Is there any function for colicinogeny in the post-weaning diarrhoea of piglets?

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ABSTRACT: Using seven experimental approaches, we attempted to solve the question of possible participation of colicinogeny and colicin-sensitivity in the pathology of the post-weaning diarrhoeic enteritis of piglets. In our research, both enterotoxic *E. coli* strains (ETEC) and normal, commensal *E. coli* strains in the intestinal microflora of 803 weaned piglets were followed. In diarrhoeic piglets, colicinogeny was more frequent in the ETEC strains than in the simultaneously isolated commensal ones. ETEC strains were largely insensitive to the most frequently appearing colicin types and the inhibitive effect of their colicins on commensal *E. coli* was less likely than the opposite (inhibition of ETEC strains by colicins of the commensal ones). Bolstering the diet of healthy piglets with a mixture of symbiotic colicinogenic strains and colicin-sensitive ETEC strains could not prevent diarrhoeic enteritis due to established dominance of ETEC. In all parts of the intestinal tract during the post-weaning diarrhoea (PWD), mostly non-colicinogenic strains of the commensal flora survived. In six serotypes of ETEC strains, the frequency of colicinogenic strains ranged from 7% (in the serotype O139) to 66% (in serotype O141). From 9 frequent colicin types, colicin V (mainly against the serotypes O8 and O147), E2 (against O139 and O8), D (mainly against O8) and E3 (mainly against O138) met most sensitive strains among ETEC. Hence, colicinogeny was no pathogenetic factor of PWD. Nevertheless, colicinogenic commensal strains gradually regained dominance during the decline phase of the disease in surviving piglets.

Keywords: piglets; diarrhoea; enteritis; *E. coli*; colicinogeny

Many *Escherichia coli* strains produce specific antibacterial proteins, called colicins; their bacteria are called colicinogenic. So far 27 colicin types have been classified, each of them exerting one of four molecular modes of killing sensitive bacterial cells: formation of ion-permeable pores through the cell inner membrane, non-specific DNase activity, specific RNase activity (against rRNA or tRNA) or interference with the wall peptidoglycan synthesis (Smarda and Smajs, 1998). The killing activity of each colicin type is started by the binding of its molecule onto a specific receptor present in the cell wall of a sensitive bacterial cell. For this purpose, colicins misuse bacterial receptors engaged in important vital functions.

Colicin action is generally limited to receptor-bearing strains of the same species, *Escherichia coli*, and to closely related species of Enterobacteriaceae. It is questionable to what extent colicins can be active in the natural medium they are produced in, i.e. in the intestine contents.

From this point of view, the possible role of colicins in dysbacteriotic conditions of the intestinal system appears interesting. An intriguing – and also in its consequence economically greatly undesirable – condition like that is the post-weaning diarrhoeic (PWD) enteritis of piglets.

Newborn piglets are gnotobiotic: their intestinal system is sterile. During the first days of life, their commensal intestinal microflora gets established; in most cases, colicinogenic *E. coli* strains are accomodated, frequently in pure cultures. This physiological flora prevails during the first three weeks of piglets' life. At the end of this period, colicinogenic strains disappear, being gradually replaced by non-colicinogenic ones (Willinger and Trcka, 1973). During the mother' s milk feeding period, a definite bacterial flora is completed; in this, the colicins of producing bacteria usually do not influence other coliform bacteria present in the same flora, due to colicin-unsensitivity of the latter. In the fifth week of piglets' age, colicinogenic strains appear in

their faeces with about the same frequency as not colicinogenic ones. At this age, piglets are usually weaned. Starting with the sixth week of piglets' life, the occurrence of colicinogenic strains in their established intestinal microflora gets decreased and in the seventh week these commensal colicinogenic strains appear only sporadically.

Weaning interrupts abruptly the supply of maternal antibodies and the piglets are exposed – at the same time as to the stress due to a profound dietary change and often also due to unfavourable environmental conditions – to risks of infection by enterotoxic *E. coli* strains (ETEC, haemolytic in most cases) or by other enteric pathogens. The ETEC strains are causative agents of a severe post-weaning diarrhoeic enteritis (PWD) of piglets with a high lethality. Antibodies circulating in blood are of very limited importance as to enteric *E. coli* infections.

