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The Use of Chicken Embryos as a Model for *Brucella abortus* Strains Isolation from Milk Collected Samples

[1]C.E. Pulido, [1]H.D.I. Martinez, [1]V.M. Villanueva, [1]O.V. Amador, [1]C.A. Peniche, [1]S.V.T. Pardio, [1]G.L.A. Landin, [2]C.R. Flores, [2]A.J.F. Morales and [3]M.A. Lopez
[1]Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Mexico
[2]Centro Nacional de Investigacion Disciplinaria en Microbiologia Animal inIFAP, Mexico
[3]Escuela Nacional de Ciencias Biologicas, IPN, Mexico

Abstract: A total 45 conventional Chicken Embryos (CE), 3-7 days old were divided in three groups of 15 to evaluate CE as an infection model to increase *Brucella abortus* concentration from milk samples. Group I was inoculated with RB51 strain, group II with S19 and group III was used as a not inoculated control. About 100-120 Colony Forming Units (CFU) of microorganisms/0.5 mL of ultra pasteurized milk were inoculated into the yolk sac. All groups were incubated for 72 h at 37°C and then slaughtered to collect liver, spleen, kidney and bone marrow. Organs were inoculated by duplicate on Tripticasein Soy Agar culture medium (TSA) and half were incubated under aerobic conditions and the other half with 5-10% CO_2 environment during 8 days. The results obtained were analyzed using Chi-square. After incubation, 9/15 (60%) CE from group I, 11/15 (73.3%) from group III and 13/15 (86.7%) from group III were alive, respectively. Inoculated groups did not show any significant differences with the control (p>0.05). Isolated concentrations in organs were higher than 100,000 CFU g^{-1} of RB51 strain from 6/9 CE of group I and S19 strain from 7/11 of group II while in group III, all *Brucella* sp. strains were isolated. RB51 strain was harvested from 5/6 livers, 2/6 bone marrows, 1/6 spleens and 1/6 single kidney. S19 strain was harvested from 3/7 livers, 2/7 spleens and 1/7 single kidney. It was concluded that the CE model is a suitable alternative to increase *Brucella abortus* concentration from infected milk.

Key words: Inoculation, concentration, RB51 and S19 strains infection, *Brucella abortus*, chicken embryo, Mexico

INTRODUCTION

Specific bacteriologic diagnosis for the genus *Brucella* has always been limited to disease control in man as well as in domestic animals. In livestock for milk production, bacterial isolation is much more difficult due to restrictions to get quality samples for processing at the laboratory.

Since, the end of the XIX century when Frederick Bang proposed the ring test as an indirect alternative to demonstrate infection at affected herds, several serological tests have been developed. The main aim is to identify reactor animals to different *Brucella* sp. antigens while assuming exposure to similar antigens during their entire life. All recognized serological tests used today for brucellosis control campaigns in different countries despite their high sensitivity, allow undetected individuals representing a continuing risk of infection to the rest of animal population and for man if they are not eliminated from the herd.

It is generally accepted that brucellosis is confirmed by isolation since *Brucella* sp. is a fastidious bacteria with low growth and development in culture media. In addition, it is also known that to prove the presence of *Brucella* sp. in a stained smear by either by using Gram or a modified Ziehl Neelsen or Koster staining method, the bacterial load has to be at least 1,000,000 bacteria or Colony Forming Units (CFU)/mL or g of collected sample. Also in order to growth in selective or differential culture media, the minimal load is 1,000 CFU mL^{-1} or g of collected sample.

