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Review Article

Assessment of F41 valence in *E. coli* vaccine strain EC/17. Immunological consequences.

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Interpretative summary

Research confirmed the presence of the F41 antigen in Escherichia coli EC/17 vaccine strain, used in commercial vaccines, and the EC/42 strain used for virulent challenge. The study also pointed out that F4, F5, F6, and F41 genes were present in forty-two European ETEC strains, and found that 23.8% of E. coli strains were positives for both the F5 and F41 fimbria. EC/17 effectively triggered an immune response to F41 in laboratory animals and adult cows. These findings prompted a change in marketing authorizations of some commercial vaccines used in the EU to reduce severe diarrhea from E. coli in newborn calves.

Abstract

This report details the molecular-biological detection of the *Escherichia coli* F41 fimbrial gene in both the vaccine strain *E. coli* EC/17 and the challenge strain *E. coli* EC/42. F41 fimbria production was identified via Polymerase Chain Reaction (PCR) utilizing oligonucleotide probes specific to a region of the gene encoding F41 fimbria. This research first assessed the specificity of the F41 primers through in silico comparative analyses with established genomic databases and evaluates the sequence identity of the targeted *E. coli* F41+ gene against previously published sequences. Additionally, the prevalence of the F4, F5, F6, and F41 fimbrial genes across a collection of forty-two *E. coli* strains is examined, and revealed that F5 and F41 fimbriae occur together in 23.8% of strains. Finally, vaccination was performed to evaluate a commercial inactivated vaccines formulated with *E. coli* strain EC /17. This vaccine was able to evoke an antibody response against both F5 and F41 adhesins in laboratory animals (rabbits) and in adult cows (the target species). These findings support updating the marketing authorization for commercial vaccines (e.g. BOVIGEN® SCOUR, Virbac, France) sold in the European Union. These vaccines can now be recommended for reducing the severity of diarrhea caused by *E. coli* $F5^+$ (K99) and $F41^+$ in newborn calves.

Key words: Neonatal calf diarrhea; ETEC; F41; Adhesin; Fimbria; Vaccine

Abbreviations

a) NCD: Neonatal calf diarrhea

b) ETEC: Enterotoxinogenic Escherichia coli

c) BLAST: Basic Local Alignment Search Tool

d) EMBOSS Cons: European Molecular Biology Open Software Suite; bp: base pairs

Introduction

Currently, the average incidence of perinatal mortality in calves born to cows and heifers varies between 2 and 20% across dairy industries internationally, with the majority of countries between 5 and 8% [15]. In the newborn calf, Neonatal Calf Diarrhea (NCD) is recognized worldwide as one of the biggest challenges for both the beef and dairy industries [12]. Enterotoxinogenic *Escherichia coli* (ETEC), Cryptosporidium parvum, the type-A bovine Rotavirus (RVA) and Coronavirus (BCoV) are usually seen as the most common infectious causes of NCD. ETEC usually only cause secretory diarrhea in the first days of life. Vaccination of the pregnant dam has been shown to upgrade the protective value of colostrum by increasing colostrum titers of specific vaccine-related immunoglobulins, particularly immunoglobulin G [2,6,10,23]. Currently, commercial vaccines for the most important pathogens that cause NCD are available and are intended for vaccination of either the dam or the calf [14]. The virulence features of ETEC are strongly dependent on the production of adhesins (fimbriae) and exotoxins [19]. Without neglecting the role of exotoxins (Heat-Labile [LT], or Heat-Stable Toxins [STa and STb] and Verotoxins [VT]), the first step in the pathogenic process of ETEC is the adhesin–ligand interaction on the small intestinal microvilli, resulting in a morphologically non-destructive attachment of bacteria to the microvilli. The most common adhesive surface antigens of ETEC are fimbria [20], now designed by a capital F followed by a number. It seems that F5 (formerly K99) and F41 are frequently produced simultaneously by bacteria of the same ETEC strain [19]. Although of similar nature (proteinaceous appendages originating from the outer membrane of the bacterial cells.) and characteristics (mannose-sensitive haemagglutining fimbriae), there are different receptors for F5 and for F41. F5 and F41 also differ in their genetic regulation (F5 is regulated by a plasmid while F41 is regulated by a chromosome). Objectives of this work were to characterize the presence of F41 in *E. coli* EC/17 and EC/42 strains used as vaccine antigen in anti-E. coli K99 vaccines, and as challenge strain used to assess vaccine efficacy, respectively, and to check whether immunization with a commercial vaccine formulated with EC/17 elicits an immune response to F41 adhesin in vaccinated animals.