At present, two major groups of virulence factors of ETEC strains are in favour of their dominance in the intestinal flora of weaned piglets, completely suppressing the primary commensal *E. coli* strains: heat-labile or heat-stable enterotoxins and adhesins. Most of the porcine ETEC isolates belong to serological groups (types) O8, O64, O101, O138, O141, O147, O149 and O157 (most of which are haemolytic); at least some of the serotype O149 carry the K88 (F4) adhesin and produce heat-labile or heat-stable enterotoxins (Alexa *et al*., 1995). The genes for the heat-stable enterotoxin and for colicin B production are mostly located on a common plasmid (Franklin *et al*., 1981).

PWD lasts about a week, after which enterotoxic *E. coli* strains gradually disappear again in piglets which survived, owing to the onset of active local immunity of the mucosa. So the time interval from weaning to the onset of mucosal immunity is the critical period for PWD enteritis.

Since the era of Fredericq (1957) it has been generally accepted that the production of colicins in any bacterial community is a selective advantage favouring the dominance of producing strains. Nevertheless, Tadd and Hurst (1961) did not succeed in proving this assumption in PWD piglets. They tried to prevent the outbreak of ETEC-caused diarrhoea of piglets by feeding them with non-pathogenic colicinogenic *E. coli* cultures: pathogenic, haemolytic ETEC strains remained dominant in the microflora of all animals – with typical consequences.

Also Sarmanova and Salajka (1971) reported, based on an their experimental research of the rectal flora of 45 weanlings, that production of colicins did not function as a determinant of prevalence of colicinogenic strains over pathogenic non-colicinogenic ones in the intestinal tract. These results extended by Salajka and Sarmanova (l971) arrived to the same conclusion. Pathogenic strains causing PWD prevailed irrespective of not being colicinogenic and in spite of being inhibited by colicins from the primary commensal microflora *in vitro*. 34% of ETEC strains of pathogenic serotypes isolated from piglets diseased of heavy diarrhoea were colicinogenic, while in non-pathogenic strains, these authors found 49% colicinogenic. However, they also noted that in 10 recovering piglets ETEC strains were replaced by non-pathogenic ones, which produced colicins killing the ETEC strains *in vitro*.

A bit later, De Alwis and Thomlinson (1975) suspected that pathogenic (haemolytic) ETEC strains colonized the intestinal tract of weaned piglets irrespective of the presence of endogenous saprophytic colicinogenic *E. coli* strains in it; this could be due to proteolytic digestive enzymes present, decomposing colicins.

In accordance, Djønne (1986) found 308 analysed *E. coli* strains of pathogenic serotypes to be resistant to a variety of 9 frequent colicin types. She suggested that the actual relation of colicin production and colicin non-sensitivity of *E. coli* flora in the intestinal tract was decisive of establishing infection with "enteropathogenic" *E. coli* in newborn piglets.

Apparently, colicin-sensitive strains can be pushed out from any microbial community by the colicins produced by producer strains only, if having an overall selective advantage in the environment given (Riley and Wertz, 2002).

So the role of colicinogenicity of ETEC strains in PWD of piglets appeared open to a thorough revision by a variety of examinations and experiments on numerous animals of several herds. At the same time, the question on the role of colicinogeny as of a predisposing factor of non-pathogenic, saprophytic *E. coli* strains for their dominance in the re-establishment of a commensal intestinal Coli-microflora in piglets recovering from PWD, was followed.

MATERIAL AND METHODS

In this section, only general (basic) materials and methods are stated. All supplementary, particular

data and subtle modifications differing in the parts of the Results section are given in their context.

Experimental animals

Piglets from the pig farming establishment of the Veterinary University, Vienna, from several herds were used throughout our studies; they were 5 weeks of age and just weaned at the start of each examination. Groups of 10–20 animals were examined in most experiments in the same way, to achieve representative results. Altogether, 803 animals were used for this research.

