Diagnostic testing of different stages of avian tuberculosis in naturally infected hens (*Gallus domesticus*) by the tuberculin skin and rapid agglutination tests, faecal and egg examinations

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ABSTRACT: Avian tuberculosis (ATBC) is a significant cause of morbidity and mortality in birds in zoos and breeding establishments. The primary sources of *Mycobacterium avium* subsp. *avium* (*MAA*) of serotypes 1, 2 and 3 are infected animals (esp. birds), who shed *MAA* in different ways and thus contaminate the environment. The first aim of this work was to compare the efficiency of the diagnostic methods that are routinely used for the diagnosis of ATBC (skin test, serology and culture of faeces and eggs) in naturally infected hens with different levels of infection. The second aim was to determine the excretion rate of *MAA* in faeces and eggs. The tuberculin skin test gave a positive result in nine (42.9%) infected hens of which four (57.1%) and one (14.3%) were heavily and slightly infected hens, respectively. A positive serological response to *MAA*-b antigen (water bird isolate of serotype 1) was observed in five (23.8%) and to *MAA*-p antigen (pig isolate serotype 2) in seven (33.3%) hens. No correlation between serological and skin-test data was found. The results show that both techniques, serological and skin-test data are inadequate for the diagnosis of ATBC. In consecutively euthanized hens, with heavy infection and tuberculous lesions, serological positivity was significant ($P < 0.05$) in comparison with slightly infected hens lacking tuberculous lesions. Faecal culture detected *MAA* in 50 (29.8%) of 168 samples collected for eight days before euthanasia. *MAA* excretion in faeces was intermittent, but significantly ($P < 0.01$) higher in heavily infected hens. No mycobacteria were detected in any of the 43 examined eggs, which implies that the shedding of *MAA* and/or transmission of ATBC through eggs may not be frequent events.

Keywords: avian mycobacteriosis; IS*901* PCR; IS*1245* PCR; food safety; zoonosis

Avian tuberculosis (ATBC) is an insidious, chronic, wasting disease with a worldwide distribution affecting all species of birds, domestic and wild animals, and is caused by *Mycobacterium avium* subsp. *avium* (*MAA*) of serotypes 1, 2 and 3 and genotype IS*901*+ and IS*1245*+ (Pavlik et al., 2000; Mijs et al., 2002). In particular, the infection is a significant cause of morbidity and mortality in birds in zoos and breeding establishments (Thoen et al., 1981). Apart from this, *MAA* also causes zoonotic infections in humans as "avian mycobacteriosis" in immunocompromised patients and can lead to pneumonia, lymphadenitis, meningitis, miliary tuberculosis, generalised mycobacteriosis (Wayne and Sramek, 1992) and other forms of the disease. All this means that the infection is an important one.

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Due to the fact that *MAA* is spread in large part by birds, it is difficult, if not impossible, to protect the host organism from exposure and the environment from contamination. *MAA* is not present in large scale-production hen farms which have introduced well and functioning production practice based on a system of "closed" technology and whose layers have a relatively "short-life", not exceeding 12 months. The number of hen farms with "ecological agriculture" has increased in the Czech Republic and other European countries. In ecological farms, hens are usually kept at pasture for the production of so called "green eggs". This way of keeping hens increases the risk of hen contact with infected domestic and free living birds (Hejlicek and Treml, 1993).

MAA is generally transmitted by direct contact with infected birds, ingestion of contaminated feed or water and contact with a contaminated environment. Birds infected with *MAA* shed the organism via their faeces and it can survive in environments for years (Thornton et al., 1999), although the frequency of shedding is not known. The possibility that *MAA* might be transmitted through eggs from infected hens has long been considered. It has been demonstrated many times that some artificially inoculated eggs will hatch and that chicks from such eggs will be infected with *MAA*. However, in contrast to this, *MAA* has not been observed in many hundreds of chicks hatched from eggs of naturally infected hens (Thoen, 1997). On top of this, instances describing the excretion of *MAA* and/or transmission of *MAA* via eggs from naturally infected hens are scarce in the literature.

