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Pathogenic potential and virulence genotypes of intestinal and faecal isolates of porcine post-weaning enteropathogenic *Escherichia coli*

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) are frequent causes of diarrhoea in infants and in young mammals by inducing attaching effacing (AE) lesions of the intestinal epithelium. EPEC bacteria have also been implicated in cases of porcine post-weaning diarrhoea but their pathogenicity for conventional weaned pigs remains less elucidated.

This present study investigates differences in pathogenic potential and virulence genotypes of intestinal and faecal isolates of EPEC from newly-weaned pigs. For this we inoculated ligated ileal loops of four weeks old weaned pigs to assess EPEC adherence to enterocytes by histology and immunohistology. Virulence gene patterns were identified by using a PCR-microarray. Intestinal EPEC isolates of sero-/intimin types O45:H11:*eae-β,* O49:NM:*eae-β*, O84:H7:*eae-γ*, and O123:H11:*eae-β* formed adherent microcolonies of EPEC with AE lesions on ileal villi more frequently than faecal isolates of O28:H28:*eae-NT*, O108:H9:*eae-β,* O145:H28:*eae-γ* and O157:H2:*eae-β* ($p \leq 0.05$). The PCR-array analysis of both groups detected all together 25 virulence genes of LEE (Locus of Enterocyte Effacement), and of non-LEE pathogenicity islands, of plasmids and phages characteristic to EPEC. Intestinal isolates carried significantly more virulence genes than faecal isolates ($p \leq 0.05$). Intestinal isolates possessed *efa1, lpfA*, and *tsh* genes most likely contributing to enterocyte adhesion while faecal isolates did not carry these genes ($p \leq 0.05$). Overall, the ileal loop model in weaned pigs combined with virulence genotyping PCR-array indicated a greater pathogenic potential of intestinal isolates over faecal isolates of porcine post-weaning EPEC. Differing virulence genotypes of the intestinal and faecal isolates as demonstrated here suggests dynamic evolutionary events within the population of porcine EPEC.

Keywords: porcine post-weaning EPEC, faecal/intestinal isolates, ileal loop, PCR-microarray

1. Introduction

Colonization of the intestine and adhesion to the intestinal epithelial cells is the first step in pathogenesis of diarrhoeagenic *E. coli*. One specific feature of adhesion is the intimate bacterial attachment to intestinal villous epithelium by enteropathogenic *E. coli* (EPEC) and by enterohemorrhagic *E. coli* (EHEC), both being responsible for severe diarrhoeal diseases worldwide. EPEC infections most commonly induce watery diarrhoea of infants and young children in the developing world, while EHEC most frequently causes hemorrhagic colitis of all age groups through Shiga-like toxins (Nataro and Kaper, 1998 and Kaper et al., 2004). Typical EPEC strains possess a large virulence plasmid known as the EPEC adhesion factor (EAF) plasmid encoding the bundle-forming pilus (Bfp) to mediate initial adherence to the intestinal epithelial cells while atypical EPEC do not possess Bfp. Typical EPEC strains are pathogenic for humans and have not been found in animals while atypical EPEC and EHEC are known as pathogenic for humans and for young animals. For atypical EPEC, both animals and humans can be reservoirs. Atypical EPEC is more closely related to EHEC and both groups seem to be emerging pathogens (Trabulsi et al., 2002 and Tozzoli and Scheutz, 2014).

EPEC and EHEC share a common mechanism of binding to the intestinal epithelium and injecting virulence proteins into the host cells through their type III secretion system (T3SS) resulting in “attaching and effacing” (AE) lesions of the infected enterocytes. AE lesions are characterized by the localized attachment of bacteria to the enterocytes with effacement of microvilli and cytoskeletal changes, including the accumulation of polymerized actin, leading to the formation of a pedestal-like structures underneath the adherent bacteria (Moon et al., 1983, Knutton et al., 1989, Frankel et al., 1998, Vallance and Finlay, 2000 and Chen and Frankel, 2005). This intimate attachment is essential for EPEC/EHEC pathogenicity with two key bacterial proteins: the outer-membrane adhesin molecule intimin (Eae) and its translocated intimin receptor (Tir) acting in harmony in order to cause AE lesion and disease

in both humans and animals (Frankel et al., 2001). Subtypes of intimin and Tir are known to be responsible for the host-, and tissue tropism of certain EPEC (Oswald et al., 2000, Zhang et al., 2002 and Lacher et al., 2006). Chromosomal genes encoding intimin and Tir of EPEC are located on a 35.6 kb pathogenicity island named as locus of enterocyte effacement (LEE). Further genes of the LEE are related to the T3SS. The effectors of T3SS known as EPEC secreted virulence proteins such as EspA, EspB, EspD and EspF are also encoded on the LEE and are essential for protein translocation and subversion of host cell signal transduction pathways to form AE lesion (McDaniel et al., 1995, Elliott et al., 1998, Kaper, 1998 and Chen and Frankel, 2005). In addition to the effectors encoded by the LEE, there are a number of non-LEE-encoded effector genes located mostly on prophage-associated elements and contributing to the intestinal pathology of EPEC infections in man and in animals (Deng et al., 2004, Schwidder et al., 2011 and Mainil and Fairbrother, 2014). They subvert further host cell signalling pathways during infection such as inhibition of apoptosis (NleH and NleD), interference with inflammatory signalling pathways (NleB, NleC, NleE and NleH) or with phagocytosis (EspJ) (Wong et al., 2011). In case of cycle inhibiting factor (Cif), there is an interference with the proliferation of eukaryotic cells (Samba-Louaka et al., 2009).

