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Review Article

Journal Impact factor
What would recommend to get the Impact Factor for a journal?

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Abstract
Hundreds of electronic and non-electronic journals have been established exponentially in the scientific community since the last two decades. The impact factor (IF) is only one of three standardized measures created by the Institute of Scientific Information (ISI) which can be used to measure the way a journal receives citations to its articles over time. The impact factor and other bibliometric indicators are currently utilized in most countries to evaluate institutions, scientific research, entire journals, and individual articles. Some of periodicals journal has been succeeded in developing their IF and citation reports (CR), while the others journals are still trying to develop those important indicators. This article deals with the most frequently asked questions about the impact factors and its genesis, the methods of calculating it are included. It discusses also the ways to get the IF for the journal and the effects of the impact factor on the journal and article quality and ISI journal selection criteria. Journals dealing with animal health and veterinary sciences with high impact factor are also included.
Keywords: Impact factor, citation reports, self-citation, veterinary sciences.

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Introduction
The journal impact factor (JIF) is now an important quality driver in academic publication and is commonly used to compare the quality of journals, and to assess the quality of publications from individual academics. The JIF and Journal Citation Reports (JCR) are an old issues developed since 1927 (Gross and Gross, 1927). Moreover, interest in this indicator and its derivatives has grown exponentially in the scientific community since 1995 (Eric and Vincent, 2009). JIF and JCR are indicators used to measure the way a journal receives citations to its articles over time. It is created by the Institute of Scientific

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Information (ISI) as one of three standardized measures. The impact factor for a journal is calculated based on a three-year period, and can be considered to be the average number of times published papers are cited up to two years after publication. For example, the impact factor 2012 for a journal **X** would be calculated like this:

A = the number of times articles published in 2010 and 2011 were cited in indexed journals during 2012

B = the total number of ‘‘citable items’’ published in 2010 and 2012(.the number of articles, reviews, proceedings or notes published in 2010-2011)

Journal **X** Impact factor 2012 = A/B

And according to this equation, the impact factor 2011 will be actually published in 2012, because it could not be calculated until all of the 2011 publications had been received. Impact factor 2012 will be published in 2013.

Importantly, not all published content is considered citable. Citable items include articles, reviews, proceedings or notes, but not editorials or letters to the editor.

The h-index is another recent measurement (Hirsch, 2005), it was introduced in 2005 by Professor Jorge Hirsch. It is used to measure the productivity of an individual, group or institution. It is calculated by taking into account the balance between the number of publications and the number of citations per publication. For example, an h-index of 3 tells us that an author or group of authors have 3 publications which received 3 citations or more.

Recently most researchers and scientists have been pointed out to the impact factor. Are there really impact factor fevers? What are the reasons for growing this fever? , intensive and continuous debates have been elicited regarding the roles of the journal impact factor (Davies, 2003; Raff, et al, 2008).

In 2008, Min Zhuo (Min Zhuo, 2008), proposes the Z factor, which is a new and useful way to measure the recent academic performance. He mentioned also to the many reasons that lead to misuse the impact factor. First, the impact factor does not provide any evaluation for the quality of science in the research articles. Although the majority of papers published in high impact journals are generally novel and of highest quality, there are a few 'bad apples'. Second, the impact factor represents the mean citation rate of papers published in one journal, and it does not represent the citation of the specific article itself. And third, a paper published in a low impact factor journal may end up well cited, while conversely, a paper published in a high impact factor journal may garner very few citations.

History of the journal impact factor

Journal impact factor had been created and developed for the first time in USA due to the needs of US University and college librarians who wanted to use an objective method to select journals for their holdings. The tool was not initially developed for research evaluation, and the approach was clearly optimized for the US context. Earlier, the methods was allowed for the identification of high impact journals in specific fields, and this subsequently raised issues of misuse in research evaluation and outright abuse in the promotion of researchers.

ISSN 2307-8073

Despite the concept of an impact factor was first proposed in 1955 (Garfield, 1955), its ancestry can be originate much further back. Since 1873, *Shepard's Citations* was a research tool conceptually similar to the impact factor which was benefited the US legal profession. It named after its founder; the Frank Shepard Company of Colorado. Shepard's Citations was a list of American court cases and judgments, with the complete history of each being recorded in a simple code. Aside from including many law reviews and law journals, Shepard's Citations also listed some specialty publications such as the Journal of the Patent Office Society (Adair ,1955), which would later stimulate the interests of a young chemistry student named Eugene Garfield. In 1927 Gross and Gross(Gross and Gross1927) of Pomona College in the United States (US) first suggested counting references as a means to rank the use of scientific journals (Garfield , 1996). Gross and Gross, 1927, were the first to develop this method [Allen , 1929; Mcneely and Crosno, 1930; Gross and Woodford, 1931; Henkle, 1938; Brodman, 1944; Garrfield, 1955; Raisig, 1960] when they sought to address rising problem of small colleges at a time when one "of the biggest of these had the problem of adequate library facility." Gross and Gross, 1927, elicited a question that is still highly relevant today: "What files of scientific periodicals are needed in a college library successfully to prepare the student for advanced work, taking into consideration also those materials necessary for the stimulation and intellectual development of the faculty?

Then in 1934, S.C. Bradford (Bradford, 1934), head of the Science Museum Library in London, described how scientific articles on a given topic were being unevenly distributed across the journal literature. Back to Garfield, who was the son of a successful newspaper magazine distributor and ran a firm known as the Garfield News Company (Bensman 2007). Garfield's first inspirations came after reading a 1945 article by Vannevar Bush (Bush ,1945) where the idea of making previously collected information more accessible and recording people's information trails, was first proposed (Anonymous 2007). Garfield worked later at the Welch Medical Indexing Project at Johns Hopkins University,(Hopkin, 2005). The Welch project itself was a venture funded by the US Army Medical Library to examine systems for medical information retrieval and new methods of indexing the biomedical literature, which would later evolve into the *Index Medicus*. During working for the Welch project, Garfield became more interested in using machines to help generate indexing terms that would describe a document's contents without the need for human intervention (Garfield Library, 2007). Interest in the concept slowly accumulated and grew, and in 1953, Garfield obtained some major press coverage after organizing the first symposium on machine methods in scientific documentation at Johns Hopkins University (Broad, 1978). Later on, Garfield visited a public library in Baltimore to see Shepard's Citations for himself, confirming that it was indeed, well suited to such a role (Thomson Scientific Website. 2007) and encouraged William Adair a retired Vice President from Shepard's Citations, to write an article about Shepard's Citations, which was subsequently published in 1955 (Adair WC 1955), and in the same year, Garfield published his landmark article in *Science* (Garfield , 1955), where it was first proposed that counting references could help measure, what he termed was, the "impact" of a particular journal

In 1961, Garfield and his colleague Irving Sher produced an experimental *Genetics Citation Index*, which would later lead to the *Science Citation Index* (SCI®). The term

ISSN 2307-8073

"impact factor" was first used in 1963, when the inaugural (1961) SCI was published by Garfield's newly formed ISI company (Garfield ,1996), although it would take some years before the SCI actually made a profit (Hopkin ,2005). In 1965, Price (Price,1965) published his classic article on the network properties of scientific papers, and by 1967, Garfield had noted that as the field of science grew, its commitment to the handling of scientific information must also increase (Garfield ,1967). *Journal Citation Reports* (JCR®) were subsequently launched as a byproduct of the SCI, and between 1975 and 1989, appeared as a supplementary volume in the annual SCI (Garfield ,1996). Garfield's invention started with a listing of 200 journals in roughly 32 pages per issue in 1958 (Thomson Scientific Website 2007), growing to 600 journals in 1964 and 2,400 journals by 1972 (Garfield ,1972). By 1972, approximately 1 million scientists were accessing the ISI database worldwide, and by the SCI's 40th anniversary in 1998, over 8,000 titles were being listed across 35 languages (Thomson Scientific Website 2007) . In 2005 it was estimated that the SCI database contained 550 million citations (Perkel ,2005), and in 2006, the JCR was including around 15 million citations from approximately one million source items per year (Garfield 2006) Table (1).

Table (1) shows the historical development of the journals impact factor.

Date	Events	Reference	Scientist or researcher
1873	Shepard's Citations is first used by the US legal profession	Adair WC (1955) Citation indexes for scientific literature? Am Document **6**, 31–2.	William Adair
1927	first suggested counting references as a means to rank the use of scientific journals	Gross PLK, Gross EM (1927) College libraries andchemical education. Science **66**, 385–9.	Gross PLK, Gross EM
1934	publishes his article on the distribution of scientific manuscripts	Bradford SC (1934) Sources of information on specific subjects. Engineering **137**, 85–6.	Bradford SC
1945	publishes his article on recording people's information trails	Bush V (1945) As we may think. Atlantic Monthly **176**, 101–8.	Bush V
1951	Eugene Garfield joins the Welch Medical Indexing Project at Johns Hopkins	Hopkin K (2005) Most highly cited. The Scientist **19**, 22–3. Garfield Library Website. Eugene Garfield Ph.D. Career Overview. http://www.garfield.library.upenn.edu/ overvu. html. Accessed May 24, 2007.	Eugene Garfield
1955	Garfield publishes his idea for a citation index in *Science*	Garfield E (1955) Citation indexes for science; a new dimension in documentation through association of ideas. Science **122**, 108–11.	Eugene Garfield
1961	The SCI's precursor, the Genetics Citation Index, is founded		Garfield and Irving Sher
1963	The term *Impact factor* is first used in the inaugural Science	Garfield E (1996) How can impact factors be improved?	Garfield E

	Citation Index (SCI®)	BMJ **313**, 411–3.	
1964	The ISI now covers at least 600 journals in its databases	Thomson Scientific Website. Thomson Scientific: Company timeline. http://scientific.thomson.com/isi/ timeline. Accessed May 24, 2007. Garfield E (1972) Citation analysis as a tool in journal evaluation. Science **178**, 471–9.	
1965	Price publishes his article about the network properties of scientific papers	Price DJ (1965) Networks of scientific papers. Science **149**, 510–5.	Price DJ
1972	It is estimated that one million scientists are accessing the ISI database		
1997	The first journal is accused of manipulating its impact factor80)	Smith R (1997) Journal accused of manipulating impact factor. BMJ **314**, 463.	
1998	40th anniversary of the ISI, which is now covering over 8,000 titles35)	Thomson Scientific Website. Thomson Scientific: Company timeline. http://scientific.thomson.com/isi/ timeline. Accessed May 24, 2007.	
1999	The concept of "topic-based" impact factors for occupational health is proposed17)	Takahashi K, Aw TC, Koh D (1999) An alternative to journal-based impact factors. Occup Med (Lond) **49**, 57–9.	Takahashi K, Aw TC, Koh D
2005	Approximately 550 million citations are contained in the SCI database37)	Perkel JM (2005) The future of citation analysis. The Scientist **19**, 24–5.	Perkel JM
2005	The Agony and the Ecstasy—The History and Meaning of the Journal Impact Factor	International Congress on Peer Review And Biomedical Publication Chicago, September 16, 2005	Eugene Garfield Chairman Emeritus, Thomson ISI

Applications of the journal impact factor

The original concept of the impact factor was used as a marketing tool for publishers, who could charge higher advertising rates and cover costs for highly cited journals. It was also intended to guide librarians on how to select journals that should be included within their catalogue, however, the applications of the impact factor rapidly expanded until it became widely regarded as a shorthand measure of a journal's quality (Harter and Nisonger, 1997). Inevitably, and recently these indicators of quality were used to criticize the quality of individual researchers output and to be widely used in academic appointments and as an evaluation tool for promotion and tenure review committees (Currie and Wheat, 2007).

http://mirrorofresearchinveterinarysciencesandanimals.com/issue 1,2012,1-60
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The effect of the impact factor on the scientist's publication and journals operations

There can be no doubt that any applications have strengths and weaknesses. Journal impact factor has become extremely important and moved in recent years from an obscure bibliometric indicator to become the chief quantitative measure of the quality of a journal, its research papers, the researchers who wrote those papers, and even the institution they work in. This importance has, in many ways, changed the way that scientists publish and the way journals operate. The impact factor becomes a major impact on scientists. Employers and review committees faced with competing Curriculum vitae (s) may evaluate scientists on the ''quality'' of the journals in which they have published. Researchers building their research profile know that they will be evaluated by the impact factor of the journals in which they publish. Consequently, researchers take the impact factor of a journal into account. The impact factor also has major implications for publishers.

At the same time many conflicting opinions about impact factors were appeared, in literature. Hoeffel (Hoeffel 1998) expressed the situation succinctly, "Impact Factor is not a perfect tool to measure the quality of articles but there is nothing better and it has the advantage of already being in existence and is, therefore, a good technique for scientific evaluation. Experience has shown that in each specialty the best journals are those in which it is most difficult to have an article accepted, and these are the journals that have a high impact factor. Most of these journals existed long before the impact factor was devised. The use of impact factor as a measure of quality is widespread because it fits well with the opinion we have in each field of the best journals in our specialty." While J.K. Vanclay 2012 mentioned that Thomson Reuters impact factor (TRIF) suffers so many weaknesses, that a major overhaul is warranted, and journal editors and other users should cease using the TRIF until Thomson Reuters has addressed these weaknesses. Urgent improvements include the adoption of a 'like-with-like' basis (i.e., citations to articles, divided by the count of articles only), the use of verified one-to-one links only (this would unite authors, editors and Thomson Reuters in quality control); the adoption of a more appropriate reference interval (the present two year interval is too short for many disciplines), the introduction of confidence intervals, and the rounding of reported indices to a more appropriate number of digits. Failing action by Thomson Reuters, journal editors should collaborate as they have come with committee on publication ethic (COPE) to introduce a journal certification system that acknowledges procedures to maintain quality: procedures that add value and restrict plagiarism and fraud. The future of quality science communication lies in the hands of editors.

ISSN 2307-8073

In 2000, Amin, M. and Mabe, M (Amin and Mabe,2007) summarized that the value of the impact factor is affected by the subject area, type and size of a journal, and the "window of measurement" used. As statistical measures they fluctuate from year to year, so that great care needs to be taken in interpreting whether a journal has really "dropped (or risen)" in quality from changes in its impact factor. Use of the absolute values of impact factors, outside of the context of other journals within the same subject area, is virtually meaningless; journals ranked top in one field may be bottom in another. Extending the use of the journal impact factor from the journal to the authors of papers in the journal is highly suspect; the error margins can become so high as to make any value meaningless. Professional journal types (such as those in medicine) frequently contain many more types of source item than the standard research journal. Errors can arise in ensuring the right types of article are counted in calculating the impact factor. Citation measures, facilitated by the richness of ISI's citation databases, can provide very useful insights into scholarly research and its communication. Impact factors, as one citation measure, are useful in establishing the influence journals have within the literature of a discipline. Nevertheless, they are not a direct measure of quality and must be used with considerable care.

Creating and increasing journal impact factor.