Material and Methods

Use of Animals

The results on laboratory animals (rabbits) were obtained from routine quality control testing of the finished product, as part of the Good Manufacturing Process license of the vaccine manufacturer (Pharmagal, Slovak Republic), and strictly complied with the requirements of the European Pharmacopeia. Vaccination of target animals (cows) was performed as routine vaccination of commercial livestock, motivated by medical necessity.

Study Aims

Two groups of studies were carried out in parallel. On the one hand the objective was to confirm the existence of genes coding for the F41 fimbria by molecular-biological means, in EC/17 and other E. coli strains. On the other hand, the objective was to verify whether EC/17-based commercial vaccines from distinct batches were able to mount an immune response to the F41 adhesin, in laboratory and target animals (cow).

Detection of *E. coli* F41 Fimbria Gene

Identification of production of F41 fimbria was done by PCR using oligonucleotide probes designed by others [13] and specific to a region of gene encoding F41 fimbria. Bacterial DNA was extracted according to the manufacturer´s instructions using QIAamp DNA Mini Kit from 200 µl samples, eluted with 100 µl of elution buffer, and until use, stored at -20°C. After PCR run, the amplicons were sequenced using F41-forward primer (sequence AGTATCTGGTTCAGTGATGG) or F41-reverse primer (CCACTATAAGAGGTTGAAGC). From the same bacterial DNA, real time PCR was performed to evaluate sequences coding for F4, F5 and F6 fimbriae. Primers devised by other authors [11,13], were used for this step.

This method was applied to forty-two E. coli strains originating from European repositories (Slovak Republic, Czech Republic, Poland and Denmark), including EC/17 and EC/42. Strains primarily selected for the study covered *E. coli* $F5^+$ but also included strains expressing F4 and F6 adhesins.

Immunization of Laboratory Animals

The principle of this part of the study was to evaluate of F41 expression and antigenicity by means of serological response to F41 evoked in rabbits. The animals were the same as used for routine batch control of an existing commercial EC/17-based vaccine (Bovigen®Scour, Pharmagal, Slovak Republic). The trial enrolled healthy rabbits that have not been previously vaccinated and were seronegative against vaccine antigens (bovine rotavirus, bovine coronavirus and *E. coli* $F5^+F41^+$) before vaccination. Twelve distinct routine manufactured batches of vaccine were tested.

Each vaccine batch was administered to 5 rabbits. Animals were dosed 2 ml intramuscularly into the thigh muscle, and were revaccinated 17-21 days after first injection, same dose, same route. Rabbits were bled before vaccination and 14 days after revaccination. Blood serum samples were stored at -20°C, avoiding repeated freezing-thawing cycle. The serum samples were examined by indirect ELISA (measure of the Optical Density (OD), 450 nm) for detection of F41 antibodies, according to the applicable standard operating procedure.

Immunization of Target Animals

The goal of this ultimate step was to verify that target animals injected with the same commercial vaccine mounted an immune response against the F41 adhesin. Thirteen clinically healthy, local Holstein-Friesian heifers, 15–28-month-old, were acquired from a commercial farm stock. Animals were ear tagged by the farmer. Before enrolment, all animals were vaccinated against

bovine herpesvirus (BHV1), bovine parainfluenza 3 (PI3) and Pasteurella multocida type A, all vaccines registered for use in cattle in the Slovak Republic. None of the animals included in the study was injected by a vaccine containing bovine rotavirus, bovine coronavirus and *E. coli* $F5^+F41^+$ antigens before testing.