Experimental strains of bacteria

Strains producing type colicins, just as the universal colicin indicator strain *E. coli* K12-Row (58-161 met B1 rpsL λ^+) sensitive to most colicin types, were gained from Prof. P. Fredericq, Service of Microbiology and Hygiene, Liège, Belgium.

Experimental procedures

Faeces probes were taken from the rectum of experimental animals at intervals, by means of a sterile, blunt plastic spoon. Each sample was suspended and diluted 10^{-1} to 10^{-6} in 0.1M phosphate buffer, pH 7.2. A loop of each dilution was evenly inoculated – in parallel – on blood agar, McConkey and Trypticase-soya agar with the addition of penicillin (10 000 U/ml). The plates were kept at 37°C overnight and then overlaid with a thin layer of nutrient agar containing the indicator strain *E. coli* Row. After a further overnight incubation, round-shaped inhibition zones could be noticed around colicinogenic colonies. (The three culture media served as controls to each other.) In this way, the occurrence of colicinogenic bacteria in the samples, their numbers and proportions in the *E. coli* flora could be ascertained. Several (at least two) different colony types of *E. coli* were isolated from each sample and their colicin production analysed separately.

To verify the results, the three-layer-agar method of Trcka and Willinger (1973) was applied. Strips of cultures of isolated *E. coli* colonies inoculated on Tryptic-soya agar were overlaid with a thin 1.5% nutrient agar layer containing the standard indicator strain Row, plates kept overnight at room temperature and overlaid with the third layer of sterile 2% nutrient agar. After the solidification, perpendicular strips of strains tested for colicin sensitivity were applied on the surface. Colicin-caused inhibition zones were read after a 24 hour incubation at 37°C.

To test simply the colicin production and colicin sensitivity of isolated strains, the standard double-layer-agar method (Fredericq, 1948) was used. The strain to be tested for colicin production (or a standard producer strain) was inoculated with a needle stitch into a plate of standard Trypticase-soya agar. The plate was incubated at 37°C for 48 hours. The grown-up rounded bacterial lawn was killed by chloroform vapour and the plate overlaid with 0.7% Trypticase-soya nutrient agar inoculated with the strain tested for colicin sensitivity or with the standard indicator strain Row. Colicin-caused inhibition zone around the colicinogenic lawn was read after a further overnight incubation of the plate at 37°C.

RESULTS

Mutual inhibitory effects of colicinogenic *E. coli* strains from simultaneous commensal and enterotoxic flora of diarrhoeic weaned piglets

The rectal flora of 10 piglets, weaned at the age of 5 weeks and suffering from PWD enteritis, was followed daily during 22 days. Colicinogeny of each isolate was followed quantitatively on Trypticase-soya agar plates, using the standard indicator *E. coli* K12-Row. The spectrum of sensitive strains was ascertained in the three-layer test using the same agar.

In this way, strains of the primary, commensal (non-haemolytic) flora, just as of the simultaneously occurring strains of the secondary flora of haemolytic ETEC, were tested. First, the frequency of colicinogenic strains was ascertained in both the commensal and toxigenic flora, using the indicator *E. coli* Row. See the results in Table 1. Colicinogenic strains appeared in the enterotoxigenic flora more frequently by 46% than in the commensal one. Second, the frequency of colicinogenic strains was ascertained again in both the commensal and simultaneous ETEC flora, but using strains detected in the opposite flora as possible indicators. Numbers

Table 1. Inhibitory effect of colicinogenic *E. coli* strains in the primary commensal and in the simultaneously appearing secondary (enterotoxic, haemolytic) flora in post-weaning piglets, using the strain Row as indicator

Piglet Nr.	Colicinogeny of the primary, commensal *E. coli* strains isolated		Colicinogeny of the secondary, enterotoxic *E. coli* strains isolated		Statistical significance of the difference for *P* value
	number	percentage of colicinogenic	number	percentage of colicinogenic	
1+	18	61	7	100	0.052
2	31	36	15	67	0.048
3	39	23	21	95	<0.001
4+	9	78	5	100	0.258
5	58	21	25	28	0.488
6	23	35	25	48	0.362
7	35	43	21	95	<0.001
8	39	28	21	100	<0.001
9	30	73	20	90	0.142
10	40	35	20	80	<0.001
	total 322	mean 43	total 180	mean 80	<0.001

+deceased during the research

of strains tested for sensitivity in individual piglets and the results see in Table 2.