Hundreds electronic and non-electronic journals have been established exponentially in the scientific community since the last two decades. Because of the contemporary modern fascination with impact factors and their potential effects on advertising, subscription rates and author attractiveness, it is not wondering that editors have often tried ways to create or How to Get an Impact Factor for his/ her journal, (Table 2), and in case of the existence of the impact factor they would work to increase their own journal's indicator scores. They are believed with few common ways that are seemed to be helpful in this regard (Gowrishankar and Divakar 1999). One of the most strategies which lead to create and increased the journal impact factor is the publication of a journal's entire contents such as abstract, tables, figure, full-text and conclusion online, for free. This technique has been shown that sharing detailed research data may be associated with an increased citation rate in some journals (Lawrence , 2001; Piwowar, et al 2007) .

Table (2) Shows the criteria to get an impact factor.

Criteria that journal must has it to get an Impact Factor
1. Have a basic level of citation activity 2. Address a niche area with the journal 3. Be 'international' or very popular regionally

ISSN 2307-8073

4. Conform to journal publishing norms
- Regular publication
- Peer reviewed

There is no doubt that open access to scientific literature online provides significant advantages for the scientific community. The free online access speeds the distribution of scientific and research information via links and other kinds of communication (Lawrence , 2001) like research gate which is very popular recently. Free access helps address an important equity issue for countries that cannot afford expensive journal subscriptions, but would nevertheless benefit from the information they contain. For developing countries in particular, the internet provides many scientists' only chance of accessing literature that would otherwise be too expensive to purchase (Khan ,2001).

Despite, free access is not always elixir, as technical access to scientific information remains a serious problem for scholars in developing countries such as Africa (Keese,2001) , some countries in Asia due to computer scarcity, limited bandwidth and difficulty in accessing the internet). Indeed, the need for greater exploitation of the internet has been previously suggested by Seringhaus and Gerstein, 2007, as a future technique to help truly integrate scientific information on a global scale.

Self-citation is another way which has been used as a technique. Self-citation tends to cite of other articles previously published in the same journal. Such behavior appears to have been first reported in the late 1990s80), although the rate at which it is attempted often varies between authors, research fields and between journals of the same special field (Miguel and Marti-Bonmati ,2002). Regardless of how regularly a particular journal or its authors may actually attempt it, blatant editorial encouragement of self-citation has generally been discouraged by the academic and scientific community (Hemmingsson, 2002; Hemmingsson, et al 2002). Publishing of review article is another technique often used for increasing the impact factor. In this strategy, a journal can choose to publish a higher proportion of literature reviews compared to original research articles, as reviews will contribute to generate a higher number of citations from other authors (Anonymous, 1998). Although review-only journals often have reasonably high impact factors, at least 40,000 review articles are published in the world each year, and not all of them will achieve high impact (Garfield , 1996); suggesting that it is not a foolproof methodology. The other way that journal may used to increase their impact factor, is choosing to focus on research topics that naturally generate a high number of citations, such as molecular biology, and eliminate research topics that do not (Anonymous, 1998). Journals may prefer to publish articles that are particularly controversial or deal with inherently controversial issues (Garfield , 1996). Controversy generates interest among readers, who may then be inclined to cite the article, regardless of its actual scientific or academic value.

ISSN 2307-8073

In this manner, it has been shown that even erroneous articles may continue to receive citations after being retracted (Budd, et al, 1998).

Currently, scientific journals exist in very competitive publishing environment; an enthusiasm with this goal may invariably become inefficient. As a fact that a publication in highly impact factor is very difficult, it should also be remembered that publication of one's research in low impact factor journals is not necessarily a disaster. Researcher must think that his useful scientific material is not only limited to the high–impact periodicals. Nakayama and colleagues (2003), in his a study regarding guidelines for evidence-based practice, found that journals with low impact factors were still frequently cited as providing important evidence. It is appeared that self-evident is the only way to improve a journal's impact factor and we do agree with Garfield's statement that he mentioned previously, "a journal impact is simply a measure of its ability to attract the best papers available" (Garfield , 1996), then it would appear that the ideal way to increase journal's impact factor is simply to attract and publish better material. The world revolution in information technology (IT) , the rapidly distribution of the internet and the domination of multiple scientific gates and links lead to increase in the impact factor of most journal as a fact that the IT open the gates for the authors to access and cite a wider variety of references per one article. Some researchers looked recently at trends for seven major medical journals and found that most impact factors had risen over the previous 12 yr Chew and colleagues for example (Chew, et al 2007).

Impact Factors and veterinary medicine

Nowadays there are vast amounts of easily accessible information relating to animal health available to veterinarians and others veterinary professionals. As in other fields, use of the Internet and the Web has greatly expanded information, research articles and communication process among veterinary professionals. Different research fields in the veterinary medicine and animal health have always depended on the exchange and citation of several items which lead to raised variations in the impact factor of the journals in veterinary medicine. There has been an increasing level of debate regarding the overall usefulness and relevance of impact factors for veterinary medicine. Impact factors for specialist animal health and veterinary medicine journals have historically been reported by Red Jasber Limited. 178"typical" veterinary sciences journals and its impact factors have been listed since1981. These data showed some journal revealed high impact factor reach to 17.42 .Table (3). (http://www.in-cites.com/research/2007/december_17_2007-2.html).

Table 3: Some Veterinary Sciences Journals Ranked by Impact

ISSN 2307-8073

Rank	2006 Impact Factor	Impact 2002-06	Impact 1981-2006
1	Vaccine (3.16)	Vaccine (4.84)	Adv. Veterinary Sci. (17.42)
2	Veterinary Research (3.15)	Veterinary Research (4.57)	Animal Production (13.75)
3	Fish & Shellfish Immun. (2.73)	Veterinary Microbiology (4.01)	Vaccine (12.61)
4	Veterinary Microbiology (2.07)	Fish & Shellfish Immun. (3.98)	Am. J. Veter. Res. (12.43)
5	Med. Vet. Entomology (2.03)	Med. Vet. Entomology (3.66)	Theriogenology (11.77)
6	Medical Mycology (2.01)	Vet. Immunol. Immunop. (3.58)	Equine Veterinary J. (11.42)
7	Comp. Immunol. Microb. (2.00)	ILAR Journal (3.55)	Veterinary Radiology (10.91)
8	Vet. Immunol. Immunop. (1.99)	Theriogenology (3.40)	Vet. Immunol. Immunop. (10.86)
9	J. Medical Entomology (1.95)	J. Fish Diseases (3.39)	Veterinary Microbiology (10.85)
10	Veterinary Parisitology (1.90)	Equine Veterinary J. (3.35)	Vet. Immunol. Immunop. (10.81)

Conclusion

The Journal Impact Factor (JIF) and Journal Citation Reports (JCR) are old issues developed since 1927. The actual concept of an impact factor was first proposed by Eugene Garfield in 1955. Moreover, interest in this indicator and its derivatives has grown exponentially in the scientific community since 1995. The original concept of the impact factor was used as a marketing tool for publishers. The impact factor rapidly expanded until it became widely regarded as a shorthand measure of a journal's quality and recently is used to criticize the quality of individual researchers output and to be widely used in academic appointments and as an evaluation tool for promotion and tenure review committees . Impact factor has many supporters as well as many conflicting opinions. Publish the journal's contents online, for free Self-citation , Publish a greater proportion of review articles ,Publish "hot topics" articles, and Focus on research areas which naturally generate more citations, are different ways and techniques used to create and increased the journal impact factor. Recently there are more than 178 journals in the field of animal health and veterinary sciences showing different impact factor scores.

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CASE REPORT

An unusual presentation of locally invasive sequamous cell carcinoma in the fatty tail of Awassi sheep

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Abstract

This report describes a squamous cell carcinoma (SCC) in the inner surface of a fatty tail of a 2.5 year old male Awassi sheep. The tumor started as a small ulcerated mass failed to respond to antibiotics treatment and reached 15 cm in diameter. The mass was removed surgically and histopathologically diagnosed as a squamous cell carcinoma. To the best of our knowledge, this is the first report of a SCC in the fatty tail of an Awassi sheep in Jordan in the veterinary literature.

Keywords: Squamous cell carcinoma; Awassi sheep; fatty tail; Jordan.

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Introduction

Cutaneous Squamous cell carcinoma in sheep has been reported in different countries throughout the world (Hawkins et al., 1981, TiLbrook, 1992, Foreyt et al., 1991, Tustin et al.,1982; Ramadan et al., 1991). This neoplasm has been described in different breeds of sheep and in different locations throughout the body (Foreyt et al., 1991, Mendez et al., 1997 and Ramadan et al., 1991). There are different factors associated with the induction of the neoplasm such as, solar radiation, papillomavirus, genetics and other undetermined factors (Vanselow and Spradbrow, 1982, Hawkins et al., 1981and Uzal et al., 2000). In this report, clinical and pathological findings of the SCC in Awassi sheep are described.

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Case history and case handling

A 2.5 year old male Awassi sheep with adequate nutritional body condition was presented to the Veterinary Health Centre (VHC) at Jordan University of Science and Technology. The animal had a 15 cm ulcerated mass at the base of the inner aspect of the fatty tail. As per owner statement, the mass was small and firm 2 months prior to presentation and continued to grow despite of antibiotic treatments and disinfection .The mass then was surgically removed, fixed in a 10% formalin solution before being routinely processed and paraffin-wax embedded. Sections (4-5µm) were stained with haematoxylin and eosin (H&E).

RESULTS AND DISCUSSION

Microscopically,the mass was unencapsulated and composed of highly infiltrative neoplastic squamous cells encompassing mainly the epidermis, dermis and lesser extent the subcutaneous adipose tissue. The neoplastic cells formed sheets, cords and islands with or without keratin pearls and were embedded within a dense connective tissue (desmoplasia) in the underneath dermis (Figure. 1). Clusters of neoplastic cells were seen infiltrating the subcutaneous adipose tissue (Figure. 2). The neoplastic cells ranged from well differentiated squamous cell with prominent desmosomes to poorly differentiated cells with often distinct cytoplasmic boundaries,moderate to large amount of eosinophilic cytoplasm and variable shaped; round, oval to irregular shaped nuclei with one or more prominent nucleoli. Mitotic figures were 1-2 per high power field. No vascular invasion was observed and the surgical margins were clean.

Extensive diffuse full thickness epidermal necrosis that was covered with a thick serocellular crust was present. The desmoplastic dermis was moderately infiltrated with mixed, predominantly neutrophils, inflammatory cells.

The clinical and pathological findings of the examined mass were consistent with SCC. Squamous cell carcinoma in sheep usually occurs in depigmented skin and in areas deprived of wool (Del Fava *et al*., 2001). Also this neoplasm occurs frequently in adult animals exposed to high solar radiation (Lagadic et al., 1982 and Lloyd, 1961). The majority of reported SCC cases in sheep involved eyelids, vulva, mucocutaneous junctions, nose and perineum (Tustin et al., 1982, Lloyd, 1961 and TiLbrooket al., 1992). In our case, SCC occurred in the skin of the inner surface of the fatty tail with local subcutaneous invasion. Awassi is a breed of sheep that is characterized by a huge fat tail. The ventral aspect of the tail lacks of wool; however, this area is not exposed to ultraviolet light or a solar radiation. Hence, it is less likely to develop SCC in this area secondary to solar radiation. Papilloma virus infection has a close relation with SCC development in sheep (Del Faval et al 2001). Neither intranuclear nor intracytoplasmic inclusion bodies were seen microscopically in this case, however, the possibility of papilloma virus infection cannot be completely rule out.

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In our report, the definite cause for SCC was undetermined. Squamous cell carcinoma has not been reported previously in the fatty tail of Awassi sheep despite more than 3000 different cases of Awassi sheep had been received by the VHC during previous years.

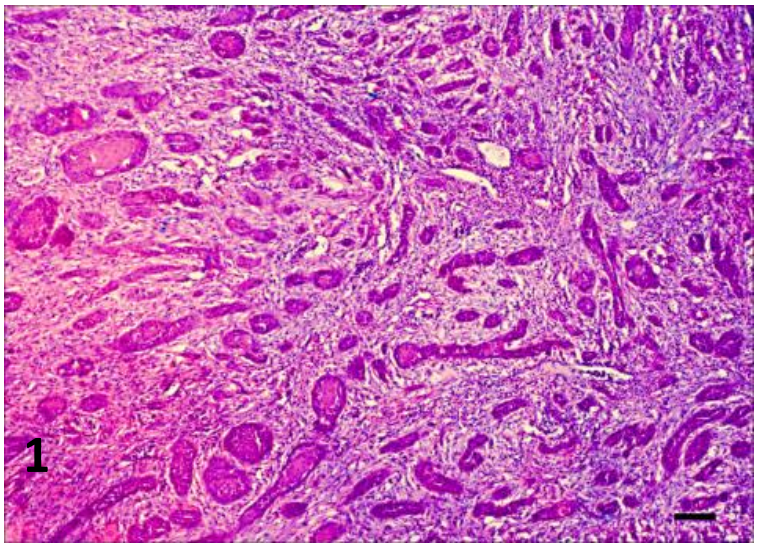
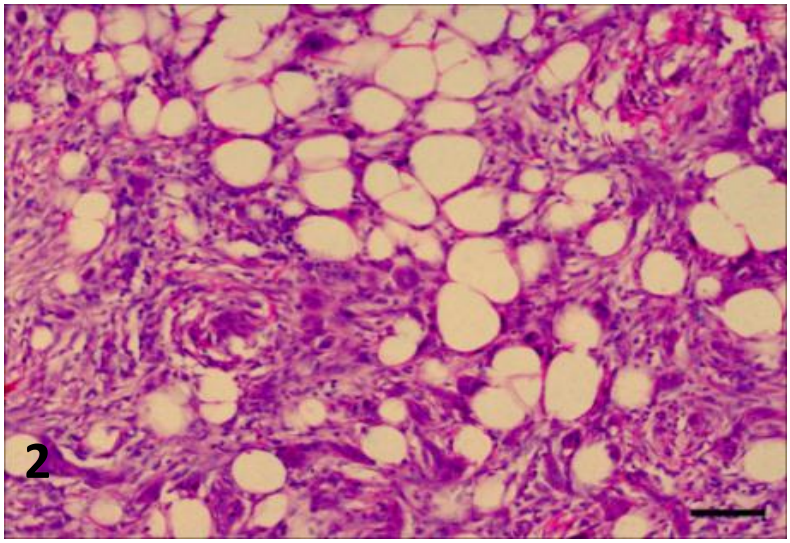


Figure.1: Shows highly infiltrative neoplastic squamous cells embedded in marked connective tissue matrix. H&E. Bar = 50µm.



Figure(1) Shows highly infiltrative neoplastic squamous cells infiltrated deeply into the underlying fat tissue. H&E. Bar = 20µm.