As stated above, atypical EPEC may produce diarrhoeal diseases in different young mammals, like calves, pigs, lambs, and dogs (Janke et al., 1989), as reviewed byTrabulsi et al. (2002), and by Mainil and Fairbrother (2014). Pathogenicity and AE lesion producing capacity of animal EPEC has been experimentally proven in rabbits (Cantey et al., 1981, Moon et al., 1983, Milon et al., 1990, Milon et al., 1999 and Dow et al., 2005), mice (Savkovic et al., 2005 and Dupont et al., 2016), and in newborn colostrum deprived pigs (Moon et al., 1983, Helie et al., 1991 and Zhu et al., 1994) as well as in severely immunosuppressed weaned pigs (Girard et al., 2005). However, porcine EPEC could only

colonize approx. 20% of intragastrically infected conventional weaned pigs (Malik et al., 2012).

Here we aimed to replace the weaned pig oral infection model by ligated ileal loop infection experiments on four weeks old weaned pigs in order to investigate differences in pathogenic potential between intestinal and faecal strains of EPEC isolated by us earlier from newly-weaned pigs (Malik et al., 2006a). Furthermore we aimed to study the relationships between molecular characteristics and enterocyte-adhesion in the above two groups of porcine intestinal and faecal EPEC by using a PCR-microarray for of *E. coli* virulence genes. As a result, we have shown significantly more frequent bacterial adhesions with ileal AE lesions induced by intestinal than by faecal isolates. In harmony with these findings we found significantly more adhesion related virulence genes among intestinal isolates than among faecal counterparts indicating different degrees of pathogenic potential of intestinal versus faecal post-weaning EPEC in pigs.

2. Materials and Methods

2.1. Bacterial strains

EPEC bacteria were isolated between 2001-2004 from the intestine of five pigs that died due to post weaning diarrhoea and from faecal samples of one diarrhoeal and three non-diarrhoeal weaned pigs of eight geographically distant "farrow-to-finish" type Hungarian farms (Malik et al., 2006a). All together 10 independent EPEC strains were studied here including six intestinal isolates and one faecal isolate representing diarrhoeal weaned pigs and further three faecal isolates from clinically normal weaned pigs. The farms represented by these EPEC strains reported post weaning diarrhoea. The intestinal EPEC strain # 86-1390 (O45, *eae-β*) (Zhu et al., 1994) was also included as a reference porcine post-weaning EPEC. This strain was provided by Dr. J.M. Fairbrother (University of Montreal, Faculty of

Veterinary Medicine, Quebec, Canada). Non-pathogenic *E. coli* strain 123 (O43:H28), isolated from the intestine of a healthy piglet was used as a negative control (Moon et al., 1968). This was provided by Dr. H.W. Moon (USDA, ARS, National Animal Disease Center, Ames, Iowa, USA). Serotypes and origin of isolation of the EPEC strains are listed in Table 1. The EPEC strains were stored at –70 °C in Tryptic Soy Broth (TSB) containing 15% glycerol.

2.2. Infection of ligated ileal loops of weaned pigs

The *in vivo* pathogenicity of strains was assessed in ligated ileal loops of healthy four weeks old freshly weaned healthy pigs purchased from clinically healthy groups of two unrelated commercial farms without any major diarrhoeal disease history. Licences for these experiments were issued by the Veterinary and Food Control Office, Budapest, Hungary (No.25-4-2/2000 and 1249/002/2004). Prior to surgery, the pigs were kept in conform, supplied with drinking water but deprived from food for 24 hours and their faeces were sampled for EPEC as described earlier (Malik et al., 2006a). For abdominal surgery, pigs were premedicated with a combination of diazepam (1 mg/kg body weight) (Seduxen, inj., Richter Gedeon RT, Budapest, Hungary), and xylazine (4 mg/kg body weight) (Primazin 2% inj., Alfasan, Woerden, The Netherlands). Approximately 10 minutes later, anaesthesia was achieved with ketamin (2 mg/kg body weight) (CP-Ketamin 10%, Cp-Pharma, Burgdorf, Germany) and maintained by inhalation of halothan (Narcotan, Léciva, Praha, Czech Republic) using an evaporation-inhalation apparatus under constant clinical control during surgery. Right sided laparotomy was performed in deep anaesthesia under sterile, surgical conditions. Ileal test loops were 10 cm long, separated from each other by 5 cm long non-infected interloops. Ligation of loops started 15-20 cm proximal from the ileocaecal valve.

