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Development of ELISA techniques for haemorrhagic septicaemia

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Abstract: Haemorrhagic septicaemia (HS) caused by *Pasteurella multocida* serotypes B:2 and E:2 in Asian and African countries respectively is a major epizootic disease of cattle and buffaloes with heavy morbidity and mortality. Being simple, rapid, inexpensive and easy for automation, ELISA has emerged as an important tool for diagnosis as well as monitoring the immune status of animals vaccinated against HS in laboratories. In the present review, development of ELISA techniques for diagnosis, sero-surveillance of immune status in vaccinated animals and DIVA strategy for the evaluation of various HS control programmes have been discussed. Among the various variants, indirect-ELISA has been found most commonly used format for sero-surveillance against HS vaccines and quantification of antibody responses in different vaccine formulation trails. The development of monoclonal antibodies based ELISA have increased the specificity and sensitivity of the test.

Key Words: ELISA; Haemorrhagic septicaemia; *Pasteurella multocida*; Sero-surveillance

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute, highly fatal, septicaemic disease of bovines occurring in most tropical regions of Asia and Africa. The disease is characterized by high fever, oedema of sub-mandibular region and respiratory rales followed by death of animal if not treated at early stage. It is classified as List B disease by Office International des Epizooties (OIE) and considered to be one of the most economically important diseases of livestock because of high morbidity and mortality in endemic areas[1-2]. Among bovines, buffaloes have been reported to be more susceptible than cattle following natural infection[3]. HS is primarily caused by two specific serotypes, B:2 (Asian serotype) and E:2 (African serotype) of *Pasteurella multocida* (a Gram-negative, non-motile, non-spore forming, cocco-bacillary organism with characteristic bipolar staining), however, some other serotypes *viz* A:1, A:1,3, A:3, A:4, B:1, B:2,5, B:3,4, E:2,5, F:3, F:3,4 have also been reported to be isolated from HS outbreaks[4]. Although the organism does not survive outside the animal body for long time, it can survive up to several days in moist soil and water leading to wide transmission during monsoon season[5]. Further, the infected animals may remain carrier for long period (as *P. multocida* persists in tonsilar crypts for several months even after antibiotics treatment) and shed the organisms intermittently in nasal secretions[6-7].

Enzyme immunoassays are broadly classified into homogenous and heterogenous assays, among which heterogenous assays are widely used. Heterogenous assays are further classified into competitive and non-competitive assays. When the antigens or antibodies adsorbed to solid phase are detected, these assays are termed as enzyme linked immunosorbent assay (ELISA)[8]. Since its development in 1971[9], ELISA has gained high importance in laboratories for diagnostic and sero-monitoring purposes and considered as serological test of choice due to its speed, sensitivity, specificity, potential of automation and ability to run large number of samples with less time. It is based on the basic principle of monitoring the changed colour with spectrophotometer after reacting an antigen or antibody conjugated with enzyme with its substrate[10]. As vaccination of livestock in the endemic areas is still the method of choice for control of HS, ELISA is commonly used for sero-monitoring the vaccination status at field level. Also, the HS working groups of FAO and Animal Production & Health Commission for Asia (Bangkok, Thailand, 1987 and 1990) has recommended ELISA for the evaluation of immune responses against *P. multocida* B:2[11]. In the present review, role of ELISA in diagnosis of the disease, monitoring of immune status of vaccinated animals and quantification of antibodies in vaccine trials has been discussed along with various protocols and modifications applied for enhancing the sensitivity and specificity of the test.

ELISA in diagnosis

As the disease is of utmost importance in the tropical developing world, a simple, reliable and inexpensive ELISA test was developed in 1990 for rapid identification of HS causing strains of *P. multocida*. Different dilutions of bacteria were tested using rabbit anti *P. multocida* immunoglobulin (Ig) fractions as coating antigen. The assay showed specificity of 99% and sensitivity 86% on 124 type strains and field isolates of *P. multocida*[12]. However, due to low sensitivity and development of polymerase chain reaction (PCR) based molecular diagnostic tests (*P. multocida* specific PCR assay, Multiplex PCR, Serotype specific PCR assay, PCR fingerprinting), ELISA are not presently used for diagnosis of disease and also not recommended by OIE[2]. A new molecular method (HS-est-RT-PCR) has been developed with more accurate diagnosis of HS isolates as compared to previously developed PCR methods[13]. Recently, an ELISA test employing somatic and capsular antigens (coating antigen) was compared with culture, microagglutination test (MAT) and indirect haemagglutination test (IHAT) in a serodiagnosis study of HS. ELISA test using capsular antigens was reported to be more sensitive to diagnose *P. multocida* in apparently healthy, diseased and emergency slaughtered animals with 42%, 92.9% and 80% positive samples respectively[14].