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Original Article

Immune Efficacy of *Salmonella ohio* Somatic antigen in mice

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Abstract

This study was designed to evaluate the effect of *Salmonella ohio* Somatic antigen on humoral and cellular immunity in mice. Two groups of mice (thirty in each) were used, first group was immunized twice at two weeks intervals subcutaneously (S/C) with 0.5 ml of somatic antigen (prepared by heat inactivation of *S. ohio*) containing 1×10^8C.F.U (protein content 200 µg); second group was injected S/C with phosphate buffer saline(PBS). Blood samples were collected at 2, 4, and 6 weeks post booster dose. Humoral immunity was detected by ELISA test, while cellular immunity detected by E. rosette and delayed type hypersensitivity test (DTH).The immunized and control mice groups were challenged with $5LD_{50}$ of virulent *Salmonella ohio* six weeks post booster dose. IgG was increased significantly ($P<0.05$) at 2, 4, and 6 weeks in the immunized group, and the maximum increase of antibody titers was determined at fourth week (651.7 ± 21.3) in comparison with the control group which remained within the normal value in all times of the experiment. E.rosette test showed a significantly increase in the mean of the activated lymphocyte of the immunized group at fourth week of immunization while control group gave normal range of active lymphocyte. In DTH test, immunized group showed a significant increase in footpad thickness after 24 hours post inoculation with soluble antigen in comparison with control group. Immunized mice were resist the challenge dose $5LD_{50}$ {5x ($1.5x10^7$)}of virulent *Salmonella ohio* and all mice of control group died within (3- 4) days.

In conclusion, immunization of mice with somatic *S. ohio* antigen was induced humoral and cellular immune response against Salmonellosis.

Keywords: *Salmonella ohio*, somatic antigen, Cellular Immunity, humoral immunity

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Introduction

Salmonella species are a leading bacterial cause of acute gastroenteritis. Although the global human health impact of *Salmonella* infections has not been estimated, gastroenteritis is

a major cause of morbidity and mortality worldwide both in children under 5 years old and in the general population (Bern et al ,1992,Kosek et al 2003 ,Scallan et al,2005).

During the summer of 2005, an increase in reports of human cases of Salmonella enterica serovar Ohio infection was observed in Belgium . During 11 weeks, 60 cases of laboratory-confirmed Salmonella Ohio infection were reported to the National Reference Centre for Salmonella. All clinical isolates caused self-limiting gastroenteritis in both genders (males and females) and all age groups (children to adult) were affected.(Bertrand et al, 2010).

In Iraq, Al Zubuidy and Yousif (2012) isolated four *Salmonella* species (*Salmonella enteritidis,Salmonella newport ,Salmonella anatum* and *Salmonella ohio*) from different organ of cows at slaughter house especially from which is used for human consumption.

S*almonella* generally exhibit an invasive potential and they can survive for extended periods within cells of the immune system. *In vivo Salmonella* infections are complex with multiple arms of the immune system being engaged. Both humoral and cellular responses can be detected and characterized, but full protective immunity is not always induced, even following natural infection. The murine model has proven to be a fertile ground for exploring immune mechanisms and observations in the mouse have often, although not always, correlated with those in other infectable species, including humans. (Dougan et al, 2011).

Vaccination is potentially an effective tool for the prevention of Salmonellosis. Whole-cell killed vaccines and subunit vaccines were used with variable results for the prevention of *Salmonella* infection in humans and animals **(**Mastroeni et al,2001).

This study was designed to evaluate the humoral and cellular immune response in mice following exposure to somatic antigens of *S.ohio* against challenge with virulent strain.

Materials and Methods

Salmonella ohio was isolated from cows (bile and mesenteric lymph node specimens) at slaughter house in Iraq (Al zubaidy and Yousif,2012), by culturing of on different selective media, biochemical and API tests (Quinn et al 2004).This isolate was confirmed in the National Center of Salmonella /Ministry of Public Health**.**

Preparation of somatic antigen for immunization

Samples of the stock culture of *S. ohio* were used for the preparation of somatic antigen. The culture was inoculated into brain heart infusion broth, and harvested during the early-logarithmic-growth phase, then the somatic antigen was prepared as followed :-

Bacterial suspension was inactivated by heating at 100°C for 30 minutes. Then washed extensively in phosphate-buffered saline (PBS) before use (Smith et al, 1984). Protein content of the antigen was determined by a method of (biurat). The antigen were tested for sterility and safety before use according to (OIE, 2004).

Preparation of soluble antigen

Soluble antigen which used for DTH (skin test) prepared according (Mitov et al,1992) Briefly, three to five colonies from the bacterial isolates on selective medium were inoculated into trypticase soy broth and incubated overnight agar. after washing three times with PBS, the cultures were harvested by centrifugation at 10.000Xg for 30 minutes. The sediment was sonicated for 50 minute at intervals in a water cooled sonicator oscillator at 40 MHZ per second full power. The homogenate was centrifuged twice by using a cooling centrifuge at

8000 Xg for 30 minutes each time to remove cellular debris. The supernatants were passed through a 0.22 μm Millipore filter and stored at (-20^{o}C) until used. Protein content was determined by biuret protein assay.

Immunization of mice

To evaluate the efficacy of the prepared antigen, sixty adult healthy mice aged 4 to 6 weeks were selected. All mice had negative faecal bacteriological culture for salmonella. They were reared in separate cages in the Animal House of Veterinary College, University of Baghdad. The mice were divided equally into two groups.

i-The first group(immunized group) was Immunized with somatic Ag subcutaneously twice at two weeks intervals at a dose of 0.5 ml containing 1x10^{8} CFU/ml and protein concentration 200μg.

ii- The second group (control group) was injected S/C with 0.5 ml of PBS at the same time. Blood samples were collected from all groups at 2nd, 4th and 6th week post-injection. Sera were separated and stored at -20^{O}C. This study was approved by the ethical and research committee of Veterinary Medicine College/University of Baghdad.

Estimating the LD_{50}

The viable count of the bacteria in eight fold dilution (10^{-1},10^{-2}.10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8}) was made by bacterial plate count method according (Quinn et al 2004).

The LD50 was estimated according to (Reed and Muench 1938). Forty eight healthy mice of both sexes were divided into (8) groups (6 in each group). Seven groups of mice were injected intraperitonially with 0.5 ml of calculated CFU diluents, and the eighth group was considered as a control group injected with PBS. All groups were monitored for 30 days to calculate total live and dead mice .

Immunological tests

1- **Enzyme-linked Immunosorbent assay (ELISA)** test for detection of IgG in the serum. This test was done according to manufacturer **(immunological consultents laboratory, Inc.)**
2- **DTH-skin** test was done 21 days after immunization as described by (Hudson and Hay 1980). Briefly, 0.1 ml of soluble antigen of *S. ohio* was injected intradermally in the right footpad of the immunized and control groups while the left side was injected by 0.1 ml of sterile PBS (pH=7.2). The thickness of skin was measured by vernier calliper before injection and at 24, 48 and 72 hours post injection.
3- **E –Rosette test :** It is a test that used for calculates the percentage of viable and non viable T-lymphocytes and estimated activity of T-lymphocytes which formed after immunization of mice with the antigens .This test was done according to (Braganza et al,1975).

a-Preparation of RBCs suspension: Three ml of blood was withdrawn from the jugular vein of a ram at the livestock of animals of the Veterinary Medicine College /Baghdad University . The blood was mixed at once with equal volume of Al-severs - solution in order to prevent clotting or lyses of RBCs ,the mixture was left for 18 hr at a refrigerator . Then the mixture was centrifuged at 1500 rpm for 5min and then 1 ml of the precipitate (cells) resuspended in 100 ml (RPMI-1640).

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b- Preparation of Lymphocytes suspension: It was prepared by taken the spleen of a mouse and cut to tiny pieces, the pieces were crushed with a mortar on stainless steel seeped on Petri dish then washed twice with 3 ml (RPMI-1640).The suspension was centrifuged at (1200) rpm for (10) min .

c- Test: A tube of mixture of 0.25 ml RBCs suspension and 0.25 ml of lymphocytes suspension was prepared, the precipitate was incubated at 37° c for 15min and a drop was taken by pasture pipette and mixed with a drop of Trypan blue stain on a slide and was examined ,unstained lymphocytes connected with 3 or more RBCs forming the rosette shape was calculated (200 cells).

Challenge of immunized mice

At 6 weeks after the second immunization (booster dose), all mice were challenged intraperitonially with 5 LD^{50} of virulent *S. ohio* in 0.5-ml PBS. The relative degree of protection afforded by the antigen was assessed by the number of mice surviving 30 days after infection.

Statistical Analysis

Statistical package for social science (SPSS) version 17 was used to calculate the means, standard error and ANOVA test was conducted to test the significance of effects of groups and periods post injection on the examined traits.

Results

The results of ELISA test

All mice before immunization (at zero time) showed the same means of IgG titers (191± 11.3). After two week of immunization with the booster dose the serum IgG titers of immunized group was (383.4 ± 55.3) and the peak appeared at fourth and six weeks (651.7 ± 21.3; 533.4± 40.1) respectively. the results showed a significant increase of antibody titers (P<0.05) at (2, 4, and 6) weeks, as compared with the control group(Table 1).

Table (1): Means of the antibody (IgG) titers in the immunized and control groups of mice .

Time(weeks)	Immunized group with somatic Ag Mean ± SE*	Control group Mean ± SE*	P.value
0 time	191± 11.3	191± 11.3	P> 0.05
2nd	383.4 ± 55.3	203± 11.1	P< 0.05**
4th	651.7 ± 21.3	189.1± 12.1	P< 0.05**
6th	533.4± 40.1	191± 11.3	P< 0.05**

SE*: Standard error. **Means significant different (P< 0.05) between groups.

The results of delayed type hypersensitivity have showed increases in the thickness of the foot pad skin of the immunized mice and the highest means of the thickness appeared after 24 hours post immunization. DTH tests indicated that the values were significantly high((P< 0.05) in the immunized group compared to the control group and there is a significant

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effect of the antigen injected on the thickness of the foot pad skin of mice after 24 and 48 hours as shown by table (2).

Table (2): Showing the thickness of skin reaction in mice before and 24,48 &72 hours after injection with *S.ohio* antigen.

Periods after injection of soluble antigen	Immunized group Footpad Skin thickness Mean ± SE*	Control group Footpad Skin thickness Mean ± SE*
Befor test/mm	1.65±0.129A	1.58±0.011A
After 24hours/mm	2.66 ± 0.19^{A}	1.59±0.013B
After 48hours/mm	2.43±0.211A	1.57±0.012B
After 72hours/mm	1.916 ± 0.098A	1.58±0.008B

*SE=standard error. A-B Means in the same row with different (capital letter) superscripts differed significantly at $P<0.05$

The E. rosette test showed the maximum reaction with mean (68.80 ± 2.02) after 4 weeks from booster dose in the immunized group while active lymphocyte remained within the normal range during experiment in the control group (Table 3).

Table (3) : Active E. rosette means of immunized and control groups:

Time (weeks)	Immunized group	Control group
	Mean ± SE	Mean ± SE
0 time	21.1 ± 1.11A	20.8 ± 1.478A
2^{nd}	32.5 ± 1.88A	21.1 ± 1.11B
4^{th}	27.9 ± 3.70A	21.2 ± 1.314B
6^{th}	26.70 ± 1.04A	20.8 ± 1.478B

A-B/ Means in the same row with different (capital letter) superscripts differed significantly at $P<0.05$

Results of estimating LD_{50}

The results of estimating LD_{50} of *Salmonella ohio* in mice injected intraperitonially with bacteria have revealed that the LD_{50} is (1.5×10^{7} cells) which estimated by calculating the dead and alive mice in each group during (30) days (table, 4). The calculation of mortality percent as followed in this equation :- Percent Mortality = total dead / sum of (total a live + total dead).

Table (4): Results of LD^{50} of *S. ohio* in mice.

(6 mice in each	Dose	Alive	Dead	Total alive	Total dead	Percent mortality
1	1.5×10^{10}	0	6	0	21	100 %
2	1.5×10^{9}	0	6	0	15	100 %

3	1.5×10^{8}	2	4	2	9	81 %
4	**1.5×10^{7}**	**3**	**3**	**5**	**5**	**50 %**
5	1.5×10^{6}	4	2	9	2	18 %
6	1.5×10^{5}	6	0	15	0	0 %
7	1.5×10^{4}	6	0	21	0	0 %
8	BPS	6	-	-	-	0%

No. of mice in each group = 6, Total No. of mice = 48

Clinical signs post challenge

All mice were challenged with 5 LD50 ($5\times1.5\times10^{7}$) 6 weeks post immunization, the immunized group exhibited moderate signs for 2-3 days while the control group exhibited these signs included listlessness, anorexia, severe diarrhea, rough coat, hunched posture and crowding near the water supply. Death occurred within 3 to 5 days after the challenge

Discussion

The important role of antibody producing B cell in protection against salmonellosis has been reported in many studies (Smith et al., 1993; Lindberg et al., 1993; Mastroeni et al.,2000). In the current study, immunization of mice with somatic Ag of *S. ohio* resulted in stimulation of significant antibody titers in the immunized group compared with control group. This is in agreement with study of (Yousif and Al-Mansory, 2011) reported that immunization with *Salmonella enteritidis* somatic Ag resulted in increasing of antibody titers.

Our result is agreed with that mentioned by (Shallal**,**2011**)** which noticed that the experimentally infected mice were able to induce humoral immune response which represented by producing antibody against *Salmonella* after two weeks and reached the peak after four weeks post infection. also our result were agreed with result mentioned by Matsiota –Bernard et al,(1993) who reported that IgG in mice during (7 to 35) days, raised on day 15 and continued to increase slightly until day 35. and with Kusumawati et al,(2006) who measured IgG titers from serum samples of mice at 2 weeks after infection with *Salmonella typhimurium*. Similar results obtained by Hur et al (2011) indicate the effective of live and killed salmonella vaccine in inducing IgG titers in the serum of mice.

It is obvious that *Salmonella ohio* is able to induce cellular immune response during experimental infection with somatic antigen. the result of the skin test in our study is in agreement with (Strindelius et al, 2002) used delayed-type hypersensitivity – skin test as a measure of cellular immunity in mice immunized with different types of *salmonella* antigens, the immunized mice showed a significant increase in the skin test.the positive result of skin test in this study is in agreement also with result of others(Mitov et al,1992;Yousif and Al-Naqeeb;2010; Yousif and Al-Mansoryo, 2011).

Many investigations have led to the conclusion that cellular immunity is the primary mechanism of protection against Salmonellosis, especially when vaccines are employed (Mastroeni et al,1993). The results of the present study have showed that antigen of *Salmonella Ohio* induce a high cellular immunity, this is compatible with other studies used E. rosette test to detect cellular immunity against other intracellular organism (Talal,2007). E rosette test is consider one of the most important discoveries that T-lymphocytes form

spontaneous E-rosettes with sheep erythrocytes (S RBCs), proving one of the simplest biological markers for identifying T lymphocytes (Kumar ,2010)

The LD_{50} dose of *S. ohio* ($1.5x10^{7)}$ is similar to *Salmonella hadar* LD_{50} dose mentioned by(Al Naqeeb ,2009) isolated from goat in Iraq. Inconrast to (Al-Hashimi, 2005) who recorded the LD_{50} of *S. enteritidis* in mice was (1.4×10^{6} C.F.U./ml).

