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ESCHERICHIA COLI GENOTYPING AND RELATION TO HISTOPATHOLOGY

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ABSTRACT

Post-wean diarrhea due to *Escherichia coli* continues to be a concern in swine production. Disease is characterized by reduced growth rate, diarrhea, dehydration, toxemia, or death. *E. coli* isolates from nursery pigs submitted to Iowa State University Veterinary Diagnostic Laboratory with diarrhea were characterized. The objective was to determine correlations with colony morphology, presence of virulence genes and histological lesions.

We expected to find virulence genes (toxin or pilus) in smooth/hemolytic colonies. Another expectation was that isolates associated with adherent bacilli on the intestinal mucosa would be positive for pilus genes.

One hundred and three *E. coli* isolates were characterized. Colony morphology was characterized using blood agar and Tergitol-7 media. All isolates were analyzed by multiplex PCR for the presence or absence of toxin and pilus genes. Intestinal mucosa from which the *E. coli* was isolated was examined microscopically for presence or absence of adherent bacilli to the intestinal mucosa.

The morphology of the isolates of *E. coli* on T7 was as follows: smooth (38/103), intermediate (36/103), smooth/mucoid (18/103), mucoid (8/103) and rough (3/103). There were 32/103 hemolytic isolates as determined on blood agar. Twenty-seven of 32 hemolytic isolates were positive for genes. Eighteen of 71 non-hemolytic isolates were positive for virulence genes. Forty-three of all isolates contained genes for at least one toxin gene, with two exceptions; one isolate was positive only for F18 pilus and another positive for K88 and F18. Isolates positive for genes represented each category of morphology. The most common toxin and pilus gene were Stb (33/44 isolates) and FI8 13/44 (isolates); respectively. Fifty-nine isolates did not contain any virulence factors of the multiplex PCR, again represented each category of morphology. Adherent bacilli were present in the intestinal sections associated with nine isolates possessing pilus genes. Nine of *E. coli* isolates did not have pilus genes present; however, there were bacilli present in their respective sections of small intestines histologically.

From this project, it was learned that colony morphology, hemolytic activity, or presence of adherent mucosal bacilli are not greatly predictable of presence or absence of virulence genes in *E.coli* isolated from nursery pigs.

INTRODUCTION

The bacterium *Escherichia coli* is responsible for post-weaning enteric colibacilosis in pigs [1]. This disease is responsible for a lot of loses to the pig industry each year due to mortality, morbidity, low growth rate and treatment of the sick animals [1].

Escherichia coli was discovered by the German bacteriologist *Theodore von Escherich,* in 1885 [2]. There are hundreds of different strains of *E.coli,* some of which are pathogenic, many are just normal inhabitants of intestinal flora of animals and humans. *E. coli* is two microns in length and one micron wide. It is rod-shaped, gram-negative, facultatively anaerobic and covered with small pili. There are two classes pili - sex pili are organelles of drug-resistance and plasmid transfer factors and somatic pili used for mobility and adhesion to vertebrate cells

(colonization and virulence factors) [2]. Using different media most of the *E.coli* population produces gas, ferments dextrose, ferments lactose and is indole positive; variable motility, H_2S and urease negative.

There are two main diseases in post-wean pigs due to *E. coli:* edema disease (ED) and post weaning diarrhea (PWD). ED can occur in both weaned pigs and grower pigs and is characterized by subcutaneous and subserosal edema, a progressive ataxia, paralysis and a high mortality [3]. The hemolytic *E. coli* are most of the strains responsible for both of them [4]. The main etiologic agent causing PWD in pigs is the Enterotoxigenic *E. coli* (ETEC) [5]. Hemolytic enterotoxigenic *Escherichia coli*, has been considered as the main etiologic agent causing PWD for about 4 decades [6].