The most important source for infection with *Brucella* sp. in man and most mammals occur in general by consumption of milk and contaminated derivates (Luna-Martinez and Mejia-Teran, 2002). It is believed that milk and derivates contain high bacterial concentrations enough to be infective at around 10^6 CFU concentration mL^{-1} or g of sample. However, this is not due to the intermittent shedding or for the concentration in every mL of produced milk (Yeager *et al*., 1967;

Corresponding Author: H.D.I. Martinez, Circunvalacion Esq. Yanez S/N, Col. Unidad Veracruzana, Veracruz, Ver., CP. 91700, Mexico

Hobby *et al*., 1973). Intracellular parasites, viruses and related bacterial microorganisms such as Chlamydophila and Rickesia are isolated by cell culture, CE or laboratory animals (Hitchner *et al*., 1980; Carter and Wise, 2004). Since, bacteria of the genus *Brucella* share ownership of behaving as facultative parasites (Tizard, 2004), several alternatives could be developed to increase multiplication and concentration to be used for posterior isolation in culture media.

There is evidence that CE had been used for bacteriologic diagnoses in animal brucellosis (Detilleux *et al*., 1988; Samartino and Enright, 1993). However, there is not information regarding if <100 CFU (Yeager *et al*., 1967) could be enough to infect them and if *Brucella* sp. can multiply in adequate quantity to be then isolated in selective or differential culture media. Thus, this could be successful if bacteria is recovered at infected herds and if the infective agent is in milk samples. This represents by itself a public health risk because minimal bacterial titer.

It will be useful to know if low virulence *Brucella abortus* strains such as RB51 and S19 inoculated to CE at 100 CFU dose^{-1} that is known as the minimal infection dose could be enough to infect 3-7 days old C. his would allow if successful to be used as an alternative assay to recover field strains from milk herds classified by official serologic tests as brucellosis affected and to demonstrate active infection in them.

MATERIALS AND METHODS

A total 45, 3-7 days old CE were used for this study. Upon arrival at the laboratory, the eggs were left to stand at least 12 h in an incubator previously fumigated with formaldehyde at 36.5-37.5°C with a Relative Humidity (RH) between 50-60% (Hitchner *et al*., 1980).

After the 12 h standing period, CE viability was observed by egg candling in order to assess survival to stress by transport (Hitchner *et al*., 1980). Observational criterion was used to determinate quality of CE movement to check if each one was dead or alive. RB51 and S19 *Brucella abortus* commercial vaccines (Nova Litton de Mexico, S.A. de C.V.) were used to inoculate CE. Vaccines titers were certificated by the manufacturing laboratory and then tittered again by the Microbiology Laboratory of the Faculty of Veterinary Medicine from Universidad Veracruzana in order to know the final concentration and to adjust the inoculation dose. Once the titre was identified, ten fold serial dilutions in Tripticase Soy Agar culture media (TSA) were made with commercial vaccines in order to obtain a final dose of 100-120 CFU contained in 0.5 mL of ultra pasteurized milk. In this respect, a ten fold serial dilution was considered valid. The bacterial growth for both vaccines was 1×10^{-8} and since 1×10^{-9} dilution was without visible bacterial growth on TSA, this was selected as the desired inoculation dose.

A total of 45 CE were divided in 3 groups of 15. Group I was inoculated with RB51 vaccine, group II with S19 and group III was used as control. Using an egg candler, the air cell for each egg was observed and marked using a carbon pencil (Hitchner *et al*., 1980). The marked area was disinfected with 70% alcohol prior to inoculation. Using a sterile lancet, the shell was drilled above the center of the air cell and then the yolk sac of the CE was localized in order to inoculate it with a the infection dose. After inoculation, the perforation of the shell of each CE was sealed with white wax. Every inoculated CE and controls were identified properly and incubated at 36.5-37.5°C with 50-60 RH for a 72 h period and checked every 24 h three consecutive times.

At the end of the incubation period, all CE were destroyed by refrigeration at 4°C for 3 h (Hitchner *et al*., 1980). Inspection of the CE was carried out by transversal cut around the air cell in order to check physically the egg membranes and to observe changes in morphology or colour. After physical inspection the CE were extracted to identify macroscopic alterations and the shells and membranes were deposited in a dish containing 5% phenol. Every CE was placed on a sterile 10 cm petri dish on the dorsal position and a longitudinal incision was made with a pair of dissecting scissors in order to expose the abdominal organs.

