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New aspects on transferable drug resistance

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Infectious drug resistance in bacteria represents just one of the ways by which micro-organisms can adapt themselves to new situations. This necessity to overcome rapid and often drastic changes in the environment is especially applicable to bacteria which inhabit the intestinal tract. These bacteria have to withstand frequent alterations in the composition of the intestinal contents and they also have to be capable of surviving the effect of substances specially prepared either to inhibit their growth or even destroy them. There are several ways by which bacteria can withstand the effects of unsuitable surroundings: they can produce spores, mutants can be selected that will resist toxic agents and certain bacteria can utilize alternative biochemical pathways in order to overcome a metabolic inhibitor but the ability to acquire protection against several inhibitory substances at one step through transferable drug resistance is perhaps the most significant of all.

The recognition [1] and subsequent study of infectious drug resistance over the past ten years has proved to be of great value to both veterinary and medical bacteriologists. Such a study has provided the means by which we can now examine some of the more fundamental properties of disease-producing organisms. For instance we now have a better understanding of haemolysin [2] and enterotoxin [3] production by strains of *Escherichia coli*. Bacterial genetics have benefited greatly from the discovery of infectious drug resistance and this is especially so in the field of cytoplasmic inheritance. The phenomenon of bacterial conjugation [4], recognised about ten years before infectious drug resistance, is rather limited in its application to extra-chromosomal inheritance because of a lack of cytoplasmic markers associated with the F factor. With R factors at least twelve markers have been recognised and this has enabled a very detailed examination to be conducted on the genetics of heritable cytoplasmic particles.

Apart from the advantages obtained in bacterial genetics various other branches of microbiology have benefited from the discovery of infectious drug resistance. The multiplicity of R factors produced in strains of Proteus [5] has enabled significant amounts of plasmid DNA to be obtained and purified for biochemical and radiographic examinations. Stimulus has also been provided to search wild type enteric bacteria for naturally occurring transfer factors and in one study these have been found in about 30% of strains examined [6]. All this evidence on transmissible plasmids indicates that bacteria have

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probably possessed the ability to exchange genetic information for an appreciable length of time and that transferable drug resistance is just one indication of the means by which this information is transmitted to other members of the bacterial population. Let us now turn to some recent developments in transferable drug resistance which have a direct bearing on this Conference on "active substances in animal production". I have been looking at some of the properties of bacteria which make them more efficient receptors of resistance factors than other bacteria under identical conditions, then using these findings to explain why transfer of antibiotic resistance is so inefficient in the intestinal tract. Two factors have already been shown to affect transferability and establishment of resistance factors in the recipient organism. The presence of modification and restriction loci on the chromosome of a bacterium will inhibit the entry of an R factor and if it gains entry will modify it sufficiently to prevent expression of its properties [7]. I have now shown that another factor appears to be involved in the transfer of R factors from resistant to sensitive cells. Work done at Princeton on the transfer of drug resistance in pigs indicated that although R factors were transferred with high efficiency in the laboratory little to no transfer occurred experimentally in the intestine of pigs [8]. At the same time another report indicated that despite a high efficiency of *in-vitro* transfer no transfer of resistance was occurring in the intestine of human patients from strains of Klebsiella picked up in a hospital surgical unit [9]. Taking these two reports into consideration I began looking at the effect of deficiences in the cell wall of bacteria on the transfer of resistance factors. Before discussing my findings I would like to mention a few points about the structure of the cell-wall of *Salmonella typhimurium* (diagram 1). Basically the cell-wall consists of three parts, O antigen, core side chain and inner core, any wild type smooth strain of *Salmonella typhimurium* possesses all these three layers. If any one of these layers is missing then the organism is no longer smooth and is classified as a rough organism, the degree of roughness will depend on how much of the cell-wall is present.