Colicins of the commensal (primary) *E. coli* strains showed fewer inhibitive effects on the enterotoxic *E. coli* strains than on the standard indicator Row. With the exceptions of piglets Nrs. 1, 5 and 8, the commensal flora of these appeared to produce some additional colicin not inhibiting the Row

Table 2. Inhibitory effect of colicinogenic *E. coli* strains in the primary (commensal) and in the secondary (enterotoxigenic, haemolytic) flora of post-weaning piglets, tested against strains of the simultaneously appearing opposite flora as indicators

Piglet Nr.	Isolated strains *E.coli*		Tests performed		Isolated strains *E.coli*		Tests performed	
	commensal colicin+	ETEC tested as colicin sensitive	number	percentage of positive	ETEC colicin+	commensal tested as colicin sensitive	number	percentage of positive
1+	11	7	77	77	7	18	126	6
2	11	15	165	26	10	31	310	0
3	9	20	180	16	20	39	780	3
4+	7	5	35	14	5	9	45	0
5	12	25	300	22	7	58	406	0
6	8	25	200	10	12	43	516	2
7	15	21	315	19	20	35	700	3
8	11	20	220	45	20	39	780	8
9	22	20	440	11	17	30	510	9
10	14	20	280	20	16	40	640	10
	total120	178	2 212	mean 26	134	342	4 813	mean 4

+deceased during the research

strain. A decreased number of tests that could be performed with the flora of piglets Nrs. 1 and 4 was due to their death during the experiments.

At the same time, 1–2 ten-orders less strains of the primary commensal flora were inhibited by colicins of the enterotoxic microflora than the standard indicator strain Row (see Table 1). The total difference between the frequency of colicinogeny in the commensal and ETEC bacteria inhibiting the opposite flora appearing simultaneously (i.e. 43% vs. 80%) was statistically highly significant ($P < 0.001$).

Distribution of colicinogenic strains of *E. coli* in the commensal flora of main intestinal compartments of diarrhoeic weaned piglets

20 diarrhoeic piglets of 5 weeks of age were killed and their intestinal tract dissected. Content samples were taken in jejunum, ileum and colon. Isolated *E. coli* strains of the commensal flora were examined for colicinogeny, using the strain Row as an indicator. Non-colicinogenic flora prevailed in all animals, with admixtured colicinogenic strains in most of them. Results are summarized in Table 3.

Dynamics of the incidence of commensal colicinogenic *E. coli* strains in the intestine of piglets during post-weaning diarrhoea

The rectal microflora of a group of weaned piglets from several herds was followed during their post-weaning diarrhoea. Their faeces samples were taken 2–3 times a week and the quantitative proportion of colicinogeny in the primary, commensal *E. coli* strains investigated each time, using the universal colicin indicator strain Row. Only the results of 116 piglets which survived the PWD enteritis were considered for evaluation.

In 20 piglets (17.2%) the proportion of colicinogenic commensal strains remained unchanged during all the enteritis phases. In 32 animals (27.6%) these strains dominated in all the phases of the disease. In 41 piglets (35.4%) a striking frequency increase in commensal strains was noted in the phase of top manifestation of the disease symptoms. And in 23 piglets (19.8%) of the group, the physiological commensal, colicinogenic population reached dominance during the decline phase of the disease or of starting recovery of surviving piglets.