ELISA in sero-surveillance

An ELISA was developed in 1989 for the evaluation of antibody responses to HS vaccine in bovines using heat stable antigen (coating antigen) and horse radish peroxidase labelled anti-cow Ig and reported elevated levels

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of antibodies to crude lipopolysaccharides in vaccinated animals. The study suggested ELISA, being specific, sensitive and low cost method, an ideal method of monitoring the immune response of animals after vaccination[15]. Presently, non-competitive indirect solid phase assays (indirect-ELISA) have been commonly used in sero-surveillance studies in which specific antibody (primary antibody) in the serum sample bind to the antigen present in solid phase (coating antigen) and are detected with an enzyme labelled anti-Ig secondary antibody. In India, an ELISA antibody kit has also been developed in 2005 for sero-surveillance studies by evaluating antibody levels in sera of cattle and buffaloes vaccinated againstHS[16]. In a district-wise sero-monitoring survey of Haryana (India), 3695 sera samples from cattle and buffaloes vaccinated with HS alum precipitated vaccine (APV) were tested with indirect-ELISA using outer membrane proteins (OMP) of *P. multocida* P_{52} strain and reported more than 85% samples of buffalo sera showing protective levels of antibodies while in cattle sera only 50% samples showed protective antibody levels indicating stepping up needs in vaccination programme for cattle[17]. Sero-monitoring of 156 buffalo and 64 cattle sera samples for HS was done using monoclonal antibody based indirect ELISA (Mab-ELISA) four and six months after vaccination with APV. The ELISA titres showed protective antibody levels at four month but marginal protection at six month post vaccination[18]. Mab-ELISA has also been used for estimation of duration of immunity of APV commonly used in field against HS. The levels of antibodies were high up to four month post vaccination and antibody levels start decreasing afterward, however remained protective up to six months indicating six months duration of immunity of HS-APV[19]. To improve the specificity of test, a Mab based blocking ELISA has been developed and standardized. The antibody titres were detected in sera of cattle and buffaloes vaccinated with HS-APV using Mab developed against whole cell lysates (WCL) of *P. multocida* B:2. The Mab based blocking ELISA developed was found to be more specific than conventional ELISA used for estimation of immunity status against HS[20]. Indirect-ELISA using OMP demonstrated the elevated antibody responses than MAT and IHAT in cows vaccinated with HS-APV and reported the shorter duration of immunity of presently used APV[21]. As the sero-surveillance study is a very cumbersome job, emergence of ELISA that can perform large number of samples at one time with high specificity and sensitivity has revolutionized the monitoring status of vaccination control programmes.

ELISA in quantification of antibodies

Various vaccines used in field (APV, oil adjuvant vaccine) and experimental vaccines (live attenuated, sub-unit, recombinant vaccines) are trialled in experimental animals for their protective efficacy and duration of immunity. To estimate the protective efficacy and duration of immunity of different type of vaccine formulations, indirect-ELISA on sera samples collected at different time intervals from vaccinated animals is commonly used. The protective efficacy of different antigens *viz* whole bacterium, antigen heated at 56 and 100°C, sonicated whole cells, capsular and lipopolysaccharide antigen, potassium thiocyanate extract and sodium salicylate extract of *P. multocida* B:2 were evaluated on buffalo calves sera samples by using indirect-ELISA and reported that capsular antigens were superior for assessing protection status in buffalo calves against HS with indirect-ELISA[22]. In a comparative study on buffalo calves vaccinated with OMP and WCL vaccine, ELISA showed superiority over IHAT[23]. An indirect-ELISA with OMP as coating antigen and skimmed milk powder (1%, w/v) as blocking reagent was used to study the antibody response to OMP vaccines adjuvanted with montanide and liposome complex[24]. In another study on immunogenicity of OMP-enriched fractions (in normal and iron-deficient conditions) of *P. multocida* B:2, indirect-ELISA with WCL as coating antigen and skimmed milk powder (3%, w/v) as blocking reagent was developed and employed to determine the antibody titres in mice[25-26]. Indirect-ELISA has also been used to study the antibody responses of various recombinant sub-unit vaccines in mice as rOmpH[27-28], rOmp87[29], rVacJ[30], rTbpA fragments[31], genetically engineered mutant vaccines as AroA vaccines in mouse model[32-33] and cattle[34-35] and live intranasal aerosol (serotype B:3,4) vaccines[36]. The types of vaccines from 1990s (bacterins with or without adjuvants) to 2010s (sub-unit and recombinant vaccines) have advanced but the test (ELISA) for quantification of antibodies to vaccine formulations remained same with slight modifications, indicating how important ELISA is for sero-monitoring as well as quantification of antibodies in vaccine trials.