The immunized groups in our study resisted the effect of lethal challenge and all were live after immunization with somatic antigen and due to its ability to reduce the appearances of severs clinical signs of salmonellosis while the control group showed sever clinical signs of salmonellosis and died within 3-4 days after challenge. These results are in agreement with Karasova (2009) who reported that mice with *S. enteritidis* induced strong cellular immunity and resisted the lethal challenge.

In conclusion, our results in the present study indicate that the *S.ohio* antigen can be a safe and effective tool for prevention of *Salmonella* infection. It can induce a protective cellular and humoral immune responses.

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Original Article

Detection of Verotoxigenic *E. coli* O157:H7 in Raw milk Using Duplex PCR in Basrah City- Iraq

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Abstract

One hundred fifty milk samples were collected from three different markets in Basrah city during a period extend of from November 2010 to March 2011. All samples were cultured in selective media to detect the presence of non-sorbitol fermenting colonies (NSF) 86 (57.34%) isolates were found non-sorbitol fermenting *E. coli*. Latex agglutination test was used to detect serotype O157:H7 in non- sorbitol fermenting isolates for 13 (27.08%) isolates. Multiplex PCR were done to all *E.coli* O157:H7 isolates and the result showed 7/150 (4.67%) from raw milk were positive to this test. All the multiplex PCR positive *E. coli* O157:H7 isolates were positive to *VT1* gene which was observed in 100% except one isolates which was positive to (*VT1*and *VT2*) genes at 14.28% from 7 isolates of raw milk samples.
Key words: Basrah , *E. coli* O157:H7, milk, PCR

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Introduction

Milk and its products are considered a kind of proteins, fats, salts rich nutrient with sweet taste, easy digested, cheep markets, and consumers for raw milk and their products have existed in many parts of the world. Milk is a highly nutritious medium for growth and transmission of several types of microorganism especially *E. coli* O157:H7. Such contaminants may render the milk and its products unsafe to use and expose the consumers to risk of infection (USDA, 2009; Robert, 2008). In recent years, Since the identification of *E. coli* O157:H7 as a human pathogen in 1982 in Oregon and Michigan, others are now commercially available for the major VTEC serogroups (O157, O26, O111, O103 and O145) previously identified as being commonly associated with human disease and have become a

very important milk-borne pathogen which constitute a public health hazard (Fratamico and Smith, 2006). *Escherichia coli* O157:H7 serotypes are identified as enterohaemorrhagic *E. coli* EHEC and categorized as verotoxin-producing *E. coli*. verotoxin is also known as shiga-like toxin, human and bovine *Escherichia coli* O157:H7 elaborates two potent phageencoded cytotoxins, known as Shiga-toxins *Stx1* and *Stx2* or verotoxins *VT1* and *VT2* (EFSA, 2007 and Jamshidi *et al*., 2008).

Detection methods for the isolation and identification of *E. coli* O157:H7 in food samples are categorized into two types conventional and rapid methods. These methods are based on cultural, serological, and biochemical properties of *E. coli* O157:H7. Selective agar media with inhibitors to reduce growth of unwanted species is done for the presumptive identification of organisms on the basis of distinct biochemical reactions and the isolates are further tested serologically for the presence of the O157 and H7 antigens using commercial available latex agglutination kits or antisera (Chow *et al.,* 2006). Multiplex PCR high sensitivity, specificity, its availability in many formats, rapid-screening tests have been used extensively for the identification and characterization of target bacteria in food samples, Including meat and dairy products based on immunological or nucleic acid technologies developed for food testing can provide results within hours (Ercolini *et al.,* 2004 ; Alarcon *et al.,* 2006).

Materials and Methods

A total of one hundred fifty random raw milk were collected from local retail markets of three different markets of Basrah city which were (market 1, market 2, and market 3). Fifty samples from each market were collected through period extended from November 2010 to March 2011.

Latex agglutination Test for *E. coli*O157:H7 was used for more specific identification of *E. coli* O157:H7 by using commercial kit (Wellcolex *E.coli* O157:H7, Remel) to detect the somatic antigen O157 and flagellar antigen H7.

Molecular Detection of verotoxine gene ((VT1 and VT2) gene by using Multiplex PCR technique was done by using commercially available DNA extraction and purification kit (Geneaid, USA). The purified DNA was detected by electrophoresis in 1% agarose gel with addition of ethedium bromide. Methylene blue stain added to the DNA sample and visualizes the DNA by U.V. light.

VT1a: GAAGAGTCCGTGGGATTACG 130 bp (Pollard *et al.,* 1990)

VT1b: AGCGATGCAGCTATTAATAA

VT2a: TTAACCACACCCACGGCAGT 346 bp (Pollard *et al*., 1990)

VT2b: GCTCTGGATGCATCTCTGGT

The verotoxin genes were studied according to protocol of (Pollared *et al*., 1990). This was done by using customize primers. The PCR reaction mixture contains 5 µl of green master mix (contains bacterially derived *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 5 µl of purified bacterial DNA, 1 µl of each forward and reverse primers, then the volume completed to 20 µl

ISSN 2307-8073

by deionized water. All tubes were centrifuged in microcentrifuge for 10 seconds. The PCR tubes were transferred to the thermalcycler to start the amplification reaction according to specific program for each gene. The agarose gel was prepared according to the method of (Sambrook *et. al.*, 1989). Two concentrations of agarose gel were prepared (1% and 2%) as we needed. The concentration of 1% agarose was used in electrophoresis after DNA purification process, while 2% agarose was used after PCR detection.

The results of the PCR were performed in post amplification process.10 µl from amplified sample was directly loaded in a 2% agarose gel containing 0.5 µl /25ml ethidium bromide with the addition of loading buffer and DNA size. Marker as standard in electrophoresis and the gel was run at 75 V. at 1 hr, then the products were visualized by UV transilluminator. The results were analyzed statistically by chi-square test (SPSS, 11). (Niazi, 2000).

Results

According to the results of culturing on TC- SMAC, identification and biochemical conformation, 86 out of 150 tested samples analyzed 57.34% were NSFEC positive. The suspected colonies of *E. coli* on SMAC were small, circular and colorless with smoky center (1-2) mm in diameter. While on MacConkey agar, the colonies were pink in color (lactose fermenter), on Eosin–Methylene Blue (EMB) agar the colonies had metallic sheen appearance. Microscopic examination of Gram's stained to the suspected VTEC isolates revealed Gram negative small bacilli. Biochemical tests (IMViC, TSI, and cellobiose) of NSF colonies showed that 55.81%, 47.87% of raw milk, and soft cheese isolates were positive (*E. coli*) respectively (Table 1).

All of the *E. coli* isolates were tested by latex agglutination test for the somatic O157 antigen. The results showed that 56.25% of *E. coli* isolates from raw milk were positive for O157 somatic antigen. The O157 positive isolates were tested for the flagellar H7 antigen .The Frequency of H7 antigen positive isolates were 27.08%.

Table (1) The Frequency of Biochemically Confirmed *E. coli* O157:H7 among *E.coli* isolates

*** = IMViC pattern ++-- , TSI & cellobiose tests**

No Samples	Nonsorbitol fermenter (NSF) (%)	No. of biochemically* positive (%)	O157 +ve (%)	H7 +ve (%)	O157:H7 +ve (%)
150	86 (57.34)	48 (55.81)	27 (56.25)	13 (27.08)	13 (27.08)

Distribution of *E. coli* O157:H7 Serotypes According to Period of the Study

The results of the present study showed that the highest rate of *E. coli* O157:H7 isolation was in March (12.5%) followed by February (8.5%), December (8%), November (6.6%) and

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January (5%). There were no significant differences in the isolation rat according to the period of study, (Tablel 2).

Table (2) : The Distribution of O157:H7 in Raw Milk Samples According to the Period (month) of Collection.

Month	No. of samples	No. of positive O157:H7	%
November / 2010	30	2	6.6
December / 2010	25	2	8
January / 2011	20	1	5
February / 2011	35	3	8.5
March / 2011	40	5	12.5
Total	150	7	4.67
X^2 =1.248 (p > 0.05)			

Distribution of *E.coli* O157:H7 Serotype from Raw Milk Among the Different Regions of the Study.

According to the the results of isolation and identification of *E.coli* O157:H7. There were 13 samples out of 150 tested samples were positive for the above isolate. The percentage of isolates in raw milk were 53.84% .The high rate of isolation was observed in market 1 (57.14%) followed by market 2 and market 3, 50% for each one. There were no significant differences (P>0.05) in the rate O157:H7 isolation among the regions of the study (Table3).

Table (3) : Distribution of VTEC O157:H7 in *E coli* isolates according to the regions of the study.

Market	No. of samples	No of *E. coli* O157:H7	%
Market 1	50	7	
Market 2	50	2	
Market 3	50	4	
Total	150	13	
	X^2= 23.2 P>0.05		

Distribution of PCR positive VTEC Isolates in raw milk Samples

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The DNA of all isolates was extracted and purified by using genomic DNA purification kit. Out of 27 *E. coli* O157 or O157:H7 isolates which were previously confirmed by biochemical and serological testing, 7 isolates (25.92%) were identified having VTEC genes. The overall prevalence of PCR positive VTEC was 4.67% (7/150) of the tested raw milk. All the isolates which were positive for *E.coli* O157:H7 latex agglutination test were amplified to detect of *vt1* and *vt2* genes**.** *vt1* gene (about 130bp) was observed in percentage100 % (7/7) of the tested isolates while only one isolate of *E.coli* O157:H7 was observed having both genes (*VT1* and *VT2* (about 346bp) in a percentage 14.28 % (Table, 4 and figure 1).

Table (4): Distribution *VT1* and *VT2* genes in *E.coli* O157:H7 isolates from raw milk samples

PCR + ve VTEC No.	Genes					
	VT1	***%***	***VT2***	***%***	***VT1+VT2***	***%***
7	**7**	**100**	**1**	**14.28**	**1**	**14.28**

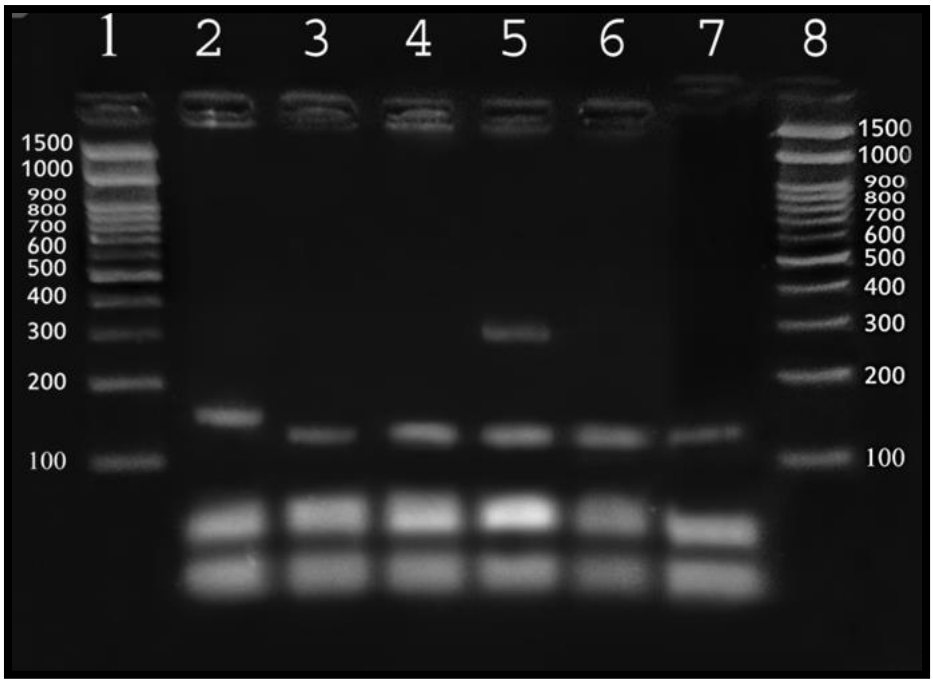

Figure (2): PCR amplification of *VT1*gene, 130bp (Lan 2-7) and *VT2* gene, 346bp (Lane 5). Lane 1&8 ladder.

Discussion

The frequency of NSF isolates in raw milk samples (57.34%) was higher than the prevalence reported by Roopnarine *et al*., (2007) who recorded that NSF isolates from milk was 37.5%, also much higher than Daood, (2007) and Adesiyun *et al*., (2007) who showed that isolation rate was 10%, 14.2% respectively. The rate of milk positive isolates in the present study was 57.34% which agrees with the result obtained by Soomro *et al*., (2002) which was 57%. Other studies such as Abdul-Raouf and El-safey, (2003), Mansouri-Najand and Khalili.(2007) who mentioned much lower rates of NSF isolates in raw milk (5%, 4.46% respectively) in comparison with the rate in the present study. The explanation of differences in isolation rate between this study and other studies may be related to the difference in the serotypes of NSF *E. coli* and to facilities techniques used in detection and diagnosis of thisbacteria. In the present study NSF *E. coli* was isolated on selective enrichment (TSB-V) supplemented with vancomycin (De-Boer and Heuvelink, 2000). This medium permits the growth of *E. coli* and inhibit a wide range of contaminants including *Proteus spp.* Also it is necessary for the increment of bacteria to the level in which easily detected as low (10-100 cells) infection dose (Reissbrodt, 1998). On the other hand, the selective medium (TC-SMAC) in this study was supplemented with cefixime (0.05mg/L) and potassium tellurite (2.5mg/L). Those inhibited other enteric organisms which compete overgrow the targeted organism of the present study.

In the present study, the overall prevalence of *E. coli* O157:H7 latex agglutination test was 27.08%. This is concordant with the results showed by(Al-Aidi and Najim, (2009), Murinda *et al*.,(2002) and Daood, (2007) which were 23.6%, 26.7%, 30.90% respectively, while it is much higher than results obtained by Al-Hasnawi, (2010), Karns *et al*., (2007), Belickova *et al* .,(2008) and Stephan *et al*., (2008) which were 11.1%, 0.23%, 1.02%, 5.0%, 2.5% respectively.

In All these previous studies, the prevalence rates were less than the results in the present study, this indicated the presence of O157:H7 of tested samples included in the present study with higher prevalence due to unhygienic measurement that lead to higher contamination rates, suggested that spread of *E. coli* O157:H7 in the raw milk which were serve as a main source of infection and the risk of acquiring is high (Jamshidi *et al*., 2008).