Despite this, ATBC can exhibit diversified clinical signs in birds; pathognomic signs are limited and no clinical symptoms specifically identify ATBC in birds (Painter, 1997; Tell et al., 2001), which makes the diagnosis procedure difficult. Hence, the diagnosis of ATBC is most commonly based on serological, haematological and allergy skin tests, and on histopathological, culture and molecular biological techniques. Although from a technical point of view its use is impractical for wild and free living birds, and its use in turkeys, geese and duck species is also limited (Bush et al., 1978; Lumeij et al., 1980; Pavlas et al., 1993); nevertheless, intradermal skin tests have been widely used for a long time in poultry industries (Pavlas et al., 1993).

For the detection of antibodies, serological tests such as rapid agglutination tests (RAT), ELISA (Jorgensen, 1978; Thoen et al., 1979), complement fixation tests (CFT) and haemoagglutination tests (HAT) are most commonly used. HAT can also be performed for the purpose of differentiation between para-allergic (non-specific) and specific reactions to avian tuberculin (Cromie et al., 1991). A whole-blood RAT, one having possible diagnostic value for ATBC in fowl has long been described. The method is applied by using whole blood for the identification of birds with a mycobacterial infection (Hawkey et al., 1990; Cromie et al., 1991; Pavlas et al., 1993).

Our two previous studies were focused on the distribution of *MAA* infection in the bodies of various grallatorial bird species: four species of the Ardeideae family – little egret (*Egretta garzetta*, buff-backed heron (*Bubulcus ibis*), great white egret (*Egretta alba*) and bittern (*Botaurus stellaris*) and two species of the Threskiornithidae family – sacred ibis (*Threskiornis aethiopicus*) and spoonbills (*Platalea leucorodia;* Dvorska et al., 2007) and domestic fowl (*Gallus domesticus*; Shitaye et al., 2008). We established that any differences between species with regard to distribution of *MAA* infection were non-significant in cases where a bird was infected with *MAA*.

Gross examination and laboratory examination of 14 different tissues from four body parts (intestinal tract, parenchymatous organs, reproductive system and blood related organs) in 21 euthanized hens for the presence of mycobacteria have been described by Shitaye et al. (2008). In the present study, individual samples of faeces and laid eggs from the previously investigated 21 hens were examined in the laboratory on eight separate days and allergological and serological testing was performed before slaughter. In order to answer the question of how reliable these tests were in respective hens, we compared the efficiency of the diagnostic methods that are routinely used for the diagnosis of ATBC (skin test, serology and culture of faeces and eggs) in naturally infected hens with different levels of infection. In addition, the second aim of this study was to determine the intensity and excretion rate of *MAA* in faeces and eggs in naturally infected hens with different levels of infection to asses the risk of transmission to other animals and humans.

MATERIAL AND METHODS

Based on their emaciation status, skin testing with avian tuberculin and the RAT of their blood with *MAA* antigen, 18 naturally infected hens from

a flock of 64 hens in one aviary were selected. Furthermore, one hen which was negative according to the above mentioned test with the exception of RAT, in which the reaction was dubious, and two hens which were shown to be totally negative in all above mentioned tests were also included in this study. Totally negative hens served as a negative control. Each hen was placed into a separate standardised cage with feed and water available *ad libitum* until euthanasia. Eight days before euthanasia faeces and eggs from all 21 hens were collected for cultivation and microscopic examination. The *post mortem* diagnosis of these hens has been described in our previous study (Shitaye et al., 2008).

Tuberculin skin test with avian tuberculin

The skin test was carried out with 0.1 ml of avian tuberculin Avitubal (28 000 TU/ml) produced from the D 4 ER Strain of *MAA* (Bioveta, Ivanovice na Hane, Czech Republic). For the administration into the ventral end of the comb of hens a very thin needle was used to avoid causing a small wound orifice. The other uninfected comb was used for comparison and/or control purposes. After the administration of tuberculin to all hens, a small swelling can develop at the site of inoculation. The reaction was evaluated 48 h after inoculation; a positive reaction is indicated by inflammation and swelling of the injected comb, which is clearly observed by comparison with the control one/uninfected comb.

Rapid agglutination test (RAT)

Blood was collected before the skin testing and transported to the laboratory. Serum was removed and stored at –20°C until examination. A RAT for the presence of antibodies against *MAA* was carried out according to a previously described method (Pavlas et al., 1993) and two different *MAA* antigens were used:

***MAA*-b** from a bird isolate of serotype 1 and genotype IS*901*+ and IS*1245*+; the antigen described previously (Dvorska et al., 2007) was produced from an isolate from little egret (*Egretta garzetta*).