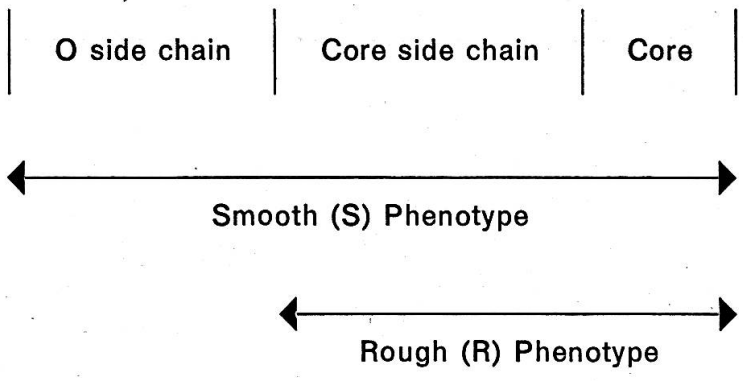

Diagram 1 Representation of the lipopolysaccharide fraction of the cell-wall of *Salmonella typhimurium*.

It is not always possible to detect roughness in a bacterial strain simply by colonial characteristics nor is it possible to say that because a culture does not auto-agglutinate in saline or acriflavine that the culture is smooth. The only sure way of detecting roughness in bacterial strains is with biochemical means or by specific bacteriophages. In general if only the O side chain is missing then the strain is classed as "superficial rough", but if the greater part of the core side chain is missing then the strain is classed as "deep rough". Some workers have shown that certain types of roughness in strains of bacteria are determined by a specific region on the bacterial chromosome [10] (diagram 2). Many of the different types of roughness can be experimen-

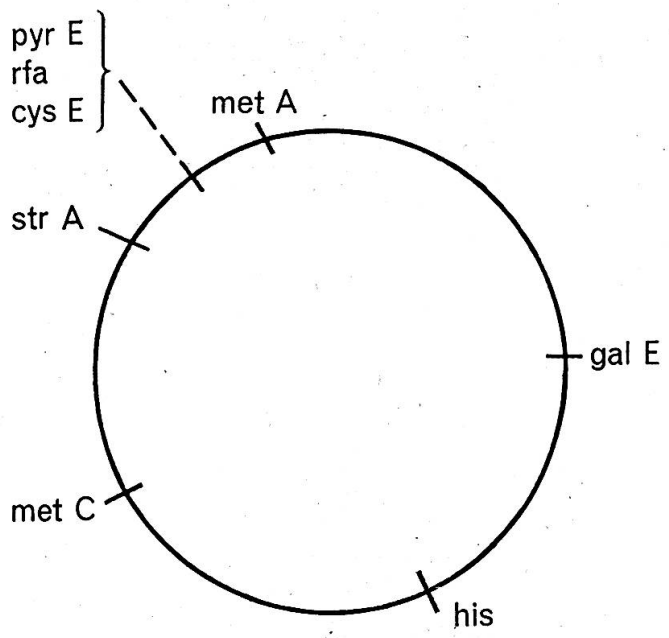

Diagram 2 Portion of the chromosome of *Salmonella typhimurium* in which the rfa gene cluster is located. This cluster contains genes that are responsible for the assembly of the core side chain.

tally produced in bacteria and if this is carried out using a single parent strain then an isogenic series of bacteria can be produced that differ only in the degree of roughness of their cell-wall. I obtained from Professor Stocker such an isogenic series of bacteria and used them to determine the effect of cell-wall components on the transfer of drug resistance factors. Using several types of R factor my experiments showed that the best receptors of R factors were strains of bacteria in which only the O side chains were missing [11]. When the O side chains are present or when most of the cell-wall is absent, as in deep rough bacteria, then the efficiency of transfer of R factors is very much reduced. Before discussing this finding I want to mention briefly certain aspects of the processes involved in the transfer of drug resistance factors in the laboratory (diagram 3). When a mating mixture consisting of donor and

