in this study were only able to induce superficial damage in the large intestine of mice, which encompassed <10% of caecal tissue, and <13% of colonic tissue overall for all isolates tested (Figure 3).

The integrity of the gut epithelial barrier is essential in the health and homeostasis of the gastrointestinal system. The cell-cell adhesion provided by intercellular junctions is vital for the maintenance of epithelial integrity by creating a physical barrier between the lumen of the gut and deeper tissues (34). Gut pathogens therefore often target these

junctions to allow penetration through the gut mucosa and dissemination of infection (34). Disruption to epithelial barrier integrity was therefore analysed in this study by the detection of the adherens junction proteins, E-cadherin and β-catenin. Disruption to these proteins was induced by all isolates tested, most severely affecting the epithelial monolayer lining the villi within the small intestine and presenting as either a loss of staining to both E-cadherin and β-catenin, or β-catenin alone (Figure 4). Disruption to adherens junctions observed in the large intestine appears to involve β-catenin only. Since disruption to E-cadherin does not appear to occur in the absence of disruption to β-catenin, this suggests that damage is occurring *via* an intracellular pathway, since β-catenin is an intracellular component of the adherens junction that interacts with the actin cytoskeleton *via* α-catenin (35). Another important factor in homeostasis of the gut epithelium is cellular polarity. The integrity of the apical domain in polarised epithelial cells is mediated by ezrin, which facilitates protein interactions at the membrane-cytoskeleton interface in this domain (36). It has therefore been suggested that through its interactions with the actin cytoskeleton, ezrin could also contribute to the maintenance of intercellular junctions (36). In line with this hypothesis, the pattern of disruption to ezrin induced by the isolates in this study was similar to that seen for E-cadherin and β-catenin, with most severe damage observed in the small intestine (Figure 5). Further work is required to ascertain the mechanism of damage to both ezrin and adherens junctions, and the contribution of this damage to gastrointestinal infection by these isolates.

Although damage was observed primarily in the villi of the small intestine, changes in cellular proliferation within the crypts of the proximal small intestine were also assessed *via* staining for PCNA, a protein which is expressed in the nucleus of dividing

cells (37). In comparison to uninfected mice, proliferation appears to be significantly higher in mice infected with *P. aeruginosa* DLL7525, and marginally higher in mice infected with either *K. pneumoniae* isolate, but a slight reduction was observed in mice infected with *E. coli* DLL7531 (Figure 6). In addition to this, a significant reduction in crypt density was observed in areas of histological damage for all isolates tested (Figure 6). The intestinal epithelium is constantly self-renewing, a process which is driven by stems cells at the base of crypts, and has the capacity rapidly repair following damage (38). Therefore, an increase in epithelial proliferation during infection is likely to represent a regenerative response following damage. A reduction in cellular proliferation, however, as seen in mice infected with *E. coli* DLL7531, might suggest that the regenerative capacity of the gut has been impaired due to infection with this particular isolate.

Taken together, our results suggest that the organisms isolated have the potential to cause gastrointestinal disease, which was confirmed for *E. coli*, *P. aeruginosa*, and *K. pneumoniae* in the mouse infection model, and are likely to cause AAD in humans. From this study, it is apparent that current standards for diagnostic screening of patients presenting with diarrhoeal disease and a recent history of antimicrobial use may be preventing the identification of many bacterial pathogens with the capacity to cause AAD. Therefore, to avoid unnecessary treatment and achieve a positive clinical outcome, other suspected organisms should be tested for in cases of *C. difficile*-negative AAD. Isolation of these organisms from AAD patients also suggests that the gut could be an important reservoir for more potentially invasive infections, since many of these organisms are opportunistic pathogens with the ability to translocate across the mucosal barrier. This study contributes to a more comprehensive understanding of

AAD, which is required if we are to control this multifactorial disease. Our development of an infection model also provides a platform for the development of therapeutic strategies that will be of clinical relevance. Emerging antimicrobial-resistance in many of the bacteria involved, as identified in this study, lends urgency to this work, epitomised by the worldwide epidemics caused by fluoroquinolone resistant *C. difficile* in the last decade (39).