The spleen, bone marrow, liver and kidneys were extracted from each CE and then each organ was sectioned in two parts. Using one half of each organ bacteriological inoculation was made by duplicate in TSA and with the other half, impression smears were fixed with absolute methanol and then stained using a modified Ziehl Neelsen and Gram staining procedures. One half of inoculated TSA was incubated in conventional bacteriological incubation and the other one with 5-10% CO_2 concentration at 37°C during 8 days.

The criteria to select growing *Brucella* sp. colonies were based on observing amber, translucent and small colonies on TSA. Colony identification was made by biochemistry tests Detilleux *et al*. (1988). Also, trough a colony counter, the number of CFU was counted for each inoculated organ on TSA. Once identified isolated colonies as RB51 or S19 *Brucella abortus* strains, they were re-inoculated on TSA and subsequently stained over using culture media with Violet Cristal staining in order to corroborate rough or smooth phase, respectively. Obtained results were statistical analyzed for significance by chi square and association by Odds Ratio (OR).

RESULTS AND DISCUSSION

About 72 h after the incubation period, 9/15 (60%) CE from group I, 11/15 (73%) from group II and 13/15 from group III remained alive as shown in Table 1. After slaughter the CE were inspected (Fig. 1) to extract liver, spleen, kidneys and bone marrow and then inoculated by duplicate on TSA using the pure culture procedure. As it can be shown in Table 2, *Brucella abortus* RB51 strain was isolated from nine CE of group I, *Brucella abortus* S19 strain from 11 of group II and none from group III. It should be noted that from groups I and II, bacterial concentration observed was higher than 100,000 CFU g^{-1} processed organ while from group III *Brucella* sp. growth was not observed or any bacterial contaminant.

From group I, RB51 strain was recovered from five livers, two from bone marrow and one from spleen and single kidney, respectively. From group II, S19 strain was recovered from 3 livers from two spleens and from one single kidney as it could be shown in Table 3.

According with the results shown in Table 1, the hypothesis that RB51 and S19 vaccine strains possessed residual virulence responsible for embryonic mortality was rejected because differences were not observed ($p>0.05$) between groups I and II compared with group III. This indicates that both strains are safe as it has been indicated by other researches. Also, temperature and RH conditions were maintained constant during the incubation period and therefore did not contribute as factors that alter the present results.

It has been noted that experimental infection models could be useful for understanding bacterial multiplication within animal tissues (Gast *et al*., 2002) particularly if they are lower than the minimal dose to be used with conventional culture media as it happens with the genus *Brucella*. Therefore, CE inoculation could be an infection alternative to increase bacterial concentration in samples (Detilleux *et al*., 1988; Samartino and Enright, 1993). It is important to note that in present study every inoculated CE received 100-120 CFU dose of different *Brucella abortus* strains. In addition, it is also noted that there were not bacterial growth on TSA with inoculums applied to any CE because multiplication of strains inside of embryonic selected tissues allowed that initial concentration estimated on $1\text{-}1.2\times10^2$ increased to 1×10^5 CFU g^{-1} of processed organ as it could be shown in Table 2 and 3.

Fig. 1: CE inoculated with *Brucella abortus* strains

Table 1: CE viability after inoculation of two *Brucella abortus* strains

Groups	Alive	Dead
I (RB51)	9 (60.0%)[a]	6 (40.0%)[a]
II (S19)	11 (73.3%)[a]	4 (26.7%)[a]
III (control)	13 (86.7%)[a]	2 (13.3%)[a]

*Same literals between cells do not differ for p>0.05

Table 2: *Brucella abortus* development from CE inoculated with vaccine strains

Groups	Positive	Negative	Total
I (RB51)	6	3	9
II (S19)	7	4	11
III (control)	0	13	13

Table 3: *Brucella abortus* RB51 or S19 strains isolated from different organs

Strain	Liver	Spleen	Kidney	Bone marrow
RB51	5	1	1	2
S19	3	2	1	4

There are several disposable diagnostic tests to diagnose brucellosis around the world today (Banai, 2002; Halling, 2002; Lopez, 2006) ranging from conventional serological agglutination, ELISA, complement fixation and others. Molecular diagnostic tests such as PCR allow the detection of the causal agent without the need to isolate it. However, even the most sophisticated tests have to be recognized from international organizations (e.g., WHO, OIE) so bacteriologic procedures remain viable due to technical and human limitations at different laboratories located in different countries.