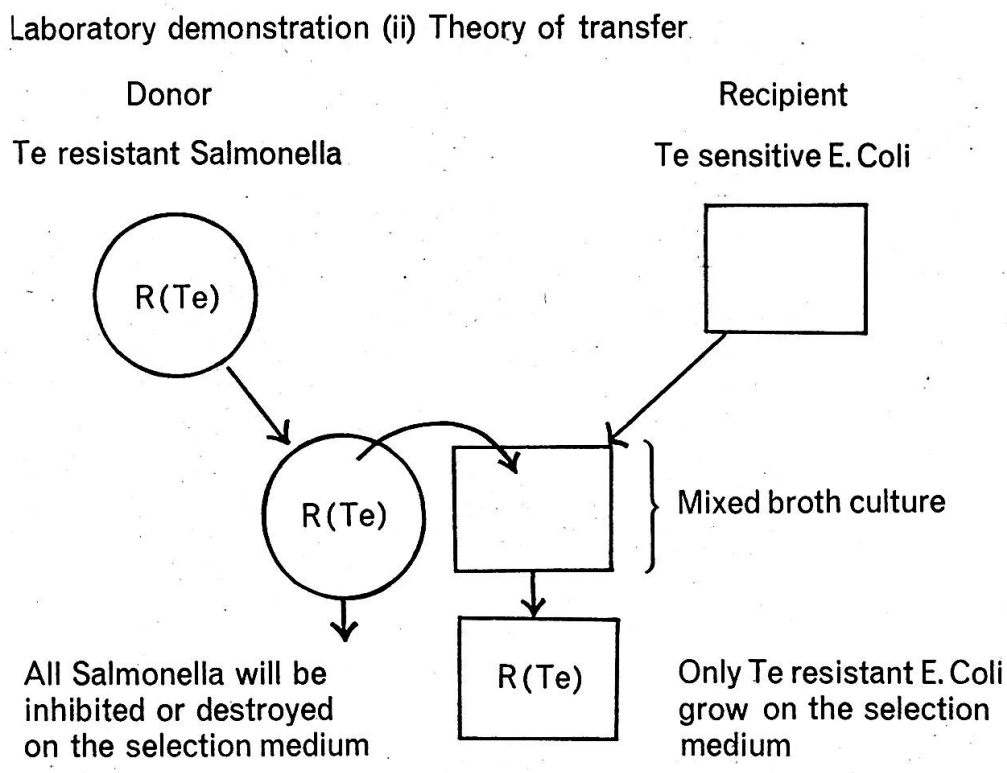

Diagram 3 Illustrates the theory of transfer of infectious drug resistance in the laboratory.

recipient organisms is prepared then the transfer of an R factor is dependent upon an effective contact being made between the two bacterial cells. Various experiments have indicated that a conjugation tube is produced by the resistant cell during the logarithmic phase of growth, but so far no one has shown the presence of an R factor inside one of these conjugation tubes. This is a very difficult procedure and perhaps we have not yet found the proper method (diagram 4). There is still some doubt about the actual role of the conjugation tube in the transfer process. An R factor may enter the conjugation tube and pass along the inside to the recipient cell or perhaps the conjugation tube is simply acting as an anchor to hold the two cells together whilst the R factor passes through the cell-wall by other means. In either case current evidence suggests that conjugation tubes are an essential part of the transfer process. Returning now to the finding that "superficial rough bacteria" are the best receptors of R factors we could conclude that loss of the surface O antigen uncovers the attachment sites for the conjugation tubes and if the greater part of the cell-wall is missing, as in "deep rough" bacteria, then the attachment sites may only be present on a very small number of bacteria. This finding that "superficial rough" bacteria are efficient receptors of R factors has an important bearing on the interpretation of experimental results. Most of the experimental work with infectious drug resistance has involved the use of *Escherichia coli* K12 as a common recipient of R factors. This particular