***MAA*-p** from a pig isolate of serotype 2 and genotype IS*901*+ and IS*1245*+; the antigen described previously (Pavlik et al., 2007) was produced from an isolate from a domestic pig (*Sus scrofa* f. *domestica*).

A reaction was considered to be positive if a marked agglutination, i.e. the formation of a white complex of an antigen-antibody reaction, was observed in 1 to 2 min. Samples were considered as "dubious" if a slight agglutination was observed after the lapse of 2 min. A negative control (distilled water) was tested simultaneously in reactions with all of the used antigens.

Faecal and egg collection

A total of 168 faecal samples were collected with a sterile wooden spatula each morning; laid eggs (a total of 43 eggs) were also collected. The examination of faeces was performed immediately after sampling and eggs were stored in a cool place (temperature between 5 to 6°C) before examination which took place no more than three weeks after this.

Laboratory examination for the detection of mycobacteria

Microscopic examination of faeces and eggs. Approximately 1 g of faeces was homogenised in 1 ml of sterile distilled water. After the sedimentation of large particles within 30 min, 10 µl of supernatant was placed on a microscopic slide. Smears from all egg samples [yolk, egg white and egg shell (grinded using mortar with some drops of water)] were also prepared and examined for the presence of acid-fast bacilli (AFB), after Ziehl-Neelsen staining. The detection of AFB was performed using a light microscope (Olympus BT17, Japan) under oil immersion at a magnification of 1 000×; at least 200 fields of view were examined for each sample.

Culture examination of faeces and eggs. For decontamination, about 1 ml of faecal suspension, 1 ml egg-shell, 1 ml egg-white or 1 ml egg-yolk was taken from each sample. The suspension was then decontaminated in 1 N HCl for 15 min; subsequently the examined sample was neutralised by 2 N NaOH until the colour changed to light purple (2% phenolphthalein was used as a pH indicator). Egg white and egg yolk were cultured simultaneously without decontamination. Eighty µl of the suspension was inoculated with sterile disposable tips and dispensed to each of the following culture media in duplicates: egg based solid medium according to Stonebrink, Herrold's egg yolk medium

Table 1. Intravital diagnostic testing of avian tuberculosis in 21 hens eight days before euthanasia