Composition of *E. coli* flora in intestines of piglets with experimentally provoked diarrhoeic enteritis

The standard diet of 27 just weaned, so far healthy piglets of the minipig race was enriched with 500-ml daily doses of freshly grown-up (24 hours old) cultures of *E. coli* in nutrient broth. In each dose bolstered, a mixture of two *E. coli* strains was applied, i.e. of one standard, commensal colicinogenic strain from a normal flora and of one haemolytic, non-colicinogenic strain of an enterotoxic O-serotype, sensitive (*in vitro*) to the colicin produced by the former. The optical density of both cultures was the same, representing approx. 1×10^9 CFU/ml of bacteria. Each piglet received the two cultures in one of five different volume ratios, varying from 1 : 10 to 10 : 1. This bolster was applied for 21 days, during which the incidence of the standard flora, just as of the haemolytic ETEC one, in samples of each animal' s faeces was followed daily.

All experimental piglets developed a typical diarrhoea, irrespective of the standard-to-haemolytic *E. coli* ratio applied; three piglets died of the disease during the experiment. The haemolytic flora prevailed in all animals (see Table 4). The frequency of col^+ *E. coli* strains in the faeces was the same as in cases of spontaneously diseased animals.

Table 3. The occurrence of commensal colicinogenic strains *E. coli* in the main parts of the intestinal system of dyarrhoeic weaned piglets

Intestinal compartment	Number of piglets with strains				
	only col^-	prevailing col^-	prevailing col^+	only col^+	alltogether
Jejunum	11	8	1	0	20
Ileum	2	15	3	0	20
Colon	0	13	7	0	20

Table 4. Incidence of commensal colicinogenic *E. coli* in piglets fed with a diet bolstered with a mixture of them and of enterotoxic serotypes (indicator: *E. coli* Row)

Enterotoxic serotype applied	Number of piglets	Percentage of samples with col$^+$ strain*	Number of piglets deceased
O8	5	48	1
O141	16	42	2
Not determined	6	49	0
	total 27	mean 46	total 3

*enterotoxic *E. coli* proved in 100% of samples

Table 5. Frequency of colicinogeny in 6 enterotoxic (and haemolytic) *E. coli* serotypes causing post-weaning diarrhoea of piglets

Pathogenic serotypes	Number of strains investigated	Percentage of colicinogenic strains among them
O8	14	50
O138	93	19
O139*	62	7
O141	38	66
O147	39	46
O149	132	17
Not determined	74	22
	total 452	mean 32

*presumably verotoxinogenic *E. coli* (VTEC)

Frequency of colicinogeny in O-serotypes of ETEC strains causing post-weaning diarrhoea of piglets

6 serotypes (including the verotoxinogenic serotype O139) of 452 haemolytic ETEC strains isolated from piglets diseased of post-weaning diarrhoea were determined. Production of colicins by all these strains was tested in vitro using the universal indicator strain *E. coli* Row in a standard test. The results are given in Table 5. Frequency of colicinogeny ranging from 7% to 66% was found for each of the O-serotypes, with the mean value for enterotoxic (haemolytic) strains of 32%. Strains without provable colicinogenic activity dominated in 5 of the 6 serotypes.

Sensitivity of enterotoxic, haemolytic strains of *E. coli* to some frequent colicin types

178 haemolytic strains *E. coli* of 6 serotypes enterotoxic for piglets (including the verotoxinogenic serotype O139) were isolated from diarrhoeic faeces and tested *in vitro* for their sensitivity to 9 frequently occurring colicin types in a standard agar test. The results are summarized in Table 6.

The ETEC strains tested showed the highest sensitivity to colicin V (100% of tested O8 and O147 strains, just as 95% of O138 and 94% of O139 strains etc. were sensitive) and to colicin E2 (91% of O39 strains etc. were sensitive) revealing that they – if at all – only rarely produce these colicins. On the other hand, none of the O8 or O141 strains and just 2%–4% of O149 and O147 strains were sensitive to colicin Ia, indicating that this colicin may be frequently produced by them.