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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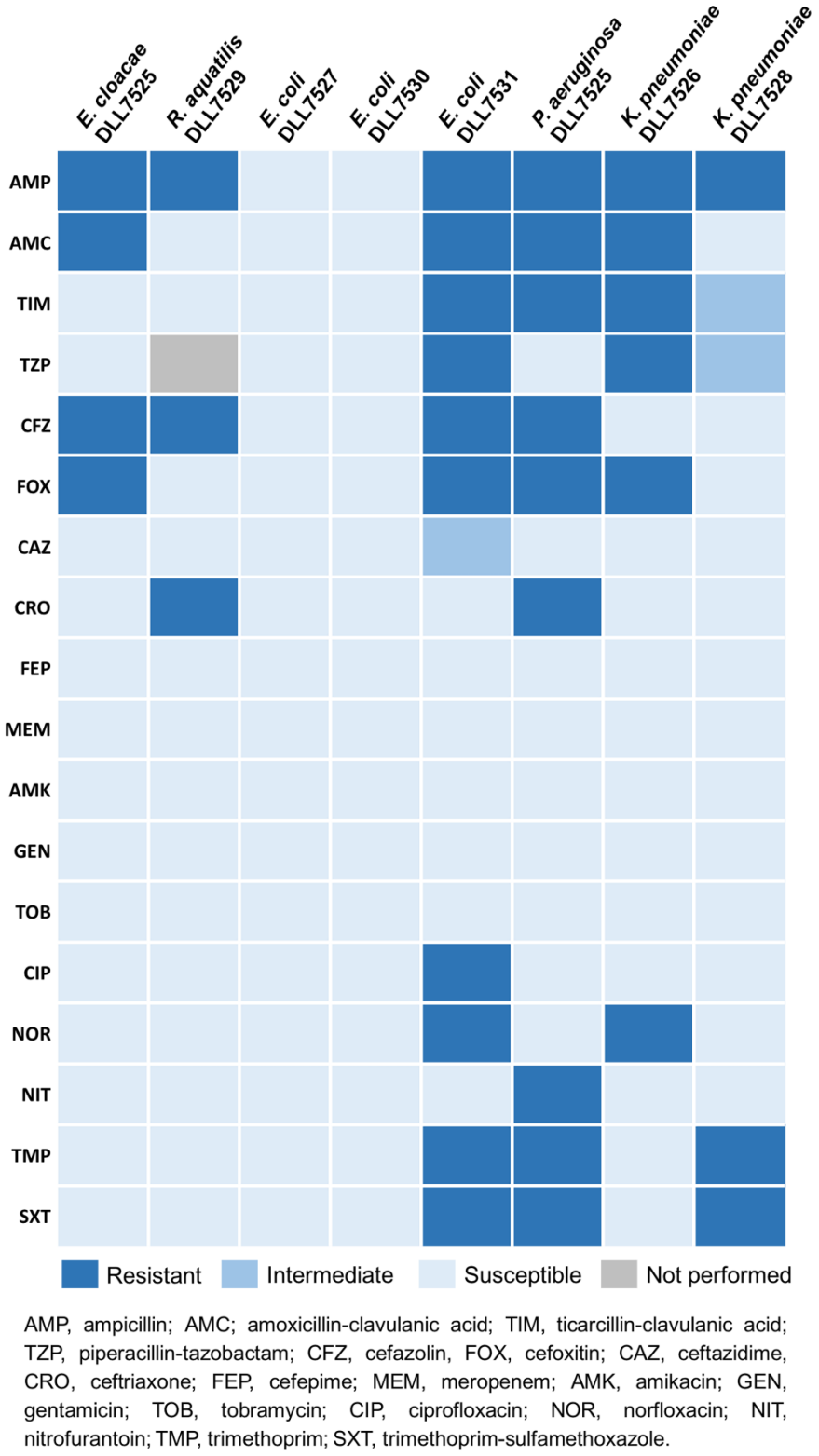

Figure 1. Antimicrobial susceptibility testing of clinical AAD isolates. Antimicrobial susceptibility was determined for each isolate using the VITEK 2 system and MICs were interpreted using EUCAST breakpoints. For full MIC results, see Supplementary Table 1.

