APPLICATION OF THE SUCKLING MOUSE BIOASSAY TO ASSESS THE ENTEROTOXIC PROPERTIES OF YERSINIA ENTEGRLOCITICA STRAINS ISOLATED FROM FATTENING PIGS

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Abstract

Enterotoxic capacities of 95 Yersinia enterocolitica strains isolated from fattening pigs by the use of the suckling mouse bioassay were investigated. A positive result was received for 14 strains (14.74%), doubtful for 33 strains (34.74%), while the rest of 48 strains (50.52%) did not induce the production of enterotoxins. The received results show that in the pig population there are almost 15% of Yersinia enterocolitica strains, which are directly dangerous to public health because of their enterotoxic capacity. It is also worth paying attention to those strains, which in the suckling mouse bioassay provided doubtful results (around 35%) because their ability to produce enterotoxins was not explicitly declared as impossible.

Key words: swine, Yersinia enterocolitica, suckling mouse bioassay, enterotoxins.

Material and Methods

Material. The study directed at the ability to produce enterotoxins in vitro by the use of the suckling mouse bioassay was carried out on 95 Y. enterocolitica strains isolated from fattening pigs from large pig farms. Thirty-nine strains were from warm culture and 56 were from cold culture.

Animals. The suckling mice, which were used in the biological test, were obtained from a husbandry of which the core was a brood flock: 10 males and 44 females of the BALB/c lineage, bought in the Mossakowski Medical Research Centre of the Polish Academy of Sciences in Warsaw. Permission for all experimental procedures was granted by a Local Ethical Commission for Experiments on Animals in Olsztyn.

Infection procedure and interpretation of results. The initial phase for the biological test was to inoculate Y. enterocolitica strains in a TSB broth, containing 0.6% yeast extract, at 28°C for 48 h. This culture was centrifuged for 10 min in an Eppendorf Centrifuge 5804 R with a speed of 3,100 x g.
Fig. 1. Scheme of the interpretation of biological test results directed at the production of enterotoxins by *Y. enterocolitica* according to Giannella (6).

Fig. 2. Results of the suckling mouse bioassay demonstrating the ability of *Yersinia enterocolitica* to produce enterotoxins.

Fig. 3. Percentage diversity of results in the biological test depending on the culture type of *Yersinia enterocolitica*.
The obtained supernatant was transported in the amount ranging from 1 ml to 1.5 ml to a test-tube, where 2 drops of a 2% Evans blue dye (bromothymol blue) were added. The prepared ex tempore mixture was administered intragastrically through the abdominal linings with a sterile disposable insulin syringe of 0.1 ml graduation and equipped with a needle to 2-4 d old suckling mice grouped into groups of three – with one examined strain. Two hours after the infection, the mice were put to sleep by overdosing the anaesthesia with isoflurane (Aerrane, Baxter S.A., USA). In the next phase of the experiment a dissection of the mice with a sterile scalpel with a no. 13 blade, surgical scissors, and veterinary ophthalmic tweezers was performed. After opening up the abdominal linings, first, the effectiveness of the intragastrical administration of the supernatant with a dye was assessed. Sucklings, whose stomach was white (milk) or slightly blue were excluded from further procedures. In case of a dark blue or navy blue colouring of the stomach, the sucklings’ intestines were prepared measuring from the duodenum to rectum. Tweezers were tightened in a way, which prevented the intestinal contents from getting out. The intestines of three sucklings from the given examination group were weighed on an analytical scale with a precision of up to four places after the decimal point. Next, the remains of the body mass were weighed and the middle ratio of the intestine’s mass to the rest of the body mass was quantified. Control markers were obtained through marking the ratio of intestinal mass to the rest of the body mass, to which 0.1 ml of the TSB broth, containing 0.6 % yeast extract, dyed with 2 drops of 2% Evans blue for every 1 ml of broth, was administered intragastrically. The results were interpreted according to Giannella’s scheme (6), presented in Fig. 1.

Results

Enterotoxin production was found in 14 (14.74%) strains, a doubtful result occurred in 33 (34.74%) cases, whereas the rest of 48 (50.52%) strains did not produce enterotoxins (Fig. 2).