Bacteria for the loop inoculations were incubated in TSB at 37 °C overnight. Ligated ileal test loops were inoculated with 1.0 ml of this stationary TSB culture with ~ 5×10^8

CFU/ml of EPEC bacteria. All together seven loops were tested for each EPEC strains, prepared in 7 different pigs (i.e. one loop for each strain per animal) in random assignment. Besides, two negative control loops (one inoculated with the non-pathogenic *E. coli* 123 and the other remaining uninoculated) were also prepared in each of the seven pigs.

Surgery was finished by replacement of intestinal segments and closing the abdominal wall. Postsurgical discomfort of pigs was minimised by application of pentobarbital sodium (15-20 mg/kg body weight) (Nembutal inj., Ceva, Paris, France) and by providing additional heat for 16-18 hours as described (Moon et al., 1983 and Nagy et al., 1992). Loop tests were terminated through an intravenous overdose of Nembutal.

2.3. Assessment of pathogenic potential of EPEC by histopathology, immunofluorescence-, electron microscopy

Sections of intestine from the loops were washed 3 times with phosphate-buffered saline (PBS) to remove unattached bacteria and placed for preservation into in 10% formaldehyde. Formalin-fixed tissues were embedded in paraffin for preparation of haematoxylin-eosin (HE) stained sections for light microscopy. Degree of pathogenic potential of the EPEC bacteria was determined by examination of 5 µm thick HE stained sections for adherent bacterial clusters (microcolonies) inducing AE lesions on the intestinal villous epithelium. For this purpose 50 villi in each loop sections were scrutinized for adherent microcolonies (bacterial clusters). The percentage (%) of these 50 villi per loop showing adherent microcolonies inducing AE lesions was determined as an adhesion score (AS) of the inoculated EPEC in the given loop. Identification of the adherent EPEC bacterial strains was done on indirect immunofluorescence stained frozen sections of the same loops using homologous OK sera raised against the inocula strains in rabbits (Edwards and Ewing, 1972). For this, intestinal tissues were embedded into methylcellulose immediately after

washing and frozen in dry ice. Cryostate sections of frozen samples were fixed in methanol and subsequently covered by the OK antisera. This was followed by a second reaction using goat anti-rabbit IgG FITC (Sigma-Aldrich) as described for indirect immunofluorescence (Nagy et al., 1992). Sections were washed, counterstained with Evan's blue, and examined under UV light for the presence of fluorescence stained microcolonies of EPEC bacteria attached to the intestinal epithelium. For transmission electron microscopy ileal loops inoculated with representatives of EPEC O45 (strain # 86-1390) and of EPEC O123 (strain # 17) bacteria were selected to provide glutaraldehyde-fixed tissues. Samples were prepared by standard techniques for toluidin blue stained 1 µm thick sections for selection of adherent bacterial microcolonies and AE lesions by light microscopy. Ultrathin sections of the selected foci were stained by uranyl acetate and lead citrate for electron microscopy, to study ultrastructural characteristics of the AE lesions, using a Hitachi H7100 electron microscope as described (Moon et al., 1983).

2.4. Identification of virulence genotypes of porcine EPEC by PCR-microarray

Previous to the present studies a basic virulence genotyping of these EPEC strains has been performed. This included detection of genes *espD* (Goffaux et al., 2000) and *paa* (Batisson et al., 2003) and determination of subtypes of *eae* genes (from *eae-*α to *eae-*θ) (Schmidt et al., 1993, Oswald et al., 2000 and Zhang et al., 2002) by PCR as reported earlier (Malik et al., 2006a). Here we further tested these strains for the presence of 62 virulence genes of *E. coli* by using the Ec03 PCR-microarray Pathotyping Kit (Alere Technologies GmbH, Jena, Germany). Microarray probes were specific to identify the main virulence genes of pathogenic *E. coli*, majority of them encoding T3SS effectors, toxins, fimbria and other adhesins and SPATE (serine protease autotransporters of *Enterobacteriaceae*) proteins respectively (Anjum et al., 2007). The array also contained probes for seven colicin and

microcin genes which were, however not regarded as virulence genes and therefore not included in this report. The PCR-microarray assay was performed according to the manufacturer's instructions, and array spot signals were measured and evaluated by the use of IconoClust 2 software (CLONDIAG) as described earlier (Szmolka et al., 2012).

2.5. Statistical analysis

For statistical analysis of data obtained, the unpaired Student's *t*-test was used. Analysis of data aimed at differences of adhesion scores (determined in the ileal loop sections as described in Materials and Methods), and prevalence of virulence genes between the groups of intestinal and faecal EPEC, as well as relationships of specific virulence genes with increased adhesion scores. The minimum degree of probability indicating significant difference was $p \leq 0.05$.