Brucella sp. demonstration from samples is a major limiting factor for bacteriological diagnosis because it is a facultative intracellular parasite (Carter and Wise, 2004; Lopez, 2006). Thus, most bacteria are located inside the host cells and therefore, the available microorganisms in the environment is low as it occurs in tuberculosis preventing to be observed by impression smears directly from tissues and other samples (Yeager *et al*., 1967; Hobby *et al*., 1973). In addition, to be isolated for the 1st time from samples of affected animals or humans,

Fig. 2: *Brucella abortus* RB51 strain isolated from organs

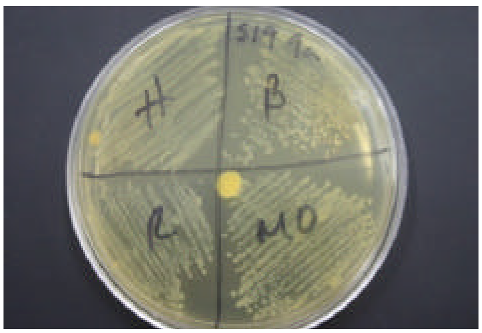

Fig. 3: *Brucella abortus* S19 strain isolated from organs

bacterial concentration must be >1,000 CFU g^{-1}. This is may prove difficult for diagnostic laboratories processing organic fluids such as milk from infected animals because almost always *Brucella* sp. concentration is low. This implies the use of alternatives (Gay and Damon, 1950; Detilleux *et al*., 1988) to increase bacterial concentration from samples such as CE used in the present study in order to expand bacterial count favouring infection of their internal tissues. This proved successful with *Brucella abortus* RB51 and S19 in the present study.

Brucella melitensis was the first bacterium of the *Brucella* genus isolated (Bruce, 1924) from livers and spleens of patients who died due to Malta fever. It means that mononuclear phagocyte system cells (Tizard, 2004) present in the liver (Kuppfer cells) were the most important source to recover these bacteria as it could be observed in CE used in present study with the ones inoculated with RB51 strain. However, for S19 strain most of the isolates were from bone marrow than in other selected organs, reflecting apparent differences between strains for tissue tropism. This also explains that monocytes or macrophages are correctly present in the right quantity and distributed in distinct organs but that there is a slower organic tropism for S19 than RB51 strain (Fig. 2 and 3).

Diverse results had been observed with biological tests using laboratory animals such as mice, guinea pigs or rabbits to demonstrate *Brucella* sp. infection. However, liver, spleen and lymphatic nodes were the most used organs for bacterial recovery consistent with the findings of the present study.

If accepted like that a large number of bacterial infections are caused by intracellular facultative parasites, the most limiting factor is microorganism concentration available as it has been previously reported (Yeager *et al*., 1967; Hobby *et al*., 1973; Moore and Curry, 1998; Mastorides *et al*., 1999) since organs with a higher quantity of phagocyte cells are the major bacterial recovery alternative due to the internal multiplication of *Brucella* sp. and then as a dead cellular response, facilitating uptake of viable bacteria (Tizard, 2004). Thus, CE lymphatic organs have slow development due to age (Hitchner *et al*., 1980) allowing *Brucella* sp. multiplication without limiting immune persistent responses that affects growth (Baldwin, 2002).