Ten animals were intramuscularly vaccinated with a single dose (3 ml) of the vaccine, from a single commercial batch. Three animals were not vaccinated and were used as control. Then, blood samples were taken twice, before vaccination and 21 days after vaccination. Blood serum samples were examined by indirect ELISA for detection of F41 antibodies.

Results

Evaluation of Occurrence of F4, F5, F6 and F41 Genes in Various *E. coli* Strains

(Table 1) summarize occurrence of genes encoding *E. coli* fimbriae F4, F5, F6, and F41in a set of 42 strains collected across Europe. Results of real-time PCR targeted at F4, F5, F6 and F41 genes confirmed the frequently detected simultaneous occurrence of F41 with F5 fimbria; ten $F5^+$ strains were confirmed positive also for F41 fimbria (23,81%).

Vaccination in Laboratory Animals

Results in rabbits are presented in Table 2. For every single batch of vaccine, difference in OD before and after vaccination, were compared using a student t-test. All the 12 batches of vaccine elicited a significant ($0.005<P<0.023$, t-test, one tail) immune response to F41 adhesin.

Vaccination in Cattle

Data related to livestock, as well as the content of F41 antibodies in their sera before and after vaccination are summarized in the Table 3. Statistical evaluation of results confirmed significant differences (P=0,0006, t-test assuming unequal variance, one tail) in F41 serology between the vaccinated and control cows at the end of the trial on the one hand, and in vaccinated cows before and after vaccination (P=0,0005, t-test paired samples, one tail) on the other hand, suggesting F41 seroconversion evoked by the commercial vaccine.

Table 1: Frequency of occurrence of genes encoding *E. coli* fimbriae F4, F5, F6, and F41 in various strains (isolates) in Europe from diarrheic calves.

Fimbria type	Total identified	%
F4	17	40,48
F5	10	23,81
F6	5	11,90
F41	10	23,81
F5+F41	10	23,81
none	10	23,81

Table 2: Serum antibody response (recombinant F41 protein-based indirect ELISA) in rabbits used in regulatory batch potency testing, for 12 commercial batches. The table displays ELISA Optical Density (OD, 450 nm) readings before and 21 days after a single vaccination. Data are shown for individual animals and as the group mean (± Standard Deviation). The P-value represents the result of a paired statistical test comparing the first and second sampling points within each group. Gray areas enlighten situations where a four-time increase of OD has been observed (seroconversion).

BOVIGEN®scour batch number												
	12012301		18022301		21022301		26032301		30032301		35042301	
Rabbit	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.
1	0,108	0,553	0,106	0,345	0,071	0,609	0,078	1,162	0,093	0,885	0,079	0,511
2	0,095	0,095	0,088	1,125	0,127	1,738	0,113	0,690	0,090	1,026	0,087	0,676
3	0,090	0,883	0,112	0,132	0,086	0,860	0,091	0,684	0,096	0,741	0,115	0,944
4	0,088	0,818	0,075	1,349	0,115	0,660	0,087	0,151	0,080	0,128	0,118	1,056
5	0,116	1,008	0,093	0,966	0,093	0,264	0,099	1,065	0,115	0,639	0,111	0,132
Seroconversion	80%		80%		100%		80%		80%		80%	
Mean (SD)	0,099 (0,012)	0,671 (0,363)	0,095 (0,015)	0,783 (0,521)	0,098 (0,023)	0,826 (0,553)	0,094 (0,013)	0,750 (0,399)	0,095 (0,013)	0,684 (0,343)	0,102 (0,018)	0,664 (0,367)
P value	0,012		0,023		0,020		0,011		0,009		0,013	

BOVIGEN®scour batch number												
	41042301		42042301		47051201		53052301		55062301		55062301	
Rabbit	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.	Before vacc.	After vacc.
1	0,082	0,423	0,098	1,280	0,111	0,145	0,083	0,493	0,120	1,339	0,122	0,151
2	0,076	0,483	0,110	0,379	0,083	0,757	0,079	0,664	0,113	1,288	0,103	0,922
3	0,118	0,949	0,124	0,717	0,123	0,823	0,102	0,109	0,099	0,314	0,084	1,147
4	0,093	0,129	0,097	1,014	0,106	0,543	0,112	0,430	0,084	0,522	0,085	1,289
5	0,111	0,401	0,106	0,742	0,093	0,459	0,092	1,100	0,105	1,238	0,131	1,092
Seroconversion	80%		100%		80%		80%		100%		80%	
Mean (SD)	0,096 (0,018)	0,477 (0,297)	0,107 (0,011)	0,826 (0,039)	0,103 (0,016)	0,545 (0,269)	0,094 (0,014)	0,559 (0,363)	0,104 (0,014)	0,940 (0,484)	0,105 (0,022)	0,920 (0,450)
P value	0,021		0,005		0,020		0,011		0,009		0,013	