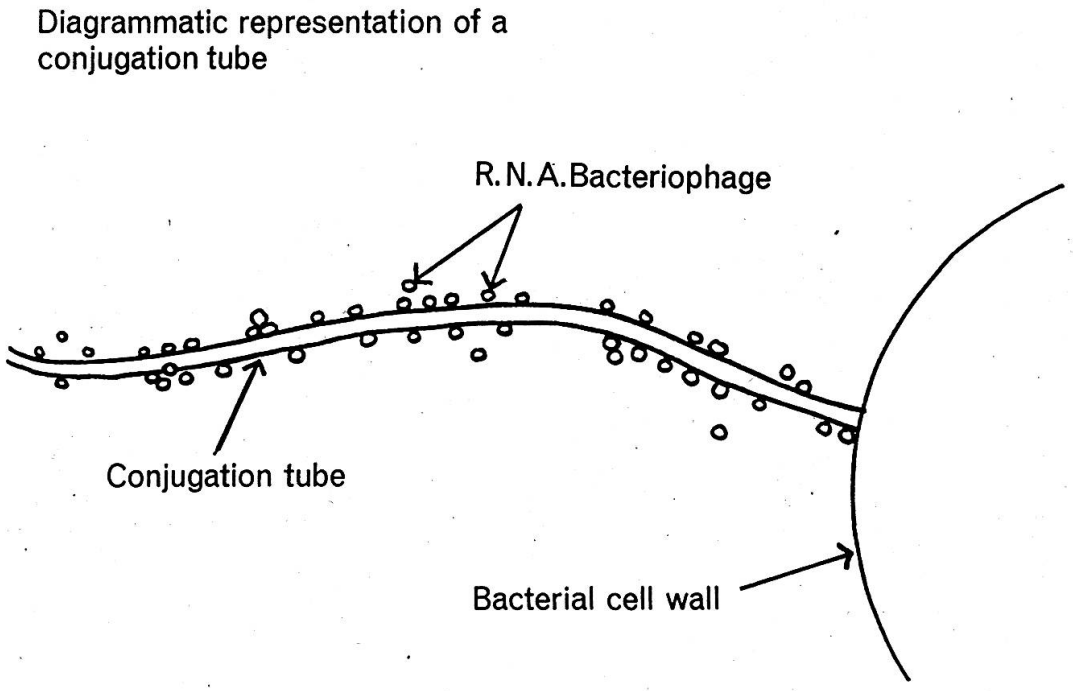

Diagram 4 Illustrates some of the features of a conjugation tube or pilus. The tube is hollow and is basically an outgrowth of the bacterial cell wall and can be detected by specific bacteriophage.

organism does not possess any detectable O antigenic side chains, therefore, using the criteria mentioned above it must be classed as a "superficial rough" organism and this provides an explanation for one of the reasons why *E. coli* K12 is such an efficient receptor of R factors under *in-vitro* conditions. Several workers have also used *E. coli* K12 as a recipient for *in-vivo* experiments and they have found that (a) the viable count of this organism in the faeces remains low and (b) that the number of recipient organisms converted to resistance after eighteen hours is very much less than when the experiment is made in the test-tube [12]; in order to explain these findings it is essential to put the *in-vivo* situation into clear perspective. Whereas in the test-tube there are only two organisms that have been specially selected for their donor and recipient ability, in the intestine there are numerous unselected organisms of different genera each having its own ecological niche. Some of these bacteria may be intracellular but the majority are found in the intestinal contents or in the folds of the intestinal mucosa. The ambient situation in the intestine is so different from that which is produced in the test-tube that it is inconceivable that anyone should attempt to deduce from their *in-vitro* findings what might occur under the conditions that exist in the living animal. Many early *in-vitro* experiments indicated that during transfer all the drug resistance determinants passed into the sensitive cell as a single unit. However, recent work has shown that this is not always so [13]. If a mating mixture is prepared then after a single mating event the culture is plated out to isolate those cells

that have received resistance determinants it will be found that some bacteria have received different resistance determinants than other bacteria. Also the type and number of determinants each recipient cell receives will vary according to the selection procedure that is used. If bacteria that have received multiple drug resistance are sub-cultured in drug free medium, then the resistance determinants that are lost will not be the same in all cases. These two experiments probably indicate that the R factor is not a simple linear structure – if it were then it would possibly transfer as a single unit and that segregation would be an all-or-none phenomenon. I draw your attention to these somewhat basic experiments in order to impress upon you the sort of complex data that is now accumulating on R factors which makes interpretation of experimental results very difficult indeed. Although aware of the complex ecological situation existing in the alimentary tract we have very little concrete evidence about the actual contiguous relationships of the different organisms within the intact alimentary canal. In order to obtain and recognise experimental transfer of drug resistance in the intestine workers have used a wide variety of techniques. One of the first available reports about *in-vivo* transfer highlights one of the problems. Germ free mice were used and because they were in an atmosphere of ultra violet radiation they developed chronic skin lesion [14]. Mice were used again by a second worker who used antibiotics prior to and during his experiments, however this author did indicate that *in-vivo* transfer could occur in the absence of antibiotics [15]. I used newly hatched chickens in my experiments and found that although transfer of resistance did occur it did not persist very long [16]. All these three experiments were trying to overcome the same difficulty that of trying to get the experimental strains to multiply and become part of the normal intestinal flora, and in order to do this various techniques were employed. – In the first case bacteria free animals were used, in the second experiment drugs were used to reduce the numbers of competitive bacteria and in my own case I used chickens before a complete intestinal flora had developed. These experiments provide absolute evidence about the difficulties associated with *in-vivo* transfer of drug resistance. In the latter stages of these experiments the recognition and isolation of strains of bacteria that had received R factors in the intestine became very difficult. Most workers that have experimented with *in-vivo* transfer of drug resistance have obtained basically similar findings and these are: (1) there is very little evidence of epidemic spread of R factors within the intestinal tract, and (2) that although transfer of resistance does occur the persistence of the strains that have acquired resistance is very short lived. Another feature of the various *in-vivo* experiments that have been reported is that in order for transfer of resistance to occur the experimental strains should colonize the alimentary tract, at least for the period of the experiment. This has generally proved to be a difficult problem, numerous methods have been used and in one recent study the experimenter had to take, by mouth, 10^9 bacteria every day for seven days in order for any significant results to be