The prevalence of PCR positive for vt_1 of *E.coli* O157:H7 isolates from raw milk samples (4.67%) was concordant with the results obtained by Karns *et al*., (2007) and Fitzmaurice, (2003) which were 4.2% and 4.7%, respectively. On the other hand, the isolation rate in this study was much higher from that registered in Spain (0.4%) by Quinto and Cepeda, (1997), in Ontario (0.87%) by Steel *et al.* (1997), in Egypt (1.10%) by El-Safey, (2001) and in Germany (3.9%) by Klie *et al.* (1997). The high rate recorded in the present study can be attributed to the use of primers designed to target genes vt_1 and vt_2 genes which encoded for *Vt1*, *Vt2* toxins respectively and these two sets of oligonucleotide primer were used in multiplex PCR assay for the detection of *Vt* genes or as a means to increase sensitivity and specificity of this technique than other assays for the detection of VTEC O157:H7 in raw milk and soft cheese (Aslam *et al*., 2003). In the present study, the highest isolation rate of *E. coli* O157:H7 from raw milk cases obtained in March (7.5%) followed by February (5.71%). These results agree with the results obtained by Murinda *et al*., (2002), Spano *et al*., (2003), Rahimi *et al*., (2008) and Al-Aidi & Najim, (2009) who found that the highest isolation rate percentage. This is an increase in viability and survival of *E.coli* O157:H7 during the warm months of the year which was nearly from optimum temperature for growth than cold months.

In contrast, a study in Scotland (Ogden *et al*., 2004) stated that the highest isolation rate was obtained in winter.

On other hand, according to the regions of study the highest isolation rate of O157:H7 from raw milk cases was recovered in market 1 (57.14%) followed by market 3 and market 2 (50%) for each one.. The results showed that season, geographical location of the farmer villages, contaminated environmental conditions and unhygienic measures had an effect on the isolation percentage of *E .coli* O157:H7 (Spano *et al*., 2003).

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Cellular immune responses induced in mice by Salmonella *hadar* O antigen

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Abstract

This study was designed to investigate the cellular immune response in mice after parenteral immunization with *Salmonella hadar* O antigen with and without Adjuvant. BALB/c mice were divided into three groups (each 25 mice). First group was injected subcutaneously with S.hadar O antigen (heat killed O antigen), second group similarly injected with (*S.hadar* O antigen suspended in alum as adjuvant) and control group injected with phosphate-buffered saline. All mice were immunized two times on days 0 and 14. Two weeks after the last immunization, cellular immune responses to *S.hadar* were assessed using E. rosette test at 2^{nd}, 5^{th} and 8^{th} week, while delayed type hypersensitivity test (DTH) skin test used after 21 days of immunization. Then all mice were challenged intraperitoneally with $4LD_{50}$ of virulent *Salmonella hadar* eight weeks post immunization. The E.rosette test in mice injected with O antigen and O antigen suspended in adjuvant showed a significant increase in the activation of lymphocyte at 2^{nd} ,5^{th} & 8^{th} week after immunization. While the control group gives normal range of active lymphocyte in all weeks of experiment. DTH skin showed significant increase in thickness of the footpad skin after 24 and 48 hours post inoculation with S hadar soluble antigen while the control group didn't show any reaction. A significant protection was observed in the immunized groups challenged with 4 LD_{50} {$4(1X10^8)$ } compared with control group of mice which died within 1-2 days.

In conclusion, this study indicate that the administration of *S.hadar* O antigen suspended in adjuvant can induce a significant cellular immunity more than non suspended *S.hadar* O antigen only, but both antigens give a good protection against salmonellosis in mice.

Keywords: cellular Immunity, O antigen, adjuvant, E.rosette, DTH skin test.

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Introduction

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Salmonella enterica are Gram-negative bacterial pathogens capable of infecting humans and animals and causing significant global morbidity and mortality (Coburn et al, 2007; Antoine et al,2008). *Salmonella hadar is now one of the five most frequently isolated serotypes in human and animals (*Valdezate *et al, 2000;* Cailhol et al, 2006*).it* is a highly prevalent food borne pathogen and therefore a major cause of human gastroenteritis worldwide *(*Snoussi et al,2012*). It is isolated firstly in the early 1950s, from a stool sample of a subject with gastro-enteritis and fever (*Hirsch *et al,1954).* In Iraq *S. hadar* was isolated from liver and bile of slaughtered goats by (Yousif et al, 2011) also *S. hadar* was isolated from feces of affected children with diarrhea in Thi-Qar province (Harab and Yousif, 2011).

Over the last century, the use of vaccines has profoundly reduced the morbidity and mortality caused by infectious diseases in both human and animal populations. In fact, vaccination has proven to be the most successful medical intervention ever developed (Schijns, 2003). It is widely accepted that cell-mediated immunity is more important than humoral responses in protection against *Salmonella* infections; but Mastroeni et al, (1993) found that both cellular and humoral immune responses are stimulated by intraperitoneally administered heat-killed and live *Salmonella* vaccines in mice. Berndt *et al*, (2007) observed that the CD8-T-cell response were as high in infected birds with *S. hadar* also they found that *Salmonella* serovars *typhimurium* and *hadar* are moderately invasive and intermediate immune stimulators. Wilson-Welder et al, (2009) report that the development of vaccine suspended adjuvant can enhance the effectiveness of vaccine, these adjuvant should have the ability to elicit a potent immune response. The efficacy of vaccines depends on the presence of an adjuvant in conjunction with the antigen. Of these adjuvants, the ones that contain aluminium, which were first discovered empirically in 1926, are currently the most widely used. Jazani et al;(2011) conclude that the administration of the alum-naloxone mixture as an adjuvant, in combination with the Heat killed *S.typhimurium* vaccine, can enhance both humoral and cellular immunity and shift the immune responses to a Th1 pattern.

The aim of this study is to investigate the efficacy of *S. hadar* O antigen of with and without adjuvant in inducing cellular immunity in mice against challenge with virulent strain of *S hadar.*

Materials and Methods

Bacterial strain

Salmonella hadar was isolated from feces of children suffered from diarrhea in Thi-Qar province in Iraq by (Harab and Yousif,2011), using selective media, biochemical tests and API 20 (Quinn et al,2004) . Finally the isolates confirmed in the National Salmonella Center in Baghdad/ Ministry of public health.

Preparation of *S hadar* O antigen for immunization

Samples of the stock culture of *S. hadar* were used for preparation of the antigen. To produce the inoculate, bacteria were grown statically for 18 h at 37°C in brain heart infusion broth, harvested by centrifugation, and resuspended in PBS. Bacteria was washed extensively in phosphate-buffered saline (PBS) three times.Colony counts were performed for all inoculate to verify the number of viable bacteria at 1×10^8 CFU, then bacterial suspension was killed by heating at 100°C for 30 minutes (Smith et al, 1984). Protein content was determined by a biurat method. The antigen was tested for sterility and safety before use according to (OIE, 2004).

Preparation of potassium alum sulphate(adjuvant)

This solution prepared from 50% of potassium alum sulphate. Fifty gram of potassium alum sulphate was dissolved in distal water and the volume completed to 100 ml. sterilized by autoclave for 15 minutes at 15 Ibs with 121 C^o, then this solution used adjuvant at 1:1 . The adjuvant was add to *S.hadar* according to (Goerge et al, 1985).

Preparation of soluble antigen

Soluble antigen which used for DTH (skin test) prepared according (Mitov et al, 1992) briefly; three to five colonies from the bacterial isolates on selective medium were inoculated into trypticase soy broth and incubated overnight. The cultures were harvested by centrifugation at 10.000Xg for 30 minutes. The sediment was sonicated for 50 minutes using ice at intervals in a water cooled sonicator oscillator at 40 MHZ per second full power. The homogenate was centrifuged twice by using a cooling centrifuge at 8000 Xg for 30 minutes each time to remove cellular debris. The supernatants were passed through a 0.22 µm Millipore filter and stored at (-20^{o}C) until used. Protein content was determined by biuret protein assay.

Immunization of mice with *S.hadar* O antigen

Seventy five, (5-8) weeks old healthy mice (BALB/c) of both gender, obtained from National center of researches and monitor of drugs in Baghdad, adapted for two weeks before started experiment in separated clean and disinfected cages ,they were fed on assorted pellets and clean water, then divided into 3 main groups (each 25 mice):

First group: Mice were immunized S/C with *S. hadar* O antigen at dose of 0.5 ml containing 1X10^8 CFU.(protein content 200µg/0.5ml).

Second group: Mice were immunized similarly with *S. hadar* O antigen mixed with adjuvant (0.5 ml mixed with 0.5 ml adjuvant. This mixture injected subcutaneously in two places.

Third group (control group): Mice were injected subcutaneouslywith 0.5ml PBS.

Detection of cellular immunity

1-**Delayed type hypersensitivity test DTH (Skin test)**

This test was done according to (Hudson and Hay 1980) after 21 days of immunization. Briefly, 0.1 ml of soluble antigen of *S. hadar* was injected intradermally in the right footpad of the mouse while the left side was injected by 0.1 ml of sterile PBS (pH=7.2). The thickness of the footpad was measured by vernier caliper before and 24, 48, and 72 h after Injection.

2-**E-rosette test**

This test was done according to (Braganza et al, 1975) with some modification. The test used for calculates the percentage of viable and non-viable T-lymphocytes and estimated activity of T-lymphocytes in three steps:

A. **Preparation of RBCs suspension** Three ml of blood was withdrawn from the jugular vein of a ram. The blood was mixed at once with equal volume of Al-severs - solution in order to prevent clotting or lyses of RBCs, the mixture was left for 18 hr at a refrigerator. Then the mixture was centrifuged at 1500 rpm for 5min and then 1 ml of the precipitate (cells) resuspended in 100 ml (RPMI-1640).

B. **Lymphocytes suspension** was prepared by taken the spleen of a mouse and cut into tiny pieces, the pieces were crushed with a mortar on stainless steel seeped on Petri dish then washed twice with 3 ml (RPMI-1640).The suspension was centrifuged at (1200) rpm for (10) min .

C. **Test A** mixture of 0.25 ml RBCs suspension and 0.25 ml of lymphocytes suspension was prepared in a test tube, the test tube was incubated at 37°C for 15min and a drop was taken by pasture pipette and stained by Trypan blue stain on a slide and was examined by microscope, for T-lymphocyte – erythrocyte rosette forming shape (lymphocyte which attached to more than 3 or more RBCs forming the rosette shape).

Estimating the LD_{50}

Tenfold dilution (10^{-1},10^{-2}.10^{-3},10^{-4},10^{-5},10^{-6},10^{-7},10^{-8},10^{-9} and 10^{-10})of Salmonella hadar were done. The viable counts of the bacteria in each diluent were made according to Quinn et al (2004**).** The diluents which had these number of bacteria :(1×10^{5} C.F.U./ml) , (1×10^{6} C.F.U./ml cells) , (1×10^{7} C.F.U./ml) , (1×10^{8} C.F.U./ml) , (1×10^{9} C.F.U./ml) , (1×10^{10} C.F.U./ml) and (1×10^{11} C.F.U./ml) were selected to injected groups of mice intraperitoneally.

Forty eight of healthy mice of both sexes were selected, the body weight was ranged from (25–30) gm and age range (6–8) weeks; they were divided into (8) groups, each group contained six mice. Seven groups out of eight groups of mice injected with (0.5)ml of the intraperitoneally calculated (C.F.U./ml) diluents and the last group injected with PBS (pH=7.2) and considered as a control group. All groups were observed for 30 days to calculate the live and dead mice and estimate the LD_{50} according to (Reed and Muench 1938).

Statistical Analysis

Statistical analysis were conducted to determine the statistical differences among different groups using ready – made statistical design statistical package for social science (SPSS).

Ethics Approval This study was approved by the ethical and research committee of Veterinary Medicine College/University of Baghdad

Results

Delayed type hypersensitivity test (DTH) Skin test

The first group showed increased in the thickness of skin footpads of the mice and the highest means of the thickness appeared after 24 and 48 hours and return back to normal after 72 hours post injection with soluble antigen of *S.hadar*, in the second group the means of the thickness appeared after 24 hours and the maximum appeared at 48 hours and. Erythematous signs showed in the footpads of all immunized group. The control group didn't show any reaction or change in the thickness or color of footpads (Table 1).

Table (1) Skin thickness (millimeters) of the immunized and control groups in DTH test

	Diameter of skin thickness	Diameter of skin thickness	Diameter of skin thickness
Times	First group O Ag	Second group O Ag +adjuvant	Third group Control
0 time	1.56±0.09	1.62±0.07	1.57±0.0597
24 hours	2.56±0.094	2.85±0.12	1.59±0.0640
48 hours	2.09±0.07	2.93±0.12	1.58±0.0611
72 hours	1.68 ± 0.08	2.33±0.13	1.58±0.0611

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E-rosette test

The first group which received O antigen without adjuvant showed a highest means of active E. rosette at 2nd and 5th weeks after injection with booster dose (59.0± 2.43&56.0 ±1.14)respectively. The second group which was received O antigen with adjuvant showed the highest increase in active lymphocyte in the 5th and 8th group, with a maximum activation at 5th weeks ranged (66.8 ± 1.83). The results of the second group appeared more increase than in first group. The control group showed a normal range of active lymphocyte (Table 2).

Table (2) Means of active E. rosette in immunized and control groups

	Time	First group	Second group	Control group	P value
Before immunization	0	20.0 ±0.70	19.2±0.86	19.6±0.67	**P> 0.05**
After immunization	2nd	29.8± 1.28	29.8± 1.28	19.8 ± 0.58	**P< 0.05****
After immunization	5th	28.8 ±0.58	33.4 ± 1.31	20.0±0.70	**P< 0.05****
After immunization	8th	26.6 ± 0.81	31.8 ± 0.91	19.4±0.24	**P< 0.05****

SE*:Standard error. **Means significant different (P< 0.05) between groups.

Result of LD_{50} dose

The estimation of *Salmonella hadar* LD_{50} in mice injected intraperitoneally have revealed that the LD_{50} is (1×10^8 C.F.U/ml) which estimated by calculating the dead and alive mice in each group during (30) days (table 3).the percentage of mortality was calculated according to the following equation :

Percent of Mortality = total dead / sum of (total a live + total dead).

Table (3) The results of estimating of LD_{50} of S. *hadar* in mice

(6 mice in	Dose	Alive	Dead	Total	Total	Percent
1	1×10^{11}	0	6	0	21	100 %
2	1×10^{10}	0	6	0	15	100 %
3	1×10^{9}	2	4	2	9	81 %
4	1×10^{8}	3	3	5	5	50 %
5	1×10^{7}	4	2	9	2	18 %
6	1×10^{6}	6	0	15	0	0 %
7	1×10^{5}	6	0	21	0	0 %
8	BPS	6	-	-	-	0%

No. of mice in each group = 6, Total No. of mice = 48 ,The dose calculated as (cells).