3. Results

3.1. Adhesion of porcine intestinal and faecal EPEC to ileal enterocytes in ligated ileal loops

Histologic study of HE sections of ileal loops inoculated with the intestinal EPEC isolates detected attaching bacterial microcolonies with AE lesions of the infected enterocytes in 6-7 loops per strain for the intestinal EPEC strains (90.4% of the test loops). In contrast faecal isolates produced attaching microcolonies in only 2-3 of the seven loops per isolate (39.3% of the test loops positive for faecal isolates) (Fig. 1). The mean adhesion scores of the EPEC strains (determined on the basis of 50 villi/loop investigated), varied between 16.6 - 71.1% (high to medium low) for the intestinal, and 1.4 – 19.4% (medium low to very low) for the faecal isolates. Statistical analysis showed significantly higher adhesion scores (%) for intestinal isolates than for faecal isolates ($p \leq 0.05$) (Fig. 1).

Fluorescence microscopy of indirect immunofluorescence stained cryostate sections of the EPEC inoculated loops validated the above results in 100% of the AE positive loops by specific staining of adherent microcolonies of EPEC bacteria on the surface of villi (not shown). In toluidin blue stained 1µm sections of adherent microcolonies of EPEC, the bacteria were seen closely embedded into the microvilli of enterocytes showing AE lesions (Fig. 2A). Transmission electron microscopy has shown the ultrastructure of typical AE lesions developing in the EPEC inoculated loops. These ultrastructural characteristics were the intimate bacterial adherence to enterocytes, effacement of microvilli, and formation of pedestal-like structures underneath the adherent bacteria (Fig. 2B). None of the negative control loops have shown adherent bacterial clusters on the intestinal villi in any of the seven pigs used for these studies confirming that they were free from subclinical EPEC infection.

3.2. Virulence genotypes of intestinal and faecal isolates of porcine EPEC

Virulence gene PCRs and PCR-microarrays array on the 10 porcine EPEC isolates detected the presence of a number of LEE and non-LEE related genes, as well as phage and plasmid mediated genes for T3SS effectors or SPATE autotransporter proteins conferring virulence in both intestinal and faecal isolates, however in an uneven distribution (Fig. 3). From the total of 64 targeted virulence genes (62 by the array plus *espD* and *paa* by previous PCR) 25 were detected in the EPEC strains tested. The intestinal isolates contained significantly more virulence genes than the faecal isolates ($p \leq 0.05$).

As shown in Figure 3, most of the LEE and and non-LEE regulated genes (*eae, tir, espA, espD, espF, nleA, nleB* and *espJ*) were detected almost equally in both groups. The genes located on LEE conferring virulence and development of AE lesions such as *eae* and *tir* were identified in all strains. Based on the presence of *espA,D* and *F* genes in almost all porcine EPEC strains seemed to have a complete LEE. Exceptions were: one intestinal and

two faecal isolates without *espB* gene, both of them carrying *eae-γ* or *eae-NT* (not typable). The lack of *espB* and the type of *eae* was not associated with a significant difference in adhesion to enterocytes (p= 0.22). The virulence genes, *nleC, cif, sepA, paa, iss* and *astA* were more frequent among intestinal EPEC, but this difference was not significant and their presence was not associated with significant differences in the enterocyte adhesion (p= 0.13 – 0.24). However, the genes *efa1*, *lpfA* and *tsh* were only detected among intestinal EPEC and not among faecal EPEC. The presence of these three genes was associated with significantly increased adherence/attachment of the intestinal EPEC strains carrying them ($p \leq 0.05$). These results suggested the potential role of *efa1* (EHEC factor for adherence), *lpfA* (fimbrial subunit gene of long polar fimbria), and *tsh* (temperature sensitive haemagglutinin) genes in the increased *in vivo* adherence/attachment of these intestinal EPEC in weaned pigs.

It was noted that bacteria of two intestinal EPEC strains (# 17, O123 and #31, O49) contained the EAF plasmid encoded regulator gene (*perA*) known to be characteristic for typical EPEC occurring only in human patients. It should also be mentioned that the only porcine EPEC of this collection that possessed the plasmid encoded enterotoxin gene (*pet*) was the faecal EPEC isolate # 72 (O157:H2) from a diarrhoeal weaned pig, and showed a relatively increased intestinal adherence (Fig. 1, Fig. 3).