Calves are passively immunized *via* transfer of maternal antibodies from dam through colostrum after birth. These maternally derived antibodies protect neonates from most infectious diseases, however, these antibodies cause hindrance in active immune responses *via* vaccination of calves. ELISA has been used to monitor the duration of maternally derived specific antibody levels against HS in calves. New born calves from Holstein Friesian dams vaccinated against HS were sero-monitered for the levels of specific maternally transferred IgG up to 6 month of age using indirect-ELISA. IgG levels were high during 8 to 16 weeks of age and then the levels started declining indicating the time of active immunization after 4 months of age[37]. In another study, indirect-ELISA using OMP evaluated approximately 3 months of age being ideal for vaccination against *P. multocida* in calves[38]. In this way ELISA is helpful in quantification of antibodies to study the immune responses against experimental and field vaccines and to monitor the maternal antibody responses, duration of protective levels and time for active immunization *via* vaccination in calves.

ELISA for differentiating infected from vaccinated animals (DIVA)

As vaccination is the method of choice to control the outbreaks of HS in endemic areas, inactivated killed vaccines are mainly used in most of Asian countries. To monitor the vaccination programmes run by different Government and non-government agencies, DIVA strategy can be an ideal tool. Among the two most economically important diseases of livestock in India, DIVA is commonly used for Foot and mouth disease using various proteins of virus in ELISA *viz* polypeptide 3ABC using Mab-based ELISA[39], protein 3D using solid-phase competitive

ELISA[40], 3ABC Mab based blocking ELISA[41-42], 3B/VPg in epitope-blocking ELISA[43] etc. However, DIVA has not been developed for HS. Recently, an attempt has been made for development of DIVA strategy by using aluminium nanoparticles and keyhole limpet hemocyanin (KLH) with formalin inactivated *P. multocida* B:2 (P_{52} strain) in mouse model. Anti-KLH antibodies produced in sera of vaccinated animals were detectable by employing indirect-ELISA for time as long as anti-bacterial antibodies indicating the suitability of KLH inclusion for DIVA strategy in HS control programmes[44].

CONCLUSION

HS, an acute, fatal and septicaemic disease of bovines, is a disease of major economic importance in Asia and Africa due to high morbidity and mortality. Though various approaches have been used for estimation of immune responses and identification of infectious agants, ELISA being simple, rapid, highly sensitive and specific, is emerged as a novel application in terms of sero-surveillance and quantification of antibodies in vaccine trials. However, in diagnosis of the disease, ELISA has been replaced by newer and faster molecular methods (PCRs). The use of Mab developed for specific bands have enhanced the specificity and sensitivity of ELISA and use of blocking reagents as skimmed milk powder have made the assays cheap for wide use of this test in laboratories.