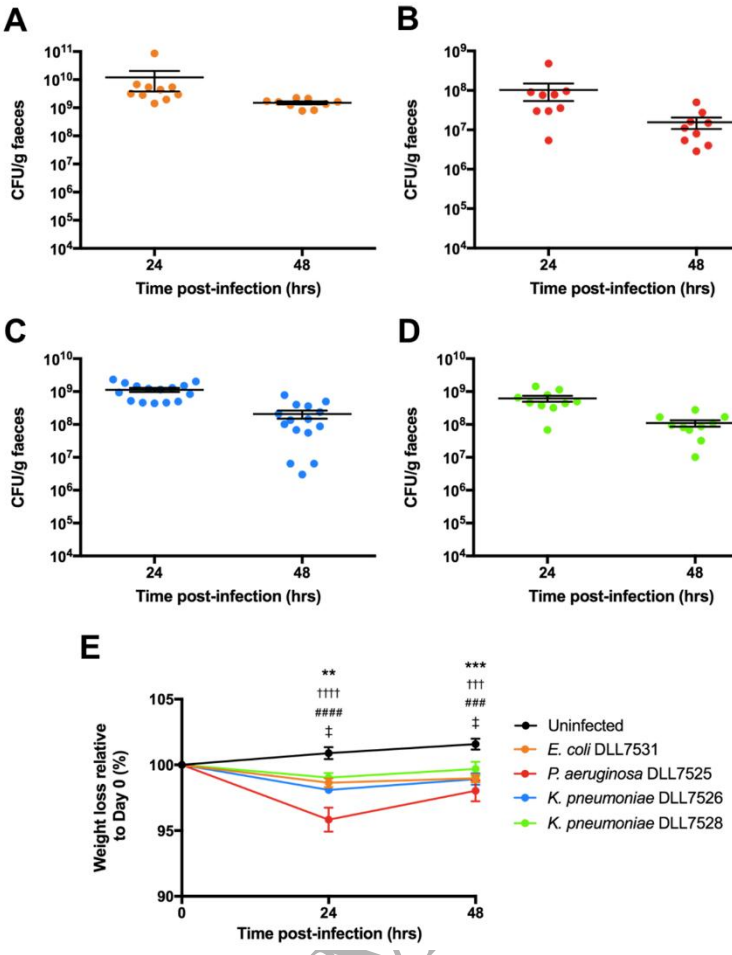

Figure 2. Faecal shedding (A-D) and weight loss (E) of mice infected with clinical AAD isolates. Faecal samples were collected at 24-hour intervals from mice inoculated with **A.** *E. coli* DLL7531, **B.** *P. aeruginosa* DLL7525, **C.** *K. pneumoniae* DLL7526, and **D.** *K. pneumoniae* DLL7528. Faecal shedding of each strain is presented as CFU/g of faeces. Error bars = Mean ± SEM; n = 9-15 mice per group. **E.** Uninfected and infected mice were weighed prior to inoculation and at 24-hour intervals post-infection. Weight loss is presented as a percentage loss relative to weight on the day of infection (day 0). Error bars = Mean ± SEM; n = 9-15 mice per group. Uninfected vs. *E. coli* DLL7531-infected = $^{**}p\leq0.01$, $^{***}p\leq0.001$; uninfected vs. *P. aeruginosa* DLL7525-infected = $^{\dagger\dagger\dagger}p\leq0.001$, $^{\dagger\dagger\dagger\dagger}p\leq0.0001$; uninfected vs. *K. pneumoniae* DLL7526-infected = $^{\#\#\#}p\leq0.001$, $^{\#\#\#\#}p\leq0.0001$; uninfected vs. *K. pneumoniae* DLL7528-infected = $^{\ddagger}p\leq0.05$.