Experimental of the challenge

All groups of mice were challenged intraperitoneally with 4 LD_{50} (4 X 10^8) of virulent *S.hadar*. Reaction in the immunized mice were revealed mild signs of illness and depression for 2-3 days without signs of diarrhea and returned normal within 7 days without mortality.

The post challenges reaction in non-immunized mice (control group) exhibited the following clinical signs: listlessness loss appetite, anorexia, severe diarrhea, increased respiration rate, severe dehydration, pregnant mice was aborted, and recumbent till death in 1-2 days after challenge.

Discussion

This study was aimed to evaluate the using of *S.hadar* O antigen with and without adjuvant in two doses to avoid the risk of live vaccines; the use of killed organisms was introduced as safer vaccines. This compatible with many researchers (Timms et al,1994; Gast et al, 1993;) using killed vaccine or subunit vaccine and the limitations of these kinds of vaccines are that their immunogenicity usually has to be enhanced by co administration with adjuvant, and, in any case, multiple doses are necessary for obtaining long-term protective immunity.

The cellular immune response induced by *Salmonella hadar* which estimated by DHT-skin test in our study appeared resemble to the results recorded by (Yousif and al Naqeeb,2010) in mice infected with *S hadar*, also with a study of (Shallal, 2009) who used delayed-type hypersensitivity (skin test) as measure of cellular immunity in mice immunized with different types of salmonella antigens .

In the present study, E rossete used for detection of immunity, that gives significant positive results with O antigen of *S.hadar* and O antigen + adjuvant. This is resemble with a study of (Yousif and Almansory,2009) which used E-rosette test to detect cellular immunity against other *Salmonella* species(*Salmonella enteritidis*) in rabbits and proved its efficacy. Kumar ,(2010) consider the E rosette test as one of the most important discoveries which showed T-lymphocytes form spontaneous E-rosettes with sheep erythrocytes (S RBCs),and proving it as one of the simplest biological markers for identifying T lymphocytes.

Our results showed that injection of the O antigen with adjuvant produce more efficient cellular immunity more efficient than that of O antigen without adjuvant, this is in compatible with (Mazloomi et al, 2012) who mix the adjuvant with killed antigen to increase the vaccine's efficacy, it shown lymphocyte proliferation in mice more than the killed vaccine alone.

the role of adjuvant in vaccine development were Increase the total antibody titer or functional titers; Decrease the dose of antigen needed; Decrease the total number of doses of vaccine necessary for complete immunization; Overcome competition in combination vaccines and Induce potent cell-mediated immunity(Dekker et al, 2008). The immunized groups in our study injected intraperitoneally with 4 LD_{50} were resist the effect of lethal challenge and all mice were live, this may contribute to the immunity that induced after immunization with O antigen and O antigen with adjuvant and it its ability to reduce the appearances of sever clinical signs of salmonellosis. While the control group showed sever clinical signs of salmonellosis and dead within 1-2 days from challenge. These results are in agreement with many researcher reported that immunization of mice with *Salmonella* species induced strong cellular immunity and resist the lethal challenge of virulent *Salmonella* (Karasova, 2009; Yousif and Al-Mansory 2009; Simon,2011) .

In conclusion, the results of this study indicate that the *S.hadar* O antigen and O antigen with adjuvant can be used as safe and effective tool for prevention of *Salmonella* infection, due to its ability to induce the protective cellular immune response.

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Original Article

Culture technique of rabbit primary epidermal keratinocytes

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Abstract

The epidermis is the protective covering outer layer of the mammalian skin. The epidermal cells are stratified squamous epithelia which undergo continuous differentiation of loss and replacement of cells. Ninety per cent of epidermal cells consist of keratinocytes that are found in the basal layer of the stratified epithelium called epidermis. Keratinocytes are responsible for forming tight junctions with the nerves of the skin as well as in the process of wound healing. This article highlights the method of isolation and culture of rabbit primary epidermal keratinocytes *in vitro*. Approximately 2cm x 2cm oval shaped line was drawn on the dorsum of the rabbit to mark the surgical area. Then, the skin was carefully excised using a surgical blade and the target skin specimens harvested from the rabbits were placed in transport medium comprising of Dulbecco's Modified Eagle Medium (DMEM) and 1% of antibiotic-antimycotic solution. The specimens were transferred into a petri dish containing 70% ethanol and washed for 5 min followed by a wash in 1 x Dulbecco's Phosphate Buffered Saline (DBPS). Then, the skin specimens were placed in DMEM and minced into small pieces using a scalpel. The minced pieces were placed in a centrifuge tube containing 0.6% Dispase and 1% antibiotic-antimycotic solution overnight at 4°C in a horizontal orientation. The epidermis layer (whitish, semi-transparent) was separated from the dermis (pink, opaque, gooey) with the aid of curved forceps by fixing the dermis with one pair of forceps while detaching the epidermis with the second pair. The cells were cultured at a density of 4 x 10^4 cells/cm^2 in culture flask at 37°C and 5% CO_2. The cell morphology of the keratinocytes was analyzed using inverted microscope.

Keywords: Culture, Rabbit, Primary, Epidermal, Keratinocytes

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Introduction

The epidermis is the outer layer of the skin (William et al, 2005) which functions as a protective covering of the mammalian body, with an average of about 0.2 mm thickness. The epidermal cells are stratified squamous epithelia which undergo continuous differentiation of loss and replacement of cells. Ninety five per cent of the epidermal cells consist of epidermal keratinocytes which is proliferated and divided in the basal layer. After the cells are mature, it will move up to the upper layer to form the cornified cells. In the epidermis, the different stages of maturation of keratinocytes are divided into four levels (horny, granular, suprabasal and basal) cell layer. In the normal skin, the turn over time of the production of daughter epidermal cells is approximately 28 days. During the process of production of new cells, the cells undergo terminal differentiation which can change the characteristic of the cells. It is reflected as highly coordinated, sequential expression of morphological and biochemical markers of cell differentiation (Green, 1980; Green et al, 1982). Many of the skin diseases whether it is benign or malignant are characterized to interfere with the normal regulation rate of proliferation and differentiation in the epidermis causing a severe disturbance in tissue homeostasis (Sybert et al, 1985; Bernard et al, 1988; Rehfeld et al, 1988; Turbitt et al, 1990). The keratinocyte cell is a specialized epidermal cell that synthesizes keratin and it is the major cell type of the epidermis. Keratinocytes can be isolated from skin biopsies and are able to undergo expansion *in vitro* which can be used in patients with deep dermal burns as cultured epidermal autograft (CEA) (Dedovic et al, 1998). Keratinocytes become activated and turn into hyper proliferative cells in the wound healing conditions which produce and secrete extracellular matrix components and signalling polypeptides. In the mean time, the production of specific keratin proteins will alter their cytoskeleton. Keratinocytes and other cutaneous cell types also contribute to the changes in growth factors, chemokines and cytokines. A culture system which enables growth of cells *in vitro* has become an important tool of study for normal and pathological cells (Jensen et al, 1991). A significant trend of methods for culturing human epidermal keratinocytes has been successfully developed during the last 26 years. These achievements have made the analysis of the growth and differentiation pattern of epidermal keratinocytes in culture open to possible new strategies for therapeutic intervention. This research describes the protocol of isolating and culturing rabbit primary epidermal keratinocytes *in vitro*.

Materials and Methods

Ethical approval

This study was approved by the animal ethics committee of Universiti Sains Malaysia vide ref. USM/Animal Ethics Approval/2010/ (54) (175) dated 22 March 2010.

Skin harvesting

Three adult New Zealand White male rabbits, (*Oryctolagus Cuniculus*) were used in this study. The rabbits were administered a premedication of atropine (1mg/kg bwt i/m) and then anaesthetized using xylazine (5mg/kg bwt i/m) and ketamine (35mg/kg bwt i/m). The surgical site was shaved and povidine iodine was applied. The rabbit was observed for reflexes and

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when the rabbit was completely unconscious, it was moved to the animal operation theatre to perform the skin surgery. Approximately, 2cm x 2cm oval shaped line was drawn on the dorsum of the animal to mark the surgical area. Then, the skin was carefully excised using a surgical blade and the target skin specimens harvested from the rabbits were placed in transport medium comprising of Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, USA) and 1% antibiotic-antimycotic solution (Invitrogen, USA). Then, the surgical wound was sutured and bandaged. The steps are shown in (Figure.1). The animal was monitored for reflexes throughout the surgical procedure.

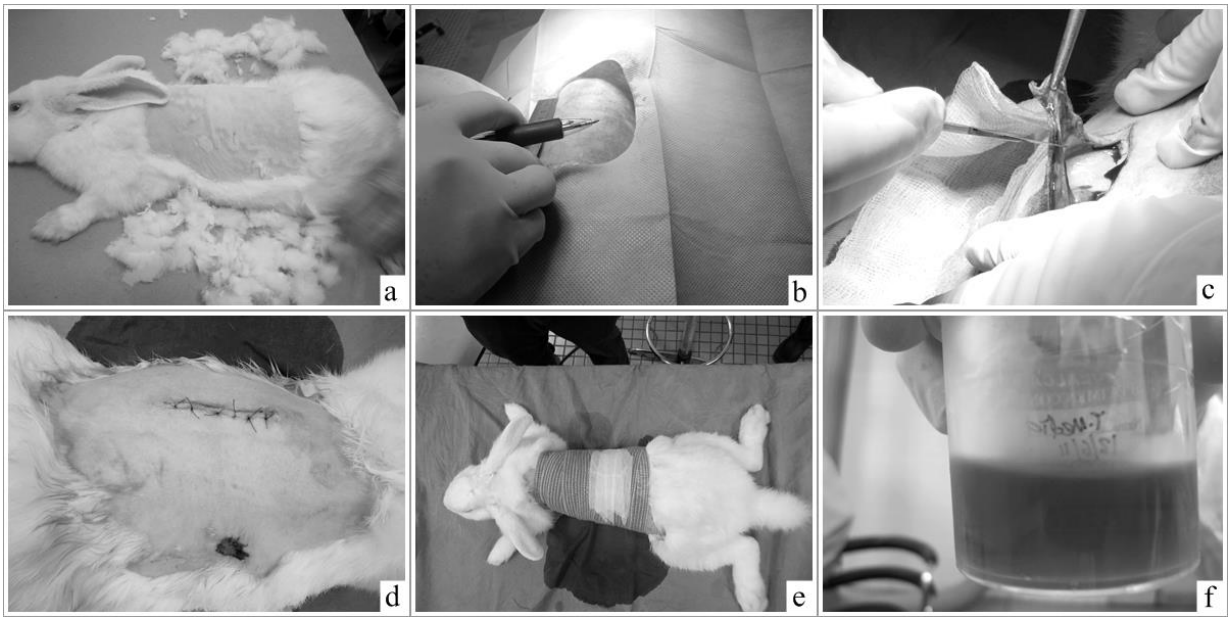

Fig 1. Skin harvesting in rabbit a) Shaved area on the dorsum of the rabbit b) 2cm x 2cm oval shaped area depicting the surgical area c) Skin excision using a surgical blade d) Sutured surgical wound e) Bandaged surgical wound and f) Transfer of target skin specimens harvested from the rabbits into transport medium comprising of DMEM and 1% antibiotic-antimycotic solution

Culture of keratinocytes

The specimens were transferred into a petri dish containing 70% ethanol and washed for 5 min followed by a wash in 1 x Dulbecco's Phosphate Buffered Saline (DBPS) (Gibco®, USA) for 5 min. Then, the skin specimens were placed in DMEM and minced into small pieces using a scalpel. The minced pieces were placed in a centrifuge tube containing 0.6% Dispase (Sigma-Aldrich, USA) and 1% antibiotic-antimycotic solution overnight at 4°C in a horizontal orientation. The next day, the specimen was transferred into a petri dish together with the dispase. Each of the specimens was transferred to a new petri dish containing CnT-57 (CELLnTEC, Switzerland) medium to wash away the excess dispase. While holding the skin submerged in CnT-57 medium, the epidermis layer (whitish, semi-transparent) was separated from the dermis (pink, opaque, gooey) with the aid of curved forceps by fixing the dermis with one pair of forceps while detaching the epidermis with the second pair. The separation of the rabbit dermis and epidermis was very difficult because the dermis was tightly bound to the epidermis by their thick and barely visible hair which serves to insulate the rabbit. The

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separated epidermis was placed in a 50 ml centrifuge tube containing 10 ml of pre-warmed trypsin solution (TrypLE^{TM} Express, Invitrogen, USA) and incubated at 37°C for 10-15 min. Later, 10 ml of CnT-57 medium was added to this 50 ml centrifuge tube to deactivate the enzyme activity. The suspension was then carefully filtered using a 70 µM nylon cell strainer (BD Biosciences, USA) into a new 50 ml centrifuge tube. This was done to separate the cells and epidermis layers. The cell suspension was then centrifuged at 1700 rpm for 7 min. The supernatant was discarded and the cell pellet was re-suspended in CnT-57 medium. The cells were counted using a haemocytometer and the cell viability was estimated using 1% Trypan blue. The viable cells were cultured at a density of 4 x 10^4 cells/cm^2 in culture flasks (Nunc, Australia). The cells were cultured at 37°C and 5% CO_2. The cell morphology of the keratinocytes was analyzed using inverted microscope (Leica Microsystems, Germany).