obtained [17]. This large number of organisms that had to be taken to obtain gut colonization is very much greater than the dose of organisms with which humans would make contact under normal circumstances.

In respect of the contamination and possible colonization of the human gut by bacteria of animal origin it has frequently been suggested that animal strains of enteric bacteria could readily form a part of the human intestinal flora. The recent findings from a study of experimental R factor transfer in the intestinal tract of man indicated that organisms of animal origin were not very efficient at colonizing the alimentary tract of man, and even when colonization did occur the frequency of resistance transfer was very low and it did not persist beyond a few days [17].

All the *in-vivo* experimental results that I have mentioned so far were obtained in healthy individuals. A few experiments have been made in sick animals that were receiving antibiotics. In these circumstances the number of R factor strains in the intestinal contents was very much higher than in normal animals – but this increase is frequently accompanied by an upset in the relative proportions of the bacteria which constitute the intestinal flora. Under these conditions animals could excrete large numbers of multiple resistant bacteria. Recent epidemiological evidence has indicated that a high percentage of human beings carry R factor-containing-bacteria [18]. Another source of R factors that can be traced to animals is to be found in processed foods like sausages [19]. In contrast to these situations, where antibiotics are readily available, R factors have also been found among native populations that have had no known contact with antibiotics [20] and also an R factor has been found in a bacterial strain that was lyophylized in 1946 [21]. All this and other evidence indicates the widespread nature of infectious drug resistance in both human and animal populations, therefore, I don't feel that one can justly accuse one population of being the source of R factors for the other.

Part of the evidence that I have presented to you indicates that the mechanism of transferring genetic information among populations of bacteria is not a recent development. In this particular context the development of antibiotics has enabled us to recognise just one of the ways in which information can be spread. Experience in the field and in the laboratory has shown that antibiotics exert a selective pressure and do help to maintain the presence of transferable drug resistance in bacteria present in the intestinal tract. But there is no evidence that antibiotics have actually caused the production of R factors although transferable drug resistance can be found in most situations that require the use of antibiotics. Thus all evidence relating antibiotics to the development of transferable drug resistance is very conflicting.

In conclusion, therefore, I consider that the discovery of infectious drug resistance has provided the bacteriologist and bacterial geneticist with a very useful laboratory tool. There is no doubt that this phenomenon has benefited many branches of biology. With regard to the implications of infectious drug resistance in the human and animal fields the mere fact that organisms of

animal origin might be found in human intestinal contents, then regardless of the presence of R factors, this situation indicates that the handling and processing of human foods in certain cases is not up to standard, so if any rigid controls of antibiotics are to be imposed on the agricultural community, then these should be supplemented with a firm insistence on improved food hygiene.

Zusammenfassung

Der Verfasser ist der Meinung, daß die Entdeckung der Resistenz von Krankheitserregern gegen Therapeutika den Bakteriologen und entsprechenden Vererbungswissenschaftern ein sehr nützliches Hilfsmittel eröffnet hat. Ohne Zweifel hat dieses Phänomen viele Gebiete der Biologie befruchtet. In Anbetracht der Rückwirkungen dieser Resistenz für Mensch und Tier und des Umstandes, daß im Darm des Menschen Organismen aus dem tierischen Darm gefunden werden, ist einzusehen, daß eine strenge Kontrolle der Antibiotika nicht genügt, sondern durch bessere Nahrungsmittelhygiene ergänzt werden muß.