4. Discussion

Enteropathogenic *E. coli* (EPEC) in weaned pigs has been reported in several studies with and without post weaning diarrhoea (Janke et al., 1989, Helie et al., 1991, Zhu et al., 1994 and Krause et al., 2005). So far the differences in pathogenic potential of porcine post-weaning EPEC have not been studied in conventional weaned pigs. The pathogenicity of EPEC # 86-1390 (O45) was experimentally proven in colostrum-deprived newborn pigs (Helie et al., 1991), but for several physiological reasons such newborn pigs can not be a

suitable model for EPEC infection of weaned pigs. An oral infection model for porcine EPEC using immunosuppressed weaned pigs has been reported by Girard et al. (2005). In this model they used the porcine EPEC #86-1390 (O45) with application of highly immunosuppressive doses of dexamethasone for seven days. Recently we reported oral infection of conventional weaned pigs using the same EPEC # 86-1390 (O45) strain and other, well characterized Canadian and Hungarian porcine post-weaning EPEC of serogroups O45, O123 and O145 with moderate predisposing factors. These factors were: therapeutic dose of dexamethasone with the mycotoxin fumonisine FB1, or enterotoxigenic *E. coli* (with or without low virulence transmissible gastroenteritis virus), as well as increasing feed protein by soybean meal. Under those conditions we could only detect colonization by EPEC in 18.5 % of the 54 experimentally infected weaned pigs indicating that this model was not adequate for comparing pathogenicity of different porcine post-weaning EPEC strains (Malik et al., 2012).

As no appropriate weaned pig model for EPEC infection has been described so far, here we aimed to develop an alternative *in vivo* model using ligated ileal loops in weaned pigs. In spite of wide ranges of biological variations, overall this system allowed us to assess and compare the *in vivo* adhesion and AE properties of intestinal and faecal isolates of porcine post-weaning EPEC strains. The intestinal isolates of porcine EPEC strains of serogroups O45, O49, O84 and O123 representing cases of post-weaning diarrhoea have shown adhesive properties to the ileal epithelium and produced AE lesions significantly more frequently than the faecal isolates ($p \leq 05$). These observations were confirmed by immunohistology, and in case of one O45 and one O123 intestinal EPEC strain by ultrastructural studies as well. The AE lesions produced by the porcine EPEC strains were highly similar to those shown in earlier studies by human EPEC and EHEC strains (Staley et al., 1969, Knutton et al., 1989, Helie et al., 1991, Booher et al., 2002, Dean-Nystrom et al., 2003 and Yin et al., 2009). Thus, the ligated ileal loop model in four weeks old weaned pigs proved to be suitable for

examining the pathogenic potential of porcine EPEC strains and to differentiate between intestinal and faecal strains.

Diverging virulence potential of these two groups of porcine EPEC has been confirmed by the *E. coli* virulence microarray showing that the intestinal isolates carried significantly more virulence genes than their faecal counterparts ($p \leq 0.05$). LEE and and non-LEE regulated genes such as (*eae, tir, espA, espD, espF, nleA, nleB* and *espJ*) were detected equally or almost equally in groups of intestinal and faecal EPEC. However, the non-LEE effector gene *efa1* adhesin (encoded on pathogenicity island OI-122) was only present in intestinal isolates in harmony with the observations about the contribution of OI-122 and the gene *efa1* to pathogenicity of human atypical EPEC (Afset et al., 2006 and Bielaszewska et al., 2007). Similar to this, the chromosomal gene *lpfA* and the plasmidic gene *tsh,* so far not known as adhesins of porcine atypical EPEC, were only present in intestinal isolates but not in faecal counterparts. These genes (*efa1*, *lpfA* and *tsh*) were all significantly associated with increased intestinal adhesion ($p \leq 0.05$), indicating their role in enhancing pathogenic potential of these intestinal isolates. Furthermore, an other adhesion related gene *paa* (porcine AE associated gene) was mostly identified in intestinal EPEC, but it's presence could not be statistically linked to an increased *in vivo* adhesion in these experiments, in contrast to the *in vitro* observations made in a pig ileal explant model (Batisson et al., 2003). On the other hand, plasmid regulated serine protease autotransporter gene *pet* was identified in one faecal but in none of the intestinal EPEC strains. The genes *pet* and *sepA* deserve special attention as serine protease autotransporters of enterobacteriaceae (SPATE) contributing to pathogenicity of not only EPEC but also of enteroaggregative *E. coli* (AEEC) and – in case of *sepA* - of *Shigella flexneri* as well (Dutta et al., 2002). They contributed to the diversity of potential virulence genes of these porcine EPEC strains, although their role in this *in vivo* weaned pig model could not be estimated. Finally the presence of gene *perA* on two intestinal EPEC should also

be noted. This EAF plasmid encoded regulator gene is known to activate genes both within the LEE and on the *bfp* operon. Therefore it is known to be characteristic for typical EPEC (Mellies et al., 2007), but recently reported to occur among human isolates of atypical EPEC as well (Teixeira et al., 2015).