ETEC strains usually cause disease of 3-- to 6-week old postweaning piglets when the immunity from the colostrum has decreased [7]. PWD, characterized by reduced growth rate, diarrhea, dehydration, toxemia and/ or death, is mostly found in pigs 7-10 days after weaning. In the spontaneous outbreaks caused by an *E.coli* the first manifestation of PWD is sudden death of one or several pigs as early as 2 days after weaning. At the same time feed consumption of affected pigs decreases significantly, and a watery diarrhea develops. The affected pigs become dehydrated and depressed. They eat irregularly, but even in the terminal stage of the disease, they try to drink. Moving around with staggering, uncoordinated movements could also be some of the signs for the disease [8]. The peak of mortality usually occurred 6-10 days after weaning. The delay in disease may be because of missing specific receptors expression in the small intestinal epithelium of young piglets for the F18 fimbriae [7].

These pathogenic bacteria can be transmitted to pigs by way of feed, aerosol, and contact with other pigs or contaminated vehicles (fomites).

Passive protection with immune colostrum has been successful in preventing infections with ETEC strains bearing the classical fimbria types (K88, K99, 987P and F41 are contained in most commercial vaccines), in most of them purified pili are used, providing specific antibodies against the ETEC adhesion factors which stops the disease on its attaching stage [9].

In intestinal *E. coli* there are at least 6 different, classified accordingly their mechanism of action *E.coli*-. Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Enterohemorrhagic (EHEC), Enteroinvasive (EIEC), Enteroaggregating (EAgEC), Attaching and effecing (AEEC) and others. The virulence characteristics of ETEC are strongly dependent on the production of adhesins and enterotoxins. After ingestion the bacteria adhere to the pilus-specific receptors in the pig intestinal epithelium, which must be available for pilus-mediated adherence [10, 11], by means of fimbrial or pilus adhesins. *E. coli* is capable of synthesizing various types of fimbriae that enable the organism to colonize the intestinal tracts of different animal species. The attached bacteria multiply and colonize the intestinal mucosa and then starts producing one or both ST (heat stable) and LT (heat labile) enterotoxins, which act on the epithelial cells and cause them to secrete large volumes of fluids and electrolytes resulting in a cholera-like diarrhea and dehydration [4]. Animal strains produce two major types of ST, designated STa (STI) and STb (STII). The first enterotoxin, which was identified on animal strain is Sta - small molecule of 2.0 kDa. Most of the animal strains also produce Stb a slightly larger ST (5 kDa). This toxin does not activate intracellular nucleotide levels and its mechanism of action is not well known. Actually, ETEC colonization factors (CFs) display remarkable species specificity, and colonization factors are clearly different from those of human and animal ETEC. The LT from animal strains, designated LT1, is similar to the LT produced by human ETEC; however, another variety designated LTII is only found in animals and is not associated with clinical disease. The ETEC CFs genetic control may be in plasmids (K88, K99, F41 or others) or in the chromosome (987P) [12]. At first they were wrongly characterized as capsule antigens, that is why some of them are marked as K - 99 and K - 88. The animal colonization factors are now be-

ing identified by F numbers (because of their fimbrial structure), such as F4, F5, and F6 instead of K88, K99, and 987P respectively [13]. The *E. coli* causing postweaning diarrhea in pigs mostly carry the F4 (K88) or the FI 8 adhesin. Because of the specificity of these adhesins, animal ETEC strains normally do not infect humans.

Different colony morphologies as determined by appearance on the T7, are characterized by smooth, intermediate, mucoid, smooth/mucoid and rough [14]. It is possible that specific morphology may be related with possession of virulence factors in the examined isolate. Additional information such as the populations of other bacteria isolated or if the *E. coli* was isolated in pure, high numbers, can also aid in the association of the recovery of *E. coli* and enteric disease. For further characterization of *E.coli* a lot of methods could be used, but the most recent and useful one is the genotyping used to identify the presence or absence of pilus and toxin genes.

Distal jejunum and proximal ileum are mostly affected by the ETEC strains the bacteria attach to the enterocytes brush border in layers. The adherent bacilli number to the villi varies with the type of adhesin and the receptors availability [7]. After the attachment of the bacteria, the already mentioned ETEC toxins act locally on enterocytes and may cause villous atrophy. Because most ETEC isolates colonize the surface of the mucosa, villous damage is because of hypovolemia, ischemia and circulatory collapse [7]. In pig cases with severe disease a microvascular trombosis could be found in the sections with congested mucosa [15].