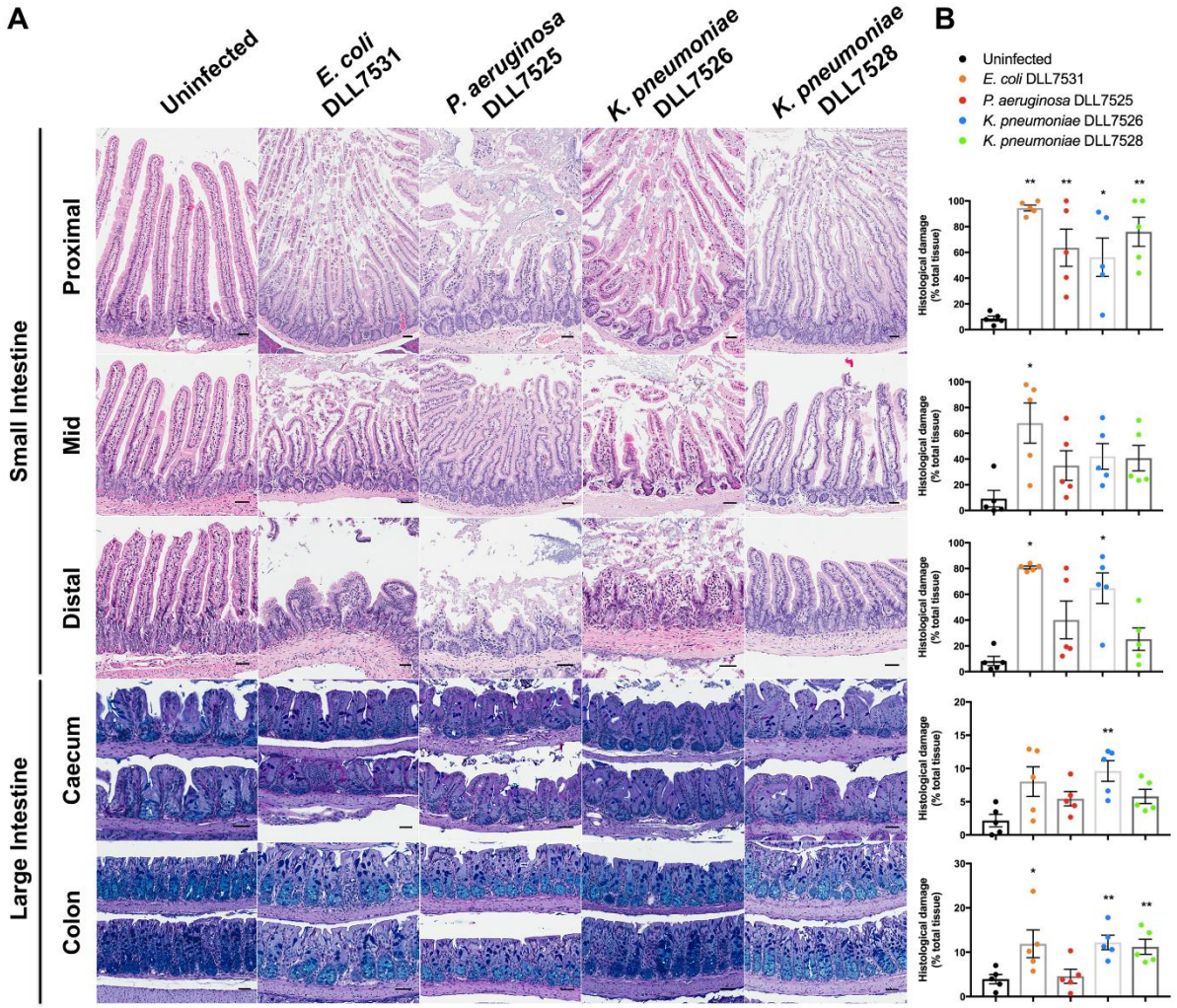

Figure 3. Histological damage of small and large intestine following 48 hours of infection with clinical AAD isolates. **A.** Small intestine (proximal, mid, and distal) and large intestine (caecum and colon) was collected from uninfected mice, and mice inoculated with either *E. coli* DLL7531, *P. aeruginosa* DLL7525, *K. pneumoniae* DLL7526, or *K. pneumoniae* DLL7528. Small intestine was stained with H&E and large intestine with PAS/AB. Scale bar = 50 μm. **B.** Histological damage was scored by measuring the length of each tissue, followed by measurements of areas showing histological damage, presented as a percentage of total tissue damaged. Error bars = Mean±SEM; n = 5 mice per group. *p<0.05, **p<0.01.