Divergence of virulence genotypes of intestinal and faecal isolates, as well as the occasional presence of genes characteristic to typical EPEC (i.e. *perA*) or to other pathotypes of EPEC (i.e. *pet* and *sepA*) are suggesting dynamic events of evolution within the population of porcine EPEC. Such events could also be ongoing between lineages of the same serogroups of EPEC. This is indicated here by a higher ileal loop adherence of the Canadian EPEC O45 strain # 86-1390 than the two Hungarian EPEC O45 strains # 5 and # 27. Indeed, our earlier pulse field gel electrophoresis (PFGE) studies have shown that the Canadian EPEC O45 strain segregated from these two less adherent Hungarian EPEC O45 strains into a different PFGE subcluster (Malik et al., 2006b). Thus, our results were in harmony with those of Bruant et al. (2009) who described two distinct groups of O45 porcine EPEC strains by comparative genomic hybridization and by microarrays, with differences in their pathogenic potential. Further reasons for differing *in vivo* adherence of intestinal and faecal EPEC isolates could be the possible differences in their virulence gene regulations as suggested for certain lineages of EHEC O157:H7 (Abu–Ali et al., 2010). This aspect would need, however further comparative *in vivo* gene expression studies on intestinal and faecal EPEC, which was not in the scope of our present work. Future *in vivo* gene expression studies would also be needed for more understanding of pathogenicity of human atypical EPEC, associated frequently with asymptomatic shedding and showing similar virulence gene patterns as animal atypical EPEC (Hu and Torres, 2015).

5. Conclusions

To our knowledge, this is the first in-depth phenotyping and genotyping study on pathogenic potential of porcine post-weaning EPEC, using ligated ileal loops in four weeks old weaned pigs. Results are suggesting that the intestinal isolates adhered to enterocytes of ileal villi of weaned pigs more frequently than faecal isolates. Intestinal EPEC carried more virulence genes known to contribute to adhesion than faecal isolates. Some of these genes (*efa1, lpfA* and *tsh*) were significantly associated with increased intestinal adhesion indicating their role in enhancing pathogenic potential of porcine post-weaning EPEC. The diverging degree of virulence of the intestinal and faecal isolates together with the great variety of virulence genes is suggesting an ongoing evolution within the population of porcine EPEC strains. Thus, our data are providing some new insights into the differences between pathogenic potential and virulence genotypes of intestinal and faecal isolates of EPEC in weaned pigs.

Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Legends to the Figures

Fig. 1.

Adhesion Scores (AS) as percentage (%) of villi with adherent attaching effacing (AE) bacterial clusters in sections of ligated ileal loops of newly-weaned pigs, inoculated with EPEC bacteria isolated from intestine and from faeces of weaned pigs. AS were determined based on 50 villi/loop investigated by light microscopy of HE sections. .Each strain was tested in one loop per pig, in seven different loops. Ligated ileal loops were inoculated with ~ 5×10^8 CFU of EPEC bacteria/loop.

Fig. 2A.

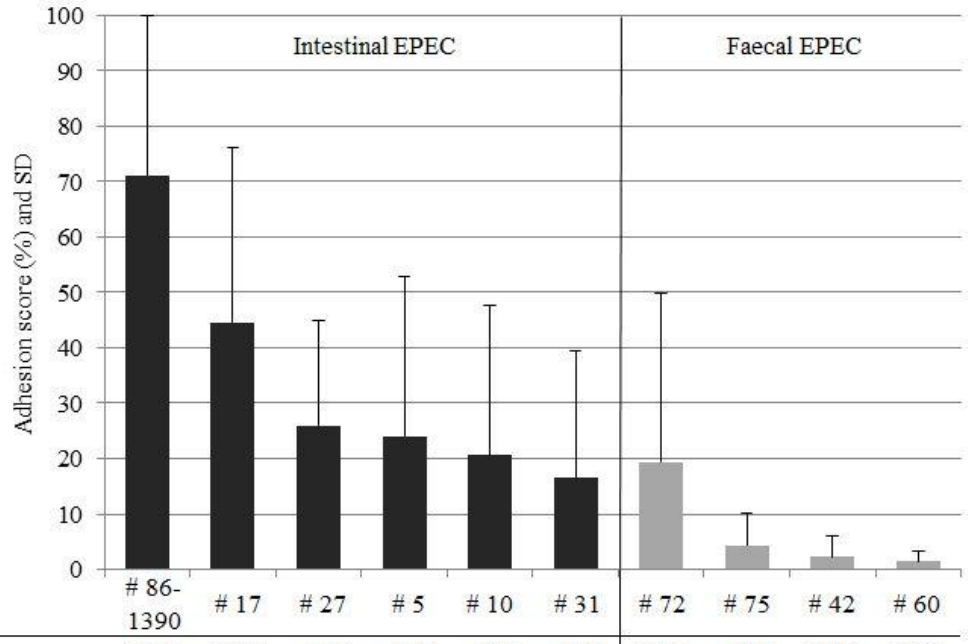
Clusters of adherent bacteria forming microcolonies closely adhering to the villous surface of pig ileal loops inoculated with the EPEC O45:*eae-β* strain # 86-1390. Arrowheads point to adherent bacterial clusters in toluidine blue stained 1 µm section. (Bar = 1 µm)

Fig. 2B.