MATERIALS AND METHODS

E. coli **strains.** Over one hundred *E. coli* isolated from almost the same number of pigs with PWD from the mid-west United States, were investigated in this study. Small intestinal sections of post-weaning pigs with diarrhea submitted to the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University between were cultured on Tergitol - 7 and blood agar for 24 hours.

Media

Tergitol 7 media with tri phenyl-tetrazolium chloride(T7 with TTC) is used. Lactose positive colonies on this media are yellow and often surrounded by yellow zones in the agar are *E. coli.* On T-7 TTC, hemolytic *E. coli* can be rust colored colonies. Five types of *E. coli* colonies are described on this medium rough, intermediate, and mucoid, smooth and smooth/mucoid, yellow to amber and produce slight yellow zones in the medium [14].

Blood agar. The hemolytic *E. coli* colonies on this media are grey, smooth, shiny and round 2.0-3.Omm.

Kligler's Iron agar is deferential medium for intestinal gram negative microorganisms; it demonstrates the fermentative ability for dextrose and lactose; and ability to produce H_2S. The targeted reactions E. coli were gas production, dextrose and lactose fermentation and negative for the H_2S producton.

SIM slant was used to detect: motility, H_2S production, and indole production. Urea agar was used the urease production to be determined. The needed reaction was negative for urease.

Triple Sugar Agar slant. TSA is a basic nutritive medium used for isolate storage.

All isolates were inoculated, incubated and read by common methods [16].

PCR reagents:

The master mix for *E. coli* mPCR to detect genes for Stb, STa, K99, LT and FI8 contained: 2.8uL Pyrogen free H_20 (Sigma-aldrich), 4,0uL Cresol Red, 2.0uL 10X PCR buffer (Qiagen), 0,4ul dNTP mixture (Qiagen), 0.15ul Primers Stb-1 (20uM), 0.15ul Primers Stb-2 (20uM), 0.15ul Primers STaP-1 (20uM), 0.15ul Primers STaP-2 (20uM), 0.2ul Primers K99-1 (20uM), 0.2ul Primers K.99-2 (20uM), 0.2ul Primers LT-1 (20uM), 0,2ul Primers LT-2 (20uM), 0,2ul Primers F18-1 (20uM), 0.2ul Primers FI8-2 (20uM), per each reaction. To this master mix 0,5ul Taq Polymerase (QIAGEN HotStarTaq™) was added for each reaction.

Master mix for *E. coli* mPCR - to detect genes for 987P, K88, F41 and Stx2e) contained: 3.6uL Pyrogen free H_2O (Sigma-aldrich), 4.0ul Cresol Red, 2.0uL 10X PCR buffer (QIAGEN), 0.4ul dNTP mixture (QIAGEN), 0.05ul Primers 987P-1

(20uM), 0.05ul Primers 987P-2 (20uM), 0.05ul Primers K88-1 (20uM), 0.05ul Primers K88-2 (20uM), O.lul Primers F41-1 (20uM), O.lul Primers F41-2 (20uM), 0.05ul Primers Stx2e-1 (20uM), 0.05ul Primers Stx2e-2 (20uM), per each reaction.

To this master mix l.Oul Taq Polymerase (QIAGEN HotStarTaq™) was added for each reaction.

Table 1 include the primer sequences, the position of the primer in the open reading frame of the gene, and the predicted sizes of amplified products. The primers were designed to have similar annealing temperatures and minimal interactions and resulted in different-sized products [17].

Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). The Taq Polymerase that was used was QIAGEN HotStarTaq™.

Bacterial extraction for PCR:

All isolates were subjected to a polymerase chain reaction (PCR) by the following procedure: bacterial DNA was obtained by suspending bacteria from the TSA slant, in **200MI** distilled, sterile water. The samples were vortexed and boiled at 100°C for 10 min, vortexed again and centrifuged at 13000 rpm/min for 1 min.