Results and Discussion

The rabbit primary epidermal keratinocytes were cultured in the Cnt-57 progenitor cell targeted media. This medium favored only the growth of rabbit primary epidermal keratinocytes (Figure. 2). CnT-57 media is a low calcium (0.07 mM) formulation containing a low bovine pituitary extract (BPE) concentration of 6 µg/mL. A study by Kuo et al, (2005) has shown that the addition of BPE aids attachment and migration and results in improved colony forming efficiency and maximum cell yield. BPE, derived from the pituitary gland (a small endocrine gland) that produces and secretes various hormones is important for the regulation of various bodily functions. It contains a full spectrum of putative mitogens and growth factors that have been used effectively in promoting robust growth in a wide range of cells *in vitro*, especially those of epithelial origin and stem cells. This medium has been used in humans (De Kock et al, 2011) and mouse epidermal keratinocytes (Yazdi et al, 2010). However, in a previous study by De Kock et al, (2011), their differentiation strategy on human foreskin-derived precursor cells showed that the CnT-57 did not contribute to their cell differentiation with or without addition of CnT-02 and Epilife. Hence, it can be postulated that the BPE present in CnT-57 medium could be the factor promoting the growth of rabbit primary epidermal keratinocytes in addition to the components present therein. There are a limited number of reports suggesting the technique in isolation the rabbit primary epidermal keratinocytes. A study by Davison et al, (1980) described the procedure for the isolation and cultivation of endothelium from the marginal vessels of the rabbit ear. The endothelial cells were isolated by slow perfusion with a trypsin solution and were cultured in minimal essential medium supplemented with 10% fresh rabbit serum for up to 6 months. They found that the fibroblast growth factor was not mitogenic for rabbit marginal vessel endothelium *in vitro.* In other study, Lapi et al, (2008) found that for culturing rabbit bone marrow mesenchymal stem cells, DMEM showed significantly lower plating density compared to alpha minimal essential medium (α-MEM). Rutten et al, (1990) developed a method for rabbit skin organ culture in a two-compartment model. The skin discs were cultured on a Millicell-HA insert unit with a microporous membrane which allowed transport of culture medium via the dermis into the epidermis, whereas the epidermal side remains free of direct contact with culture medium. They concluded that at the end of the 7 days of culture period, the distance between single dermal collagen fibrils had increased as compared to non cultured skin. In contrast, there are many different methods reported for cultivating human epidermal keratinocytes which suggests two major categories (Fusenig, 1986; Watt, 1987); a simple system which retains some of the properties of the epidermis *in vivo* after serial passage of cells, while the second

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major categories are elaborating on reconstructing a true epidermal tissue in primary culture by approximating the *in vivo* epidermal environment. Between these two major categories of the culture systems, the system developed by Rheinwald and Green (1975) has been the most successful system. The feeder layer of irradiated mouse 3T3 cells which was seeded by disaggregated keratinocytes enhances the plating efficiency and stimulates the keratinocytes growth (Green et al, 1977; Rheinwald & Green, 1977; Watt and Green, 1981). In contrast, other studies have reported that irradiated human dermal fibroblasts can substitute for the 3T3 cells as a feeder layer (Limat et al, 1989; Limat et al, 1990). The Rheinwald-Green method is now used in almost every research because the method allows serial passage for many generations. It also gives a major impact on the study of many cellular and molecular aspects of proliferation and terminal differentiation of the keratinocytes (Fuchs and Green, 1980; Watt and Green, 1981). Furthermore, it allows large-scale production of epidermal cultures suitable for the covering of skin defects such as burn wounds (Green et al, 1979; Gallico et al, 1984). Growing keratinocytes in the absence of dermal cell products by altering the conventionally used tissue culture medium has now become possible. There were also few reports on chemically defined media which were serum-free (Tsao et al, 1982; Boyce and Ham, 1983; Rikimaru et al, 1990). These defined media which comes with the low calcium concentration gives rise to undifferentiated monolayer cultures of uniform small polygonal cells. Growth in defined media may be advantageous for some applications such as the study of growth regulatory substances (Wille et al, 1984; Pittelkow et al, 1986). The development of reliable methods for recreating and manipulating normal and pathological human epidermis in culture make human keratinocytes readily accessible for exploitation in basic and clinical research. Moreover, the enzyme dispase has made the cultured epithelium easily detachable (Gallico et al, 1984). Cultured keratinocytes thus also represent an attractive model system for somatic gene therapy and the successful transfer and expression of foreign DNA have been accomplished in these cells by a number of different techniques (Teumer et al, 1990; Jiang et al, 1991). Similar to the culture of human keratinocytes as reported by the researchers described as above, more research is deemed necessary for exploiting the culture advantages of rabbit keratinocytes which could be employed in skin grafting and other therapeutic interventions.

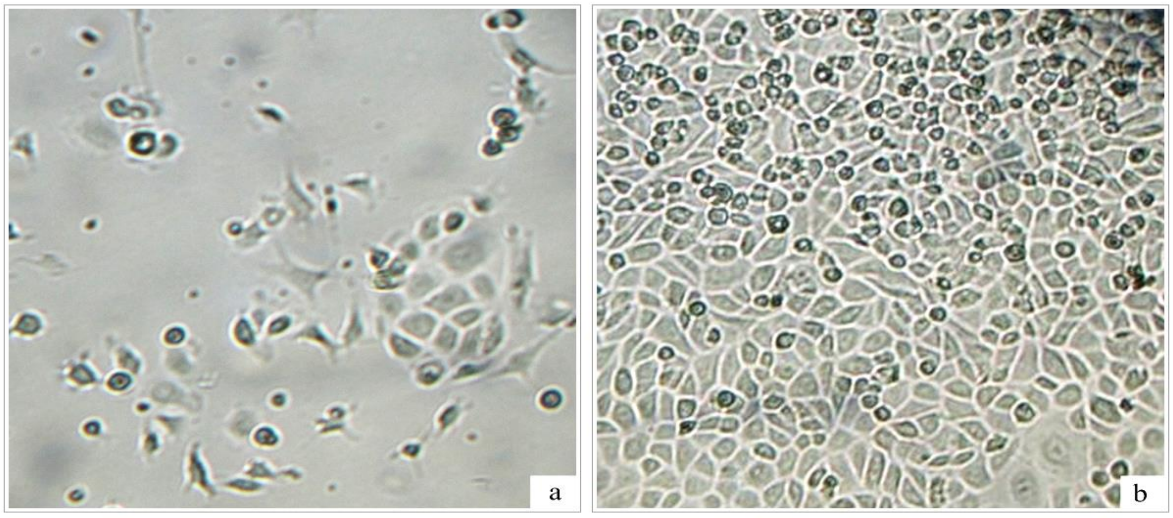

Fig 2. Rabbit primary epidermal keratinocytes a) Day 2 and b) Day 7 of the culture with magnification (1000x)

Conclusion

Keratinocyte cultures have a great potential in cell differentiation and tissue development. In particular, we are fascinated by the possibilities of establishing the culture method of rabbit primary epidermal keratinocytes. Although some valuable results have already been achieved, this research is still in its infancy. New technical developments are clearly needed to make use of the full potential of the cultured rabbit primary epidermal keratinocytes.

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We thank En. Nur Muhammad Muhd Makhatar and En. Md Faizul Ismal Che Adam from Laboratory Animal Research Unit, Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia, for their help rendered during conduction of animal experiments. We would also like to acknowledge the Universiti Sains Malaysia Research University Grant (No.1001/PPSG/811169) for funding this study.

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Morphological and histopathological study of Air Sacs (Sacci pneumatic) in Japanese *Quail* (Coturnix coturnix japonica)

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ABSTRACT

This study aimed to investigate the anatomical and histological features of air sacs in Japanese quail. Twenty healthy birds from Japanese quail (10 Males and 10 Females) were obtained for routine anatomical and histological study, 2 ml of 10% chloral hydrate was injected directly into the heart and then they were injected via trachea with a cold cure plastic mixture for corrosion cast making. The birds were immersions in 3% potassium hydroxide 40c for maceration, washing by tap water. Grossly, the quail had eight air sacs , four of these were paired, cranial thoracic, caudal thoracic, and abdominal air sacs, While the singular air sacs were the interclavicular and cervical. Histological investigation confirmed that the wall of air sack composed of a delicate single layer of squamous or cuboidal epithelial cells supported by a delicate layer of connective tissue.

Key words: air sac, maceration, epithelium, corrosion cast.

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Introduction

The avian respiratory system characteristics by the presence of air sacs that connects with lungs, most of the birds possessed nine air sacs pair of cranial thoracic, caudal thoracic, abdominal and cervical air sacs with a single clavicular air sac.(Powell,2000:Dunker,2004) The cervical and clavicular air sacs in mallard ducks consistent with previous studies, the cranial thoracic air sacs was smaller than the caudal sacs pneumatics the second to seven ribs

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by their diverticula, the caudal thoracic air sac had no diverticulum, the left abdominal air sac had two parts, the cranial and caudal sacs (Demirkan et.al. 2006). The number of the air sacs in duck and goose is nine, four paired sacs with one unpaired the clavicular sack (Brown et. al. 1985: Joines et. al. 1985, While Milson et. al. 1992 explain that the number of the air sacs, Canada's geese are eleven, four paired and three single, the clavicular, dorsal and sacral air sac. There are seven air sacs in turkey, whereas the pair caudal thoracic air sacs absence and connects the air sacs with the secondary bronchus (Cover, 1953).

Getty, 1975 mention that the air sacs of the avian connect into lungs directly by primary bronchus. The wall of the air sack was thin and lines by simple squamous epithelium supported by collagen and elastic connective tissue (Hodges, 1974) The epithelium lining of simple squamous or cuboidal epithelium such as in Penguin (Duncker, 1974) or squamous in avian, duck and goose. This study was designed to investigate the anatomical and histological features of air sacs in Japanese quail.

MATERIALS AND METHODS

This study was approved by the research committee / College of Veterinary Medicine, University of Basrah, / Iraq. The present study carried out on twenty quails (10 males and 10 Females) obtained from the Basrah local market, for anatomical study, the corrosion cast method used in ten birds injected via trachea by a cold cure plastic mixture after euthanasia by 10% chloral hydrate injected directly into the heart. They were macerated with 3% potassium hydroxide at 40^0C for 48 hours (Caja et. al, 1999), then they washed with tap water, each sac was colored by different oil tincture. Ten birds obtained for histological study, the samples immediately post-fixed in 10% neutral formalin. The specimens were washed in running water, dehydrated in a graded series of alcohol, cleared in xylol and embedded in paraffin wax, serial sections of five micrometers thick were made, mounted on slides and stained with hematoxylin and eosin (Luna, 1968).

RESULTS AND DISCUSSION

The air sacs in Quials illustrated by a corrosion cast method using cold cure plastic material, showed that quail had eight sacs, three paired and two unpaired, the paired were caudal, cranial and abdominal air sacs, while the single sacs were cervical and interclavicular air sacks (Fig;1) The results of this study is in agreement with (Duncker 2004,Getty1975,King and Mclleland 1984,Powell 2000). Our results are also compatible with (Cevik et.al,2006), who reported that the air sac related to Japanese quail were the cervical,the clavicular ,the cranial ,the caudal and the abdominal sacs . However our results are in disagreement with (Milson et.al.1992) who study the air sac in goose and found that it possessed eleven air sacs.

The interclavicular sac receiving air via the tertiary bronchi, the cranial thoracic air sack had three indirect connecting groups with tertiary bronchi while there were direct connections of the caudal thoracic air sac with the first and second lateral secondary bronchi, while the abdominal air sac connects directly with the primary bronchi.

This study revealed the following features:

ISSN 2307-8073

1. The cervical and interclavicular sacs;
Cervical sac situated on the cranial part of the thoracic cavity under the cranial cervical and cranial region of vertebral column, they bounded ventro-laterally the

clavicular bone,choroid bone and the anterior thoracic wall, the ribs and sternum (Fig 2).
2. The interclavicular sac aerated from the third abdominal secondary bronchi, the retrocardiac diverticula were observed at the caudal end of the interclavicular sac. The bronchial divercula occupying on the posterior end of the sternum, while the auxiliary diverticula (diverticulum axillare) lies around the shoulder joint (Fig; 3). This result is in agreement with (Duncker 1971) and (Mennega and Galhoun 1968) that showed similar results in chick and chine's ducks respectively.
3. Caudal and cranial thoracic sacs; The cranial thoracic sacs was opposing the thoracic wall between the second and fifth rib beneath the pulmonary hilus. They presence three surfaces: costocervical surface, pulmonary surface and ventromedial surface. The caudal thoracic sacs lie between the cranial thoracic and abdominal sacs, they aerated by the first and second lateral secondary bronchi on the middle of the lateral border of the lung medial to the fifth rib (Fig 4). And this result is compatible with Cevik et.al.(2006) ,powell (1983) and Kurtul et.al.(2004) ,While, it is incompatible with Hadeel (2003)and Khadim (1996) in duck and goose.
4. Abdominalsacs:
The abdominal sacs occupying on the abdominal cavity , its extended caudal to the lungs beneath the posterior part of the abdominal cavity, theses sacs regard as the largest and covered the kidneys and adrenal gland .The wall sac fused dorsally with the kidneys to the floor of mesentery and became free to step down into the abdominal cavity (Fig: 5). The right abdominal sac was longer than the left one (Fig: 6). And this result is in agreement with Maina (1989) and Abdullah (1989).
The histological observations revealed that the wall of the air sacs composed of a thin layer of squamous or cuboidal epithelium supported by a very thin bundle of connective tissue involves a bundle of collagen and elastic fibers, The average of air sac wall thickness was 0.028 micrometers (Fig;). This results are in agreement with King(1970),Duncker (1971) , Hodges (1974) in chicks, and in disagreement with Cook et.al.(1986) who found that the air sac wall had ciliated epithelial layers.

ISSN 2307-8073

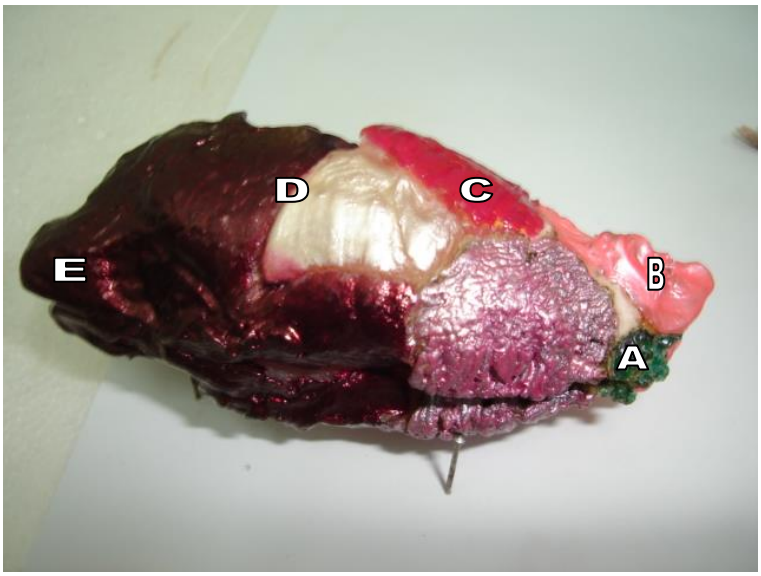

Fig .1.Corrosion cast of air sacs of Quial. A- cervical air sac . , B- interclavicular air sac , C- anterior thoracic air sac .D- posterior thoracic a. sac ,E- abdominal thoracic air sac .

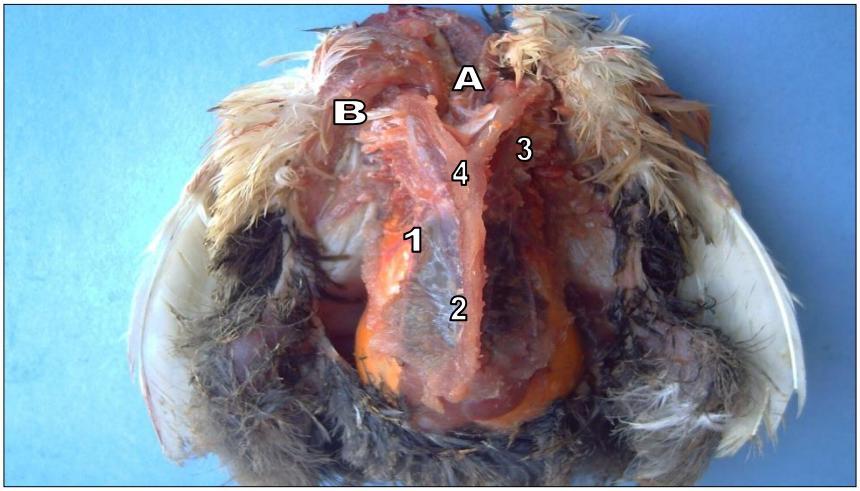

Fig (2);Ventral and lateral borders of the cervical and interclavicular air sacs
1)Ribs . 2) Sternum 3) Clavicula . 4) Hart .A) cervical air sac . B) interclavicular air sac .