Transmission electron micrograph of AE lesion in ligated ileal loop of a 4-week-old pig infected with EPEC O45, *eae-β* strain # 86-1390, showing multiple pedestal formations of the enterocyte under the attaching EPEC bacteria, with loss of microvilli. (Bar = 1 µm).

Fig. 3.

Prevalence and distribution of virulence genes detected by the *E. coli* virulence microarray and by PCR in EPEC bacteria isolated from the intestine or faeces of weaned pigs.



	# 86-1390	# 17	# 27	# 5	# 10	# 31	# 72	# 75	# 42	# 60
O groups	O45	O123	O45	O45	O84	O49	O157	O28	O145	O108
Intimin types	*eae-β*	*eae-β*	*eae-β*	*eae-β*	*eae-γ*	*eae-β*	*eae-β*	*eae-NT*	*eae-γ*	*eae-β*
Loops with adherent EPEC	7	7	7	6	7	4	3	3	2	3
Adhesion score, mean (%)	71.1	44.6	26.0	24.0	20.6	16.6	19.4	4.3	2.3	1.4
Adhesion score, range (%)	32-94	2-88	6-58	0-92	2-78	0-66	0-86	0-16	0-10	0-4

Fig. 1

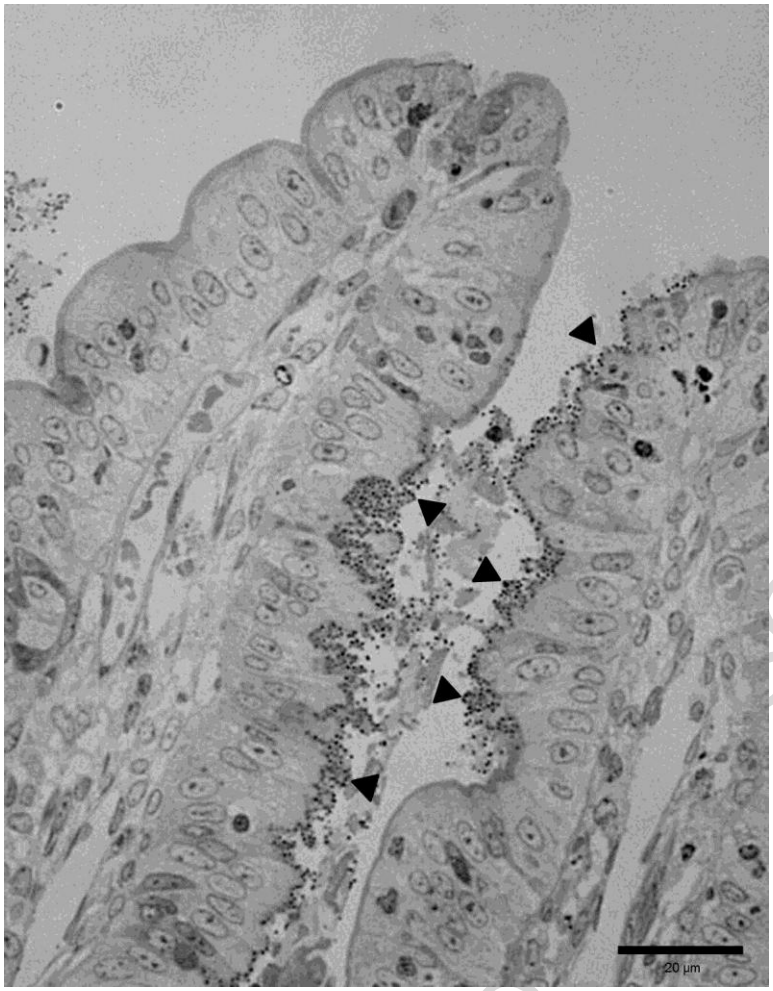


Fig. 2A

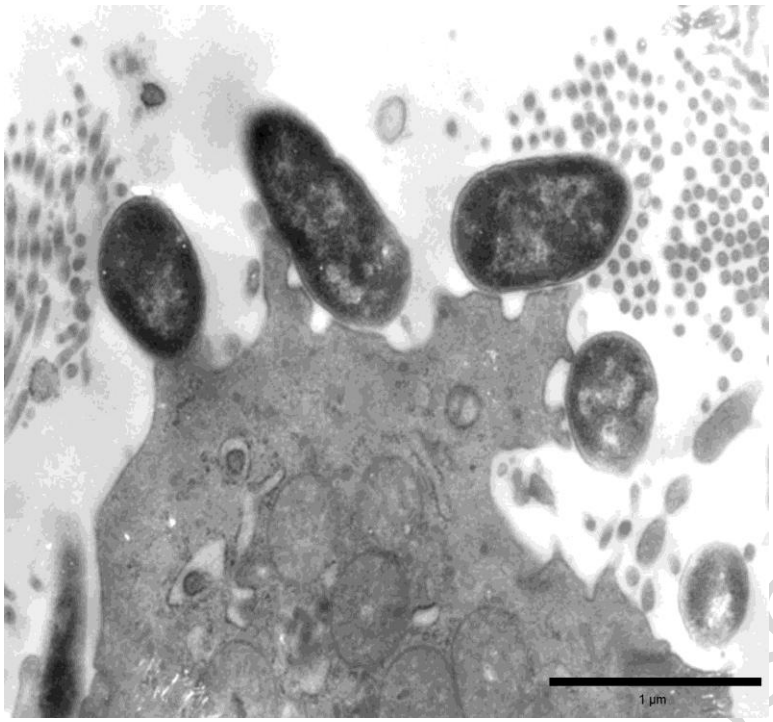


Fig. 2B

Gene	Function	Description	Location	Intestinal EPEC strains # 86-1390 O45	# 17 O123	# 31 O49	# 5 O45	# 27 O45	# 10 O84	Faecal EPEC strains # 75 O28	# 60 O108	# 42 O145	# 72 O157
*eae**	adhesion	intimin	LEE	*eae-β*	*eae-β*	*eae-β*	*eae-β*	*eae-β*	*eae-γ*	*eae-NT*	*eae-β*	*eae-γ*	*eae-β*
tir	adhesion	translocated intimin receptor protein	LEE	■	■	■	■	■	■	▒	▒	▒	▒
espA	T3SS	T3SS effector protein	LEE	■	■	■	■	■	■	▒	▒	▒	▒
*espD**	adhesion	secretion-associated protein EspD	LEE	■	■	■	■	■	■	▒	▒	▒	▒
espB	adhesion	protein EspB	LEE	■	■	■	■	■			▒		▒
espF	T3SS	T3SS effector protein	LEE	■	■	■	■	■	■	▒	▒	▒	▒
nleA	T3SS	non-LEE-encoded T3SS effector A	non-LEE	■	■	■	■	■	■	▒	▒	▒	▒
nleB	T3SS	non-LEE-encoded T3SS effector B	non-LEE	■	■	■	■	■	■	▒	▒	▒	▒
nleC	T3SS	non-LEE-encoded T3SS effector C	non-LEE	■	■	■	■	■				▒	▒
espJ	T3SS	T3SS effector protein	phage	■	■	■	■	■	■	▒	▒	▒	
cif	T3SS	T3SS effector protein	phage	■	■	■	■	■	■	▒	▒		
sepA	SPATE	serine protease sepA precursor	plasmid	■	■	■	■	■	■	▒			▒