Table 3: Serum antibody response in cows (recombinant F41 protein-based indirect ELISA). The table displays ELISA Optical Density (OD) readings from a vaccinated group (n=10) and a non-vaccinated control group (n=3). The vaccinated group was sampled before (May 22nd) and 21 days after (June 12th) a single vaccination. The control group was sampled on the same dates. Data are shown for individual animals and as the group mean (± Standard Deviation). The P-value represents the result of a paired statistical test comparing the first and second sampling points within each group. Similar superscripts in different columns indicate significant differences (P<0.001). Gray areas enlighten situations where a four-time increase of OD has been observed (seroconversion).

Vaccinated			Control		
Animal	Before vacc. (May 22nd)	After vacc. (June12th)	Animal	May 22nd	June12th
V1	0,050	0,518	C1	0,057	0,061
V2	0,053	0,063	C2	0,049	0,048
V3	0,049	0,480	C3	0,052	0,054
V4	0,050	0,498			
V5	0,052	0,469			
V6	0,053	0,064			
V7	0,048	0,466			
V8	0,050	0,065			
V9	0,049	0,549			
V10	0,056	0,529			
Mean (SD)	0,051 (6.10^{-6})[a]	0,301 (0,045)[a,b]	Mean (SD)	0,053 (16.10^{-6})	0,054 (42.10^{-6})[b]
P value	0,0006		P value	0,185	

Discussion

Enterotoxinogenic *E. coli* is a primary contributor to intestinal disease in calves, especially within the first four days of life. ETECs encode lipopolysaccharide structures that may act as endotoxins, fimbrial adhesins, and finally enterotoxins. Fimbrial adhesins F5, F17, and F41 are linked to diarrhea in calves [22]. Therefore, the need for multivalent vaccines makes sense [19]. The seek for multiple anti-fimbriae vaccines is a trend in current research with the development of Multi-Epitope Fusion Antigens (MEFAs) that carried, expressed, and displayed representative epitopes of F4, F5, F6, F18, and F41 ETEC fimbriae [5].

The concurrent presence of both antigens F5 and F41 ($K99^{+}F41^{+}$) is common and was already deductible from previous studies where the two antigens were detected in the reference strain *E. coli* B41 [3,17], or in three out of six specimens from scouring calves but none of them in four of four specimens from non-scouring calves [21]. In a recent study in Turkey, K99 and F41 antigens were detected together in 22 of the 61 *E. coli* isolates from clinical patients [1]. Typically, all strains of O serogroups 9 and 101 carry both F41 and F5 fimbriae [4], which is the case of the tested strains EC/17 (O9) and EC/42 (O101). Our findings are consistent with these results with 23,81% of the tested strains exhibiting F5 and F41 antigens, simultaneously.

Several methods are being used for detecting the presence of virulence factors in ETEC strains. They were among the first bacteria for which molecular diagnostics were developed. DNA

probes were found to be useful for detecting LT- and ST-encoding genes [18]. Gene probes are segments of the target genes that are labeled with either radioactive (^{32}P) or non-radioactive nucleotides. Bacteria are lysed and the DNA is denatured and hybridized to the labeled gene probe in situ. Positive colonies hybridize to the labeled gene probe, thus emitting radiation. Many gene probes have been found to be sensitive and specific for screening virulence factors of *E. coli* in animal isolates [4].