Into two 20µ1 tubes was added 11,5µ1 of each master mix for *E. coli* mPCR and appropriate quantity of taq polymerase for each reaction. Into both tubes was added 8.5µ1 of the supernatant of the previously centrifuged bacterial culture. Samples were amplified in an Applied Biosystems 2720 thermal cycler under the following conditions: 10 min at 95°C; 35 cycles beginning with a 30-s denaturation at 94°C, primer annealing at 55°C for 45 s, followed by extension

STb	Stb–1 (Primer) Stb–2 (Primer)	5'-TGCCTATGCATCTACACAAT -3' 5'-CTCCAGCAGTACCATCTCTA-3'	113bp
STa	Sta–1 (Primer) Sta–2 (Primer)	5'-CAACTGAATCACTTGACTCTT-3' 5'-TTAATAACATCCAGCACAGG-3'	158bp
F5 (K99)	K99–1(Primer) K99–2(Primer)	5'-AATACTTGTTCAGGGAGAAA-3' 5'-AACTTTGTGGTTAACTTCCT-3'	230bp
LT	LT–1(Primer) LT–2(Primer)	5'-GGCGTTACTATCCTCTCTAT-3' 5'-TGGTCTCGGTCAGATATGT-3'	270bp
F18	F18–1(Primer) F18–2(Primer)	5'-TGGTAACGTATCAGCAACTA-3' 5'-ACTTACAGTGCTATTCGACG-3'	313bp
F6 (987P)	987P–1(Primer) 987P–2(Primer)	5'-GTAACTCCACCGTTTGTATC-3' 5'-AAGTTACTGCCAGTCTATGC-3'	409bp
F4 (K88)	K88–1(Primer) K88–2(Primer)	5'-GAATCTGTCCGAGAATATCA-3' 5'-GTTGGTACAGGTCTTAATGG-3'	499bp
F41	F41–1(Primer) F41–2(Primer)	5'-AGTATCTGGTTCAGTGATGG-3' 5'-CCACTATAAGAGGTTGAAGC-3'	612bp
Stx2	Stx2e–1(Primer) Stx2e–2(Primer)	5-AATAGTATACGGACAGCGAT-3' 5'-TCTGACATTCTGGTTGACTC-3'	733bp

Table 1 Primer sequences, the position of the primer in the open reading frame of the gene, and the predicted sizes of amplified products.

Procedure.

The small intestinal tissues received from the necropsy floor were plated on T-7 with TTC and blood agar for 24 hours at 37°C, using sterile sticks. After that period colonies from each sample were sub-cultured onto T-7 for 24 hours at 37°C, to obtain a pure culture. Next each isolate was confirmed as *E.coli by* incubating in Kligler's, SIM and Urea agar slants. After identification, the *E. coli* were inoculated on to blood agar to detect the hemolytic isolated. Samples were stored on a TSA slant.

for 1 min 30 s at 72°C. The extension time was ramped for an additional 3 s per cycle a final extension for 10 min at 72°C. Products were electrophoresed in a 2% Ambresco 3:1 agarose gel for 1 hour at 100 V, stained with Ethidium Bromide 2%, and photographed under uv light. Each attempt contained negative controls (negative amplification and negative extraction) with all reagents except template DNA. Also, a positive control was included with bacterial DNA from three strains containing all genes of interest.

All cases except 6, were tested histologicaly. Formalin - fixed intestinal sections for each case were prepared and examined with hematoxilin and eosin stain on histological sections, to determine the presence or absence of adherent bacilli to the intestinal mucosa.

RESULTS:

The *E.coli* isolate morphology as determined on T7 were as follows: smooth (38/103); intermediate (36/103); smooth/mucoid (18/103); mucoid (8/103) and rough (3/103). On the blood agar we found 32/103 hemolytic isolates. Twenty-seven of 32 hemolytic isolates were positive for genes, of these 14 were smooth, 4 smooth/mucoid and 9 intermediate colony morphologies on T7. Eighteen of 71 non-hemolytic isolates were positive for virulence genes, representing the following category of morphologies - Smooth 7/18, smooth/mucoid 5/18, intermediate 5/18 and rough 1/18. Forty-three of all 103 isolates contained genes for at least one toxin gene, with the two exceptions the first isolate being positive only for F18 pilus and the second isolate positive for K88 and FI8. Twenty-three of all positive isolates were positive for pilus genes. Isolates positive for genes represented each category of morphology. The most common toxin genes were Stb (33/44 isolates) followed by LT (18/44) and Sta (17/44). For the pilus genes the most common one was FI8 (13/44) followed by K88 (8/44). The most common gene combinations for the hemolytic isolates was Stb:LT:K88 (4/26) and Stb:Sta: LT: K88 (3/26). For the non-hemolytic group the most common gene combination was Sta:Stb (4/18). During the project two of the genes were not found in the multiplex PCR, in these 103 isolates - 987P and F41. Histologically 18 of the cases were positive for adherent bacilli to the intestinal mucosa. Only nine of twenty-three isolates positive for pilus genes were positive for adherent bacilli to the intestinal mucosa. On the other hand one isolate positive only for toxin genes (STb:LT) and eight isolates negative for all targeted genes had adherent bacilli in the histological sections. Fifty-nine isolates did not contain any virulence factors of the multiplex PCR, again represented each category of morphology as follows: smooth 16/59, intermediate 24/59, smooth/mucoid 10/59, mucoid 7/59, rough 2/59. in the following table there is column which shows is the diagnosis of the case is related with *E.coli.* we added this column because in some cases the *E. coli* isolates are just additional, but not the main reason for the disease.