efa1	adhesion	putative adherence factor	non-LEE	■	■	■	■	■					
*paa**	adhesion	porcine attaching-effacing associated protein	chromosome	■	■	■	■	■		▒		▒	
lpfA	adhesion	long polar fimbriae	chromosome	■	■	■	■	■					
katP	cell protection	catalase/peroxidase	plasmid	■	■	■	■	■		▒	▒	▒	▒
iss	cell protection	increased serum survival	plasmid	■	■	■	■	■			▒		
tsh	SPATE	temperature-sensitive hemagglutinin; autotransporter	plasmid	■	■	■	■	■					
astA	host damage	heat stable enterotoxin 1	plasmid	■	■	■	■		■			▒	
toxB	host damage	toxin B	plasmid		■	■	■						
tccP	T3SS	tir-cytoskeleton coupling protein	chromosome		■	■				▒	▒	▒	
iha	adhesion	bifunctional enterobactin receptor/adhesin protein	plasmid		■	■							
perA	adhesion	EPEC adherence factor, transcriptional activator	plasmid		■	■							
hlyA	host damage	hemolysin transport protein	plasmid								▒	▒	
pet	SPATE	serine protease pet precursor	plasmid										▒
Total no. of virulence genes				19	23	23	20	18	11	13	14	14	12

* Genes detected earlier by PCR (Malik et al., 2006a)

Fig. 3

Table 1.

Identification, origin of isolation and serotypes of porcine intestinal (I) and faecal (F) isolates of EPEC used for ligated ileal loop inoculation and for PCR-microarray.

Intestinal (I) and faecal (F) isolates of porcine EPEC			Farm of origin
# 5	O45:H11	I	A
# 10	O84:H7	I	B
# 17	O123:H11	I	D
# 27	O45:H11	I	E
# 31	O49:NM	I	F
# 86-1390	O45:NT	I	Canada
# 72	O157:H2	F	M
# 60*	O108:H9	F	C
# 42*	O145:H28	F	C
# 75*	O28:H28	F	S

* EPEC strains isolated from non-diarrhoeal pigs

Hihlights for porcine EPEC: ligated intestinal loops and virulence microarray

- Ileal loops of weaned pigs are suitable for testing in vivo virulence of porcine EPEC

- Ileal isolates of porcine EPEC proved more virulent in loops than faecal isolates

- E. coli microarray detected more virulence genes in intestinal than in faecal EPEC

- Results suggest diverging evolutionary linkages between strains of porcine EPEC