Among the 39 Y. enterocolitica strains examined with the suckling mouse bioassay from warm culture, in eight cases a positive result was obtained, which constitutes 20.51% of the examined strains. A doubtful result was received for 19 (48.72%) strains, and the lack of the ability to produce enterotoxins was noted in 12 cases (30.77%). Among the 56 examined strains from cold culture, the majority, that is 36 (64.29%), did not show any toxigenic activity, which would be indicated by the ability to produce enterotoxins. A doubtful result was observed in 14 (25%) Y. enterocolitica strains, whereas a positive result occurred only in six cases, constituting 10.71% (Fig. 3).

Discussion

Assessment of Y. enterocolitica strains ability to produce enterotoxins was done with the use of the suckling mouse bioassay. This test was devised by Dean et al. (4) in 1972 and was meant to assess the ability to produce enterotoxins by E. coli strains, and after slight modifications it is still used nowadays. The wide range use of the method contributed to the creation by Giannella’s (6) a precise characterisation of the method as a model, and defining the optimal conditions in which examinations can be carried out in a way that would provide the most reliable and repetitive results. It was established that the production of thermostable STI enterotoxin begins not sooner than after 8 h of bacteria culture incubation, reaching its peak after 24 h. No significant increase in toxin production was noticed in case of prolonged time of culture incubation, which resulted in the fixing of an optimal time of its duration – as 24 h. The effect of the enterotoxin activity after its intragastrical administration to the suckling mice appeared not sooner than after 1 h, and optimal secretion was observed between the 2nd and 3rd h of the test duration, though some of the examined strains produced the enterotoxin, which was fully active only between the 3rd and 4th h of the test duration. The optimal room temperature, at which the suckling mice were held, was fixed to 25°C, since under the influence of 37°C test results were falsely negative or weak. The stability of the enterotoxin was also examined through freezing a centrifuged supernatant at -20°C. It was established that even after 6 months of storing, the enterotoxin showed identical properties to the one obtained straight from the bacteria culture. No influence of high temperature on the activeness of the examined enterotoxins was noticed, even after heating for 15 min to 65°C. However, a small but significant decrease in activeness was noticed after heating up to 100°C and maintaining this temperature for 30 min. The scheme of interpreting results from the suckling mouse bioassay was also established through marking sections, which were used to classify a set ratio of intestinal mass to the rest of the body mass. A ratio, which equalled 0.074 or was smaller, indicated a negative result, the ratio between 0.075 and 0.082 was interpreted as a doubtful result, whilst the ratio, which equalled 0.083 or higher as a positive result (6).

On the basis of the prototype of Dean et al. (4), as well as Giannella’s modification (6), models used to detect the production of other enterotoxins were created, including those produced by Y. enterocolitica because of a unique resemblance between the enterotoxin produced by E. coli and the enterotoxin produced by Y. enterocolitica. On the basis of the Giannella (6) publication, a procedure of carrying out the suckling mouse bioassay in our examinations, with slight modifications was devised. One of them was a way of preparing inoculate, modified by Nessbakken (10) and Singh and Virdi (19), which resulted from the fact that Y. enterocolitica are characterised by slightly different culture conditions in comparison to E. coli. In our research a 48-h culture in a TSB-YE (YE – yeast extract) broth was used. The second of the modifications
used regarded the time of the test from the moment of inoculation to the moment of anaesthetisation of a suckling and its dissecting with a fixed ratio of intestinal mass to the rest of the body mass. Nunes and Ricciardi (11) in a work devoted to detecting thermostable enterotoxin produced by *Y. enterocolitica* with the use of the suckling mouse bioassay, suggest that it should be carried out within a maximum of 2 h after the intragastrical administration of inoculate in order to avoid false results. While the peak of activeness in the case of STI enterotoxin, produced by *E. coli*, occurred during the 4th h after adding a dyed supernatant, the thermostable enterotoxin produced by *Y. enterocolitica* had its peak of activeness between the 1st and 2nd h after inoculation.