Hen		Cachexia	Skin test[2]	Days of laboratory examination[3]								No. of eggs	Faeces[4]			Serology test[5]	
Stage[1]	No.			1	2	3	4	5	6	7	8		No.	Positive	%	*MAA*–b	*MAA*–p
I	1	+	–	0^{E}	0^{E}	1^{*}	1^{E}	1^{E}	0	0	1	4	8	4	50.0	+	±
	2	+	+	1	0	0	1^{*}	0	0^{E}	0	1	1	8	3	37.5	+	+
	3	+	–	1^{*}	1	1	1	1	1^{*}	1	1	0	8	8	100	±	+
	4	+	+	1	1	0	1	1^{b}	1^{b}	0	1	0	8	6^{b}	75.0	±	+
	5	+	–	1^{b}	0^{E}	1^{E}	1^{E}	0^{E}	0^{E}	1	1	5	8	5^{b}	62.5	+	+
	6	+	+	0	0	0	0	0	1^{bE}	0	1	1	8	2^{b}	25.0	+	+
	7	+	+	1	0	1	1	1^{b}	0	0	0	0	8	4^{b}	50.0	±	±
	examined	7	7	7	7	7	7	7	7	7	7	11	56			7	7
Subtotal	positive[a]	7	4	5^{b}	2	4	6	4^{b}	3^{b}	2	6	0		32^{b}	57.1	4	5
	%	100	57.1	71.4	28.6	57.1	85.7	57.1	42.9	28.6	85.7	0				57.1	71.4
II	8	+	–	1^{bE}	0^{E}	1	0^{E}	0	0^{E}	1^{E}	0	5	8	3^{b}	37.5	±	±
	9	+	+	0	0	0	0	0^{E}	0	0	0	1	8	0	0	±	–
	10	+	+	1	0	0	1^{b}	0	0	0^{E}	0	1	8	2^{b}	25.0	–	±
	11	+	–	1	1	0	0	0	0	0	0	0	8	2	25.0	±	±
	12	+	+	1	1^{*E}	1	1^{E}	0^{E}	1^{E}	1	1^{E}	5	8	7	87.5	±	±
	13	+	+	0	0	0^{E}	0	0	0	0^{E}	0	2	8	0	0	±	–
	14	+	–	0^{bE}	0^{E}	0^{E}	1^{E}	0^{E}	0^{E}	1^{b}	1^{E}	7	8	3^{b}	37.5	±	±
	examined	7	7	7	7	7	7	7	7	7	7	21	56			7	7
Subtotal	positive[a]	7	4	4^{b}	2	2	3^{b}	0	1	3^{b}	2	0		17^{b}	30.4	0	0
	%	100	57.1	57.1	28.6	28.6	42.9	0	14.3	42.9	28.6	0				0	0
III	15	–	–	0	1	0	0	0	1	0	0	0	8	2	25.0	±	+
	16	–	–	0	1^{E}	0^{E}	0^{E}	0^{E}	0^{E}	0	0	5	8	1	12.5	–	–
	17	–	–	0	0	1	0	1	0	0	0	0	8	2	25.0	±	–
	18	–	–	0	0	0	0	0	0	0	0	0	8	0	0	–	–
	19	–	–	0	1^{b}	0	0	0^{E}	0	0^{E}	0	2	8	1^{b}	12.5	±	+
	20	+	+	0	0^{E}	0	0	0	0	0	0	1	8	0	0	±	±
	21	+	–	0	1	1	1^{E}	0	0	0^{E}	1^{E}	3	8	4	50.0	+	±
	examined	7	7	7	7	7	7	7	7	7	7	11	56			7	7
Subtotal	positive[a]	2	1	0	4^{b}	2	1	1	1	0	1	0		10^{b}	17.9	1	2
	%	28.6	14.3	0	57.1	28.6	14.3	14.3	14.3	0	14.3	0				14.3	28.6
	examined	21	21	21	21	21	21	21	21	21	21	43	168			21	21
Total	positive[a]	16	9	9^{b}	8^{b}	8	10^{b}	5^{b}	5^{b}	5^{b}	9	0		59^{b}		5	7
	%	76.2	42.9	42.9	38.1	38.1	47.6	23.8	23.8	23.8	42.9	0			35.1	23.8	33.3

Explanations to Table 1

[1]stage of infection: I = group of 7 heavily infected hens with the presence of tuberculous lesions in different organs with 4 or more positive tissue cultures for *M. avium* subsp. *avium* (*MAA*), II = group of 7 moderately infected hens without presence of tuberculous lesions and with 4 or more positive tissue cultures for *MAA*, III = group of 7 slightly infected hens without presence of tuberculous lesions and with 1 to 3 positive tissue cultures for *MAA*

[2]skin testing was carried out with avian tuberculin (PPD prepared from the *MAA* D 4 ER Strain)

[3]days on which laboratory examination of faeces by microscopy took place after Ziehl-Neelsen staining; cultivation was followed by PCR identification, serotyping and biochemical assays

[4]from each hen 8 faecal samples were collected during the investigated period

[5]rapid agglutination test was carried out with the two antigens described in the Chapter Material and Methods

[E]eggs were collected on this day

[a]*MAA* isolate of serotype 2 and genotype 1 030/180 bp fragments of 16S rRNA, IS*901*+ and IS*1245*+

[b]besides *MAA*, other mycobacterial species unidentified by biochemical assay were isolated

[c]dubious reactions were observed in 6 (14.0%) samples of the control group

*acid – fast bacilly detected after Ziehl-Neelsen staining

and the liquid media of Sula as described by Matlova et al. (2005). Incubations were performed at 25°C and 37°C and cultures were monitored during the first week of incubation to rule out fast growing mycobacteria species and early contamination and then every other week for three months (Fischer et al., 2001).