Résumé

L'auteur estime que la découverte de la résistance des germes pathogènes envers les agents thérapeutiques à fourni un moyen utile aux bactériologues et aux généticiens. Ce phénomène a certainement enrichi plusieurs branches d'activité de la biologie. En considérant la rétroaction de la résistance pour l'homme et l'animal et en tenant compte du fait qu'on a trouvé des microorganismes originaires du canal intestinal de l'animal dans l'intestin de l'homme, on est obligé de reconnaître que seul un contrôle même sévère des antibiotiques est insuffisant, il doit être complété par une meilleure hygiène alimentaire.

Riassunto

L'autore esprime l'idea che la scoperta della resistenza di agenti patogeni contro medicamenti ha aperto un aiuto molto valido ai batteriologi e rispettivamente agli scienziati che si dedicano allo studio dei problemi ereditari. Senza dubbio questo problema incide in molti campi della biologia. In considerazione delle ripercussioni di questa resistenza sull'uomo e sugli animali e per il fatto che nell'intestino dell'uomo si trovano organismi dell'intestino animale, appare evidente che un severo controllo degli antibiotici non è sufficiente, ma che occorre integrarlo con una migliore igiene delle derate alimentari.

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REFERATE

Periartikuläre, subchondrale Knochenzysten beim Pferd. Von H. Pettersson und S. Reiland, Proceedings of the Fourteenth Annual Convention of the American Association of Equine Practitioners, 1968, 245.

Wir haben bereits im Jahrgang 1954, Seite 276, über eine Knochenzyste beim Hund referiert. Die vorliegende Arbeit bezieht sich auf 14 Pferde, welche im Tierspital Helsingborg in Schweden in den Jahren 1949–1967 unter 6500 auf Lahmheit untersuchten Pferden anfielen. Die Zysten verteilten sich auf Pferde von 5 Monaten bis zu 5 Jahren, 10 davon aber unter 2 Jahren, 11 waren Hengste oder Wallachen. Die meisten dieser Pferde zeigten intermittierende Lahmheit von wechselndem Grad. Eines davon gewann während dem akuten Stadium immerhin ein Rennen. Bei einem Pferd war trotz positivem Röntgenbefund keine Lahmheit aufgetreten. Bei den meisten Pferden mit Lahmheit ergab die Untersuchung Verstärkung der Arterienpulsation an der lahmen Gliedmaße und Druckschmerz im befallenen Gelenk. Bei einzelnen Tieren waren die entsprechenden Gelenke erheblich aufgetrieben. Die Knochenzyste saß bei drei Tieren im Hufbein, bei drei im Kronbein, bei sieben im Fesselbein und nur bei einem Tier im Karpus. Im Röntgenbild sieht man eine erbsen- bis daumenbeerengroße Zone mit geringerer Knochendichte und unscharfem Rand, dicht unter dem Gelenkknorpel. Die Sektion an vier Pferden zeigte einen kleinen Knorpeldefekt über der Zyste, der eine Verbindung mit dieser herstellte. Bei vier Pferden wurden periodisch Untersuchungen und Röntgenaufnahmen gemacht, welche zum Teil zeigten, daß der Defekt im Verlaufe von Monaten kleiner werden oder verschwinden oder auch 2 bis 6 Jahre weiterbestehen kann. Die Diagnose kann gesichert werden durch Leitungsanästhesie oder noch besser durch Gelenkanästhesie. Von den 13 Pferden mit Lahmheit heilten sieben nach 3 Wochen bis 2½ Jahren ab und konnten wieder zu gewöhnlichem Reiten, Trab- und Galopprennen verwendet werden. Ein Pferd blieb während 6 Jahren intermittierend lahm, vier wurden geschlachtet, und für eines blieb der weitere Verlauf unbekannt. Die Behandlung bestand in Ruhestellung im akuten Stadium, Entwurmung, Gaben von Vitaminen und anabolen Steroiden.

In der auf den Vortrag folgenden Diskussion ergab sich, daß Knochenzysten als Zufallsbefunde auch in den oberen Gelenken beim Pferd nicht selten sind, vermutlich machen sie dort weniger Lahmheit. Über die Ursache gehen die Ansichten noch weit