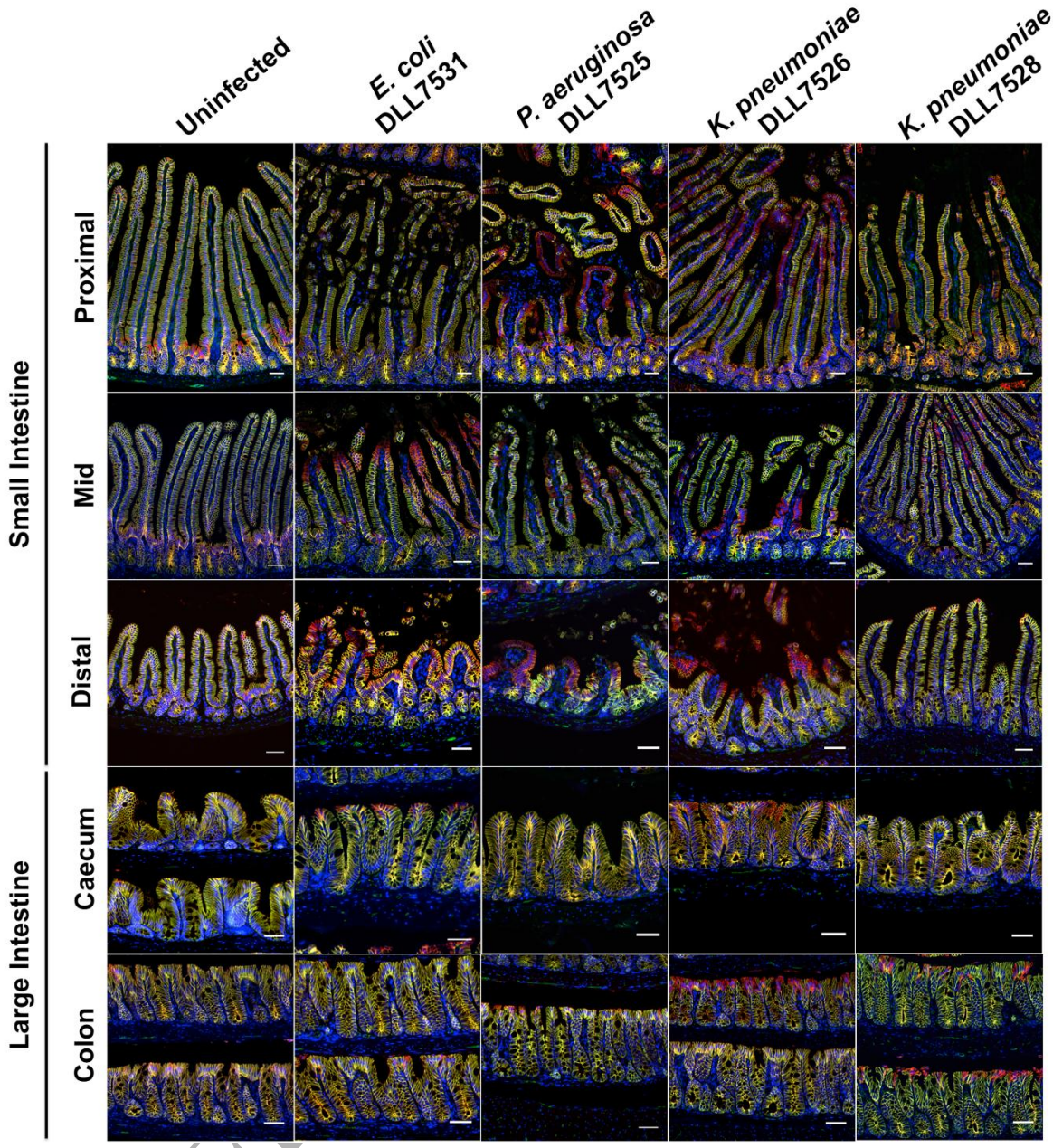

Figure 4. Detection of tight-junction proteins E-cadherin and β-catenin in the small and large intestine following 48 hours of infection with clinical AAD isolates. Small intestine (proximal, mid, and distal) and large intestine (caecum and colon) was collected from uninfected mice, and mice inoculated with either *E. coli* DLL7531, *P. aeruginosa* DLL7525, *K. pneumoniae* DLL7526, or *K. pneumoniae* DLL7528. All tissues were immunostained with E-cadherin (red), β-catenin (green), and counterstained with DAPI (blue). Co-localisation of E-cadherin and β-catenin shows as yellow. Scale bar = 50 μm.