Identification of mycobacterial isolates. All the AFB positive isolates were examined by PCR. Firstly PCR for 16S rRNA according to Wilton and Cousins (1992) was carried out. The presence of the 1 030 bp fragment identified the isolate as belonging to the genus *Mycobacterium* and the presence of the 1 030 bp fragment together with 180 bp indicated that it was a *M. avium* species. All the AFB positive isolates were also tested by single PCR for IS*901* and IS*1245* (Bartos et al., 2006). Isolates of IS*901*+ and IS*1245*+ were identified as *MAA* species (Kunze et al., 1992; Pavlik et al., 2000; Bartos et al., 2006) and isolates of IS*901*– and IS*1245*+ as *M. avium* subsp. *hominissuis, MAH* (Guerrero et al., 1995; Bartos et al., 2006). *M. avium* complex (*MAC*) isolates were serotyped according to a described system (Wolinsky and Schaefer, 1973) which has later been modified (Sussland and Hrdinova, 1976). Mycobacterial isolates that were not classified as *MAC* members were assessed by biochemical methods (Wayne and Kubica, 1986).

Statistical evaluation

The chi^2-test and Fisher test (Stat Plus) were used for the statistical evaluation of results (Matouskova et al., 1992).

RESULTS

All the results of intravital diagnostic testing performed in 21 studied hens are presented in Table 1. Studied hens were divided into three groups according to the stage of infection that was determined after euthanasia and following the pathology and culture examination of tissues published previously (Shitaye et al., 2008). *MAA* was detected in the tissues of all studied hens except hen No. 21. In the tissue of this hen no pathology or *MAA* isolates were detected.

Stage I. This group of hens was comprised of seven emaciated and heavily infected birds with the presence of tuberculous lesions in different organs (hens Nos. 1 to 7) and with the culture detection of *MAA* in at least four examined tissue samples.

Stage II. This group of hens was comprised of seven moderately infected birds without the presence of tuberculous lesions (hens Nos. 8 to 14) and with the culture detection of *MAA* in at least four examined tissue samples.

Stage III. This group of hens was comprised of seven slightly infected birds without emaciation or the presence of tuberculous lesions (hens Nos. 15 to 20) and with the culture detection of *MAA* in one to three examined tissue samples. This group also included hen No. 21 which exhibited emaciation; however no *MAA* isolate was detected in the tissues of this hen.

Skin test with avian tuberculin

The tuberculin skin test has shown a positive allergic response in nine (42.9%) hens: the same positivity (57.1%) was detected in both hen groups with

advanced stages of ATBC (infection Stages I and II). In contrast, positivity was only 14.3% in the group of hens with slight ATBC (infection Stage III).

Rapid agglutination test (RAT)

Positive RAT reactions were found with the *MAA*-b antigen in five (23.8%) and with the *MAA*-p antigen in seven (33.3%) hens only. RAT positivity with antigens *MAA*-b and *MAA*-p was significantly higher ($P < 0.05$) in hens affected by advanced ATBC with concurrent tuberculous lesions (Stage I infection) in contrast to positivity with these antigens in hens affected by Stages II and III infection where dubious reactions prevailed (Table 1).

Laboratory examination for the detection of mycobacteria

Microscopic and culture examinations of faeces and eggs. AFBs were detected in five (3.0%) of 168 faecal samples from only four (19.1%) of 21 studied hens. AFBs were not detected in egg samples (Table 1).

***MAA* isolation from faeces**. *MAA* of serotype 2 and genotype 1 030/180 bp fragments of 16S rRNA, IS*901*+ and IS*1245*+ was isolated from 50 (29.8%) of 168 samples. The faeces of nine hens (42.9%) on the day of slaughter and from a total of 17 hens (81.0%) over the previous 8-day period yielded positive results. *MAA* excretion in faeces was intermittent in 16 hens except for hen No. 3, which was positive in all faecal cultures. Four hens Nos. 9, 13, 18 and 20 (Stages II and III) did not shed *MAA* at all. *MAA* positivity (57.1%) found in the faeces of the highly infected group of hens (Stage I) was significantly higher ($P < 0.01$) in comparison with the positivity in groups of hens in Stage II (30.4%) and Stage III (19.6%; Table 1).

Other mycobacteria isolated from faeces. Besides the above described *MAA,* nine mycobacterial species other than *MAA* or *MAH* (1 030 bp fragment of 16S rRNA positive, and IS*901* and IS*1245* PCR negative), which could not be identified by biochemical testing, were isolated from the faeces of eight hens Nos. 4–8, 10, 14 and 19.

Culture examination of eggs. Mycobacteria were not detected in any of the 43 examined eggs. In 16 cases, eggs were laid on the same day when *MAA* was detected in hen faeces. The Stage II hens laid significantly more eggs ($P < 0.05$) over the investigated period than hens from the other two groups (Stages I and III).

