Stiftung zur Förderung der Erforschung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen

Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing

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Example of a project

Development of a Real Time Reverse Transkriptase-PCR procedure for the detection of *Clostridium botulinum* type A, B, E and F-neurotoxin-production in food as an alternative to the mouse bioassay

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The aim of this research project was to develop an alternative method to the mouse bioassay („mouse test “) which is the “gold standard” for the detection of botulinum neurotoxins (Bont) in food. For this purpose a Real Time Reverse Transkriptase-PCR-procedure (Real Time RT-PCR) has been developed.

Clostridium botulinum (*C. botulinum*) is an obligatorily anaerobic, spore-forming rod shaped bacterium, which belongs to the family *Clostridiaceae.* The agent is wide-spread in the environment and is able to produce seven different neurotoxintyps (Bont/A-G). Botulinum toxins are considered as the strongest naturally occurring toxins, which cause the botulism in humans and in animals. The types A, B and E, rare F, are reported to cause food poisonings in humans, while in animals predominantly the types C and D can cause clinically botulism.

The classical food-borne botulism is a severe, life-threatening intoxication of humans. After an incubation period of a few hours to several days serious illness symptoms arise which can cause the death of the patient. The main symptoms of the illness in humans are: nausea, vomiting, diarrhea, abdominal cramps, sip and language disturbances as well as flaccid paralyses of the head, neck, skeleton and respiratory muscles. The patient dies with full consciousness. The lethal dose is 0,3 ng/kg toxin for mice and 0,2 µg/kg to 2,0 µg/kg for humans. In the last years 5-15 cases in average were announced in Germany, but world-wide more than 1,000 cases have been counted. The botulism is caused predominantly by home preserved meat and vegetable tins, vacuum-packed kipper or bone ham.

A special form is infant botulism. It can be caused by consumption of honey which may carry high numbers of *C. botulinum* spores, but probably also by house dust. In the case of infant botulism the death rate is particularly high.

In accordance to the DIN standard 10102/§ 64 LFGB, L 06,00-26 the investigation of a suspicious food sample requires a number of at least 54 experimental mice. Therefore we developed a Real Time Reverse Transkriptase-PCR procedure (Real Time RT-PCR) as an alternative method for the detection of *C. botulinum* type A, B, E and F-neurotoxinproduktion in food samples. Such a molecular-based method can be a basis for the innovative development of further auxiliary and replacement methods.

We have developed a molecular-based procedure (Real Time RT-PCR) for the qualitative and quantitative detection of a gene expression for all food-associated *C. botulinum* toxintyps (type A, B, E and F) including their subtyps. As a starting point for establishment of the new method a total number of 67 *C. botulinum* strains have been used. All of them have been

characterized by biochemical, serological and molecular-biological fine differentiation and classification procedures as *C. botulinum* strains (24 type A-, 31 type B-, six type E- and six type F-strains). By the establishment of an appropriate primer probe system the Bont A-, B-, E- and F-production could be proven by the detection of mRNA and their transcription into cDNA.

The absolute quantification was based on a decadic serial dilution of Bont A-, B-, E- and F-cDNA and was calculated on the basis of a standard curve. For the relative quantification of the Bont A, B, E, and F an “housekeeping”- gene, which functioned as an endogenous control has been used and the data was analysed by using the comparative Ct-method. The expression of the Bont A-, B-, E- and F-gene was described comparatively to the expression of the selected „housekeeping“- gene. The actual expression and thus the actual quantity of the produced neurotoxin could be determined in this way. A method cascade can be finally presented, which can serve as an alternative to the mouse bioassay.

Own publications/lectures

1. AßMUS, N., ROSA, S., ABDULMAWJOOD, A., NIKOLAUS, S., GAREIS, M., BÜLTE, M. and H. EISGRUBER (Poster)
   Development of a Reverse-Transcriptase Real Time-PCR for the detection of neurotoxin-production of *Clostridium botulinum* type A, B, E and F in foodstuff as an alternative to the mouse-bioassay, *Clostridium botulinum* congress, Helsinki (16.-19.6.2008)

2. BÜLTE, M., S. NIKOLAUS
   Food-borne botulism, internal symposium for the detection of *Clostridium botulinum*-toxins, Hannover (25.03.2009)

3. ABDULMAWJOOD, A.
   Molekular-based identification of botulinum toxin, internal symposium for the detection of *Clostridium botulinum*-toxins, Hannover (25.03.2009)

4. NIKOLAUS, S., ABDULMAWJOOD, A., AßMUS, N., ROSA, S., GAREIS, M., BÜLTE, M. and H. EISGRUBER (Poster)
   „Phenotypic and genotypic characterization of food-relevant *Clostridium botulinum*-strains“, 49. meeting of the group of food hygiene of the german veterinary association (DVG), Garmisch-Partenkirchen (29.09.-02.10.2008)

5. NIKOLAUS, S., BÜLTE, M. and H. EISGRUBER (lecture)
   „To the phenotypic characterization of *C. botulinum*-strains“, 50., meeting of the group of food hygiene of the german veterinary association (DVG), Garmisch-Partenkirchen (29.09.-02.10.2009)

6. ABDULMAWJOOD, A., ROSA, S., EISGRUBER, H. and M. BÜLTE (Poster)
   „Molecular-based detection of botulinum toxin genes“, 50. meeting of the group of food hygiene of the german veterinary association (DVG), Garmisch-Partenkirchen (29.09.-02.10.2009)

7. ABDULMAWJOOD, A., DÜKER, F., BABIĆ STEGELMANN, K., EISGRUBER, H. and M. BÜLTE (Poster)
   „Quantification of neurotoxin type-A gene expression of *Clostridium botulinum* by using Reverse Transkriptase Real Time-PCR”, 51. meeting of the group of food hygiene of the german veterinary association (DVG), Garmisch-Partenkirchen (27.09.-01.10.2010)

Publications in preparation

1. Characterization of food-borne C*lostridium botulinum* strains by means of ELISA and polymerase chain reaction

2. Development of Reverse Transcriptase Real Time-PCR for the detection of botulinum toxins from food-borne strains

3. The Reverse Transcriptase Real Time-PCR as alternative to the mouse bioassay

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