The Polymerase Chain Reaction (PCR) is now the universally accepted method for the detection of virulence factors. PCR reactions are performed essentially according to standardized protocols. Template DNA is amplified in a reaction containing selected primers, DNA polymerase and other reactants. The reactions are electrophoresed on agarose gel and scanned in a way or another. Positive samples are identified on the basis of the presence of an appropriate band compared with the positive control. Many laboratories have been successful in formulating multiplex reactions [8] tailored for specific host species or pathologies, physical separation on the gel usually being the most limiting factor. This method has been used and adapted in this work. However, we paid a lot of attention to the qualification of forward and reverse primers used to produce amplicons from 10 *E. coli* $F41^+$ strains, including the vaccine strain EC/17 and the challenge strain EC/42. All resulting consensus sequences finally matched a F41 prototype sequence with a very high degree of identity (>98,92%). It is important to mention that this method helps identify the presence of the targeted gene, but does not allow a quantification of the antigen.

Latex agglutination kits, ELISA assay kits and antisera are also available for detecting K88, K99, F41 and 987P virulence factors in *E. coli [4]*. Virulence factors F5 and F41 of the *E. coli* strain CN7985 have been investigated using peptide sequencing. Antigens were detached from the bacterial body and analyzed using Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) after digestion of the denatured, reduced, and alkylated proteins using trypsin. The reported peptides were identified using published *E. coli* protein sequences. Relative protein quantification was performed by calculating the sum of the intensities of the three most intense peptides of 13 independently produced batches of the *E. coli* antigen [16]. In this study, the F41/F5 ratio was constant across the different cultures of *E. coli* strain CN7985, close to 0.7 (IQR [0.58;0.78]).

Referring to the results of molecular-biological study presented in this paper, strain EC/17 of Escherichia coli included in commercial vaccines carries the genes encoding F5 and F41 fimbriae, and genotypically complies with the prerequisite of two fimbrial valences in the vaccine. Similarly, strain EC/42 used to challenge calves born to dams vaccinated with EC/17 *[6,23]*, also bears genes encoding the two fimbriae. Other strains have been used to immunize cows against F5 and F41 adhesins simultaneously. Historically, the inactivated strain B41 has been formulated in a first vaccine in 1994 [7,9]. Recently, it has been shown that a commercial vaccine made of strain CN7985 is also able to elicit an immune response to F41 in the serum and colostrum of vaccinated dams.

For the final protective post-vaccination effects, we processed blood sera from groups of *E. coli* $F41^+$ exposure-free rabbits collected during the regulatory release tests. This ELISA test is required by the European Pharmacopoeia to assess the potency of all batches of a commercial vaccine. Twelve batches have been tested; a sharp seroconversion has been noted against the F41 adhesin for all batches. When it comes to the fluctuation between industrial batches, the differences in the results were statistically insignificant (P=0.766, one-way ANOVA). This finding is of a great importance since each of the batches was formulated from distinct, on purpose Escherichia coli cultures which were not monitored for the content of the F41 fimbria and no exact quantification for the antigen. Furthermore, vaccine batches are identified by a distinct batch code and manufactured one after the other, independently. Consequently, all batches of the commercial vaccine Bovigen®Scour carry F41 antigen, and are able to elicit a significant immune response.

Finally, the EC/17 based commercial vaccine elicited a sharp seroconversion in 7 of 10 cows, three weeks after inoculation.

Conclusion

Results of real-time PCR targeted at F4, F5, F6 and F41 genes thus confirmed the frequent association of F41 with F5 fimbria. Genes encoding F5 and F41 have been confirmed for both of *E. coli* strains (i.e. vaccination strain EC/17 as well as for challenge strain EC/42). For strain EC/17 the activity has been observed directly by the monitoring of ability to act as an immunogen and evoke the formation of specific F41 adhesin antibodies after vaccination.

Conclusion that can be drawn from this study is that, when commercial E/C 17-based vaccines currently available in Europe are administered, an immune response against F5 and F41 is therefore obtained. Since both of *E. coli* strains (vaccination strain EC/17 as well as challenge strain EC/42) have been confirmed for F41 valence, it is therefore possible to state that commercial E/C 17-based vaccines are able to protect the animals against diarrhea caused also by *E. coli* producing F41 adhesin.

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