DISCUSSION

The suitability of the use of the following three intravital techniques (i) tuberculin skin test with avian tuberculin, (ii) serological test with surface antigens of *MAA* and (iii) culture examination of faeces and eggs for diagnosis of ATBC was investigated in 21 hens.

Skin test with avian tuberculin

The detection rate of infected hens with different stages of infection by skin testing with avian tuberculin has been found to be low (42.9%). The reliability of this test to detect *MAA* infected birds especially decreases in flocks with high infection rates (Pavlas et al., 1993) was one of the findings of our study. In agreement with previous reports (Lumeij et al., 1980; Pavlas et al., 1993), false negative results were observed but generally this test was found to be more sensitive than the RAT, in particular with the *MAA*-b antigen (Table 1). Similarly, the skin test has been found to be unreliable in various species of birds including pigeons, geese, quail and other exotic species showing false negative results (Ensly et al., 1975; Bush et al., 1978; Pavlas et al., 1983).

This low number of positive reactions found by skin testing in our study may be related to the late stage of the disease process that leads to desensitisation and/or it may result from the stage of anergy accompanying miliary ATBC (Pavlas et al., 1993), which is often associated with the depression of cellular immunity. Although the reason for anergy is poorly understood (Monagham et al., 1994), Lepper et al. (1977) have previously indicated that animals with generalised severe tuberculosis are anergic; a similar scenario in poultry populations is certainly likely. On the other hand, the failure to respond to tuberculin in some hens may be due to the early stage of the infection e.g. the false negative results were mainly observed in slightly infected hens.

Rapid agglutination test (RAT)

The RAT showed its limitations for the screening of infected hens with *MAA* of serotype 2 from

the flock due to the low positivity with both *MAA* antigens (*MAA*-b of serotype 1 and *MAA*-p of serotype 2) used. The interpretation of these results is complicated by the occurrence of false negative reactions and "subjective" reading with subsequent interpretation of the results by the technician performing laboratory tests.

In general our finding agrees with previous reports that have claimed that the RAT is not a reliable method for detecting infected birds, although it has been suggested that the RAT is possibly more sensitive than tuberculin skin tests (Hawkey et al., 1983, 1990; Pavlas et al., 1983; Cromie et al., 1991). We confirmed the high sensitivity of the RAT (71.4%) with *MAA*-p antigen only in heavily infected birds. In contradiction to our finding, however, Gotz (1984) reported a high specificity and sensitivity of the RAT. Despite this, the sensitivity of the RAT may be affected by the type of sample used (Pavlas et al., 1993); in addition, this discrepancy may most likely be related to the stage of infection and reaction of the host to the infection.

Despite the presence of considerable variations in specificity and sensitivity, the RAT and tuberculin skin test remain the most frequently applied methods for *ante mortem* diagnosis of ATBC. No correlations between serological and skin-test data were found, except that in both tests, hens in Stage I gave more positive results than hens in Stages II and III. The results show that both serological and skin-test techniques are inadequate for the diagnosis of ATBC.

Egg examinations

The most common route of infection for susceptible birds is the alimentary tract; but *MAA* has also been detected in the eggs of an infected flock of domestic birds (Hines et al., 1995) and the vertical transmission of the infection to embryos through eggs has been reported (Gerlach, 1986; Kyari 1995; Prukner-Radkovcic et al., 1998). In our previous study (Shitaye et al., 2008) we isolated *MAA* from ovaries and/or oviducts and/or the uterus of seven hens (Nos. 2, 4, 5, 8, 9, 11 and 14; hen No. 4 did not lay eggs); out of which two hens (Nos. 2 and 5) were severely infected (Stage I). Nevertheless, *MAA* was not isolated from any of these 19 eggs nor from the other 24 eggs laid by *MAA* infected hens in the present study (Table 1).

In this study, moreover, mycobacteria were detected neither in egg shell membranes nor egg white/albumin and egg yolk samples. We found no discrepancies in the results from those egg sample cultures processed with or without decontamination, irrespective of the level of infection. In all cases our findings are consistent with previous reports (Fitch and Lubbenhusen, 1928 and Schalk et al., 1935 – cited by Thoen, 1997), where it has been demonstrated that no causative agent of ATBC has been observed in a single case from many hundreds of chicks hatched from eggs of naturally infected hens. Likewise, similar suggestions have been made by Francis (1958) and Hejlicek (1977).