DISCUSSION

Summing up, results of our investigations justify four principal conclusions:

1. Strains of ETEC are more frequently colicinogenic (up to 80%) than commensal *E. coli* strains. However, their colicins do not inhibit a vast majority of saprophytic strains of the commensal *E. coli* flora

Table 6. Bactericidal activity of 9 standard colicin types towards strains of 6 enterotoxic serotypes *E. coli* causing post-weaning diarrhoea of piglets

Colicin tested		Strains of ETEC serotypes tested													
		O8		O138		O139*		O141		O147		O149		total	
Type	producer strain	number	percentage of sensitive	number	percentage of sensitive	number	percentage of sensitive	number	percentage of sensitive	number	percentage of sensitive	number	percentage of sensitive	number	percentage of sensitive
A	CA31	7	43	38	32	32	13	26	12	26	15	49	8	178	20
B	CA18	7	29	38	47	32	50	26	15	26	4	49	10	178	26
D	CA23	7	86	38	79	32	64	26	69	26	46	49	24	178	61
E1	CA62	7	0	38	61	32	38	26	42	26	12	49	12	178	33
E2	CA42	7	86	38	82	32	91	26	69	26	85	49	24	178	73
E3	CA38	7	71	38	87	32	56	26	65	26	42	49	10	178	55
Ia	CA53	7	0	38	32	32	13	26	0	26	4	49	2	178	12
K	K235	7	29	38	42	32	9	26	38	26	58	49	2	178	30
V	CA7	7	100	38	95	32	94	26	81	26	100	49	53	178	87

*presumably verotoxinogenic *E. coli* (VTEC)

present simultaneously in the intestine, which are largely not sensitive to the colicins of the former. Thus, colicins cannot be regarded as a virulence factor in the pathogenesis of PWD enteritis of piglets. They have no significant influence on the establishment of the invading enterotoxic flora in any part of the intestinal tube and thus on the outbreak and clinical picture of the post-weaning diarrhoea.

2. The frequent colicinogeny of commensal *E. coli* strains towards ETEC ones may present a positive factor supporting the re-introduction of normal commensal *E. coli* microflora in the intestine and hence the recovery of piglets from the disease.

3. There is no realistic reason to expect any meaningful preventive or therapeutic effect from an introduction of a saprophytic colicinogenic *E. coli* strain into the digestive tract of piglets diseased from post-weaning diarrhoeic enteritis (as indicated earlier by Tadd and Hurst, 1961).

4. ETEC strains may produce often colicin Ia, typical for some Shigellae (Brandis and Smarda, 1971); nevertheless, this hypothetic conclusion provokes further research.

These conclusions are in accordance with those of Sarmanova and Salajka (1971) and Salajka and Sarmanova (1971) mentioned in the Introduction, and confirm the observations of Trcka and Willinger (1987). They also correspond to the results of Vasenius (1967) who analysed the *E. coli* flora of just weaned (so far healthy) piglets and found 52% of the pathogenic strains, while only 17% the commensal strains to be colicinogenic. De Alwis and Thomlinson (1975) reported a broad activity of colicins produced by commensal strains against pathogenic serotypes *in vitro*, while a comparable activity of pathogenic strains against commensal ones *in vivo* mostly failed. They attributed this striking phenomenon to a hypothetical inactivation of colicin activity in the intestine by action of proteases in the contents. This simple interpretation is probably not sufficient according to our experience (not published). Many other physical, chemical and immunological factors of the intestine contents should be taken into consideration, a.o. sugar fermentation (Horak, 1973) and, mainly, the prevailing non-sensitivity of the commensal *E. coli* strains to colicins, due to production and, hence, to immunity thereof. Colicins admit survival and multiplication only of saprophytic colicin non-sensitive strains. The extensive sensitivity of serotypes O8 and O138 to colicins E2 and D stated confirms the experience of Trcka and Willinger (1987).

Martins *et al*. (2000) followed 91 *E. coli* strains isolated from healthy and diarrhoeic newborn (0 to 11 days of age) piglets in three East-Brazilian states. Among ETEC strains, they found 54%, while among commensal ones, 24% colicinogenic. This relation is close to our proportion 80% to 43%; nevertheless, we analysed 5.5 times more animals. Finally, it is worth noting that the 43 % frequency of colicinogeny among saprophytic *E. coli* strains of piglets is very close to that in humans (41% – Smarda and Obdrzalek, 2001).

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