One of the first studies concerning the ability to produce enterotoxins *in vitro* at 4°C, 22°C, and 37°C by *Y. enterocolitica* strains and *Y. enterocolitica*-like bacteria, with the use of the suckling mouse bioassay was carried out by Kapperud (7). Among the total number of 171 examined strains, 83 were Scandinavian *Y. enterocolitica* isolates, out of which 20 were from clinical cases from humans, and 63 were environmental isolates. All the strains isolated from clinical cases from humans produced enterotoxins at 22°C. From the 63 environmental isolates, enterotoxins were produced by 30 (47.62%) strains out of which the majority, 23 strains, showed such ability only at 22°C; six strains produced enterotoxins at 4°C and 22°C, and one at 22°C and 37°C. The remaining 33 (52.38%) strains did not show the ability to produce enterotoxins, although it should be mentioned that only a ratio of intestinal mass to the rest of the body mass, which was higher than 0.09 was regarded as a positive result, according to the classical method adopted by Dean *et al.* (4).

Similar studies were conducted by Nessbakken (10) on 71 Norwegian *Y. enterocolitica* strains and *Y. enterocolitica*-like bacteria, isolated from swine palate tonsils and pork products. In this case, a positive result was a ratio of intestinal mass to the rest of the body mass, which equalled 0.083 or was higher, and the duration of the biological test was 4 h. Among 44 *Y. enterocolitica* strains, 27 (61.36%) showed biological activeness in the suckling mouse bioassay. Enterotoxins at 22°C were produced by 26 strains, and additionally one of the examined strains produced enterotoxin at both 22°C and 4°C. From pork products, nine *Y. enterocolitica* strains were isolated, and a positive result of the suckling mouse bioassay in the form of accumulated fluids in the intestinal lumen was noticed in four (44.44%) cases, though the activeness in the production of enterotoxins was only at 22°C. The study conducted by Nessbakken (10) showed that the highest number of enterotoxic *Y. enterocolitica* strains were isolated from the clinical cases from humans, which seems to confirm the main theory that the reason for clinical yersiniosis is the production of enterotoxins by *Y. enterocolitica* strains. It is worth noticing that almost 60% of the strains isolated from swine palate tonsils and over 44% of the strains isolated from pork products are capable of producing enterotoxins.

In studies of Kwaga and Iversena (8) on the assessment of the pathogenicity of *Y. enterocolitica* strains and related species from pigs and pork products, one of the phases was the suckling mouse bioassay. In the total of 67 *Y. enterocolitica* strains, a positive result was obtained in 50 (74.63%) cases.

In Poland studies on the activeness of enterotoxins with the use of the suckling mouse bioassay according to Dean *et al.* (4), with a slight modification concerning the interpretation of obtained results, were conducted by Zaremba *et al.* (20). Among 178 examined *Y. enterocolitica* strains, the ability to produce enterotoxins was shown in 23 (12.9%) cases. Therefore, results from the biological test obtained in our studies are the closest to the results obtained by Zaremba *et al.* (20), for among the 95 examined *Y. enterocolitica* strains, a positive result was noticed in 14 (14.74%) cases. A doubtful result was obtained in 33 (34.74%) cases, whilst the remaining 48 (50.52%) strains did not induce the enterotoxin production.

Through a comparison of the results of our research, obtained in the biological test for strains from warm and cold cultures, proportional to the number of examined strains, it is important to notice that the positive and doubtful results were shown twice as often in the strains from the warm than from cold culture. The cold culture was characterised by twice as big number of negative results. Thus, strains from the warm culture show their enterotoxical properties more often and they seem to be the cause of yersiniosis in humans. Only one strain from the cold culture was an exception, since it showed the ability to produce enterotoxin, while a strain isolated from the same animal but cultured in the ITC broth did not have such a property. In 32 cases (in which two strains were marked with the same number, although they were from different cultures), which underwent an examination of the ability to produce enterotoxins; 20 (62.5%) were characterised by a distinct result. This confirms the existence of mixed infections and a necessity to run both kinds of cultures in order to obtain the most reliable results.

Recently, Singh and Virdi (19) examined the enterotoxical activeness of 216 *Y. enterocolitica* strains isolated from the faeces of pediatric patients with diarrhoea (36 strains), from pig throats (162 strains), and from different water reservoirs (18 strains). Among the strains isolated from pigs, enterotoxical activeness was shown in 101 (62.3%) strains.

The results obtained in our study showed that almost 15% *Y. enterocolitica* strains existing in the population of pigs are directly dangerous to public health because of their enterotoxical capacities. It is worth noting, however, that the capacity to produce enterotoxins by strains, which gave a doubtful result (around 35%) in the suckling mouse bioassay, was not explicitly declared as impossible.

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References