CONCLUSION

As previously reported (Gay and Damon, 1950; Detilleux *et al*., 1988; Cheville *et al*., 1992; Samartino and Enright, 1993), the use of CE are a feasible option for *Brucella* sp., bacteriological diagnosis particularly if bacterial concentration is <1,000 CFU. If milk samples from infected animals with field strains are collected aseptically, successful isolation is possible because they infect phagocyte mononuclear cells and develop self defense mechanisms to avoid immune responses (Baldwin, 2002; Tizard, 2004). CE from 3-7 days old do not have an immune response that deter *Brucella abortus* infection allowing bacterial infection capacity to increase from the initial concentration allowing the recovery of bacteria from tissues in culture media. Nevertheless if milk samples directly from herds and possibly contaminated with other bacteria inoculums from milk or derivates must be treated with selected agents that allow only *Brucella* sp. multiplication previous to infect CE as it is carried out for virus isolation (Hitchner *et al*., 1980).

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REFERENCES

Baldwin, C.L., 2002. Immune response overview. Vet. Microbiol., 90: 365-366.

Banai, M., 2002. Control of small ruminant brucellosis by use of *Brucella melitensis* Rev.1 vaccine: Laboratory aspects and field observations. Vet. Microbiol., 90: 497-519.

Bruce, D., 1924. Prevencion of disease. Science, 60: 109-124.

Carter, G.R. and D.J. Wise, 2004. Essentials of Veterinary Bacteriology and Mycology. 6th Edn., Wiley-Blackwell, USA., ISBN-13: 9780813811796, Pages: 290.

Cheville, N.F., A.E. Jensen, S.M. Halling, F.M. Tatum and D.C. Morfitt *et al*., 1992. Bacterial survival, lymph node changes and immunologic responses of cattle vaccinated with standard and mutant strains of *Brucella abortus*. Am. J. Vet. Res., 53: 1881-1888.

Detilleux, P.G., N.F. Cheville and B.L. Deyoe, 1988. Pathogenesis of *Brucella abortus* in chicken embryos. Vet. Pathol., 25: 138-146.

Gast, R.K., J. Guard-Petter and P.S. Holt, 2002. Characteristics of *Salmonella enteritidis* contamination in eggs after oral, aerosol and intravenous inoculation of laying hens. Avian Dis., 46: 629-635.

Gay, K. and S.R. Damon, 1950. Use for the chick embryo for isolation of *Brucella*; multiplication of the organism in the yolk sac and selection of embryo age optimal for isolation from blood. Public Health Rep., 65: 1187-1194.

Halling, S.M., 2002. Paradigm shifts in vaccine development: Lessons learned about antigenicity, pathogenicity and virulence of *Brucellae*. Vet. Microbiol., 90: 545-552.

Hitchner, S.B., C.H. Domermunth, H.G. Parchase and J.E. Williams, 1980. Isolation and Identification of Avian Pathogens. American Association of Avian Pathologists Inc., USA., pp: 63-66.

Hobby, G.L., A.P. Holman, M.D. Iseman and J.M. Jones, 1973. Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. Antimicrob. Agents Chemother., 4: 94-104.

Lopez, M.A., 2006. Agentes infecciosos. Rev. Latinoam. Microbiol., 48: 146-153.

Luna-Martinez, J.E. and C. Mejia-Teran, 2002. Brucellosis in Mexico: Current status and trends. Vet. Microbiol., 90: 19-30.

Mastorides, S.M., R.L. Oehler, J.N. Greene, J.T. Sinnott, M. Kranik and R.L. Sandin, 1999. The deteccion of airborne *Mycobacterium tuberculosis* using micropore membrane air sampling and polymerase chain reaction. Chest, 115: 19-25.

Moore, D.F. and J.I. Curry, 1998. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by ligase chain reaction. J. Clin. Microbiol., 36: 1028-1031.

Samartino, L.E. and F.M. Enright, 1993. Pathogenesis of abortion of bovine brucellosis. Comp. Immunol. Microbiol. Infect. Dis., 16: 95-101.

Tizard, I.R., 2004. Veterinary Immunology: An Introduction. 7th Edn., Saunders, Philadelphia, PA., USA., ISBN-13: 9780721601366, Pages: 496.

Yeager, Jr. H., J. Lacy, L.R. Smith and C.A. LeMaistre, 1967. Quantitative studies of mycobacterial populations in sputum and saliva. Am. Rev. Respir. Dis., 95: 998-1004.