morphology		# isolates	positive for bacilli	diagnosis related with *E.coli*	Stb	Sta	LT	Stx2e	F18	K88	K99	987P	F41
Hemolytic	smooth	15	5	6	12	5	11	2	8	5	1	0	0
	intermediate	7	2	2	6	1	3	1	3	5	0	0	0
	mucoid	1	0	0	1	1	0	0	1	0	0	0	0
	smooth/mucoid	3	0	1	3	2	2	0	0	1	0	0	0
	rough	0	0	0	0	0	0	0	0	0	0	0	0
Non-hemolytic	smooth	7	1	3	4	1	2	1	1	0	1	0	0
	intermediate	5	2	2	4	2	1	2	1	0	0	0	0
	mucoid	0	0	0	0	0	0	0	0	0	0	0	0
	smooth/mucoid	5	0	1	4	4	1	0	0	0	0	0	0
	rough	1	0	0	0	1	0	0	0	0	1	0	0

Table 2A. Shows all results for the targeted genes and their belongings to the colony morphology, presence for bacilli in the intestinal mucosa and what was the diagnosis of the case.

morphology		# isolates	Positive for bacilli	diagnosis related with *E.coli*
Hemolytic	smooth	2	1	1
	intermediate	3	1	1
	mucoid	0	0	0
	smooth/mucoid	1	0	0
	rough	0	0	0
Non-hemolytic	smooth	14	2	2
	intermediate	21	2	2
	mucoid	7	1	1
	smooth/mucoid	9	1	1
	rough	2	0	0

Table 2B. Shows all results for colony morphology, presence for bacilli in the intestinal mucosa and what was the diagnosis of the case for the isolates without presence of any genes.

DISCUSSION

The objective of the project was to determine correlations between the colony morphology, presence of virulence genes and histological lesions. At the beginning we had some expectations that most of the positive for genes isolates will be with smooth/ hemolytic morphology, and that all of the cases with presence of adherent bacilli in the small intestinal sections will be positive for the pilus genes. Our results were not the same as our expectations; we found that except the smooth/hemolytic morphology, which could be predicted to poses virulence genes, but some of the other 4 colony morphologies were positive both for toxin and pilis genes. We found genes not only in the hemolytic *E.coli,* but also 17 of the non-hemolytic group were positive for the targeted genes. If an isolate was positive for some of the pilus genes and in the histological report was concluded that there was not any adherent bacilli to intestinal mucosa, it is still possible that adherent bacilli were present, but not in the intestinal sections examined. A significant thing for the project was that we found nine isolates negative for pilus genes and positive for adherent bacilli histologically, which means that there may be some other factors which predispose the colonization of the pigs' intestine.

From the column "diagnosis related with *E.coli* we can see that *E.coli* was not the main pathogenic factor for all the cases, virulence factors were detected only in 44 of 103 isolates. Although there were some cases diagnosed as some other disease, we found the targeted virulence genes, which suggest that the ETEC strain may have been contributing to clinical disease.

Two genes (F41 and 987P), were not found because these genes are mostly found in neonate pigs.

From this project, it was learned that colony morphology and hemolytic activity is not greatly predictive of presence or absence of virulence genes in *E.coli* isolated from nursery pigs.

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