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REFERENCES

1. De Alwis, MCL. "Haemorrhagic Septicaemia – A General Review." *British Veterinary Journal* 148.2 (1992): 99-112. Print
2. OIE. "Haemorrhagic septicaemia, Chapter 2.4.12. Terrestrial manual, Paris, France." (2009): 739-750. Print
3. Annas, S, M Zamri-Saad, FF Jesse, and Z Zunita. "Comparative Clinicopathological Changes in Buffalo and Cattle following infection by *Pasteurella multocida* B:2." *Microbial Pathogenesis* 88 (2015): 94-102. Print
4. Shivachandra, SB, KN Viswas, and AA Kumar. "A Review of Hemorrhagic Septicaemia in Cattle and Buffalo." *Animal Health Research Reviews* 12.1 (2011): 67-82. Print
5. Benkirane, A, and MCL De Alwis. "Haemorrhagic Septicaemia, Its Significance, Prevention and Control in Asia." *Veterinary Medicine Czech* 47.8 (2002): 234-240. Print
6. De Alwis, MCL, TG Wijewardana, AI Gomis, and AA Vipulasiri. "Persistence of the Carrier Status in Haemorrhagic Septicaemia (*Pasteurella multocida* serotype 6:B infection) in Buffaloes." *Tropical Animal Health and Production* 22.3 (1990): 185-194. Print
7. Harper, M, JD Boyce, and B Adler. "*Pasteurella multocida* Pathogenesis: 125 Years after Pasteur." *FEMS Microbiology Letters* 265.1 (2006): 1-10. Print
8. Carpenter, AB. "Enzyme Linked Immunoassays, In. Rose, NR, EC de Macario, JD Folds, HC Lane, and RM Nakamura, *Manual of Clinical Immunology* Ed 5th, ASM, Washington DC." (1997): 20-30. Print
9. Engvall, E, and P Perlmann. "Enzyme-Linked Immunosorbent Assay (ELISA). Quantitative Assay of Immunoglobulin G." *Immunochemistry* 8.9 (1971): 871-874. Print
10. Lequin, RM. "Enzyme Immunoassay (EIA)/ Enzyme-Linked Immunosorbent Assaay (ELISA)." *Clinical Chemistry* 51.12 (2005): 2415-2418. Print
11. Dawkins, HJS, Ramdani, RB Johnson, and TL Spencer. "Haemorrhagic Septicaemia: Correlation of Vaccinal Antibody Responses in Mice with Protection against *Pasteurella multocida* strain M1404." *Veterinary Microbiology* 27.3 (1991): 309-326. Print
12. Dawkins, HJS, RB Johnson, TL Spencer, and BE Patten. "Rapid Identification of *Pasteurella multocida* Organisms Responsible for Haemorrhagic Septicaemia using an Enzyme-Linked Immunosorbent Assay." *Research in Veterinary Science* 49.3 (1990): 261-267. Print
13. Petersen, A, M Bisgaard, K Townsend, and H Christensen. "MLST Typing of *Pasteurella multocida* associated with Haemorrhagic Septicaemia and Development of a real-time PCR specific for Haemorrhagic Septicaemia associated isolates." *Veterinary Microbiology* 170.3 (2014): 335-341. Print
14. El-Jakee, JK, SS Ali, SA El-Shafii, AM Hessain, AA Al-Arfaj, and MI Mohamed. "Comparative Studies for Serodiagnosis of Haemorrhagic Septicaemia in Cattle Sera." *Saudi Journal of Biological Sciences* (2015) (in press). Print
15. http://dx.doi.org/10.1016/j.sjbs.2015.06.011
16. Johnson, RB, HJS Dawkins, TL Spencer, AA Saharee, AR Bahaman, Ramdin, and BE Patten. "Evaluation of Bovine Antibody Response to a strain of *Pasteurella multocida* known to cause Haemorrhagic Septicaemia." *Research in Veterinary Science* 47.2 (1989): 207-209. Print
17. Anonymous, "Project completion report on Development of Monoclonal Antibody and Outer membrane Proteins based ELISA kit for Monitoring Immune Status of Animals Vaccinated with HS." submitted to Department of Biotechnology, Govt. of India (2005).
18. Kumar, A, and NK Kakkar. "Annual Report-Monitoring Immune Status of Cattle and Buffaloes Vaccinated against Haemorrhagic Septicaemia in the state of Haryana." (2006) Department of Veterinary Microbiology, CCSHAU, Hisar. Print
19. Kumari, L, S Kumar, A Kumar, NK Mahajan, BL Pander, and RS Khokhar. "Seromonitoring of Cattle and Buffaloes Vaccinated against Haemorrhagic septicaemia using Monoclonal Antibody based indirect-ELISA." *Journal of Immunology and Immunopathology* 9.1 (2007): 67-70. Print
20. Markam, SK, RS Khokhar, NK Mahajan, A Kumar, SK Kadian, and S Kapoor. "Monoclonal Antibody based ELISA for Seromonitoring of Bovines Vaccinated against Haemorrhagic Septicaemia in Haryana." *The Haryana Veterinarian* 48 (2009): 88-90. Print
21. Kumar, P, and A Kumar. "Development and Standardization of a Blocking ELISA based on Monoclonal Antibody to *Pasteurella multocida*." *The Haryana Veterinarian* 52 (2013): 90-92. Print
22. Qureshi, S, and HM Saxena. "Estimation of Titers of Antibody against *Pasteurella multocida* in Cattle Vaccinated with Haemorrhagic Septicemia Alum Precipitated Vaccine." *Veterianry World* 7.4 (2014): 224-228. Print