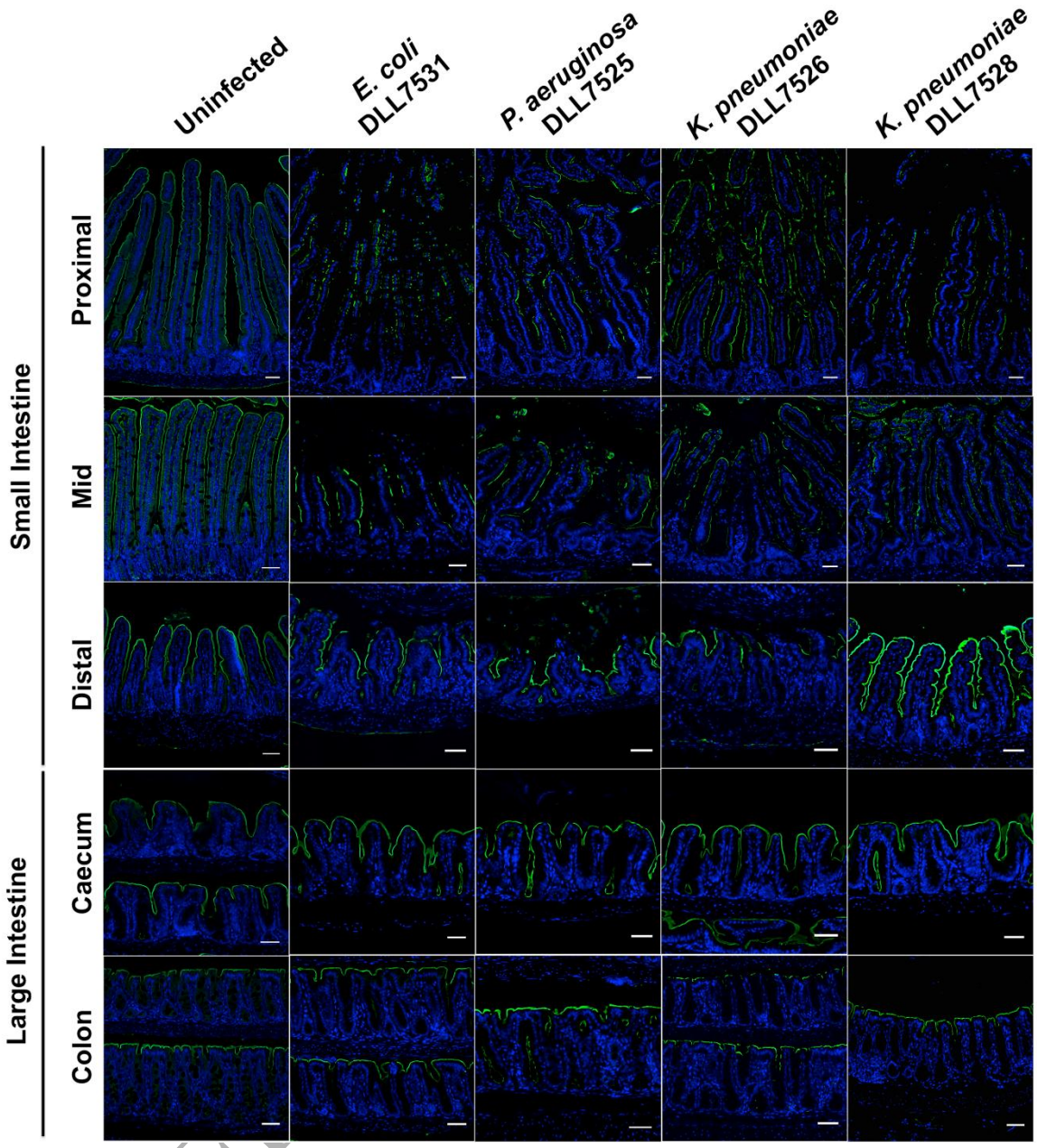

Figure 5. Detection of cellular polarity marker ezrin in the small and large intestine following 48 hours of infection with clinical AAD isolates. Small intestine (proximal, mid, and distal) and large intestine (caecum and colon) was collected from uninfected mice, and mice inoculated with either *E. coli* DLL7531, *P. aeruginosa* DLL7525, *K. pneumoniae* DLL7526, or *K. pneumoniae* DLL7528. All tissues were immunostained with ezrin (green) and counterstained with DAPI (blue). Scale bar = 50 μm.

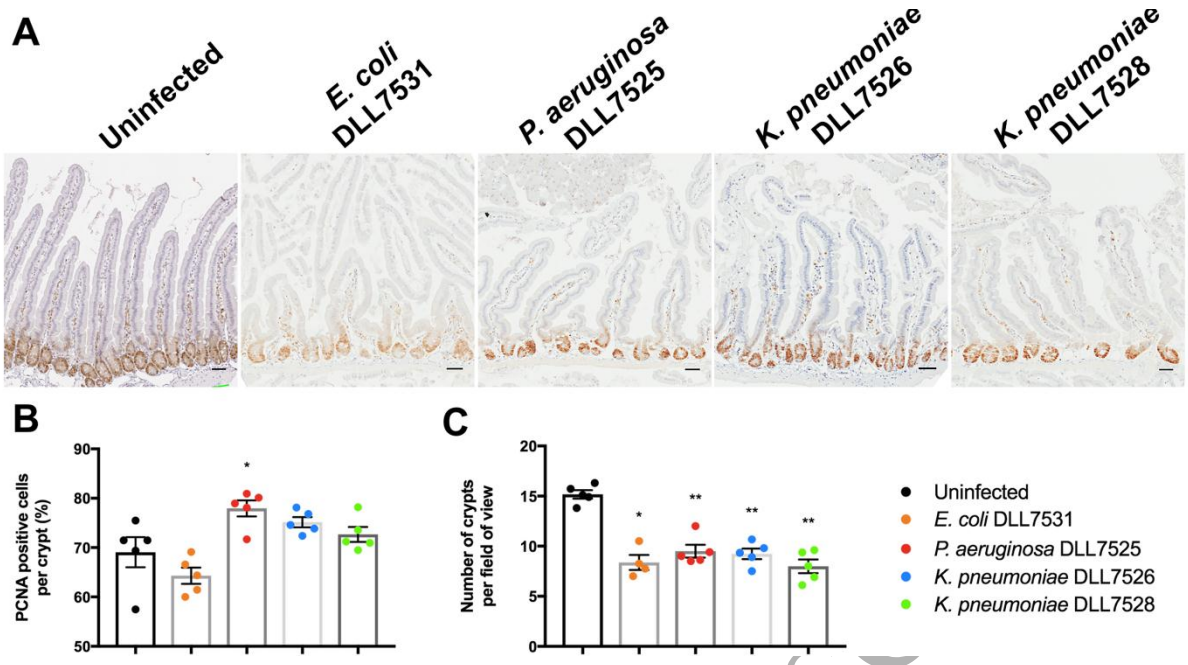

Figure 6. Detection of cellular proliferation marker PCNA in the small and large intestine following 48 hours of infection with clinical AAD isolates. **A.** Proximal small intestine was collected from uninfected mice, and mice inoculated with either *E. coli* DLL7531, *P. aeruginosa* DLL7525, *K. pneumoniae* DLL7526, or *K. pneumoniae* DLL7528.All tissues were immunostained with PCNA (brown) and counterstained with haematoxylin (blue). Scale bar = 50 μm. **B.** Cellular proliferation was quantified by counting the number of PCNA positive and negative cells per crypt for 30 crypts per mouse, and is presented as the percentage of PCNA positive cells per crypt. **C.** Crypt density was quantified by counting the number of crypts in a defined field of view for uninfected mice in comparison with fields of view from infected mice where histological damage was observed, and is presented as the number of crypts per field of view. Error bars = Mean±SEM; n = 5 mice per group. *p<0.05, **p<0.01.