In contrast to this finding Fritzsche and Allam (1965) and Bojarski (1968) isolated *MAA* by culture from the eggs of naturally infected hens in Germany and Poland, respectively. We assume that to a large extent, this discrepancy may be related to the severity and intensity of the infection and the difference in the sample processing techniques applied. These facts may impair the diagnosis of mycobacteria in eggs, and if this is not the case it is likely that the transmission of *MAA* through eggs and thus its detection may not occur frequently. These previous reports including the result that we obtained, remain to be discussed. We recommend that the "theory of trans-ovarian transmission of mycobacteria and the possibility of acquiring ATBC infection" through eggs needs further study.

Culture examination of faeces

MAA shedding in naturally infected hens in faeces over the 8-day period of investigation was irregular; the exception was one hen in whose faeces *MAA* was detected each day. *MAA* shedding through faeces ranged between 23.8% to 47.6% for one day sampling.

Based on the culture examination of tissue samples from all 21 studied hens we established that 17 (80.9%) of them shed *MAA* in faeces; this was confirmed by the detection of *MAA* as follows (Table 1):

– in 16 (76.2%) hens in the intestinal tract,
– in one (4.8%) hen No. 6 in the liver that communicates with the intestinal lumen via biliary ducts (*MAA* was also detected in the lungs and spleen of this hen),
– in one (4.8%) hen No. 14 in the kidneys that communicate with cloacae via the urethra.

MAA was detected in only 17 (80.9%) hens by repeated culture examinations of faeces on each of eight consecutive days. Moreover, *MAA* was detect-

ed in the faeces of only 17 out of 20 hens with supposed shedding (*MAA* infection present in organs). Contrary to expectations, *MAA* was detected in the faeces of hen No. 21, even though all examinations of tissues were negative. When considering these results, it is apparent that the repeat culture examination of faeces is a more sensitive diagnostic test then the serological and skin-test. With regard to the intermittent shedding of *MAA* through faeces (Table 1) and the long incubation time necessary for the *in vitro* isolation of *MAA,* however, the application of this diagnostic method in practice is difficult.

MAA shedding in faeces by Stage I individual hens with tuberculous lesions in parenchymatous organs ranged between 25 and 100% over the 8-day period of investigation. In the Stage II group, shedding was less frequent, and in two hens, *MAA* shedding in faeces was not observed at all during the eight days. In the Stage III group of slightly infected hens, *MAA* was not detected in faeces from two hens and *MAA* detection in faeces from the remaining hens was less frequent than in the two previous groups. It follows from the obtained results that (Table 1):

– *MAA* shedding in faeces is not regular, especially in hens without developed gross lesions in parenchymatous organs.
– *MAA* can be shed in faeces by birds in a flock whose tissues have not yet been infected (most likely "passive" transport of *MAA*).
– The reliability of culture examination seems to be affected by decontamination procedures (sample preparation with the aim of controlling contaminating micro-flora), which may cause devitalisation of *MAA* pathogens.

This finding strengthens our suggestion that the contaminated faeces of infected hens are the major source of infection for other susceptible birds (Hejlicek and Treml, 1995; Painter, 1997). Due to the fact that mycobacteria can survive for long periods in contaminated materials, they constitute a potential source of infection for a successive generation or new groups of flocks. These are factors that could complicate a control programme.

CONCLUSIONS

The comparison of the efficiency of three intravital routine diagnostic methods for ATBC (skin test, serology and culture of faeces and eggs) revealed that the probability of diagnosis of avian tuberculosis in infected hens increased with the later stage of infection in all tested methods. But only the faeces cultivation over the eight day period was able to identify *MAA* infection absolutely in all heavily infected hens. The probability of *MAA* isolation is lower during earlier stages of infection using this method as well. The big advantage of the RAT, however, is its speed in comparison with cultivation. The reliability of the skin test was also the lowest in the earliest stage of infection, in heavily and medium infected hens the efficiency was 57.1% compared to 14.3% in the earliest stage. In this paper we confirmed the faeces of infected hens as the main source of infection but on the other hand we did not confirm the risk of egg consumption for humans, all examined eggs from infected hens were *MAA* negative.

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