23. Afzal, M, R Muneer, and S Akhtar. "Serological Evaluation of *Pasteurella multocida* Antigens associated with Protection in Buffalo Calves." *Revue Scientifique et Technique (International Office of Epizootics)* 11.3 (1992): 917-923. Print

24. Pati, US, SK Srivastava, SC Roy, and T More. "Immunogenicity of Outer Membrane Protein of *Pasteurella multocida* in Buffalo Calves." *Veterinary Microbiology* 52.3 (1996): 301-311. Print

25. Basagoudanavar, SH, DK Singh, and BC Varshney. "Immunization with Outer Membrane Proteins of *Pasteurella multocida* (6:B) provides Protection in Mice." *Journal of Veterinary Medicine A: Physiological, Pathological and Clinical Medicine* 53.10 (2006): 524-530. Print

26. Kharb, S, and S Charan. "Immunogenicity of Iron-Regulated Outer Membrane Proteins of *Pasteurella multocida* B:2 in Mice Model." *Indian Journal of Experimental Biology* 48.12 (2010): 1181-1187. Print

27. Kharb, S, and S Charan. "Estimation of Antibodies against *Pasteurella multocida* B:2 in Serum employing ELISA: Comparision of Different Blocking Reagents." *International Journal of Bioassays* 2.5 (2013): 748-750. Print

28. Tan, HY, NH Nagoor, and SD Sekaran. "Cloning, Expression and Protective Capacity of 37 kDa Outer Membrane Protein Gene (ompH) of *Pasteurella multocida* serotype B:2." *Tropical Biomedicine* 27.3 (2010): 430-441. Print

29. Joshi, S, K Tewari, and R Singh. "Comparative Immunogenicity and Protective Efficacy of Different Preparations of Outer Membrane Proteins of *Pasteurella multocida* (B:2) in a Mouse Model." *Veterinarski Archiv* 83.6 (2013): 665-676. Print

30. Kumar, A, R Yogisharadhya, MA Ramakrishnan, KN Viswas, and SB Shivachandra. "Structural Analysis and Cross-protective Efficacy of Recombinant 87 kDa Outer Membrane Protein (Omp87) of *Pasteurella multocida* serogroup B:2." *Microbial Pathogenesis* 65 (2013): 48-56. Print

31. Shivachandra, SB, A Kumar, R Yogisharadhya, and KN Viswas. "Immunogenicity of Highly Conserved Recombinant VacJ Outer Membrane Lipoprotein of *Pasteurella multocida*." *Vaccine* 32.2 (2014): 290-296. Print

32. Shivachandra, SB, R Yogisharadhya, A Kumar, NN Mohanty, and VK Nagaleekar. "Recombinant Transferrin Binding Protein A (rTbpA) fragments of *Pasteurella multocida* serogroup B:2 Provide variable Protection following Homologous Challenge in Mouse Model." *Research in Veterinary Science* 98 (2015): 1-6. Print

33. Tabatabaei, M, Z Liu, A Finucane, R Parton, and JG Coote. "Protective Immunity Conferred by Attenuated *aroA* derivatives of *Pasteurella multocida* B:2 strains in a Mouse Model of Haemorrhagic Septicaemia." *Infection and Immunity* 70.7 (2002): 3355-3362. Print