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ISSN 2307-8073

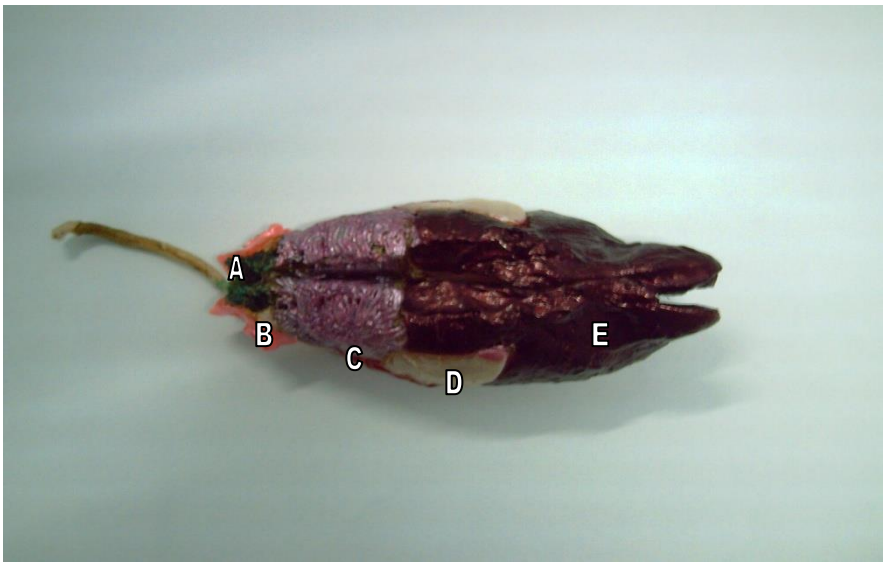

Fig (3);corrosion cold cure cast for the air sacs (dorsal view) A- cervical air sac . B- interclavicular air sac . C- anterior thoracic air sac. D- posterior thoracic sac .E- abdominal thoracic air sac .

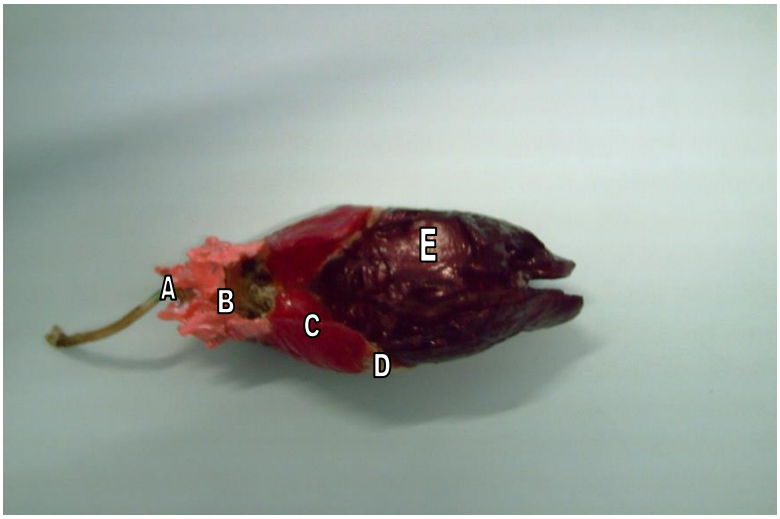

Fig (4); Cold cure corrosion cast (ventral view) A-cervical air sac. B-interclavicular air sac C-anterior thoracic air sac . D- posterior thoracic. sac .E-abdominal thoracic air sac .

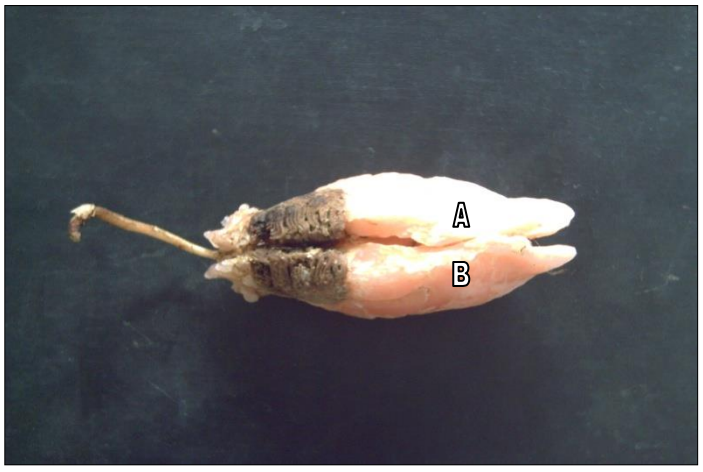

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Fig (5) Corrosion cast of air sacs show; A-femoral diverticulus of the abdominal air sac
B-Suprarenal diverticulus of the abdominal air sac

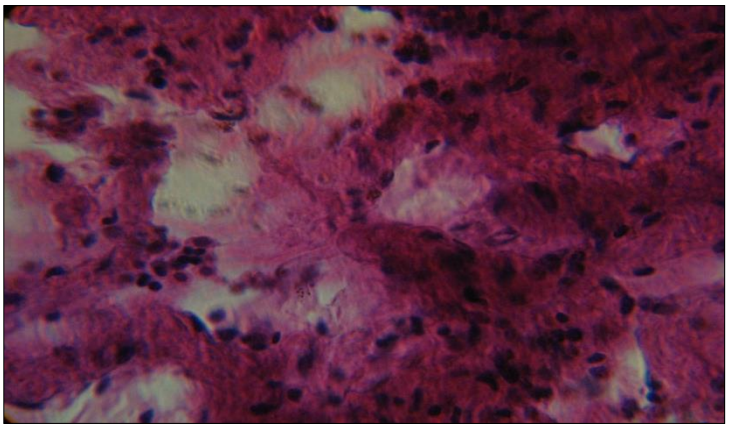

Fig (6);Squamous epithelial cells of the wall of air sac .X1000,H&E

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Survey and diagnostic study of parasitic pneumonia in cats in Al-Qasim district /Babel governorate/Iraq

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ABSTRACT

The study was carried out in Al-Qasim district/Babel governorate –Iraq, where 16 feral cats were captured and searched for parasitic pneumonia .The results showed that 5 out of 16 cats were infested with lung worm (31.25%). All infested cats demonstrated various degrees of respiratory signs in addition to obvious eosinophilia .The gross examination of infested lungs showed apparent consolidation as well as to pulmonary congestion and emphysema, while the microscopic investigation revealed emphysema, alveollar compression, congested blood vessels and presence of parasite eggs in numerous alveoli.

Keywords: cat, parasitic pneumonia, histopathology, Aelurostrongylus *abstrusus*

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Introduction

The feline lungworm *Aelurostrongylus abstrusus* is one of the most common, worldwide distributed lungworms of cats (Scott, 1973).This prostrongylid worm has an indirect life cycle (Soulsby, 1965).The adults worms live in the bronchioles and terminal ducts, and the eggs form nodular deposits in the alveoli, then the eggs developed into first stage larvae that are coughed and swallowed to pass in feces (Anderson, 2000). The cats become infected by eating an infected intermediate or paratenic hosts ,the main intermediate host is either terrestrial or aquatic snails ,while many species of birds ,rodents ,frogs and lizards serves as paratenic host (Dwight et al,2002).

Aelurostrongylus abstrusus cause symptomless infection, however in severe infection, some signs such as coughing ,sneezing and polypnea are seen (Losonsky et al, 1983).

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Since there are no references about diagnosis of Aelurostrongylosis in Iraq ,the aims of present study was designed to describe the incidence *Aelurostrongylus abstrusus* in addition to study of gross pathological and histopathological changes of infested lungs.

Materials and Methods

The study was carried out in Al-Qassim district /Babel governorate at January 2012. Sixteen feral cats aged between 2 to 5 years were captured and brought for post mortem examination in the college of Veterinary Medicine /University of Babylon This study was approved by the research committee / College of veterinary medicine /Al-Qasim green university/Iraq. All cats were anesthetized by intramuscular injection with mixture of ketamin (OBOI, laboratories /India) and xylazin (Interchemie/Holland) in dose of 10 mg and 0.15 mg/kg b.w. respectively(James ,1995). Before performing of euthanasia, the lungs of each animal were clinically examined by auscultation and the blood samples were collected from cephalic vein and transferred in cool box for haematological examination (McCumin, and Bassert , 2002). General necropsy examination was done with special attention to respiratory system ,where the later was dissected and removed entirely from thoracic cavity , examined carefully for worms and/or any pathological lesion .Tissue specimens were preserved in 10% buffered formalin and then sectioned in Al-Sader teaching hospital laboratory in Al-najaf province for preparation of histological slides of 5 µm thickness.

Results

The parasitic pneumonia was confirmed in 5 out of 16 feral cats in percentage rate 31.25%. All infested animals demonstrated abnormal respiratory sounds include crackles , wheezes and moist rales , in addition to obvious eosinophilia which observed in blood samples obtained from these animals . The results of gross examination of respiratory system revealed presence of various pathological lesions in all 5 cats, include apparent consolidation of lungs ,pulmonary congestion ,emphysema and the bronchi was filled with mucopurullent discharge (Figure. 1),while the worms were recovered from only 2 cats (Figure. 2).

The histopathological examination of lungs revealed obvious emphysema with presence of compressed alveoli in some parts of lung sections, the pulmonary blood vessels were congested and surrounded by aggregated inflammatory cells ,many alveoli filled with parasite eggs with some cross section of adult worms (figures 3- 6).

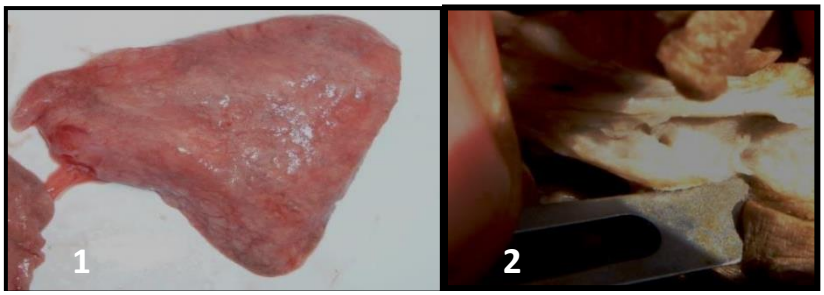

Figure 1, Shows the gross hepatization, emphysema and congestion of infected cat
Figure 2, Shows the adult feline lung worm in bronchiole of infected lung.

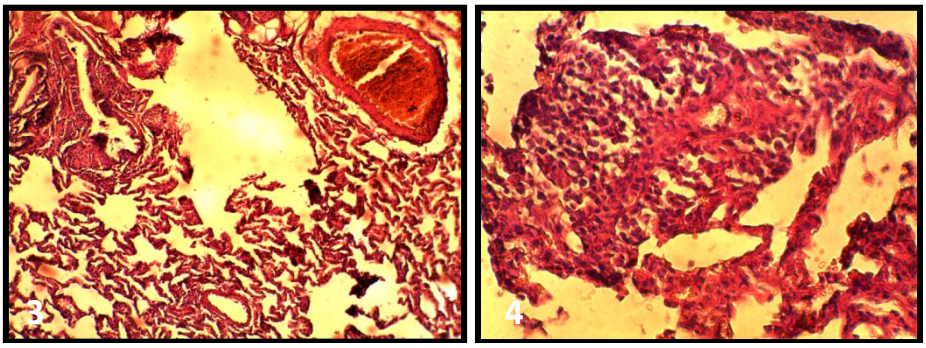

Figure 3, Shows congestion of blood vessels with inflammatory exudates in bronchiole.
Figures 4, Shows sever inflammatory reaction in infected lung with compressed alveoli.

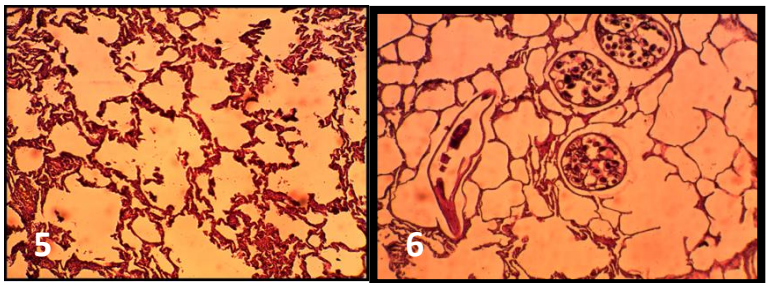

Figure 5, Shows the obvious emphysema of infected lung.
Figure 6, Shows the presence of eggs of lung worm in alveoli

Discussion

According to available references , the alone study about Aelurostrongylosis in Iraq was carried by Al-Khalidi et al ,(1988) in Mosul province, where they first recorded the *Aelurostrongylus abstrusus* in Iraq. However, the parasite was recorded in many countries around the world including Croatia. (Grabarevic et al ,1999) , Turkey (Tuzer et al ,2002) , Australia (Mackerras,1957) , Argentina. (Schiaffi ,2002) , Kenya. (Gathumbi ,1991) and Italy (Grandi,2005).

Many authors (Dwight et al, 2002; Losonsky et al, 1983) suggested that most infection with *Aelurostrongylus abstrusus*, occurs without any clinical signs while others (Rawlings et al, 1980; Smith, 1980) recorded mild respiratory signs including coughing and abnormal respiratory sounds such as crackles, wheezes and moist rales. Most of these clinical signs were in agreement with the results of present study.

In general, the eosinophila associated with allergy or parasitism (canine &feline), and the obvious eosinophilia in present study was in agreement with Center (Center, 1990), whose performed a retrospective study of 312 cases of cats with eosinophilia revealed that 2 percent of the cases were infected with *Aelurostrongylus abstrusus* while the majority of cases, 20.5%, had eosinophilia as a result of flea-bite allergy.

Identical to results of present study, Alliss et al, 2010 recorded that the main macroscopic changes in infested lungs were consolidation, congestion and purulent materials in bronchi.

The main microscopical changes in infested lungs observed in the present study were emphysema, compressed alveoli, thickening and congestion of blood vessels with presence of numerous eggs in alveoli, these changes were compatible with findings of others (Stockdale

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,1970). We conclude that feline lungworm *Aelurostrongylus abstrusus* occurred in Al-Qassim district /Babel governorate / Iraq at (31.25%). All infested cats demonstrated various degrees of respiratory signs and lead to gross and histopathological changes.

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