34. Chaudhuri, P, VP Singh, A Thamizharasan, and J Lalsiamthara. "*Pasteurella multocida* P52 AroA Mutant Conferred Protection to Rabbits and Mice against Haemorrhagic Septicaemia." *DHR International Journal of Biomedical and Life Sciences* 3.1 (2012): 127-136. Online

35. Hodgson, JC, A Finucane, MP Dagleish, S Ataei, R Parton, and JG Coote. "Efficacy of Vaccination of Calves against Hemorrhagic Septicemia with a live *AroA* derivative of *Pasteurella multocida* B:2 by Two Different Routes of Administration." *Infection and Immunity* 73.3 (2005): 1475-1481. Print

36. Dagleish, MP, JC Hodgson, S Ataei, A Finucane, J Finlayson, J Sales, R Parton, and JG Coote. "Safety and Protective Efficacy of Intramuscular Vaccination with a Live A*roA* derivative of *Pasteurella multocida* B:2 against Experimental Hemorrhagic Septicemia in Calves." *Infection and Immunity* 75.12 (2007): 5837-5844. Print

37. Saleem, L, R Munir, G Ferrari, M Afzal, and FR Chaudhry. "Efficacy and Cross-protectivity of Live Intranasal Aerosol Hemorrhagic Septicemia Vaccine in Buffalo Calves." *International Journal of Current Microbiology and Applied Sciences* 3.11 (2014): 300-307. Online

38. El-Eragi, AMS, MM Mukhtar, and SH Babiker. "Specific Antibodies of *Pasteurella multocida* in newborn calves of vaccinated dams." *Tropical Animal Health and Production* 33.4 (2001): 275-283. Print

39. Prado, ME, TM Prado, M Payton, and AW Confer. "Maternally and Naturally Acquired Antibodies to *Mannheimia haemolytica* and *Pasteurella multocida* in Beef Calves." *Veterinary Immunology and Immunopathology* 111.3 (2006): 301-307. Print

40. Brocchi, E, MI De Diego, A Berlinzani, D Gamba, and F De Simone. "Diagnostic Potential of mab-based ELISAs for Antibodies to Non-Structural Proteins of Foot-and-Mouth Disease Virus to Differentiate Infection from Vaccination." *Veterinary Quarterly* 20.2 (1998): S20-S24. Print

41. Clavijo, A, EM Zhou, K Hole, B Galic, and P Kitching. "Development and Use of a Biotinylated 3ABC Recombinant Protein in a Solid-phase Competitive ELISA for the Detection of Antibodies against Foot-and-Mouth Disease Virus." *Journal of Virological Methods* 120.2 (2004): 217-227. Print

42. Sorensen, KJ, K De Stricker, KC Dyrting, S Grazioli, and B Haas. "Differentiation of Foot-and Mouth Disease Virus Infected Animals from Vaccinated Animals using a Blocking ELISA based on Baculovirus expressed FMDV 3ABC Antigen and a 3ABC Monoclonal Antibody." *Archives of Virology* 150.4 (2005): 805-814. Print

43. Lu, Z, Y Cao, J Guo, S Qi, D Li, Q Zhang, J Ma, H Chang, Z Liu, X Liu, and Q Xie. "Development and Validation of a 3ABC Indirect ELISA for Differentiation of Foot-and-Mouth Disease Virus Infected from Vaccinated Animals." *Veterinary Microbiology* 125.1 (2007): 157-169. Print

44. Oem, JK, BS Chang, HD Joo, MY Yang, GJ Kim, JY Park, YJ Ko, YJ Kim, JH Park, and YS Joo. "Development of an Epitope-Blocking-Enzyme-Linked Immunosorbent Assay to Differentiate between Animals Infected with and Vaccinated against Foot-and-Mouth Disease Virus." *Journal of Virological Methods* 142.1 (2007): 174-181. Print

45. Ray, SM, and A Singh. "Aluminum Oxide Nanoparticle-Adjuvanted *Pasteurella multocida* B:2 Vaccine is Potent and Efficacious and is able with Keyhole Limpet Hemocyanin as a Marker to Differentiate Infected from Vaccinated Animals (DIVA)." *Research and Reviews: A Journal of Immunology* 5.2 (2015): 8-16. Online

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